PREIMPLANTATION DIAGNOSIS OF X-LINKED DISEASE

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To

George Stavridis

and

Ioanna Kontogianni

Abstract

Preimplantation diagnosis of inherited disease following *in vitro* fertilization (IVF) allows the transfer of unaffected embryos to the uterus avoiding the termination of an affected pregnancy in couples at risk of transmitting an inherited disorder. Possible approaches for X-linked recessive disease include transfer of females by identification of embryo sex, biochemical assay of enzyme deficiencies or specific detection of the mutation.

DNA amplification using the polymerase chain reaction (PCR) of Y-specific repeats from single blastomeres biopsied at cleavage stages identified male embryos. Following transfer of females, the first pregnancies were established. The sex of the remaining embryos was confirmed by PCR. Examination of single blastomeres from disaggregated embryos demonstrated that amplification failure occurs in a minority. Co-amplification of X- and Y-specific repeats from single cells was possible. Different lysis protocols were compared and a two-step method shown to minimise failure. A minimum of two cells is nevertheless necessary.

Detection of enzyme deficiencies by biochemical assay requires that embryo-derived enzyme predominates over maternal. To examine embryonic gene expression, qualitative and quantitative aspects of protein synthesis were studied. Protein synthesis was constant during cleavage, when most qualitative changes occurred, and increased significantly at the blastocyst stage. Transcriptional inhibition had no effect on rate of synthesis at early stages but blocked the rise in blastocysts suggesting persistence of maternal mRNA after activation of the embryonic genome. Lesch-Nyhan syndrome is caused by deficiency of X-linked hypoxanthine phosphoribosyl transferase (HPRT). HPRT activity was dependent on transcription and was different in males and females at the blastocyst stage, suggesting that embryo-

coded enzyme predominates over the maternal. However, diagnosis based on the product of gene expression requires further work.

Diagnosis of the predominant mutations causing single gene defects is now possible by PCR. However, many X-linked defects are heterogeneous. Identification of sex is equally applicable to all of these conditions and is, therefore, likely to predominate for the foreseeable future.

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ABBREVIATIONS

PCR	Polymerase Chain Reaction
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic Acid
mRNA	messenger Ribonucleic Acid
EDTA	Ethylenediaminetetracetic acid
hCG	human chorionic gonadotrophin
HEPES	N-(2-Hydroxyethyl)pipirazine-N'-(2-ethanesulfonic acid)
ICM	Inner Cell Mass
SDS	Sodium Dodecyl Sulfate
IVF	In Vitro Fertilization
LHRH	Luteinizing Hormone Releasing Hormone
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PVP	Polyvinylpyrrolidone
TE	Trophectoderm
TE buffer	Tris-EDTA buffer
UV	Ultraviolet
TCA	Trichloroacetic acid
rRNA	ribosomal Ribonucleic Acid
tRNA	transfer Ribonucleic Acid
CVS	Chorionic villus sampling
CF	cystic fibrosis
DMD	Duchenne muscular dystrophy

Chapter 1

General Introduction

1.1. Introduction

Genetic diseases have a prominent role in childhood illnesses and mortality. They cause about a third of admissions to paediatric wards and are an important cause of childhood deaths. Most of them are associated with mental or physical handicap, or both (Weatherall, 1991). A number of surveys have indicated that at least one in fifty newborns have a major congenital abnormality, about one in a hundred have a disorder due to a single gene defect and about one in two hundred has a major chromosomal abnormality (Emery & Rimoin, 1990). Many of the well-known genetic diseases are caused by a single gene defect. These include cystic fibrosis (CF), Huntington chorea, haemophilia, the various muscular dystrophies and the inborn errors of metabolism.

During the last few years, advances in molecular and cell biology have led to the development of methods for isolating, determining the fine structure and understanding the function of genes, making it possible to comprehend the molecular pathology of many diseases. The mapping of the human genome greatly increased the number of single gene defects identified at the molecular level. In the most recent edition of McKusick Catalogue of Mendelian disorders published in 1990 4,937 single gene defects affecting man are listed compared with 1,487 in 1966. The majority of these single gene defects are serious, none is curable and very few can be treated effectively. Therefore, the main goal of clinical genetics has been prevention, mainly by offering prenatal diagnosis to couples at high risk of transmitting the disease.

Prenatal diagnosis currently involves sampling of fetal cells either by chorionic villus sampling (CVS) or amniocentesis in the first or the second trimester of pregnancy and analysis by cytogenetic, biochemical or DNA methods. However, if the fetus is shown to be affected, the couple faces the difficult choice of whether or not to

terminate the pregnancy. The only prospect of starting a pregnancy which is known to be unaffected, is to be able to diagnose a potentially affected conceptus prior to implantation (preimplantation diagnosis) and so eliminate the need for the abortion.

Before discussing possible approaches for preimplantation diagnosis in general and for X-linked diseases in particular, the molecular pathology, treatment and current approaches to prenatal diagnosis will be reviewed.

1.2. Molecular pathology of inherited disease

1.2.1. Variety of disorders

Inherited disorders are generally recognised because they run in families and usually parents only become aware that they are at risk when they have an affected child or relative. Inherited disorders range in severity from minor defects, such as colour-blindness, to severe ones, such as cystic fibrosis (CF). For the more serious ones, usually prenatal diagnosis is requested. The metabolic defect is known in about 350 different inherited conditions (Besley, 1992) and in most cases affects the production or function of an enzyme. In many cases the biochemical basis for the condition has been identified before the gene defect was known. The number of identified diseases is increasing each year, as during the last 15 years developments in recombinant DNA technology has led to a better understanding of the molecular pathology of single gene disorders.

Inherited diseases can be divided into four main groups according to the nature of the genetic defect: i) chromosomal disorders, in which there is an abnormality in the individual chromosomes (that can be detected in many cases by cytogenetic methods) - many of these are associated with specific clinical syndromes, ii) single gene defects, in which the disease results from the presence of a single mutated gene -

these diseases are characterised by Mendelian patterns of inheritance in families and are inherited as autosomal dominant, recessive, or sex-linked, iii) multifactorial, which have partly genetic and partly environmental causes - it is responsible for about 20% of the cases of major congenital malformation, such as congenital heart disease (Young, 1992) and iv) inherited abnormalities of mitochondrial DNA, which account for some human genetic diseases (Emery & Rimoin, 1990).

1.2.2. Chromosomal abnormalities

Most chromosomal abnormalities involve anomalies in the number of chromosomes. Many of these, particularly autosomal monosomies, are lethal at various stages of pregnancy. Others are compatible with development to term. They cause a variety of syndromes, from relatively mild mental retardation and infertility, such as in Klinefelter syndrome (47, XXY), to severe conditions as Edward's syndrome (trisomy 18), in which all affected individuals survive for only short periods of time after the birth. The most common chromosomal abnormality is trisomy 21 (Down's syndrome). It is characterised by mental retardation and congenital heart disease. Two other trisomies that are also common are trisomy 18 and trisomy 13 (Patau's syndrome). They are associated with mental retardation and congenital malformations and life expectancy (for the majority of the cases) is limited to a few weeks after birth. Other chromosomal abnormalities include structural rearrangements that can be inherited or arise de novo and have a range of phenotypic effects which depend on the chromosomes involved.

1.2.3. Single gene defects

1.2.3.1. Identification of gene defects

Almost 5,000 single gene defects have been described. In many cases the biochemical basis for the condition was identified before the genetic defect itself. It may be possible to find the approximate location of a gene for a disorder of this type and then move along the chromosome until the particular locus is reached. Having found the location of the gene, the next step is to sequence it and then try to understand its function by working out its probable amino acid sequence from the DNA sequence. This has been termed reverse genetics (Orkin, 1986).

The first successful application of reverse genetics was the mapping of the gene responsible for Duchene muscular dystrophy (DMD), an X-linked recessive disease(review by Caskey, 1986). An important clue came from the observation of females affected by the disease with balanced X/autosome translocations. The autosomal breakpoints were different, but the X-chromosome breaks were localised in the Xp21 region, suggesting that this region might contain the DMD gene. The localization was tested and confirmed by linkage studies using restriction fragments length polymorphisms (RFLPs) flanking the X21 band. From a patient with a deletion within Xp21, who was suffering from DMD, clones from within the DMD locus were isolated. One of the fragments generated detected DMD deletions in a proportion of patients and was tightly linked to the disease in family studies. By chromosome walking, approximately 200 kb of DNA was isolated and it was later demonstrated that breakpoints found in DMD patients were spread heterogeneously over the region. A large mRNA species (14kb) was detected and it was shown that the size of the gene was at least 2.3 million bases. Portions of the gene sequence were expressed in vitro and reacted with a 400,000 kb protein, called dystrophin, the absence of which causes DMD (Caskey, 1986).

In a similar way the CF gene and the corresponding cystic fibrosis transmembrane regulator protein (CFTR) were found. For the moment, other diseases have an incomplete success story in reverse genetics (such as chronic granulomatous disease) and the research is being continued. The idea of reverse genetics has not been

easy to apply in many diseases, as it often depends on the chance of finding a patient with a deletion which is associated with a particular phenotype. However, the initial successes with gene mapping have focused attention on human molecular biology on the project of mapping the whole human genome. Recent technical developments have made this feasible. Since the genes of functional importance may constitute as little as one-tenth of the total genome, this goal may be achievable within the next ten or twenty years (Weatherall, 1991)

A genetic defect can be expressed at several levels. The mutation might cause a reduced rate of transcription of a gene, abnormal processing of mRNA precursors, defects of initiation, translation or termination of the synthesis of a protein product on the cytoplasmic mRNA template, abnormal processing of the protein, or abnormal structure of the gene product.

1.2.3.2. Classification

Many of the well known genetic diseases are caused by a single mutated gene, for example CF, Huntington's chorea and the various muscular dystrophies. Single gene defects can be subdivided according to the type of chromosome on which they are located (autosome or sex chromosome) and whether the disease is dominant or recessive.

1.2.3.3. Autosomal gene defects

A disorder which is coded by a gene on one of the autosomal chromosomes is said to be inherited as an autosomal disease. Autosomal diseases affect both males and females with the same frequency and they can be dominant or recessive. Unlike autosomal dominant diseases, autosomal recessives manifest only when the mutated gene that causes the disease is present in the homozygous state. Affected individuals have parents who are both heterozygous for the gene (carriers), but are generally healthy.

1.2.3.4. Sex-linked gene defects

Diseases caused by genes on one of the sex chromosomes are called sex linked or X-linked, as the majority of the genes that cause these diseases are located on the X rather than the Y chromosome. There appear to be just a few genes of significance on the Y-chromosome (Skinner, 1990). An affected male would transmit the gene on his only Y chromosome to all of his sons, but none of his daughters (Y-linked or holandric inheritance). Since males have only one X chromosome, an abnormal gene on the X chromosome cannot be paired with a normal allele; the male is said to be hemizygous for that mutant gene. Therefore all males that inherit such genes are affected by the disease, even if the gene is recessive. An X-linked dominant disease is one which affects both the hemizygous male and the heterozygous female. These conditions are very rare. Examples include the Xg blood group, vitamin D-resistant rickets and the oral-facial-digital syndrome (Skinner, 1990).

If females have a mutated X-linked recessive gene, its effects will be counteracted by the normal allele on their other X chromosome. A female would manifest a recessive gene only in the homozygous state, which is very rare. These diseases are therefore transmitted within families either by healthy female carriers (if the disease is serious and leads to early death of the affected male, such as DMD and Lesch-Nyhan syndrome), or by both female carriers and affected males (if the disease does not significantly affect fertility and allows survival into reproductive age, such as Becker muscular dystrophy).

1.2.4. Incidence of genetic diseases

A number of surveys have indicated that about one in two hundred newborns has a major chromosomal abnormality (Emery & Rimoin, 1990). The frequency of chromosome abnormalities before birth is much greater: the frequency of early spontaneous abortions is between 15 and 20% of pregnancies and about 60% of

these are associated with chromosomal abnormalities. The incidence of specific chromosomal abnormalities is much greater in spontaneous abortions than in live births: 3-4 times in the case of trisomy 21 and more than 150 times in Turner's syndrome. Other anomalies that are found frequently in spontaneous abortions are triploidy and polyploidy (Boue & Boue, 1978). Obviously the majority of fetuses that have a chromosomal abnormality in early embryonic life cannot survive and only a small proportion of these abnormalities is represented in live births.

The incidence of single gene defects has been the subject of many investigations. Early estimates were very low, as some of the disorders do not manifest until later in life and the molecular basis of the diseases were unknown. With the advances in molecular genetics and the development of new techniques, the number of identified genes that cause genetic diseases over the last decade is increasing (Mc Kusick, 1990). From 4,937 single gene defects affecting man that have been described, 3,047 are autosomal dominants, 1,554 autosomal recessives and 336 X-linked.

Individually many of the single gene defects are relatively rare, but there are many of them and as a whole, the group is an important cause of genetic disease. If late manifesting diseases, such as monogenic hypercholesterolaemia are included, the overall incidence of autosomal dominants is about 1.8-9.5 per 1,000 population, autosomal recessives about 2.5 per 1,000 and X-linked disorders about 0.5-2.0 per 1,000 population (Weatherall, 1991) (Table 1.1.). A number of surveys have shown that about one in a hundred newborns has a disorder due to a single gene defect (Weatherall, 1991). It has to be considered that there are ethnic and geographic variations in the incidence of some single gene defects (e.g. CF is more common in Caucasians and Tay- Sachs disease in the Ashkenazic Jewish populations).

Table 1.1.: Incidence of inherited diseases (from Weatherall, 1991)

Type of disease	Frequency per 1000 population
Single gene defects	
Dominant	1.8-9.5
Recessive	2.2-2.5
X-linked	0.5-2.0
Chromosomal abnormalities	6.8
Multifactorial disorders	(7-10)*
Congenital abnormalities	(19-22)*
Total	(37.3-52.8)*

(The numbers in brackets are estimations)

1.2.5. Other inherited diseases

1.2.5.1. Multifactorial diseases

Most of the single gene disorders considered in the previous section are inherited in a definite autosomal dominant, autosomal recessive or X-linked fashion. However, many diseases do not show a clear-cut pattern of inheritance and occur among the relatives of affected individuals in higher frequencies than in the general population. The increased risk to the relatives may be the result of the interaction of several genes at different loci, together with environmental influences. This type of inheritance is termed multifactorial. The effect of environmental factors on genetically susceptible individuals has been the subject of many studies. In this group of diseases fall many of the common conditions that affect the population of Western societies, such as cardio-vascular disease (as genetic factors seem to be very important in the generation of hypertension and the causation of degenerative arterial conditions), diabetes, psychiatric illnesses (such as schizophrenia and manic-depressive conditions), many forms of cancer (such as breast cancer and hereditary retinoblastoma) etc.

1.2.5.2. Mitochondrial diseases

Although the majority of the known genetic diseases are caused by mutations in genes on chromosomes in the nucleus, some are inherited in a non-Mendelian fashion and are determined by DNA in the cytoplasm. Sperm does not contain a large amount of cytoplasm but oocytes do and mitochondria are mainly inherited from the mother through the oocyte. Mitochondria have their own DNA which is separate and distinct from nuclear DNA. Mitochondrial DNA codes for 13 proteins,

which are components of the mitochondrial respiratory chain and oxidative phosphorylation system as well as two rRNAs and twently two tRNAs. Mutation in the mitochondrial DNA coding for these proteins may result in disease, for example diseases affecting the muscle such as the mitochondrial myopathies or the central nervous system (CNS) as in Leber's optic atrophy. This type of inheritance is distinguished by transmission of the mutation through the oocyte of an affected mother to all her children, irrespective of sex. However, only the daughters would transmit this trait to subsequent generations.

1.2.5.3. Fragile X, Myotonic dystrophy and heritable unstable elements

Until recently, the genetics of some relatively common inherited diseases, such as fragile X and myotonic dystrophy, have been enigmatic. Fragile X is characterised by mental retardation and it is inherited as an X-linked disease. Under certain conditions in cultured cells from affected and carrier individuals, the mutation produces a fragile site on the X-chromosome, that can be diagnosed at the cytogenetic level. However, a large percentage of female asymptomatic carriers of the disease do not have the fragile site. Also between 20-50% of males having the fragile site are asymptomatic. The asymptomatic male 'carriers' can pass the gene to their daughters, which are also asymptomatic. The children of that daughter may have the syndrome, indicated that 'something' happens to the mutation before the effects are observed in the third generation. Recent progress led to the identification and cloning of the fragile X and myotonic dystrophy genes (FMR-1 and DM respectively) and the results suggest that at least a part of the 'something' is a change in the size of a repeat in the fragile X region. In both cases the mutation involves the amplification of a trinucleotide repeat [(CCG)n in fragile X and (CTG)n in myotonic dystrophy] (Yu *et al*, 1991; Harley *et al*, 1992). An increase in the number of repeats in successive generations is responsible for the higher penetrance observed (anticipation). In the case of the fragile X, normal chromosomes have 6-60 copies of the repeat. Carrier males (which are phenotypically

normal) have an increased copy number of between 60-200 repeats. Affected individuals have more than 200 copies. Although the definitions of 'carrier', 'transmitting' and 'affected' categories have to be clarified, the identification of this type of mutation solves problems of interpreting a type of disease that does not follow strict mendelian rules. Previously unexplained terms, such as 'anticipation' (the increase in the number of affected individuals at successive generations from fragile X and myotonic dystrophy families), have a molecular explanation today (a positive correlation between the copy number and the phenotype has been established). The relationship between the copy number and the phenotype is now used to predict the phenotype in prenatal diagnosis (Fu *et al*, 1991).

1.3. Treatment of inherited disease

1.3.1. General

All the developments in molecular genetics during the last decade, although they increased our understanding of the molecular pathology of genetic defects, had little effect on the treatment of these conditions. Very few genetic diseases can be treated effectively. For some conditions with a genetic component, such as cleft palate, harelip, and some forms of congenital heart disease, surgical correction is possible. Some of the inborn errors of metabolism can be controlled by regular replacement of a missing protein or enzyme (for example long term transfusion therapy for thalassaemia) or by preventing the accumulation of toxic metabolites by appropriate manipulation of the diet . Genetic metabolic disorders that have been treated by dietary modifications with variable success include galactosaemia, phenylketonuria and inborn errors of essential amino acids metabolism. Also, in metabolic disorders in which a specific deficiency has been demonstrated, attempts have been made to replace missing enzymes. The missing enzyme has been delivered by intravenous infusion, packaged in liposomes or using red cell ghosts. Although enzyme deficiencies have been successfully corrected at the gene level in tissue culture or in experimental animals, this approach has not yet been successfully applied to humans (Weatherall, 1991).

1.3.2. Cell therapy

Bone marrow transplantation has been used to correct some genetic disorders. The principle is that donor bone marrow will produce the missing component in the host. Bone marrow transplantation has been used for the treatment of β–thalassaemia and sickle cell anaemia, disorders of lymphocyte function (such as X-linked severe combined immunodeficiency and adenine deaminase deficiency) and

inborn errors of metabolism (such as the lysosomal storage disease). The success rate has been variable.

1.3.3. Gene therapy

Another possibility that is currently being explored is that of gene replacement therapy. The replacement or correction of defective genes is a major aim in the application of genetic engineering technology to human disease. It is now possible to isolate genes together with their regulatory regions. Also gene replacement therapy is feasible in culture. However, a lot of problems are involved and it is essential that several questions are answered before patients are subjected to gene therapy: the gene must be isolated and its regulatory regions have to be defined; appropriate target cells have to be identified and harvested; safe and efficient vectors with which to introduce the new gene have to be developed; and finally, there must be clear evidence from extensive animal experiments that the inserted gene will function adequately, that the recipient cell population will have a reasonably long life span, and that the inserted gene will produce no deleterious effects in the recipient patient. Until those requirements are fulfilled, the only approach for the avoidance of genetic diseases is prevention (Weatherall, 1991).

1.4. Prenatal diagnosis of inherited disease

1.4.1. General introduction

Although all the developments in molecular genetics during the last decade have had little impact on the treatment of these conditions, they have significantly enhanced the ability to diagnose genetic diseases prenatally. The first step in prenatal diagnosis is the identification of couples at increased risk of having a child with a genetic disorder. Such couples can be identified on the basis of their family histories or through screening methods. If after screening the couple is found to be at high risk of transmitting a genetic defect, prenatal diagnosis can be offered. If the disease in question is identified in a fetus, the couple is faced with the possibility of a termination. Alternatively, the parents may choose not to have any children, to adopt, or to use artificial insemination or IVF with gamete donation. However, these alternatives are not easily accepted by most couples, who have persevered to have their own children.

1.4.2. Examination of the fetus and sampling of fetal tissues

Prenatal diagnosis procedures include non-invasive approaches, such as visualisation of the fetus and invasive ones, such as amniocentesis, CVS and biopsy of fetal tissues.

1.4.2.1. Ultrasound and fetoscopy

Direct examination of the fetus can be carried out non-invasively by ultrasound analysis for the detection of major congenital malformations. Recent

technical advances in ultrasound can also identify more delicate developmental anomalies. Ultrasonography is used to date the pregnancy accurately, in order to interpret correctly the biochemical markers (as maternal serum alpha-fetoprotein for the detection of neural tube defects or Down's syndrome); to examine the anatomy of the fetus, which can detect major malformations or minor ones that might be markers of chromosomal defects; and also to assess the safety and effectiveness of amniocentesis, CVS and biopsy of fetal tissues (Nicolaides *et al,* 1991).

The fetus can also be examined directly by fetoscopy. The technique involves visualisation of the embryo after the insertion of an endoscope. The insertion is guided sonographically. Potential applications of fetoscopy are the prenatal diagnosis of structural abnormalities, confirmation of ultrasonographic diagnosis and fetal tissue sampling (Reece & Hobbins, 1991). Today, fetoscopy has almost been abandoned as an aid for fetal tissue sampling and has been replaced by ultrasound. The main reasons are that fetoscopy is an invasive procedure, it requires more skill and longer training than ultrasound and it has a rate of miscarriage between 2.5 and 5% (Nicolini & Rodeck, 1992). Improvements in ultrasound technology enable the vast majority of invasive procedures to be monitored by ultrasonographic guidance (also see 1.4.4.1. & 1.4.4.2.).

1.4.2.2. Amniocentesis

Amniocentesis in combination with ultrasonography is the most established and widely used method for prenatal diagnosis. It was first applied for the prenatal identification of sex in the 1950s. Ten years later in was demonstrated that the technique can be used for the diagnosis of chromosomal abnormalities and the inborn errors of metabolism. Amniocentesis involves insertion of a needle transabdominally into the amniotic cavity and the removal of amniotic fluid for the diagnostic tests to be performed. Most amniocenteses are performed at 15 to 16 weeks of gestation. The cells

obtained by amniocentesis are cultured and results after karyotypic analysis are ready after 2-4 weeks (see 1.4.4.3.) (Charrow *et al,* 1990).

Complications of amniocentesis may include failure to aspirate fluid, fetal injury and minor maternal complications. The major risk is of miscarriage. However, the risks of amniocentesis are very low if the procedure is performed by a trained and experienced operator. In various studies, the rate of fetal loss is between 0.5 and 3.5%, depending on the experience of the doctor performing the procedure and other factors, such as the number of needles inserted (Charrow *et al,* 1990)

With increasing experience in amniocentesis, it has become possible to perform the technique earlier in gestation, as early as 10-14 weeks. The advantage of early amniocentesis is timing, as it is desirable to perform prenatal diagnosis as early as possible. Early amniocentesis can be useful in some twin pregnancies (in which sampling of both fetuses is not possible with CVS) or circumstances when CVS is contraindicated. Although limited experience during the last few years indicated that the safety of the procedure is comparable to traditional amniocentesis, more extensive and long term studies are required.

1.4.2.3. Chorion villus sampling

More recently a technique called chorion villus sampling (CVS) has been developed (reviews by Silverman & Wapner, 1992; Nicolini, 1992; Evans & Johnson, 1992). From about 9 to 12 weeks gestation it is possible to biopsy the chorion by the cervical route or transabdominally. Depending on the techniques used, results of a cytogenetic analysis after CVS will be available from 2 days to 2 weeks after receipt of the tissue in the laboratory versus 2-4 weeks for results after amniocentesis (see 1.4.4.3.). As the technique is performed earlier than amniocentesis and the waiting time for cytogenetic results is reduced, CVS permits an early termination of the pregnancy if the fetus is affected.

The desire to diagnose a genetic defect as soon as possible during the pregnancy led to attempts for CVS to be performed earlier in pregnancy. The development of early CVS has been stopped, as there have been some reports of severe limb abnormalities in some babies whose mothers had undergone CVS earlier than 9 weeks (Firth *et al*, 1991).

1.4.2.4. Fetal blood and tissue sampling

Fetal blood can be obtained by puncture of the fetal heart (cardiocentesis), the liver (hepatocentesis) or the umbilical cord (cordocentesis) under fetoscopic or ultrasonographic guidance (also see 1.4.2.1.). Currently, the method used most is cordocentesis (Nicolini & Rodeck, 1992). Fetal blood sampling has been used succesfully for the prenatal diagnosis of haemoglobin disorders and various red cell enzyme defects. Although the application of molecular genetics techniques makes possible the identification of many of the haemoglobinopathies in the first trimester of pregnancy by CVS and DNA analysis, in many cases fetal blood sampling is necessary for the cases which are not informative for the available probes, for confirmation of the diagnosis when it is based on linked probes or because of late clinical referral (Nicolaides & Snijders, 1992). The major disadvantage is the small amount of fetal blood that can be obtained, which makes it necessary to develop microtechniques for the various measurements that are required. Fetal blood sampling is associated with a fetal mortality of between 2 and 5 % (Nicolini & Rodeck, 1992).

Although the use of fetal blood sampling is directly useful for diagnosis of genetic blood diseases and conditions in which there are altered levels of metabolites in the blood, fetal blood sampling is also used for karyotype analysis. Karyotyping by cytogenetic analysis of blood lymphocytes can be available within 3 days from the day of sampling, compared with 1-4 weeks for amniotic fluid (Gosden *et al*, 1985) (see 1.4.4.3.). The rapid karyotyping reduces the time of parental anxiety in cases of

increased risk for a chromosomal abnormality (due to abnormal maternal serum biochemistry or ultrasound diagnosis of fetal malformation), failed amniocentesis culture, or to distinguish between true- and pseudo-mosaicism and late clinical referral (Nicolaides & Snijders, 1992).

Some enzymes are expressed only in the liver. Prenatal diagnosis by an enzyme assay is only possible after liver biopsy, which is performed under fetoscopic or ultrasonographic guidance. Fetal liver biopsy and analysis enabled prenatal diagnosis of some rare diseases, such as ornithine transcarbamylase deficiency (OTC) (Rodeck *et al*, 1982). Today the need for fetal liver biopsy is reduced as the availability of DNA analysis is increasing.

If a genetic disorder results in an abnormality which can be diagnosed histologically in a tissue (like skin), then diagnosis is possible after tissue biopsy, even when the molecular or the biochemical basis of the disease is unknown. For definite diagnosis it is required that the abnormality is detectable at the histological level at the time of sampling. An example is epidermolysis bullosa fetalis, which can be diagnosed after histological examination of skin that has been biopsied at 16-17 weeks (Nicolini & Rodeck, 1992).

1.4.2.5. Fetal cells in maternal blood.

It is known that small amounts of fetal cells circulate in the maternal blood. Detection and analysis of these cells would provide an alternative to invasive diagnostic procedures. Isolation of fetal cells was facilitated with the use of the fluorescence-activated cell sorter (FACS). Up to now, a series of studies has been published that analyse fetal cells using cytogenetic techniques, FISH and PCR in sorted and unsorted blood samples (Bianchi *et al,* 1992; Lo *et al*, 1989; 1993). Although successful results have been reported, many issues require further investigation (such as the persistence of fetal cells from a previous pregnancy in the maternal blood and

maternal contamination of the sample) before considering the method to be applied for the prenatal diagnosis of genetic diseases (for reviews see Adinolfi, 1992).

1.4.3. Risks associated with current methods

The main problem with fetoscopy, amniocentesis, and fetal blood sampling is that they cannot be performed until the second trimester. Since the analytical methods take at least a week, a decision to terminate the pregnancy cannot be made until 20 weeks gestation, resulting in a difficult termination of pregnancy and causing a great degree of maternal emotional trauma.

Although CVS has several advantages over amniocentesis, some studies show CVS to be potentially less safe. There have been reports of congenital abnormalities in infants after CVS, especially severe limb abnormalities and limb reductions (Firth *et al*, 1991, Burton *et al*, 1992). In most of these cases, CVS has been performed before 9 weeks of gestation (Rodeck, 1993). The rate of abnormalities increases 20-30-fold if the sampling is performed before the 9th week of pregnancy than after the 10th (Rodeck, 1993). The rate of limb abnormalities following CVS performed after the 10 week of pregnancy, occur in a similar rate as in the cases that limb abnormalities happen spontaneously (6 and 5.4, respectively, in 10,000 births). Despite those isolated reports, CVS is a safe procedure in skilled hands, as centres with a large experience have not seen similar abnormalities (Mahoney *et al,* 1991; Rodeck, 1993).

In comparison to amniocentesis, the accuracy of the cytogenetic results is lower after CVS (see 1.4.4.3.) despite adoption of methods ensuring the accuracy of their results, including the use of both direct and cultured preparations. The main reason for the reduced accuracy of CVS is mosaicism, which may arise as a culture artefact, or because the karyotypes of placental cells are different from the fetus (confined placental anomalies) (Kalousek and Dill, 1983; Kalousek *et al* ,1987;

Kalousek *et al*, 1989;). There also seems to be an increased risk of maternal contamination on the CVS specimens (Canadian Collaborative CVS-Amniocentesis Clinical Trial Group, 1989). A small proportion of women (4% in the Canadian Collaborative CVS-Amniocentesis Clinical Trial Group, 1989) require a second test to confirm the cytogenetic result or risk termination of a normal pregnancy following an ambiguous first CVS result.

1.4.4. Detection of genetic defects

For the identification of abnormalities in the fetus, there are two basic approaches: visualisation and analysis of fetal tissues. Visualisation can provide information about anatomical abnormalities. Analysis of fetal tissue may provide information about a genetic disease at the chromosome or gene level and certain biochemical defects. Usually the disorder for which the fetus is at risk for, dictates the type of tissue that should be biopsied and the safest timing and technique for the sampling (Charrow *et al,* 1990).

1.4.4.1. Morphology of the fetus

Ultrasound has been used to ensure the safety of invasive procedures, such as amniocentesis or CVS and to screen for fetal structural anomalies. Those anomalies might be serious enough to cause a problem, but they also might be markers for a chromosomal defect. Although the sensitivity of ultrasonography alone as a screening tool remains to be evaluated, it was shown that anatomical abnormalities detected by ultrasound increase the probability of a chromosomal abnormality (Benacerraf *et al*, 1985; Nicolaides *et al,* 1991).

The main advantage of the technique is that it is a non-invasive method. However, it is very much operator-dependent and can be unreliable under many

circumstances, unless performed by highly trained staff. Ultrasound in combination with invasive techniques is used as a primary tool to aid tissue sampling (Charrow *et al,* 1990).

1.4.4.2. Fetoscopy

Fetoscopy is a method for direct visualisation of the fetus using an endoscope which is inserted into the amniotic cavity.The method has been used for direct visualisation of the fetus to detect morphological abnormalities and also to facilitate blood sampling and transfusion, fetal skin and liver biopsies, as well as corrective therapy. However recent improvements in imaging by ultrasonography have made fetoscopic guidance almost unnecessary and most biopsy procedures of fetal tissue can be performed under sonographical imaging (Nicolaides & Snijders, 1992).

1.4.4.3. Karyotype analysis

The risk of cytogenetic abnormalities is the most frequent indication for prenatal diagnosis and approximately 90% of all amniocenteses are performed for cytogenetic analysis. The main indications are advanced maternal age, parental chromosome abnormality, previous child with cytogenetic abnormality, microdeletion syndromes and chromosomal breakage syndromes (Charrow *et al,* 1990).

The most common sources of cells for karyotype analysis include amniocytes, peripheral blood leukocytes and chorionic villi. Immediate use of uncultured amniocytes avoids cell culture, with all the associated problems of potential contamination, slow growth of the cells, infection, cost and most importantly, time. Direct preparations of amniotic fluid cells have been used for the detection of biochemical abnormalities, DNA analysis and rapid fetal sexing using X- and Y-chromatin. However, cell culture has the advantages that it separates the viable amniotic

fluid cells from dead amniotic fluid cells (which can account for 80% of the cells) and which may release proteolytic enzymes, DNAses, etc, which might degrade the viable cells and cause diagnostic problems. Also with cell culture the viable amniotic fluid cells are separated from maternal cells, which will be lost on repeated subculture. The cells obtained after amniocentesis can be cultured and after 2-4 weeks usually enough cells are present to permit reliable karyotyping. Usually a minimum of 15 cells, arrested in metaphase, are examined for the chromosome number and a smaller number are stained to reveal the characteristic banding patterns to detect structural rearrangements. One drawback with amniocytes culture is that cell cycles cannot be synchronised, and the percentage of cells in mitosis is small. Therefore, large numbers of cells are required for analysis and this is one reason why long periods of culture are required. Abnormalities of chromosome number and structure can be determined with accuracy in excess of 99%. The major problems is pseudomosaicism, which occurs in 2-3% of cell cultures and results from maternal contamination. To distinguish between true mosaicism and pseudomosaicism, a second amniocentesis or fetal blood sampling is required for confirmation of the result (Nicolaides & Snjiders, 1992)

CVS allows more rapid cytogenetic analysis due to the higher mitotic activity of trophoblast cells compared to the amniocytes. The obtained chorion is tissue which grows rapidly and cytogenetic results can be obtained from spontaneous mitoses in direct preparations or after short term culture for 12-24 h. However, the cells are cultured further for up to one week, as false results can be reported. The main reasons for these are confined placental anomalies (when the karyotype of placenta cells are different from the fetus) and pseudomosaicism. Both these problems arise after amniocyte culture, but they occur more frequently after CVS (Gosden, 1990).

Peripheral blood leukocytes is a common source of cells for cytogenetic studies. Although occasional mitotic divisions are observed in blood samples, the cells are selectively stimulated with mitogens to proliferate so that a large number can be obtained for analysis. Results after karyotyping from fetal blood cells can be obtained

as early as 2 days. Also, as fetal blood is a true fetal tissue, the results may be very helpful in cases of mosaicism in culture after CVS (Gosden, 1990).

1.4.4.4. Biochemical analysis in maternal serum

Alterations in levels of maternal serum proteins (e.g. alphafetoprotein) has been associated with genetic abnormalities, which include neural tube defects (NTDs) and chromosomal anomalies, such as Down's syndrome. Although analysis of maternal serum proteins is a screening and not a diagnostic procedure, the ability to place low risk patients (based on age) in a high risk group (which could require further investigation) has major implications for prenatal diagnosis (Charrow *et al*, 1990).

1.4.4.5. Enzyme analysis

When a genetic disease results in defective production or function of an enzyme or protein, it can be diagnosed prenatally if a specific test has been developed. About 100 autosomal recessive and X-linked enzyme deficiencies may be diagnosed prenatally by assaying enzyme activity from cells derived from amniocentesis or CVS (Ben-Yosef, 1992) (see also 4.1.1.). These include Lesch Nyhan syndrome, disorders of lysosomal enzymes etc. An important consideration for the biochemical detection of a genetic disease is that the enzyme in question has to be expressed in amniotic fluid cells, as the enzymes responsible for some biochemical disorders, for example phenylketonouria and most urea defects, are normally expressed only in liver and prenatal diagnosis for these can not be performed on amniotic fluid cells (Charrow *et al.*, 1990)

1.4.4.6. Linkage analysis

In cases where the approximate location of the gene is known but the mutation has not been identified, linkage analysis has been used as an aid for prenatal

diagnosis of genetic disease. In the human genome there are polymorphisms (small genetic differences between individuals) that can be detected by the presence or absence of a restriction enzyme recognition site (restriction fragment length polymorphisms or RFLPs) or the variation in the number of tandemly repeated sequences in a specific locus (variable number of tandem repeats or VNTRs). If in a family or a population, a polymorphism is closely linked with a gene which causes a disease, it is possible to study inheritance patterns without characterisation of the gene itself. The closer the polymorphism is to the mutated gene, the fewer the possibilities of a recombination to occur between the two loci at meiosis and the greater the chance to be inherited together (Caskey & Rossiter, 1992). Linkage analysis is being used for prenatal diagnosis, but there is a tendency for this to be abandoned as soon the mutated gene is known and characterised, as in the case of CF.

1.4.4.7. Detection of mutations

Until the availability of recombinant DNA technology, prenatal diagnosis for single gene defects was possible only for a limited number of these disorders, in which the deficient gene product was known to be expressed in a tissue which could be obtained from the fetus without great risk. The use of recombinant DNA technology revolutionised the diagnosis of genetic disorders, as it does not rely on the expression of the gene product and makes it possible to diagnose at the DNA level any inherited disease for which the molecular basis is known.

A variety of methods have been used for identifying abnormalities in specific genes. Mutations that affect only a single gene can be detected by the success or failure of a probe to hybridize to a DNA fragment or the appearance of an abnormal sized fragment after restriction digestion. The main application of recombinant DNA technology in prenatal diagnosis has been, until recently, restriction mapping followed by Southern blotting. The technique has been used to identify mutations that range from

deletions of several hundred bases to single base mutations that alter a restriction site. For mutations that do not alter a restriction site, closely linked restriction fragment length polymorphisms (RFLPs) have been used instead or oligonucleotide probes that are prevented from hybridization to a specific region when a mismatch exists. Also there are many genetic diseases for which the location and sequence of the abnormal gene are not known, or which are very heterogeneous in their molecular basis. For these diseases, diagnosis is possible indirectly, by combining DNA hybridization methods with linked markers pedigree analysis (see 1.4.5.6.). Recently it has become possible to prepare large amounts of a specific region of DNA, for example to isolate a fragment of a gene in large quantities, by the Polymerase Chain Reaction (PCR) (see 1.5.). (For methods that are used for mutation detection see 1.5.2.)

1.5. DNA amplification for detection of genetic defects

1.5.1. Principle

The Polymerase Chain Reaction (PCR) is an in vitro method for producing large amounts of a specific DNA fragment of defined length and sequence from small amounts of a complex template (Saiki *et al*, 1985). PCR is based on the enzymatic amplification of a DNA sequence that is flanked by two oligonucleotide primers that hybridize to opposite strands. The primers are oriented with their 3' ends pointing towards each other. Repetitive cycles of heat denaturation of the template, annealing of the primers to their complementary sequences, and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. Since the extension product of each primer can serve as a template for the other primer, each cycle essentially doubles the amount of DNA fragment produced in the previous cycle, resulting in the exponential accumulation of the specific target fragment. Several millionfold amplification is achieved in just a few hours. The method can be used with a complex template such as genomic DNA and can amplify a single-copy gene contained therein. Crude cell lysates can be amplified without the need for DNA purification. PCR is also capable of amplifying a single molecule of target on a complex mixture of DNAs or RNAs (Saiki *et al,* 1988) and can, under appropriate conditions, produce fragments up to 10 kbs long (Jeffreys *et al,* 1990). PCR has made possible new approaches in all areas of molecular biology. Using PCR, prenatal diagnosis can take less than one day compared with weeks using Southern blotting and hybridization approaches. PCR was initially applied for the prenatal diagnosis of sickle cell anaemia; samples containing as few as 75 cells were used for the amplification of a 110-bp fragment of the human β–globin gene spanning the polymorphic Dde I restriction site (Saiki *et al,* 1985).

1.5.2. Detection methods

Mutations involve as few as one single base change and as many as thousands of bases of DNA. They can be identified by physical, enzymatic or chemical methods. Methods for mutation detection involve i) techniques that screen for previously unknown mutations in DNA fragments up to a few kilobases and ii) techniques that identify known mutations. In the first group are techniques that detect the effect of a mutation on the stability of the double-stranded DNA under denaturing conditions (Myers *et al,* 1985), the susceptibility to RNAse cleavage of an RNA probe and a target strand (Chang & Lu, 1991), the susceptibility to chemical modification of a mismatch in the double-stranded DNA (Naylor *et al,* 1991), the effects on gel migration by mutations, as heteroduplexes that contain internal mismatches can be separated from correctly matched molecules by electrophoresis under certain conditions (Keen *et al,*, 1991) and by sequencing of a fragment of DNA of limited length (Hunkapiller *et al,*, 1991). In the second group belong techniques that can identify known mutations. A large number of methods are available: short hybridization probes can show a difference in their hybridization stability to matched and mismatched alleles (allele-specific oligonucleotide hybridization or ASO) (Saiki *et al,*, 1989), analysis of restriction site (Landergren *et al*, 1988), allele-specific priming for PCR (Kwok *et al,* 1990), mini-sequencing (Syvanen *et al*, 1990), oligonucleotide ligation assay (Landergren *et al*, 1988). As amplification techniques are being investigated, new techniques for the rapid and accurate analysis of mutations are being developed (Table 1.2.).

Table 1.2.: Detection of defects in PCR products

DIRECT VISUALISATION

Presence or absence of amplified fragment

Fragment length difference

Presence of a restriction site

Heteroduplex detection

RNAs or chemical cleavage of mismatched heteroduplexes

LABELLING (RADIOACTIVELY OR ENZYMATICALY)

1. PROBE

Allele-specific oligonucleotide hybridization

reverse-oligonucleotide dot-blot hybridization

RNAs or chemical cleavage of mismatched heteroduplexes

2. dNTPs

Incorporation of biotinylated or labelled nucleotides into the amplified fragment

Fluorescence PCR amplification / detection

allelic-specific PCR using nested PCR

3. PRIMERS

Amplification of specific alleles

Site-specific labelled nucleotides

Competitive binding of labelled primers

1.5.3. Nested amplification

After a first round of amplification, a second set of primers that are located within the first set can be used to reamplify an aliquot of the product of the first reaction (nested amplification). This procedure can enhance the sensitivity and specificity of PCR when the amount of starting material is very small (Li *et al*, 1988; Holding & Monk, 1989) or when the first round of amplification results in non-specific as well as specific products (Mullis & Faloona, 1987).

1.5.4. Multiplex PCR

It has been possible to amplify several DNA loci simultaneously (co-amplification or multiplex PCR). This can facilitate the diagnosis of a genetic disease, as it can provide information for many loci at the same time. Among several examples, the amplification of nine regions of the DMD gene in one reaction has been reported (Chamberlain *et al*, 1988). This results in one assay that can detect 81% of all the DMD deletions. Co-amplification has also been used as an internal control for amplification failure (Jeffreys *et al*, 1988). Multiplex PCR is a sophisticated technique that requires reaction conditions that permit equal amplification of all fragments, careful design of the primers so that artefacts formed between the primers and non-specific amplification are avoided, and generation of products of different size to facilitate analysis.

1.6. Preimplantation diagnosis of inherited disease

1.6.1. General introduction

Over the last 15 years methods of ovarian stimulation, oocyte collection, IVF, embryo culture and transfer have been established for the treatment of infertility. The introduction of ovarian stimulation allows several oocytes to be produced per treatment cycle, increasing the chance of pregnancy (Jones *et al*, 1984; Trounson *et al*, 1981). Furthermore, ultrasound guided techniques permit effective monitoring of the follicular growth and the formation of the corpus luteum (Hackelier *et al*, 1979). More recently ultrasound techniques have been applied to the aspiration of oocytes from follicles, minimising the trauma of egg collection procedures, which can be performed on an outpatient basis (Wren & Parsons, 1989).

If genetic defects can be identified during early stages of embryonic development before implantation occurs i.e. Preimplantation diagnosis, then embryos that are shown to be unaffected by the disease could be selected for transfer, eliminating the need for a termination. In general the diagnosis of genetic defects requires one or more cells to be biopsied from the embryo. Several methods have been developed to biopsy the preimplantation embryo.

1.6.2. Polar body biopsy

Preconception diagnosis is possible even before fertilization by analysing DNA sequences or chromosomes in the first polar body accompanying the oocyte (Preconception diagnosis) (Strom *et al,* 1990; Monk & Holding, 1990). If the

polar body from a woman heterozygous for a mutant gene causing a disease is found to be homozygous for one allele, then it can be assumed that the oocyte is homozygous for the other allele, and that crossing over did not occur. If crossing over occurs, the first polar body will be heterozygous with copies for both alleles and the genotype of the oocyte can not be predicted. Preimplantation diagnosis by polar body biopsy has been attempted for the diagnosis of a_1 anti-trypsin deficiency (Verlinsky *et al*, 1990). Polar bodies were removed from seven oocytes and have been amplified by PCR and products hybridized with allele-specific oligonucleotide hybridization (or ASO) (Saiki *et al*,, 1989) for the normal and the mutant alleles. The genotype was determined by the relative intensity of hybridization with the two allele-specific oligonucleotides. Amplification was successful in 5/7 cases. The analysis of two polar bodies showed no hybridization with either of the probes, possibly due to amplification failure. The diagnosis was confirmed in one case by analysis of an oocyte which failed to fertilize.

Polar body biopsy does not involve removal of embryonic material, however, the effects on development of such pre-fertilization manipulations are unknown. The main disadvantages of this approach are that the detection only of maternal defects is possible, the test might not be efficient for telomeric loci, as the possibility of a crossing-over is high resulting in heterozygosity in the polar body and also sex identification is not possible.

1.6.3. Methods of embryo biopsy

Preimplantation diagnosis requires the removal of one or a small number of cells from the embryo for analysis. Biopsy techniques have been developed at cleavage or blastocyst stages.

1.6.3.1. Blastocyst biopsy

In the original study of Gardner & Edwards (1968), rabbit embryos were biopsied at the blastocyst stage by trophectoderm cell removal. Blastocysts can be obtained either by IVF or, in fertile women, could be collected from the uterine cavity after in vivo fertilization by transcervical uterine flushing (uterine lavage). This technique has been used after artificial insemination to collect preimplantation embryos from egg donors, which are then transferred to recipient mothers with synchronised cycles (Buster *et al*, 1983; 1985; Formigli *et al*, 1987). There are several advantages in this method; at the blastocyst stage the embryo has the maximum number of cells before implantation and the greater the number of biopsied cells for analysis, the more reliable the diagnosis is likely to be. The total number of cells in human blastocysts in vitro increases from an average of 58 to 126 cells between days 5 and 7 post insemination (Hardy *et al,* 1989). The blastocyst consists of inner cell mass cells (ICM) and trophectoderm cells (TE). By making a slit in the zona pellucida the day before the biopsy, TE cells are allowed to herniate out (Dokras *et al*, 1990). The next day the herniated TE cells can be excised. From 47 human blastocysts in which biopsy was attempted, it was successful in about half of the cases and an average of 10-30 TE cells were recovered (Dokras *et al*, 1990).

Another important advantage is that TE cells contribute only to placental tissues after implantation. The fetus is derived exclusively from the ICM. Therefore removal of some of these cells should not affect the normal development of the fetus.

Also if preimplantation embryos are obtained with uterine lavage, they will be exposed to in vitro conditions for a minimal period. As IVF culture conditions are believed to be suboptimal, viability of flushed embryos will be less affected. Finally, it was suggested that those embryos which have developed to the blastocyst stage at lavage will have 'selected' themselves, so that preimplantation diagnosis will be performed on embryos with the highest chance of implantation (Bolton *et al*, 1990).

However, the rate of recovery of embryos at this stage of development is very poor, suggesting that uterine lavage following normal conception may not be the optimal source of embryos for biopsy. Ovarian hyperstimulation prior to lavage was suggested as an approach to increase the number of embryos recovered from a single attempt. However, preliminary results with uterine lavage in hyperstimulated cycles have been disappointing (Sauer *et al*, 1989). Also, after uterine lavage was used as a source of embryos for biopsy, retained pregnancies and infections have been observed (Buster *et al*, 1983; Formigli *et al*, 1987), and as fluid may be lost in the tubes during lavage, the theoretical risk of salpingitis and ectopic pregnancy exists.

Another major problem with biopsy at the blastocyst stage is the low pregnancy success rate following IVF and transfer at this stage. A few pregnancies have been reported after transfer of morulae and early blastocysts on day 5 (Bolton *et al*, 1991). No pregnancies were obtained following transfer of blastocysts on day 6 and 7 (Dawson *et al*, 1988). Recent work of Bolton *et al* (1992) shows that pregnancy rates after blastocyst transfers are similar to the rates after transfers on day 2. However, only a variable proportion (about 50%) of embryos reach the blastocyst stage in vitro (Hardy *et al*, 1989) which is likely to reduce the number of embryos identified as suitable for transfer and therefore reduce the chance of establishing a pregnancy. Also, the effect of removing a proportion of the TE remains to be studied, as there is the theoretical risk that it may affect implantation and the maintenance of the early pregnancy. Therefore, although there are fewer cells at earlier stages, the only practical

alternative at the present time is to biopsy cleavage stage embryos, so that there is enough time for the transfers to be carried out on day 3, as in routine IVF.

1.6.3.2. Cleavage stage biopsy

At early cleavage stages, each cell of the mammalian embryo remains totipotent and can contribute to all the tissues of the conceptus (Papaioannou & Ebert, 1987). In a number of rodent and domestic species, single cells from embryos at these stages have been demonstrated to form blastocysts and to develop into normal offspring following transfer (Papaioannou & Ebert, 1986). The effect on human embryos biopsied at the 8-cell stage was examined in a series of experiments by Hardy *et al* (1990), by assessing the proportion developing to the blastocyst stage, and the uptake of the energy substrates, pyruvate and glucose on successive days from the day of biopsy on day 3 to day 7. The proportion of normally fertilized human embryos developing to the blastocyst stage varies widely between patients, but averages are between 40 and 70% (Hardy *et al*, 1989). After biopsy of one or two cells, the proportion developing to this stage (79 and 71% respectively) was relatively high and even exceeded that of unmanipulated control embryos from the same patients (59%). Also, many biopsied embryos hatched from the zona pellucida. The analysis of cell numbers showed that total cell numbers were reduced in both 7/8 (1 cell biopsy) and 6/8 blastocysts (2 cell biopsy), especially on day 6, but only in proportion to the reduced cellular mass, demonstrating that the cleavage rate had not been affected. More importantly, since the fetus is derived from the ICM after implantation, the numbers of these cells as a proportion of the total was maintained.

Finally, viability of the embryos after biopsy was assessed by measuring the uptake of energy substrates. The uptake of glucose and pyruvate was reduced in both 7/8 and 6/8 blastocysts throughout their development after biopsy, but again only in proportion to the reduction in cellular mass. They also showed the

characteristic switch in substrate preference from pyruvate to glucose (Hardy *et al*, 1989). There was no indication of an additional effect on the viability of the embryos.

Tarin *et al* (1992) studied the effect of biopsy at the 4-cell stage (second day after insemination). They found that biopsy did not affect the proportion of embryos developing to blastocyst or the pyruvate uptake. However, the number of ICM and trophectoderm cells and the ratio of ICM:trophectoderm was reduced at the blastocyst stage in the biopsied group.

The advantages of cleavage stage biopsy include that preimplantation development of human embryos to the blastocyst stage *in vitro* does not appear to be adversely affected by biopsy at the 6- to 10- cell stage and that embryo transfer during cleavage stages is routine in therapeutic IVF. However, cleavage stage biopsy has certain disadvantages. Experiments in rodents and domestic species indicate that there is lower limit to the embryonic cellular mass compatible with implantation and development (Papaioannou & Ebert, 1986). The implications for human embryos are that the reduction of cellular mass should be minimised to avoid an increase in biochemical pregnancies and implantation failure. Also 40-60 % of cleavage stage embryos will undergo developmental arrest, so that the chance of implantation of a biopsied embryo might be limited.

1.6.4. Methods for preimplantation diagnosis of genetic diseases

The small number of cells in human embryos at these stages and restrictions on the numbers that can be biopsied without seriously affecting the viability and development of the embryos, limits the number of techniques that can be used for preimplantation diagnosis. Despite that, a number of methods are suitable and (in other species than the human) some have already been used successfully.

1.6.4.1. History

The possibility of preventing X-linked recessive diseases by identifying the sex of the embryo was initially suggested by Edwards (1965). Gardner & Edwards (1968) first achieved sex detection in rabbit preimplantation embryos by using micromanipulation to excise trophectoderm cells and examining the nuclei for sex chromatin which is detectable in the majority of female cells with an inactive X chromosome at this stage. The embryos were then transferred to recipient females and the sex confirmed later in gestation or at term.

More recently, because of the economic advantages, most work in this area has been in farm animals including the successful sexing of bovine embryos by chromosome analysis (Hare *et al*, 1976; Singh & Hare, 1980; Wintenberger-Torres & Popescu, 1980; Picard *et al*, 1985) or Y chromosome-specific DNA probes (Leonard *et al*, 1987; Matthews *et al*, 1987).

The first successful preimplantation diagnosis of genetically defective embryos was in a mouse model for Lesch-Nyhan syndrome (Monk *et al*, 1987; Monk & Handyside,1988; Hooper *et al*, 1987), using a sensitive enzyme microassay. Using the same microassay, the relative activity of X-linked HPRT activity and an autosomal enzyme was measured and this permitted sexing of biopsied embryos (Monk &

Handyside, 1988) using the 2:1 difference in the amount of X-linked gene products before X-inactivation occurs in female preimplantation embryos.

1.6.4.2. Non- invasive methods for preimplantation diagnosis

A potential approach for preimplantation diagnosis involves non-invasive techniques, in which the medium in which the embryo is growing is assessed for its contents of certain substances, which might be of some diagnostic value. Specific compounds might be released from the embryos into the medium and there are few reports, e.g. chorionic gonadotrophin (Fishel *et al*, 1984), early pregnancy factor and histamine releasing factor (Cocchiara *et al*, 1987), though none of these are relevant to inherited diseases.

The uptake of pyruvate and glucose from single mouse embryos was shown to have a correlation with their viability after transfer at the blastocyst stage (Gardner & Leese, 1986; 1987). In similar studies Leese *et al* (1986) and Hardy *et al* (1989), have measured the uptake of pyruvate and glucose at various intervals during the preimplantation development of human oocytes and embryos. Initial results suggested that non-invasive measurement of pyruvate uptake before embryo transfer may provide a functional criterion for the selection of embryos capable of developing to the blastocyst stage. However, a recent study by the same team in a large number of embryos showed that non-invasive measurement of pyruvate uptake alone is not an adequate criterion for selection of the embryos that are transferred and implanted.

The obvious advantage of a non-invasive approach is that embryos are not biopsied and the potential hazards of their future development caused by micromanipulation are minimal. However, only a few preliminary studies have investigated the release of factors synthesised by preimplantation embryos in vitro. Also sensitive assays have to be developed, to overcome the technical difficulties of detecting small amounts of molecules released in the culture media. Even when there is

evidence that these molecules are synthesised at an early stage of development, it has to be determined if they are coded by the embryonic or the maternal genome. Also, there exists the possibility that many diagnostically important proteins will be produced after the development of specific organs, making the identification of these proteins before implantation impossible.

1.6.4.3. Enzyme activity measurements

Preimplantation diagnosis of a disease by enzyme activity measurements is possible when the biochemical defect is known at the protein or enzyme level and requires: i) that the gene in question is transcribed by the embryonic genome, translated and that the level of the gene product inherited in the maternal cytoplasm of the oocyte is sufficiently low for the activity of the embryonic product to be detected above this background, ii) that a sensitive biochemical microassay is available which would detect the presence or absence of the gene product in the single cell. Today, microassays capable of measuring the activity of several enzymes involved in inherited syndromes at the single cell level are available (Monk, 1987; Benson & Monk, 1988; Leese *et al*, 1991).

The first successful preimplantation diagnosis of genetically defective embryos has been established in a mouse model for Lesch-Nyhan syndrome (Monk *et al,* 1987; Monk & Handyside,1988; Hooper *et al*, 1987). Lesch-Nyhan syndrome is caused by a deficiency of the X-linked enzyme hypoxanthine phosphoribosyl-transferase (HPRT). This condition is characterised by mental retardation and severe neurological symptoms including spastic movements and self-mutilation. Affected boys do not survive beyond their early teens (Lesch & Nyhan, 1964). Using a sensitive enzyme microassay, Monk *et al* (1987) detected the presence or absence of enzyme activity in single cells removed from embryos at the 8-cell stage while the embryos remained in culture. Normal and HPRT-deficient embryos were then transferred to

separate foster mothers and the diagnoses were confirmed by measuring enzyme activity in the resulting fetuses later in gestation. However, in the mouse it has been established that embryo-coded enzyme predominates over maternally-coded enzyme inherited from the oocyte at these stages (Monk & Harper, 1978; Monk, 1987).

In contrast, the levels of HPRT activity measured in individual human oocytes and embryos, using the same microassay as in the mouse, were low and variable at all stages (Braude *et al*, 1990). Using a more sensitive HPLC microassay and higher substrate concentrations, however, higher levels in activity were measured (Leese *et al*, 1989), and an increase in activity was observed between days 2 and 4. That increase in HPRT activity could have resulted from the synthesis of embryo-coded enzyme which would allow the diagnosis of enzyme deficiencies at that stage. Although the levels of enzyme activity in different embryos and in embryos from different patients were variable, enzyme deficiencies may be detectable if the maternal enzyme activity at these stage is sufficiently low. For the present time, however, preimplantation diagnosis by enzyme activity measurements cannot be attempted in human embryos, as very little is known about human gene expression during preimplantation development.

1.6.4.4. Karyotype analysis

Karyotype analysis for chromosomal abnormalities is a well-established diagnostic method, usually performed on chorionic villus and amniocentesis samples. Karyotype analysis of amniocytes and CVS cells generally involves analysis of 15 to 30 banded metaphase chromosome spreads which are selected for optimal quality. Angel *et al* (1986) examined metaphase chromosome spreads of nuclei from human 8-cell embryos derived by IVF. The method requires staining of the chromosomes to reveal the banding patterns that are characteristic for each chromosome pair. This has proved universally very difficult with preimplantation embryos, mainly because the

chromosomes are short and often clumped. Therefore, information about chromosome defects has been limited to analysing abnormalities of chromosome numbers and gross genetic defects such as chromosome fragmentation. To examine the feasibility of karyotype analysis in single cells, cleavage stage embryos were disaggregated and the chromosome spreads studied (Handyside & Delhanty, 1993). The quality of preparations was variable and in most cases poor spreading or fixation did not allow exact analysis. Chromosomes from some embryos appear to be in a triploid or haploid range and chromosome breaks were seen in many cells. Therefore, it is unlikely that preimplantation identification of sex by karyotype analysis from a single metaphase would be reliable, mainly because of poor quality chromosome preparations or loss of chromosomes during spreading.

Another way to identify the sex of a biopsied embryo by karyotyping is by the number of small acrocentric chromosomes, a group which includes the Y chromosome. Females have four chromosomes in this group, males have five. However, the method is not very efficient as the heterochromatic regions of other chromosomes are also labelled to a lesser extent, which makes the analysis difficult (Handyside & Delhanty, 1993).

1.6.4.5. In situ hybridization

In situ hybridization (ISH) of Y-specific probes has been used on biopsied trophectoderm cells to sex cattle embryos, and the predicted sex verified by karyotype analysis of the remainder of the embryo. In situ hybridization techniques with a human Y-specific radiolabelled probe have also been used to sex whole human embryos (West *et al,* 1987; Penketh *et al*, 1989). However, detection of hybridized probe by autoradiography takes several days. Detection by a non-isotopic method using biotinylated Y-specific probe and a streptavidin-linked alkaline phosphatase detection

system was achieved in less than a day (Penketh *et al*, 1989), but the efficiency of hybridization with human embryo nuclei was low.

In situ hybridization (ISH) with radiolabelled or biotinylated probes was successful in sexing intact human embryos or cells excised from them. West *et al* (1987) identified the sex of preimplantation embryos by ISH. Similar studies using different Y-specific DNA probes showed that it would be feasible to sex embryos using this method. Such methods though have the disadvantage of needing 24 hours even with biotinylated probes (Jones *et al*, 1987; West *et al*, 1987; Penketh *et al*, 1989). Fluorescent methods for detecting ISH can also be carried out within a day and have the added advantages of increased specificity and sensitivity, and simultaneous detection of several probes is possible using different fluorochromes (Nederlof *et al*, 1989).

However, the main barriers that have prevented in the past the use of non-isotopic ISH or FISH, were the incidence of embryonic nuclei that fail to hybridise with either of the probes and the time involved, since 12 hrs was needed to arrest the majority of cells in metaphase, if required, and 24 hrs for detection of hybridization. However, recent methods have been described that overcame these problems and require less than a day to give a result (they are discussed at Chapter 7).

1.6.4.6. Polymerase Chain Reaction

In 1985, Saiki *et al* described a procedure which allowed the amplification of specific DNA sequences *in vitro* using DNA polymerase and short oligonucleotide primers flanking the DNA region of interest polymerase chain reaction (PCR). Repeated cycles of enzymatic amplification were found to increase the quantity of the target DNA fragment several hundred thousand fold.

For preimplantation diagnosis, PCR has two major advantages: i) sensitivity, which makes it possible to detect a single copy sequence in a single or a few

cells. A variety of sequences have been amplified from single cells, including human sperm, fibroblasts, human unfertilized oocytes and polar bodies (Li *et al*, 1988; Jeffreys *et al*, 1988; Holding & Monk, 1989; Monk & Holding, 1990). ii) speed, that permits specific DNA fragments to be amplified within a few hours (Saiki *et al,* 1985; 1988) so that analysis can be completed within the time available for transfer later the same day.

1.7. X-linked disease

1.7.1. Introduction

Over 300 X-linked diseases, the majority of which are recessive and typically affect males, have been described. These diseases are transmitted within families either by healthy female carriers, if the disease is serious, leads to early death of the affected male (such as DMD and Lesch-Nyhan syndrome), or by both female carriers and affected males, if the disease does not significantly affect fertility and allows survival into reproductive age (such as Becker muscular dystrophy).

1.7.2. Approaches to prenatal diagnosis

Many X-linked diseases have been characterised at the molecular level and detection of the specific defect by DNA analysis is feasible prenatally. Also, when the underlying biochemical defect is known, prenatal diagnosis is possible by a biochemical assay. Many of these conditions are due to heterogeneous new mutations (Caskey, 1986). In severe X-linked disorders such as DMD, in which affected males die before reproducing, the disease would be eliminated if the lost genes were not replaced by new mutations. Approximately one-third of all cases of DMD result from a new mutation, and the potential sites of the mutations span several hundred kbs of the locus. In those cases analysis by standard Southern blotting techniques is extremely difficult (Koenig *et al*, 1987).

Many X-linked diseases have not been fully characterised at the gene level, are very heterogeneous or biochemical diagnosis is not possible for a number of reasons. For these diseases, prenatal diagnosis is very restricted or impossible. For

couples at risk of an X-linked disease, identification of the sex of the embryo and selective abortion of a potentially affected male fetus is the only alternative to a specific diagnosis.

The routine method for the identification of sex prenatally is cytogenetic analysis of embryonic cells, to show whether an XX or XY karyotype is present. Karyotype analysis is a very reliable method for sexing, with the additional advantage that it examines the genotype of the fetus and can detect major and minor chromosomal anomalies. However several weeks are required for cell culture after amniocentesis, and this increases the duration of parental anxiety in cases where the couple are at risk of transmitting an X-linked disease.

Another classical method for distinguishing between male and female embryos is the identification of the Barr body. The Barr body represents the condensed inactive X-chromosomes in suitably stained female cell nuclei. Although this is a quick method for fetal sexing, it is not very reliable, as less than 30% of female nuclei show a typical Barr body.

On the Y-chromosome, a region on the long arm (Yq) can reveal a fluorescent pattern after quinacrine treatment. Fluorescent spots (F-bodies) can be seen in male nuclei, and this method have been used for quick prenatal diagnosis of sex. As with the Barr-bodies, this method is not reliable enough to be used routinely, as some men have Y-chromosomes that lack the fluorescent region (producing false-negative results) and also other fluorescent heterochromatic regions in autosomes can produce false-positives.

DNA sequences that are present on the sex chromosomes have also been used to distinguish between a male and female fetus. Fetal sexing after CVS using Y-specific probes has been achieved by Southern blotting (Gosden *et al,* 1982), by dot blotting (Gosden *et al,* 1984) and ISH (West *et al*, 1988) in 2-4 days. Kogan *et al* (1987) used PCR to diagnose haemophilia A and to detect the presence of Y sequences

in chorionic villi. Other sequences and methods for PCR have been described for prenatal sexing. The importance of the PCR-based methods are the speed and reliability.

1.7.3. Strategies to be used for preimplantation diagnosis

For couples at risk of transmitting X-linked diseases, identification and transfer of female preimplantation embryos avoids the implantation of a potentially affected male embryo. The number of techniques that can be used for preimplantation diagnosis are limited by i) the small number of cells that can be biopsied safely from a preimplantation embryo and ii) the limited time available for analysis of the sample (see also 3.1.3.).

If the genetic defect which causes the disease is X-linked, it typically affects males. In cases of non-characterisation of the gene, heterogeneity, new mutations or inability to use a biochemical assay, the only alternative is to identify the sex of the embryo and to transfer to the uterus female embryos that will not be affected by the disease. This approach has the advantage of being applicable to all X-linked recessive conditions.

For couples at risk of transmitting X-linked diseases, identification and transfer of female preimplantation embryos avoids the implantation of a potentially affected male embryo. Two different approaches to preimplantation diagnosis have been investigated; at the gene level, by DNA amplification and at the post-transcriptional level, by enzyme assay.

The amplification of a pericentromeric sequence (Y-alphoids) will be assessed to the single cell level. The efficiency and reliability of DNA amplification by PCR of the DYZ1 and the Y-alphoid sequences in single blastomeres will be investigated. To improve the rate of successful amplification, co-amplification of X- and Y-specific

sequences and improvement of blastomere lysis has been attempted. To reduce the percentage of amplification failure, different lysis procedures were compared. The consistency of amplification has been investigated to assess the reliability of preimplantation diagnosis. Preliminary results show that the reliability is high enough to permit clinical application. Preimplantation identification of sex will be attempted in couples at risk of transmitting X-linked diseases, after appropriate counselling for the novelty of the method.

To perform preimplantation diagnosis by enzyme activity measurements, it is essential to understand the timing of gene expression in early human development. As a general approach to examine early embryonic gene expression, the pattern of polypeptides synthesized by single human embryos cultured in the presence or absence of the transcriptional inhibitor α–amanitin, has been investigated by one-dimensional polyacrylamide gel electrophoresis. Also the quantitative aspects of protein synthesis will be studied by measuring the uptake and the incorporation of ^{35}S-methionine into protein. The profile of HPRT activity has been examined in normally fertilised human embryos developing *in vitro* between the morula stage on day 4 and blastocyst stage on day 6. In order to investigate the persistence of the maternal gene products, the effect of transcriptional inhibition on a group of embryos will be studied.

In order to sex by quantitative assays of X-linked products, the assay must be performed at a time when the residual oocyte-coded activity is insignificant compared to the embryo-coded activity, but before X inactivation. To study the timing of X-inactivation in humans, the profile of HPRT activity has been measured in male and female embryos. The lysed embryonic ghost will subsequently be sexed by PCR.

Chapter 2

Materials and Methods

2.1. Source of human preimplantation embryos

The unfertilized oocytes and preimplantation embryos used were donated from patients undergoing therapeutic *In Vitro* Fertilization (IVF). As the chance of establishing a pregnancy after IVF is increased if more than one embryo is transferred into the uterus (Edwards & Steptoe, 1983), regimes for superovulation have been developed to maximise the number of oocytes recovered in one IVF cycle. However with increased numbers of embryos replaced, the chance of multiple pregnancy and the risks associated with it also increase and for this reason, the embryos transferred into the uterus are limited to a maximum of three. As a consequence of the treatment, a number of surplus embryos are available and, with the consent of the couple and appropriate ethical approval, were used for research.

Before and during superovulation induction with human menopausal gonadotrophin (hMG), patients undergo pituitary desensitisation with a luteinising hormone releasing hormone (LHRH) agonist (Buserelin, Hoechst) (Rutherford *et al*, 1988). Superovulation was induced with hMG (Pergonal, Serono). The administration of hCG (Pregnyl, Organon) and the oocyte collection were timed according to endocrinological and ultrasound criteria.

Mature oocytes were collected using ultrasound visualisation of the ovaries, preincubated for 4-7 hours and inseminated (day 0) with a prepared sample of sperm. The cum ulus cells were removed by aspiration 15-18 hours later under a stereo dissecting microscope, to aid visualisation of the oocytes and two pronuclei, to confirm normal fertilization. The embryos were cultured in 1 ml Earles medium in 5 ml Falcon tubes (Becton Dickinson), containing 10% maternal serum at 37°C inside a Flow modular incubator chamber in 5% CO_2-in-air incubator. The module was flushed with 5% CO_2, 5% O_2 and 90% N_2 gas mixture and sealed during use. The atmosphere in the module was humidified by the presence of an open petri dish filled with distilled

water. On day 2, each embryo was examined and up to 2 or 3 normally cleaving embryos with optimal morphology were selected for transfer. Any remaining surplus embryos, after written confirmation of the couples consent were allowed to develop further in vitro in an 5% CO_2-in-air incubator until use, in the same 1 ml culture medium or in 20-50 µl drops of medium under pre-equilibrated paraffin oil (Light Liquid Paraffin Oil, BDH, 29436).

After approval of the ethics committee of the Royal Postgraduate Medical School, Hammersmith Hospital, and by the Voluntary Licensing Authority for Human In Vitro Fertilization and Embryology the patients were informed about the possibility of donating surplus embryos for studies aimed at diagnosis of genetic diseases at the preimplantation stage.

2.1.1. Classification and grading

Oocytes were checked for the presence and number of pronuclei about 16 hours after insemination. If two pronuclei were seen, the embryos were classified as normally fertilised; if there were three or more pronuclei as polyspermic embryos and as unfertilised oocytes if they had one or no pronuclei.

On day 2 or 3 fertilized embryos were selected for transfer. The criteria used were the cleavage rate, the eveness of blastomeres and gross morphology (fragmentation and presence of cellular debris). Embryos were scored into four grades, ranging from grade 1 for embryos with clean, even blastomeres with no extracellular material to grade 4, when at least one normal blastomere existed, with obvious extracellular material and clear fragmentation (Dawson *et al*, 1992). Unfertilised oocytes which cleaved over that period were reclassified as parthenogenetic embryos.

2.2. DNA amplification of sex chromosomes specific repeated sequences

The sequences that have been used for amplification by PCR are (Table 2.1.):

I. Y long arm (DYZ1): the 3.4 kb EcoRI fragment, repeated 800-5000 times on the long arm of the Y chromosome (Cooke, 1976; Nakahori, 1986). This sequence is in rare cases deleted in normal males or translocated and carried on an autosome in normal females (Kogan *et al*, 1987; Cooke & Noel, 1979).

II. Y alphoid repeat: the 5.5 EcoR1 fragment, present in ~100 copies in the pericentromeric regions of the Y chromosome. The monomers (subunits) of ~170bp in length are amplified, resulting in ~3200 target sequences. The Y alphoid monomers show 70-90% homology to each other (Wolfe *et al*, 1985).

III. X alphoid repeat: The 2.0 kb Bam III fragment, repeated ~5000 times, resulting in ~60,000-70,000 target sequences. The 2.0 kb consists of twelve satellite monomers (subunits), arranged in imperfect repeats. Each of the monomers is ~170 bp in length. The X monomers are 65-85% identical to each other (Waye & Willard, 1985).

Table 2.1.: Y- and X-chromosome specific repeat sequences amplified by PCR

Sequence	Fragment length	Copy number	Reference
DYZ1	3.4 kb EcoRI	800-5000	Cooke, 1976 Nakahori, 1986
Y alphoid	5.5 kb EcoRI	100	Wolfe *et al*, 1985
X alphoid	2.0 kb Bam HI	5000	Waye & Willard, 1985

2.3. Preparation of oligonucleotide primers for PCR

Oligonucleotide primers were synthesised on a Cyclon Milligene synthesiser, were eluted from the column, deprotected with ammonium hydroxide and precipitated with ethanol, as advised by the manufacturer. All manipulations were performed under a class II cabinet. Tubes were siliconised and positive displacement pipettes and tips used (Gilson Microman positive displacement pipettes and tips). All solutions used for elution, precipitation and dilution of the oligonucleotide primers were filtered through a 0.22 filter (Millex-GS, Millipore).

2.3.1. Elution from column

The oligonucleotide primers were eluted from the column within 1-2 days of synthesis. If immediate elution was not possible, they were stored at 4°C. Frozen aliquots of millipore-filtered ammonium hydroxide (4:1, ammonia : distilled water) were thawed and allowed to reach room temperature before use. A disposable 2ml syringe was inserted into one end of the column. Another 2ml syringe containing 1ml of ammonium hydroxide was inserted into the other end. Ammonia solution was slowly drawn into the column by depressing the plunger on one syringe and releasing the other and this was repeated 4-5 times. The column was left at room temperature and the elution was repeated after 45 min and 1 hr 30 min. The fluid was taken up into one syringe, transferred to a sterile 1.5 ml screw-top eppendorf tube and incubated for at least 16 hrs or overnight at 55°C.

2.3.2. Ethanol precipitation

The oligonucleotide primers were transferred to siliconised centrifuge tubes (Corex). Three volumes of absolute ethanol and 0.1 volume of 2M sodium acetate (millipore-filtered and precooled on ice) were added and gently mixed. The tubes were left at -70°C for at least 2 hrs or overnight. The precipitate was centrifuged in a precooled centrifuge (rotor temperature approximately 4°C) at 14,000 rpm for 1 hr. The supernatant was kept in a sterile tube at 4°C until the yield had been measured. The pellet was resuspended in 1 ml of distilled water by carefully mixing the tube. The precipitation was then repeated .

2.3.3. Dilution of oligonucleotide primers

Ten microliters of the primers resuspended in distilled water were diluted into 1 ml of water and the optical density (OD) was measured at 260 and 280 nm. The yield for 20mer oligonucleotides is generally 0.3 to 0.9 mg. If the yield was greater than 1.2 mg from a 0.2 µmole synthesis, the primers were reprecipitated, as they can be contaminated with ammonia, which absorbs UV light. The primers were aliquoted (20-50 µl aliquots) in 0.5 ml tubes (Eppendorf) at a concentration of 0.5 or 0.250 mg / ml

The sequence of the primers is shown on Table 2.2..

Table 2.2. : Oligonucleotide primers sequences

Sequence Amplified	Oligonoucleotide Primers	Expected fragment sizes (bp)
DYZ1	5'-ATTACACTACATTCCCTTCCA-3' 5'-AGTGAAATTGTATGCAGTAGA-	149
DYZ1 nested	5'-ATTACACTACATTCCCTTCCA-3' 5'-AGTGAATTGTATGCAGTAGA-3'	109
Y-alphoid	5'-ATGATAGAACGGAAATATG-3 5'-AGTAGAATGCAAAGGGCTCC-3'	170
Y-alphoid nested	5'-CTTCACATGATAACCACACA-3' 5'-GTTTCAAATCTGCTCAATCA-3'	110
X-alphoid	5'-AATCATCAAATGGAGATTTG-3 5'-GTTCAGCTCTGTGAGTGAAA-3'	130

2.4. Preparation of genomic DNA from human blood

Human DNA was isolated from blood as described by Bell *et al* (1981). Blood was collected in a heparinized tube and if necessary, stored at 4°C for no longer than 48 hrs. Ten mls of blood were mixed with 90ml ice-cold lysis buffer (0.32 M sucrose, 10mM Tris-HCl (pH 7.5), 5mM $MgCl_2$, 1% Triton X-100). The white cell nuclei were collected by centrifugation at 10K rpm for 10 min. The nuclear pellet was suspended in 4.5 ml of suspension buffer (0.075M NaCl, 0.024M EDTA (pH 8.0)). Five hundred microliters of 5% SDS and proteinase K at 2 mg/ml were added to the suspended pellet and the mixture was incubated at 39°C for approximately 16 hrs. The digest was mixed with 5 ml of phenol saturated with 20 mM Tris-HCl (pH 8.0). The mixture was centrifuged for 15 min at 10K rpm. The upper, aqueous phase was removed and the phenol extraction was repeated twice. Then 5 ml of chloroform / isoamyl alcohol (24:1, vol / vol) was gently mixed with the aqueous phase and centrifuged for 15 min at 10K rpm. The aqueous phase was added to 11 ml of 99.4% ethanol and 0.5 ml of 3M sodium acetate (NaOAc) pH 5.2. The tube was inverted gently several times until the DNA precipitated. The precipitate was removed using a hooked pasteur pipette, dipped into 70% ethanol, drained on the side of the tube and then dissolved into 1 ml TE buffer. The DNA was allowed to dissolve for 2-3 days at 4°C before measuring the concentration by optical density. From 10 mls of blood, approximately 300-500 µg of DNA were obtained. Serial dilutions of male DNA (20ng, 200pg, 2pg) were used for positive controls. Negative control tubes were prepared with 10µl of wash drop medium or 10 µl of distilled water (Table 2.3.).

Table 2.3.: Buffers and solutions for preparation of genomic DNA from human blood.

TE BUFFER

Conc.	Comp.	Amount (g/l)	Molecular Weight	Final Conc.	Supplier (catalogue number)
1X	Tris-HCl (pH 7.5)	1.21	121.1	10mM	Sigma T-1503
	EDTA (pH 8.0)	0.38	380.2	1 mM	BDH 10093

SUSPENSION BUFFER

Conc.	Comp.	Amount (g/l)	Molecular Weight	Final Conc.	Supplier (catalogue number)
1X	EDTA (pH 8.0)	0.00912	380.2	0.024mM	BDH 10093
	NaCl	0.003	40.1	0.075mM	Sigma S-0923

Table 2.3. (continued): Buffers and solutions for preparation of genomic DNA from human blood.

LYSIS BUFFER

Conc.	Comp.	Amount (g/l)	Molecular Weight	Final Conc.	Supplier (catalogue number)
1×	Tris-HCl (pH 7.5)	1.21	121.1	10mM	Sigma T-1503
	$MgCl_2$	1.01	203.3	5mM	BDH 10149
	Sucrose	109.44	342.3	0.32M	Sigma S-0389
	Triton X-100	10ml		1%	Sigma

2.5. Blastomere preparation

Single cells biopsied from cleavage stage embryos were first examined for nuclei using phase or interference contrast optics and if there was any doubt labelled with Hoechst 33342 (Sigma). Cells with single nuclei were washed twice in PCR buffer (Table 2.4.) and transferred to 0.5 ml Eppendorf tubes loaded with 10 µl distilled water under a dissecting microscope which allows observation of the transfer of the cell into the tube using a drawn out capillary tubing. Tubes loaded with water were kept at 4°C until immediately before use to minimise condensation on the sides of the tubes and help ensure the cell was placed at the bottom of the tube. The caps on the tubes were closed and left at room temperature for 10 minutes to allow the cells to lyse before freezing and thawing twice to -20°C. Each sample in 10 µl of water was overlaid with 50-100 µl of filtered paraffin oil and heat-denatured at 99°C for 6 min for the DNA controls and at 95°C for 35 min for cells. After addition of the DNA polymerase to the master mix, 10-90 µl was added to each sample, at the top of the oil. The tubes were then spun and transferred to the thermal cycler programmed as shown in Table 2.5.

2.6. DNA amplification

Samples lysed in water were overlaid with 50 µl oil and heat-denatured at 95°C for 35 min before PCR. Ten µl of PCR mix was then added to each sample to give a final concentration of 1.5µM each primer, 200 µM each dNTP and 0.5 units Taq polymerase. The buffer used was a modification of a buffer designed for multiplex PCR which optimises the yield of the specific fragment (16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8.8, 17 mg/ml bovine serum albumin, 3.35 mM $MgCl_2$, 10 mM ß-mercaptoethanol). For samples prepared in lysis buffer, a potassium and Tris-free buffer was used (3.0 mM $MgCl_2$) as the lysis and neutralisation buffers contain sufficient

amounts of potassium and Tris for a 50 µl reaction volume. In this case, therefore, 10 µl of a PCR mix with the same final concentrations of primers and other components was used. The cycling conditions include an initial denaturation step at 94°C for 4 min, followed by 40 cycles of denaturation at 94°C for 30 sec and by annealing and extension at 65°C (DYZ1) or 55°C (Y-alphoid repeat) for 1 min 30 sec with a final extension at 72°C for 10 min (Table 2.5.) (DNA thermal cycler, Perkin Elmer Cetus). In addition to the blastomeres, control wash medium blanks and male genomic DNA diluted in sterile water or neutralised lysis buffer were amplified concurrently.

Table 2.4.: Composition of concentrated PCR buffers

Standard 10xPCR buffer

Stock	Comp.	Molecular Weight	Final Conc.	Supplier (catalogue number)
A (10X)	Tris-HCl (pH 7.5)	121.1	100 mM	Sigma T-1503
	KCl	74.55	500 mM	BDH 10198
	gelatine		0.01% (w/v)	
B (10X)	MgCl2	203.3	1 5	BDH 10149

Each stock was made up in autoclaved distilled water, reautoclaved, filtered, aliquoted and stored at -20° C. Equal volumes of stocks A and B were added to the PCR mix to give final dilution of 1 in 10.

Table 2.4. (continued): Composition of concentrated PCR buffers

Multiplex 10x PCR buffer

Stock	Comp.	Amount (g/l)	Molecular Weight	Final Conc.	Supplier (catalogue number)
A (10X)	Tris-HCl (pH 8.8)	8.11	121	67mM	Sigma T-1503
	$(NH_4)_2SO_4$	2.19	132.2	16.6 mM	Sigma
	BSA	0.170		0.17mg/ml	Sigma
B (10X)	$MgCl_2$	1.36	203.3	6.7mM	BDH 10149
C	β–mercapto-ethanol	2ml	78.13	100mM	Sigma B-6131

Each stock was made up in autoclaved distilled water. Stocks A and B were autoclaved. Sufficient stock C was added to stock A, a to give a final concentration of 10 mM. This combined stock and stock B were filtered, aliquoted and stored at -20 C. Equal volumes of the combined stocks A and C and stock B were added to the PCR mix to give a final dilution of 1 in 10.

Table 2.5.: PCR cycling conditions for each set of primers and various combinations.

Oligonoucleotide Primers	Initial Denaturation Temperature (time)	Denaturation Temperature (time)	Annealing / Extension Temperature (time)	Number of cycles
DYZ1	94°C (4 min)	94°C (30 sec)	65°C (1 min 30 sec)	40
DYZ1 nested	94°C (4 min)	94°C (30 sec)	65°C (1 min 30 sec)	40
Y alphoids	94°C (4 min)	94°C (30 sec)	55°C (1 min 30 sec)	40
Y alphoids nested	94°C (4 min)	94°C (30 sec)	55°C (1 min 30 sec)	40
X alphoids	94°C (4 min)	94°C (30 sec)	65°C (1 min 30 sec)	40
X alphoids and DYZ1	94°C (4 min)	94°C (30 sec)	60°C (1 min 30 sec)	40
X alphoids and Y alphoids	94°C (4 min)	94°C (30 sec)	55°C (1 min 30 sec)	40

2.7. Labelling of blastomere nuclei

One mg/ml stock of Hoechst 33342 (Sigma) in M2 supplemented with 4mg/ml BSA (M2+BSA) was prepared, aliquoted, frozen immediately in liquid nitrogen and stored at -20°C. Single blastomeres were placed in drops of Hoechst 33342 diluted 1:200 in M2+BSA to give final concentration of 5 µg/ml on heat-sterilised glass slides under oil, and cells were incubated at room temperature until the nucleus was visible (for 2-4 min). Normal nuclei were clearly visible with a distinct outline and examined by fluorescent microscopy.

2.8. Restriction digestion of PCR products

PCR products were digested with restriction enzymes to confirm their identity: i) the restriction enzyme EcoRI, for the DYZ1 149bp fragment (Cooke, 1976) ii) the restriction enzymes DdeI, HinfI, HpaII, NciI, EcoRI, for the Y-alphoid 170bp fragment (Wolfe et al, 1985).

The amplified fragment was digested with a series of endonucleases at known restriction sites and the size of the digested fragments analysed by gel electrophoresis. Ten µl of the amplified product was diluted to a final volume of 20 µl in restriction buffer containing 2-5 units of the enzyme appropriate for the sequence and incubated at 37°C according to the manufacturer's guidelines.

The expected fragment sizes after digestion of the DYZ1 sequence were as follows; EcoR I: 95bp, 54 bp; HinfIII: 27 bp and 122 bp. The expected fragment sizes after digestion of the Y-alphoid sequence were as follows; EcoR I: 170 bp (no cutting sites); Hinf I: 137, 33 bp ; Nci I: 87,83 bp; Hpa II: 88, 82 bp; Dde I: 122, 48 bp.

2.9. Polyacrylamide gel electrophoresis (PAGE)

Ten µl of the amplification products were mixed with 2 µl of loading buffer. The sample was loaded on 12% polyacrylamide minigel (10cm x 8cm x 0.1cm), which were prepared and allowed to set for at least 30 min. The samples were loaded carefully and were subjected to electrophoresis at 100 V for 1 hr (Biorad Mini Protean II minigel apparatus, LKB Power supply Bromma 2197). After electrophoresis, the gel was stained for 15 min in electrophoresis buffer containing ethidium bromide (0.5 µg/ml), rinsed in distilled water, viewed under UV illumination and photographed (Table 2.6.).

Table 2.6.: Electrophoresis Buffers

TBE buffer

Conc.	Comp.	Amount (g/ 500 ml)	Molecular Weight	Final Conc.	Supplier (catalogue number)
10 X	Tris-HCl	60.6g	121.1	1M	Sigma T-1503
	Boric acid	30.9 g	61.8	1M	BDH 10058
	EDTA	7.6g	380.2	20mM	BDH 10093

Loading buffer

Conc.	Comp.	Amount (g/100ml)	Molecular Weight	Final Conc.	Supplier (catalogue number)
10X	Sucrose	50g	342.3	50%	Sigma S-0389
	EDTA (pH 8)	0.038g	380.2	0.1M	BDH 10093
	Bromophenol blue	0.05g	692.0	0.05%	Sigma B6131

2.10. Contamination

To control contamination, the physical transfer of DNA must be prevented. For this to be achieved, laboratory coats and gloves were worn at all times. The PCR reaction was prepared under a laminar flow hood (class II cabinet). PCR samples were prepared in a separate room, which was free of PCR product. No tube containing PCR product was opened in the room where the PCR was prepared and not a single piece of equipment (including tube racks, ice buckets and pens) was shared between the laboratories where the PCR was prepared and the PCR product was analysed. Different laboratory coats were worn in the two places. Gloves were worn all the time and during the preparation of PCR mixture they were changed frequently. All the buffer solutions and water were autoclaved, filtered and aliquoted to minimise the number of times that each reagent was used. Paraffin oil, which was used to overlay the reaction mix, was filtered and aliquoted. For the preimplantation diagnosis cases, only unopened aliquots of reagents (from batches tested previously) were used. Disposable plastic tubes were kept in the room where the PCR reaction was prepared, the tubes were washed, dried and siliconised wearing gloves. Pipette tips and microcentrifuge tubes were autoclaved. For single blastomere work and for the preimplantation diagnosis cases, eppendorf tubes have been selected before use as some of them do not permit visualisation of the blastomere in the tube. Three different sets of pipettes were used for preparation of the DNA dilutions, preparation of PCR mixture and handling the PCR product. As the barrel of some pipettes can become contaminated with aerosols containing DNA, only positive displacement pipettes were used for the preparation of PCR mixture (Gilson Microman positive displacement pipettes and tips). Also, positive and negative controls were used. As negative control, samples with all the reagents except DNA were used, that have gone through the same sample preparation steps. The samples were handled very carefully, to avoid any spillage of liquid. Pipette tips were used only once and then discarded.

2.11. Radio-labelling of newly synthesized proteins

After two washes in Earles medium (Hepes buffered) containing 0.4 w/v Polyvinylpyrrolidone (PVP), the embryos were transferred individually to 30µl drops of Earles medium containing 0.4 w/v PVP under oil, containing 3 µl of ^{35}S-methionine of specific activity 1000 Ci/mM (cell-culture grade, Amersham) and incubated at 37°C for two hours. They were then washed three times in protein free Earles medium and transferred individually in a 10 µl Earles medium drop, into 10 µl of 2X sample buffer (Table 2.7.) (Laemmli, 1970) and stored at -70 C .

α–amanitin was diluted and stored as described in Chapter 6 (6.2.1.)

2.12. Inhibition of transcription

Drops of Earles medium (30 µl), containing 0.4 w/v Polyvinylpyrrolidone (PVP) plus or minus 10 or 100 µg/ml α–amanitin, were prepared under paraffin oil in culture dishes and equilibrated in a humidified 5% CO_2-in-air atmosphere at 37°C for at least one hour. Embryos were washed twice, added individually to the culture droplets and cultured for 24 hours. Their morphology was assessed before and after treatment.

2.13. Uptake and incorporation of ^{35}S-methionine

Individual embryos, stored frozen in lysis buffer, were thawed and heated at 95°C for 2 min. After 5 min at room temperature, two 2µl aliquots of the sample were taken. For assessment of uptake, one aliquot was placed directly into scintillant and counted. For assessment of the incorporation into protein, 3 µl of 0.5% BSA and 195 µl of 10% trichloroacetic acid (TCA) was added to each aliquot and the sample was allowed to stand for 2 hrs at 4°C (Howlett, 1987). The samples were then individually filtered through glass fibre discs (Whatman CF/C), wet with ice-cold 10% TCA using a multiple sample filtration harvester (Millipore). After 3 washes with 10% TCA, the filter discs were dried and placed in scintillant (Cocktail T, BDH, Scintran) in an LKB Wallac 1209 Rackbeta Liquid Scintillation Counter at 90% efficiency .

2.14. SDS-Polyacrylamide gel electrophoresis

For one-dimensional analysis, polypeptides were separated using uniform 10% or 8-15% gradient SDS-polyacrylamide gels containing 0.1% SDS and 0.5 M Tris-HCI (pH 8.8), with a stacking gel of 4.5 acrylamide containing 0.1% SDS and 0.125 M Tris-HCI (pH 6.8 (Laemli 1970) (Table 2.7.). The gels were run for 12-18 hrs at 100-200 Volts (LKB Power supply Bromma 2197, Biorad Protean II gel apparatus) and were fixed afterwards for 30 min in 10% acetic acid, 30% methanol. The gels were soaked in Amplify (Amersham) for 15 min before drying down on to filter paper (Whatman) and exposure to Hyperfilm MP (Amersham) at -70°C for between 1 week and 2 months.

Equal counts were loaded in each lane of the gels that are presented in Fig. 5.9. and 5.10. (Chapter 5) : 18000cpm in gel 5.10. and 3700 cpm in gel 5.9. In the other gels presented in Chapter 5, one embryo per lane was loaded.

2.15. Cell number analysis

The cell number of the embryos that were at cleavage stages were counted directly. For the blastocyst stage, the cell number was approximated from the study of Hardy *et al*, (1990), from counts made with spare embryos at the same age as those used for protein synthetic studies.

Table 2.7.: Stock solutions and buffers for SDS-PAGE

SAMPLE BUFFER FOR SDS-PAGE

Conc.	Comp.	Amount (g/100ml)	Molecular Weight	Final Conc. (in 1X buffer)	Supplier (catalogue number)
2X	Tris-HCl (pH 6.8)	1.512	121.1	62.5mM	Sigma T-1503
	SDS	4	288.4	2%	Sigma L-4390
	β–mercapto-ethanol	1ml	78.13	5%	Sigma M6250
	glycerol	20 mls	92.09	10%	BDH 10118
	bromophenol blue	0.004	692.0	0.002%	Sigma B6131

STACKING GEL BUFFER

Conc.	Comp.	Amount (g/100ml)	Molecular Weight	Final Conc.	Supplier (catalogue number)
1X	Tris-HCl (pH 6.8)	60.55	121.1	5mM	Sigma T-1503
	SDS	4	288.4	4%	Sigma L-4390

Table 2.7. (continued): Stock solutions and buffers for SDS-PAGE

SEPARATING GEL BUFFER					
1X	Tris-HCl (pH 8.8)	181.65	121.1	1 5mM	Sigma T-1503
	SDS	4	288.4	4%	Sigma L-4390

2.16. Measurements of HPRT activity

2.16.1. Sample preparation for HPRT measurements

Extracts of individual embryos were prepared between days 4 and 6 after insemination. Embryos were washed twice with Tris / $MgCl_2$ buffer, pH 7.4, containing 275 mM Tris, 244mM $MgCl_2$ and 0.5 mg / ml BSA. Single embryos were transferred under a stereo dissecting microscope, into 2.5 µl of the same buffer in a 5 µl capillary tube (Microcap; Drumond Scientific). The ends were sealed with a small piece of parafilm and the tubes were immediately frozen to -70°C for storage, before analysis.

2.16.2. Enzyme assay

Embryo extracts were thawed and their contents immediately expelled on a microscope slide under mineral oil. One µl extract was removed and added to 2 µl assay mixture to give final concentration of 50 µM adenine, 50 µM hypoxanthine and 1 µM-5-phosphorybosyl 1-pyrophosphate in Tris / $MgCl_2$ buffer, pH 7.4. The resulting 3µl solution was than loaded in a 5 µl capillary tube, the ends were sealed with parafilm and the tube was incubated at 37°C for 3 hrs. At the end of the incubation the contents of capillary tube were expelled into 27µl of 25mM ammonium phosphate and the resulting 30µl volume was analysed for inosine monophosphate (IMP) and adenine monophosphate (AMP) on a Kotron 400 series high performance liquid chromatography (HPLC) apparatus, using a Hypersil 5 ODS (25cm X 2mm) column. The eluant was 25 mM ammonium phosphate pH 4.6 at a flow rate of 0.3 ml / min. The eluant was passed through a variable wavelength scanning detector set at 254 nm.

Using this system, IMP, AMP, hypoxanthine and adenine were readily separated. Enzyme activities were calibrated against IMP and AMP standards. Under these conditions, enzyme activity was linear for at least 3 hrs.

2.16.3. HPRT activity measurements and embryo sexing

Before the embryo extract was expelled for analysis (as described on 2.16.2.), the lysed embryo (embryonic ghost) was removed with a fine pulled pasteur pipette and transferred in a 0.5ml eppendorf tube, loaded with 10µl of distilled water. The tubes with the embryonic ghost were kept on ice for a day. They were then sexed by PCR, as described in 2.5.

Chapter 3

Clinical trial for preimplantation diagnosis of X-linked disease

3.1. Introduction

3.1.1. Current methods for prenatal diagnosis

For couples at risk of an X-linked disease, when a specific diagnosis is not available, the only alternative is identification of the sex of the embryo and selective abortion of a potentially affected male fetus. The routine method for the prenatal identification of sex is cytogenetic analysis for the presence of XX or XY karyotype (see 1.7.2.). More recently, rapid sexing was possible after CVS using ISH and PCR (West *et al*, 1988; Kogan *et al* , 1987) (see 1.7.2.)

The main disadvantage of prenatal diagnosis is that some couples are faced with the dilemma of whether or not to abort an affected fetus. Some couples find the choice of an abortion unacceptable for religious or moral reasons. For couples at risk, a great number of fetuses may be affected, and this often results in the physical and emotional trauma of repeated pregnancy terminations (Penketh & McLaren, 1987). The only prospect of starting a pregnancy which is known to be unaffected is to be able to diagnose a potentially affected conceptus prior to implantation.

3.1.2. Preimplantation diagnosis

Over the last 15 years, methods of ovarian stimulation, oocyte collection, *In Vitro* Fertilization (IVF), embryo culture and transfer have been established for the treatment of infertility. To maximise the chances of a pregnancy, these techniques allow increased numbers of oocytes to be recovered and fertilized and preimplantation embryos to be cultured for several days before transferring one or two of them back to the patient. Couples at risk of transmitting genetic diseases inherited as mendelian traits produce unaffected as well as affected embryos. Therefore, detection of

genetic defects at these very early stages (preimplantation diagnosis), would make it possible to transfer only unaffected embryos, eliminating the need for a termination.

3.1.3. Limitations of single cells and time

In general, diagnosis of inherited diseases involves either measuring the absence or abnormal expression of the gene product responsible for the condition or detecting genetic defects directly at the DNA level (in those cases in which the molecular basis is known). Human preimplantation embryos have a small number of cells at cleavage stages and from those only a limited number can be biopsied without seriously affecting the viability and development of the embryo. Conventional approaches to DNA analysis such as Southern blotting involve the digestion of approximately 5µg of DNA, followed by electrophoresis, Southern transfer, hybridization to a probe which is most often radioactivel y labelled and exposure of the filters to the film (Ivinson & Taylor, 1991) (see also 1.4.5.7. in Chapter 1). The whole procedure takes around 4 days. For preimplantation diagnosis, the very small amount of sample (1 or 2 cells) contains the equivalent of 5-10pg of DNA and prevents DNA analysis by Southern blotting. However, the development of the polymerase chain reaction (PCR) has allowed genetic analysis from small number of cells (Saiki *et al*, 1985). Apart from its great sensitivity, a very important advantage of PCR is that DNA fragments can be amplified within a few hours making it possible to transfer the embryo the same day, without the need for cryopreservation. Also, sensitive enzyme microassays have been developed, that can detect enzymatic activity levels in a single cell (Monk *et al*, 1987; Benson & Monk, 1988; West & Green, 1983; Sermon *et al*, 1991).

Instead of performing sexing and embryo transfer the same day, an alternative option which would permit more time for genetic testing would involve cryopreservation of the biopsied embryos and transfer in a later cycle. Biopsied 4-cell

mouse embryos have been cryopreserved with an ultra-rapid protocol and have developed to term after thawing and transfer (Wilton *et al*, 1989). However the slit left after the biopsy on the zona might increase the possibility of the embryo being damaged, as the zona is thought to protect embryos from damage caused by ice crystal formation in the medium. Although pregnancy rates after transfer of frozen embryos is equal to those without freezing, around 20% of the frozen embryos are lost in the freezing process (Trounson, 1990). Until the feasibility of cryopreserving biopsied human embryos is examined, the best alternative method was considered to perform the diagnosis and transfer during the same day that IVF transfers are routinely performed.

3.1.4. Preliminary work for preimplantation diagnosis in the human embryo

Amplification of a 149bp fragment of the 3.4Kb repeated sequence DYZ1 (Cooke, 1976; Nakahori *et al*, 1986) has been described by Kogan *et al* (1987) for rapid sexing of fetal DNA from chorionic villi. Using the same primers, Handyside *et al* (1989) examined the possibility of identifying the sex of human preimplantation embryos by DNA amplification of biopsied blastomeres. In this study 38 embryos (30 normally fertilized, 5 polyspermic and 3 parthenogenetic embryos) were examined by single blastomere biopsy at the 8-cell stage. Of these, 13 were lost because of technical problems. Twenty five embryos were sexed by single blastomere biopsy and amplification of the DYZ1 sequence (as described by Kogan *et al*, 1987). The biopsied embryos were returned to culture to test their viability after the biopsy. Of these, 37% reached the blastocyst stage, which is a rate similar to the blastocyst rate of unmanipulated embryos. Of the 25 embryos sexed by PCR, in 19 the sex was confirmed by examining the biopsied embryos by ISH and / or fluorescent F-body staining with spermidine bis acridine. In 4 males, 11 females and 2 parthenotes, the sex (identified in the whole embryo by ISH) was as predicted by single cell PCR. In two cases in which PCR showed males, ISH identified female embryos. However, both

embryos were polyspermics, and therefore the contradiction in the results can be explained by the potential mocaisism of the polyspermic embryos. With the exception of the 2 polyspermic embryos, diagnosis was correct in all 15 normally fertilized embryos that had been sexed. These results were very encouraging in two ways: firstly, they showed that biopsy of the human embryo does not affect the development to the blastocyst stage in that the blastocyst rate after biopsy was similar in biopsied and untreated embryos. Secondly, they demonstrated that single blastomere amplification by PCR is a reliable method to identify the sex of the biopsied embryo. Also the whole procedure (biopsy and PCR) does not exceed 8 hours, transfer of the sexed embryo is possible on the same day as the diagnosis.

3.1.5. Clinical trial for preimplantation diagnosis of X-linked disease

Based on these results, ethical approval for a clinical trial with couples known to be at risk of X-linked diseases was granted. The patients followed the protocol for IVF that was routinely used at the Hammersmith Hospital. Normally fertilized human embryos were biopsied at cleavage stages early on day 3. The single biopsied blastomeres have been amplified by PCR using two DNA repeated sequences; the DYZ1 and Y-alphoid repeats. For the 10 first cases for preimplantation diagnosis, the DYZ1 repeat was used. The DYZ1 repeat was initially described as Y-specific repeated DNA family, which is recognised as an EcoRI fragment of 3.4 kb in male DNA (Cooke, 1976). Male DNA contains 800-5000 copies of the 3.4kb fragment (Nakahori *et al*, 1986). A sequence of 149 bp is amplified, which spans the EcoRI restriction site, and is present in 800-5,000 target sequences per cell (Kogan *et al*, 1987). The use of DYZ1 amplification, although was successful, was complicated with contamination problems. Also, as the DYZ1 repeat is located on the long arm of the Y-chromosome (Cooke et al, 1976) and in rare cases can be deleted in normal fertile males (Bobrow *et al*, 1971) or translocated to autosomes and carried by normal females

(Cooke & Noel, 1979), there is the possibility of misdiagnosis. Therefore, the parents had to be screened for the presence or the absence of the specific sequence.

As an alternative, another Y-specific sequence was then examined. The alphoid sequences are located mainly in the centromeres of autosomal and sex chromosomes and it is the only repetitive DNA family that is shown to have chromosome specificity. The advantage of using a pericentromeric sequence instead of a telomeric one is that the parents do not need to be screened beforehand. The Y-alphoid repeat has been described by Wolfe *et al* (1985). The Y-specific alphoid DNA consists of a basic unit of 5.5 - 5.7 kb fragment. There are about 100 copies of this 5.5kb unit on the Y chromosome (Wolfe *et al*, 1985). Each unit contains subunits or monomers of 170-171bp. The monomers of ~170bp are amplified, resulting in ~3200 target sequences. These individual monomers are 70-90% homologous to each other and 87-92% homologous to the consensus sequence. Between the Y and the X chromosome, the monomers share an homology of 89% (Waye & Willard, 1985).

Witt & Ericson have designed primers that amplify the X- and Y-alphoid repeats. As a source of DNA dried blood specimens were used. They demonstrated that the technique was sensitive enough to detect the Y-specific fragment from as little as 10µl of dried blood and the X-specific fragment from 5µl of blood, which is equivalent to 100-200ng of DNA or 10^5-$2x10^5$ cells (Ivinson & Taylor, 1991). However, they mention that the PCR product from 10µl of blood was faint on an agarose gel.

PCR conditions for DYZ1 were further optimised from those used by Handyside *et al* (1989) as described in Chapter 4. The conditions for amplifying the Y-alphoid repeat at the single cell level required more extensive preliminary work, also described in detail in Chapter 4.

3.1.5.1.. Contamination

The main advantage of PCR, the ability to produce large numbers of copies of a sequence from minute quantities of DNA, is also one of its greatest drawbacks, as contamination often occurs (Strom *et al*, 1990), which could lead to false positives and result in misdiagnosis. Contamination can be caused from DNA from cells (from the skin or hair of the operator) (sporadic contamination) and also by the amplification of product from a previous reaction (carry-over contamination).

In the work of Handyside *et al* (1989), the problem of contamination was highlighted, as in 2 embryos that have been shown to be males by PCR were identified as females by ISH. Although in these cases the problem could have been mosaicism (as the embryos were polyspermics), the possibility of contamination could not be excluded. Also the authors mention that a cell was judged as female if the Y-specific band was absent or faint, which again indicates contamination problems.

3.2. Methods

3.2.1. Y-chromosome specific repeat amplification from single blastomeres and DNA dilutions

For control blanks, distilled water was used instead of DNA. As positive and negative controls, 20ng, 200pg and 2pg male and female DNA have been used. (DNA was extracted from blood, as described in 2.4. and diluted in distilled water.) For the biopsied blastomeres, the blastomere's washdrop was used as a negative control. All the reagents (including the Taq polymerase and the primers) were aliquoted to minimise the number of times an aliquot was frozen and thawed.

Biopsied blastomeres were first examined for nuclei using phase contrast optics or were vitally labelled with Hoechst 33342 (Sigma), as described in Chapter 2 (2.2.8.). The blastomeres were washed twice in PCR buffer and transferred to 0.5 ml Eppendorf tubes loaded with 10 µl distilled water under a dissecting microscope which allows observation of the transfer of the cell into the tube using drawn out capillary tubing. Tubes have been selected previously that have smooth surfaces, before attempting transfer of the cells. They were loaded with water and kept at 4°C until immediately before use to minimise condensation on the sides of the tubes and help ensure the cell is placed at the bottom of the tube. The caps on the tubes were then closed and left at room temperature for 10 minutes to allow the cells to lyse before freezing and thawing twice to -20°C. The tubes were spun for 20 sec, overlaid with oil and transferred at 95°C for 35 min. After the preparation of the master mix, 10-90 µl was added to each sample, at the top of the oil. The tubes were then spun and transferred to the thermal cycler.

3.2.2. Confirmation of single blastomere PCR

To confirm the diagnosis from single cells, biopsied embryos that had not been transferred were disaggregated and each or several cells were amplified with the Y-specific sequences (DYZ1 - or Y-alphoids). The blastomeres were first examined for nuclei as described in Chapter 2 (2.2.8.).

3.2.3. Contamination

For precautions against contamination see 2.8 in Chapter 2. Extra precautions for contamination were taken for the preimplantation diagnosis cases: all the reagents (including the Taq polymerase and the primers) were aliquoted to minimise the number of times a tube was opened. An aliquot of each reagent was tested before each diagnosis was performed and only unopened aliquots were used.

3.3. Results

3.3.1. IVF and embryo biopsy

Thirteen couples at risk of X-linked conditions had a total of 21 treatment cycles (Table 3.1.). The diseases included adrenoleukodystrophy, X-linked mental retardation (non fragile-X type), Lesch-Nyhan syndrome, retinitis pigmentosa and Duchenne muscular dystrophy (Table 3.1.). Many patients had affected children, for example patient D had a boy affected by Lesch -Nyhan syndrome and patient B had a boy affected by X-linked mental retardation (non-Fragile-X type) (Table 3.1).Except patients A and M, all the others were fertile and the majority had undergone previous abortions after amniocentesis or CVS to prevent the birth of an affected child.The mean maternal age was 32.9 per cycle, ranging from 24 to 39 years. Each couple had been counselled about all the alternative reproductive options. They were also warned about the low success rate after IVF and that no baby had been born after embryo biopsy at preimplantation stages, so that the procedure was still experimental. In the event of a pregnancy, the couples were encouraged to consider having CVS to confirm the diagnosis.

Table 3.1. Maternal age, pregnancy history and pregnancy outcome after preimplantation diagnosis in patients who were carriers for X-linked disease.

Cycle No	Patient	Disease	Maternal Age	Pregnancy History*	No embryos transferred	Pregnancy Outcome
1	A1	ALD	28	0	1	Bio
2	B1	X-linked M. R.	34	2+3		NT
3	C1	DMD	36	1+4	2	NP
4	D1	Lesch Nyhan	29	1+7	2	NP
5	B2	X-linked M. R.	34	2+3	2	P
6	E1	DMD	33	0+3	1	NP
7	A2	ALD	28	0	2	P
8	C2	DMD	37	1+4	1	NP
9	F1	DMD	37	0+1		NT
10	D2	Lesch Nyhan	29	1+7	1	NP
11	E2	DMD	33	0+3	2	NP
12	C3	DMD	37	1+4	2	P
13	G1	DMD	37	0+1		NT
14	H1	SNMD	24	0+2	2	P
15	I1	X-linked M. R.	39	0+1		NT
16	E3	DMD	34	0+3		NT
17	J1	RP	31	1+1	1	P
18	K1	DMD	30	2+1	2	NP
19	L1	X-linked M. R.	37	0+2	0	NT
20	I2	X-linked M. R.	39	0+1	1	NP
21	M1	DMD	36	0	2	NP
		Average	32.9±0.9		1.5±0.1	
		Range	24-39		0-2	

ALD: Adrenoleukodystrophy
SMND: type II hereditary sensory motor neurone disease
X-linked M.R.: X-linked mental retardation (non fragile-X type)
RP: Retinitis pigmentosa
DMD: Duchenne muscular dystrophy
*: livebirths + abortions
NT: no embryo transfer
P: pregnant
NP: non-pregnant
Biochemical pregnancy
± standard error of the mean

After superovulation, a total of 200 oocytes were collected (4-20 per patient, 9.5 +/- 0.9 average). One hundred and fifteen oocytes were fertilized (57.2%) (Table 3.2.). Nineteen of the 21 cycles got as far as biopsy. In two cycles only one oocyte was fertilized (B1, G1 in Table 3.2.) or no fertilization occurred (E3 in Table 3.2.) and biopsy was not attempted. From the normally fertilized embryos, 86 embryos (80%) developed to the six to ten-cell stage early on day 3.

One or two blastomeres were biopsied from cleavage stage embryos as described by Handyside (1990). The isolated blastomeres were then examined for nuclei using phase contrast optics and/or were vitally labelled with Hoechst 33342 (Sigma), as described in Chapter 2 (2.8.8.). Detailed notes were kept on the presence and the morphology of the nuclei. If an embryo was partially lysed or otherwise damaged during biopsy, it was disaggregated after the removal of the zona pellucida and the cells were used as controls for the amplification. Eighty six cleavage stage embryos were biopsied, 8 on day 2 (at about the 4-cell stage) and 78 on day 3 (at the 6- to 10-cell stage). On average, five (5.4) normally fertilised embryos were available for biopsy in each treatment cycle (Table 3.2.). Of these, 78 embryos were succesfully biopsied (86% of biopsied embryos and 67.8% of the normally fertilized embryos). The number of blastomeres that were biopsied was 147 (2-14 per patient, 8.1 average) and 101(70%) were succesfully biopsied (without lysing the blastomere) (1-12 per patient, 5.6 average). In one case (F1) with 2 normally fertilized embryos, neither was biopsied succesfully and further diagnosis was impossible (Table 3.2.). Amplification was attempted in 95/101 blastomeres in which a nucleus had been observed (Tables 3.2., 3.3.). Blastomeres which lysed during biopsy or in which no nuclei was obvious, were also amplified, but the result was not considered as diagnostic. In three cases the sex identified from a lysed blastomere was the same as that identified by amplification of the whole biopsied embryo. Conversely, five anucleate blastomeres biopsied from male embryos showed no signal.

Table 3.2. Number of embryos and cells biopsied for preimplantation diagnosis of X-linked disease.

No	Patient	No. of oocytes	No. fertilised	Day biopsy	Embryos biopsied	Embryos succesfully biopsied	No cells. biopsied	Cells succesfully biopsied
1	A1	8	2	3	2	2	3	2
2	B1	5	1	N B				
3	C1	8	7	3	7	6	11	6
4	D1	12	9	3	5	5	6	5
5	B2	14	10	3	7	7	15	7
6	E1	11	4	3	3	3	4	3
7	A2	9	6	3	6	5	6	5
8	C2	8	4	3	3	3	6	3
9	F1	5	2	3	2	0	3	0
10	D2	20	11	3	5	3	6	3
11	E2	10	7	3	6	6	14	9
12	C3	11	5	3	5	5	10	9
13	G1	4	1	N B				
14	H1	18	7	3	6	6	8	7
15	I1	6	3	3	3	1	2	1
16	E3	9	0	N B				
17	J1	7	4	3	2	2	5	2
18	K1	7	7	3	5	4	10	5
19	L1	10	8	2	8	8	11	11
20	I2	10	9	3	8	6	13	11
21	M1	9	8	3	6	6	14	12
	Total	200	115		86	78	147	101
	Average	9.5±0.9	5.4±0.6		4.7±0.5	4.3±0.5	8.1±1.0	5.6±0.9

ALD: Adrenoleukodystrophy
X-linked M.R.: X-linked mental retardation
DMD: Duchenne muscular dystrophy
±: standard error of the mean
SNMD:
RP: Retinitis pigmentosa
N B: Not biopsied

3.3.2. Preimplantation embryo sexing by DNA amplification of the biopsied blastomeres.

Seventeen out of 21 cycles reached the stage for analysis of the biopsied blastomeres. In the first ten, the embryos were sexed by amplification of the DYZ1 sequence and in the following seven, the Y-alphoid repeat (Table 3.3.). When the DYZ1 sequence was used for sexing, the parents were screened beforehand for the presence or absence of the DYZ1 sequence in a preparation of their blood lymphocytes (Fig. 3.1.). A total of 77 embryos (95 blastomeres) were amplified. Thirty three (42.3%) were identified as males and 34 (43.6%) as females (Table 3.3.). In Fig. 3.2. the result of the first clinical application of preimplantation diagnosis is shown (A1 in Table 3.1.). Overall, 95% of embryos have been diagnosed as males or females. In 6 embryos identification of the sex was not possible; in two cases the amplification levels were between those expected for a male and a female (C2, D2); in two cases, multiple bands appeared that did not permit identification of the sex (B2, K1); and in two embryos from which two cells had been removed, amplification of the two biopsied cells gave opposite results (E2, M1) (Table 3.3.) .

The mean number of male and female embryos that were identified with both sequences was 2 males and 2 females average with DYZ1 and 1.4 males and 2 females with Y-alphoids. There was a smaller number of male embryos detecting with the Y-alphoids, but the number of embryos examined is too small to make that difference significant. The mean number of embryos transferred per cycle was the same in both groups (1.6 average). In 11 cycles involving 57 embryos, 1 cell per embryo was analysed by PCR. In the 6 remaining cycles from in 20 embryos, 2 cells per embryo were analysed. In these two sets of cycles, 15 and 9 embryos were transferred and 3 and 2 pregnancies were established respectively (Table 3.3., 3.4.).

Figure 3.1.

Amplification product of lymphocyte preparation of the candidates for preimplantation diagnosis, to screen for the presence or absence of the 149 bp DYZ1 fragment (top arrow) in males and females. Lanes 1, 3 & 5: Male lymphocytes. Lanes 2 , 4 & 6: Female lymphocytes. Lanes 7, 8 & 9: Negative controls. Lane 10: Positive control: amplification of 2ng of male DNA. Middle arrow: primer-dimer. Bottom arrow: Primers.

Figure 3.2.

Preimplantation diagnosis of X-linked disease by sexing: First clinical application. M: molecular weight markers (pBR322 Hae III digest). Lanes 1 & 2: Amplification product of single blastomere biopsied from a female and male embryo, respectively. Lanes 3, 7 : Negative controls (blanks). Lane 4, 5 and 6: 20 ng, 200pg and 2 pg of male DNA, used as positive control. Lanes 8-12:Amplification of blastomeres biopsied from different embryos that have been identified by single blastomere biopsy and PCR to be males. Top arrow: the 149 bp DYZ1 fragment. Middle arrow: primer-dimer. Bottom arrow: Primers.

Sizes of DNA fragments after Hae III digest of pBR 322: (bp) 587, 540, 504, 458, 434, 267, 234, 192, 184, 124, 123, 104, 89, 80, 64, 57, 51, 21, 18, 11, 8.

The same markers have been used through Chapters 3 and 4.

Figure 3.1.

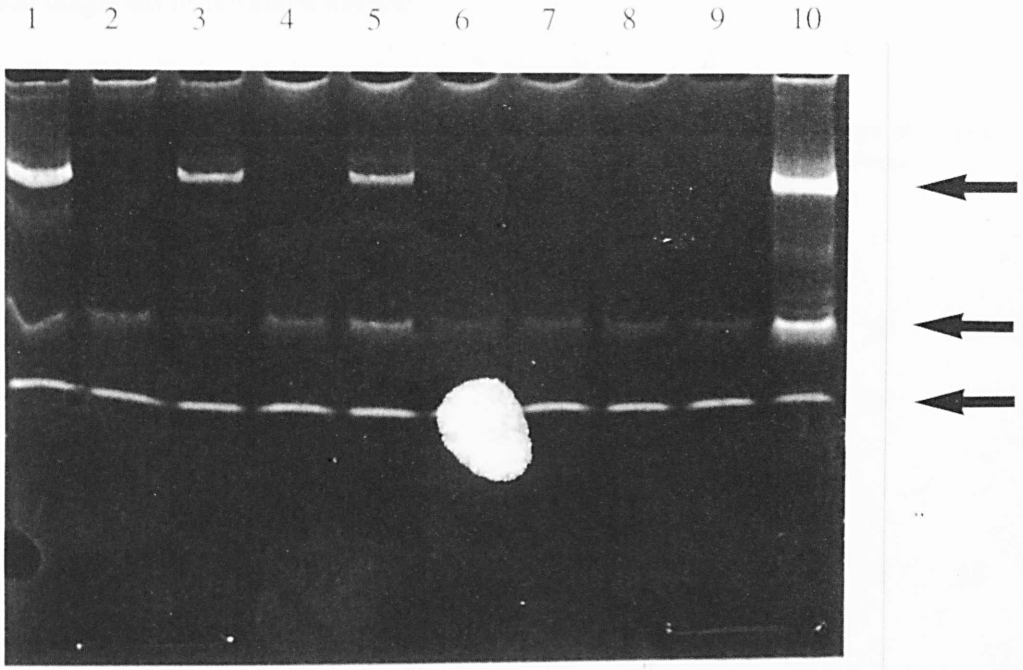


Figure 3. 2.

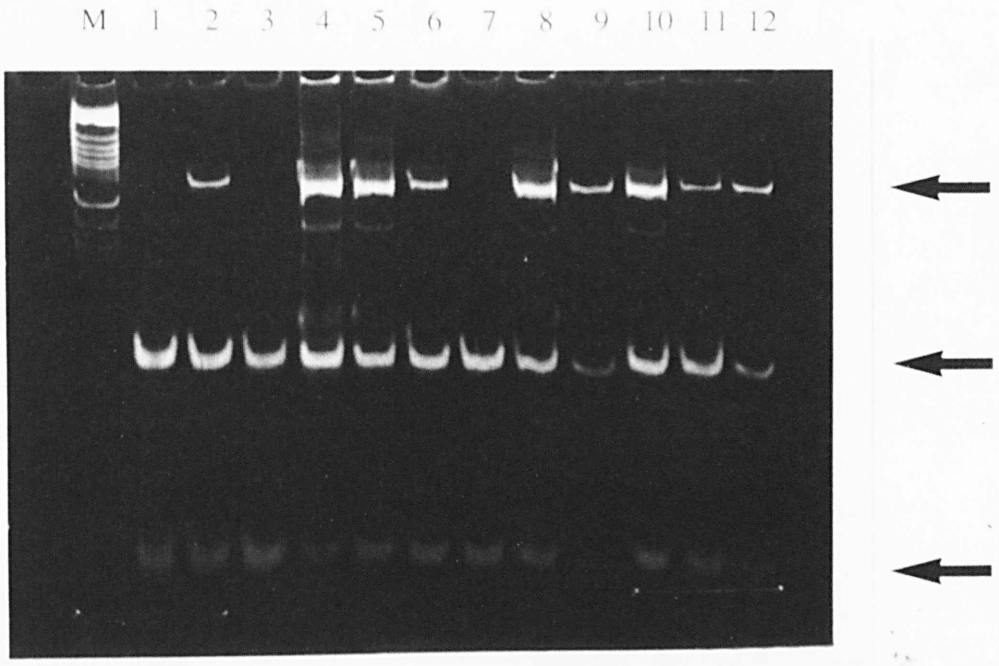


Table 3.3. Sexing biopsied blastomeres by PCR using 2 Y-specific sequences for preimplantation diagnosis of X-linked disease.

Pat.	Sequence	No. embryos	No. cells	Males	Females	Not sexed	No. transferred	Pregn.	Sex of Fetus	Del.
A1	DYZ1	2	2	1	1		1	Bio		
B1								NT		
C1	DYZ1	5	5	2	3		2	NP		
D1	DYZ1	5	5	2	3		2	NP		
B2	DYZ1	7	7	4	2	1 *	2	P	2F	2F
E1	DYZ1	3	3	1	2		1	NP		
A2	DYZ1	5	5	2	3		2	P	2F	2F
C2	DYZ1	3	3	1	1	1 **	1	NP		
F1								NT		
D2	DYZ1	3	3	1	1	1 **	1	NP		
E2	DYZ1	6	9	3	2	1 @	2	NP		
C3	DYZ1	5	8	3	2		2	P	1F	1F
	Average	4.4±0.5	5±0.7	2±0.3	2±0.3	0.4	1.6±0.1			
	Total	44	50	20	20	4	16			
G1								NT		
H1	Y ALPHOIDS	6	7	2	4		2	P	1M	Aborted
I1	Y ALPHOIDS	1	1	1	0			NT		
E3								NT		
J1	Y ALPHOIDS	2	2	1	1		1	P	1F	1F
K1	Y ALPHOIDS	4	4	0	3	1 *	2	NP		
L1	Y ALPHOIDS	8	11	PCR/n	PCR/n		0	NT		
I2	Y ALPHOIDS	6	8	5	1		1	NP		
M1	Y ALPHOIDS	6	12	1	4	1 @	2	NP		
	Average	4.7±0.9	6.4±1.6	1.4±0.7	2±0.7	0.3	1.6±0.3			
	Total	33	45	10	14	2	8			
	Grand Total	77	95	30	34	6	24			
	Grand Average	4.5±0.5	5.6±0.8	1.7±0.3	2±0.3	0.4	1.5±0.2			

*: multiple bands
**: +/-
@: 1 cell male, 1 cell female
NT: no embryo transfer
P: pregnant
NP: non-pregnant
Preg.: Pregnancies

NT: not transferred
P.: Patient
±: standard error of the mean
Biochemical pregnancy
± standard error of the mean
Del.: Delivered

Table 3.4. Outcome after analysis of 1 or 2 cells per embryo.

	1 cell analysed	2 cells analysed
Single ET	5	1
Double ET	5	4
Non-transferred	1	1
Total No of cycles	11	6
No of embryos	57	20
No of embryos transferred	15	9
Pregnancies	3	2

E T: embryo transfer

3.3.3. Confirmation of the diagnosis by amplification of biopsied embryos

With the patients informed consent, those biopsied embryos in which the sex has been identified by biopsy and amplification of a single cell and were not selected for transfer, were used to confirm the assignment of sex (Table 3.5.). The morphological development of most of the embryos that were not selected for transfer was observed each day until they were tested. An exception to this involved a carrier for Lesch-Nyhan syndrome (D in Table 3.5.). In this case the embryos were used for measurements of HPRT activity (see Chapter 4). The sex of 34 biopsied embryos that were not transferred was checked by DNA amplification of the disaggregated embryonic cells or the whole embryo itself. Thirty three embryos (18 males and 15 females) analysed this way demonstrated that the sex predicted by single cell amplification was correct (Table 3.6.). In one case, an embryo that was shown to be male by single cell amplification, showed no amplification when the whole biopsied embryo was amplified (Table 3.6.). Additional confirmation of the diagnosis in seven of the embryos that were transferred and were implanted by CVS and cytogenetic analysis, showed 6 females and 1 male. Overall, out of 41 embryos sexed by single cell PCR (their sex has been confirmed by DNA amplification or cytogenetic analysis, for the pregnancies) in one case one male embryo was misdiagnosed as female and in another case a female embryo showed Y-specific amplification in a single biopsied cell.

Table 3.5. Confirmation of the assignment of sex of biopsied embryos

No	Patient	No. embryos	Males	Females	Not sexed	No. transferred	Not-transferred embryos confirmed by PCR
1	A1	2	1	1		1	1M
2	B1						
3	C1	5	2	3		2	2F, 1M
4	D1	5	2	3		2	HPRT measurements
5	B2	7	4	2	1 *	2	5M
6	E1	3	1	2		1	1F, 1M
7	A2	5	2	3		2	1F, 2M
8	C2	3	1	1	1 **	1	1F, 1M
9	F1						
10	D2	3	1	1	1 **	1	1M
11	E2	6	3	2	1 @	2	1F, 2M
12	C3	5	3	2		2	
13	G1						
14	H1	6	2	4		2	2F, 1M
15	I1	1	1	0			
16	E3						
17	J1	2	1	1		1	1M
18	K1	4	0	3	1 *	2	
19	L1	8	PCR/n	PCR/n		0	
20	I2	6	5	1		1	4F, 1M
21	M1	6	1	4	1 @	2	3F, 1M

*: multiple bands
**: +/-
@: 1 cell male, 1 cell female
PCR/n: PCR failed to give result

Table 3.6. Confirmation of diagnosis

	Male embryos	Female embryos	Males misdiagnosed as females	Females misdiagnosed as males
Surplus embryos	18	15	0	1
Pregnancies	0	6	1	0

3.3.4. Pregnancy outcome and confirmation of sex by CVS

Out of seventeen cycles that reached the stage for preimplantation diagnosis of sex from biopsied blastomeres, in fifteen cases one or two embryos were transferred. In one case that no embryos were transferred, it was because the only embryo available for sexing was shown to be male (I1 in Table 3.3.) and in another case PCR failed to give results both in the biopsied blastomeres and the controls, due to technical failure (L1 in Table 3.3.). In 6 cycles one embryo was transferred and in nine cycles, two embryos (Table 3.3., 3.4.). Five women became pregnant, two with twins and three with singleton pregnancies. One and four pregnancies occurred after one and two embryos were transferred respectively. Pregnancy rates were 23.8% per egg collection (EC), 33.3% per embryo transfer (ET), 38.4% per patient. When one embryo was transferred, pregnancy rates were 16.6% and when two embryos were transferred, 44.4% (Table 3.7.).

All patients had CVS to confirm the identification of the sex. All fetuses were female, except in one singleton pregnancy that CVS and cytogenetic analysis showed a male fetus (H1 in Table 3.3.). The disease in question was type II hereditary

sensory motor neurone disease, for which no specific diagnosis is available. The parents chose to terminate the potentially affected pregnancy. Four other embryos from this patient which were not transferred, were cultured for three days after the biopsy (up to day 5), to assess their development. One reached the blastocyst stage and then collapsed, two embryos showed fragmentation and did not show cavitation and one had several small cavities. On day 5, the sex of the embryos was examined by PCR. The sex was confirmed (two males and two females) as predicted by PCR. In one male embryo 2 cells were biopsied and the Y specific fragment was clearly detectable in both.

The twin and the two singleton pregnancies have gone to term. The second of one set of the twins (patient B2 in Table 3.3.) was stillborn, probably due to intrapartum anoxia. Post-mortem examination did not reveal any gross abnormality in the stillborn baby girl. All the other children born are normal girls.

Figure 3.3.

Preimplantation diagnosis of X-linked disease by sexing: The misdiagnosis case (H1 at Table 3.3.). M: molecular weight markers (pBR322 Hae III digest). Lanes 1 & 2: 2 and 20 pg of male DNA. Lanes 3 & 4: negative controls (blanks). Lanes 5 & 6: amplification product from single blastomeres. The Y-specific band in lanes 6, 7, & 8 indicate male embryos. In lanes 7 & 8, blastomeres that were biopsied from the same embryo are amplified. In lanes 5, 9, 10, & 11, no evidence of amplification product indicate female embryos. The single blastomere analysed on lane 9 belonged to the embryo that when transferred resulted in a male pregnancy.

Lane 7: the extra band below the Y-specific band is propably the result of non-specific amplification.

Figure 3. 3.

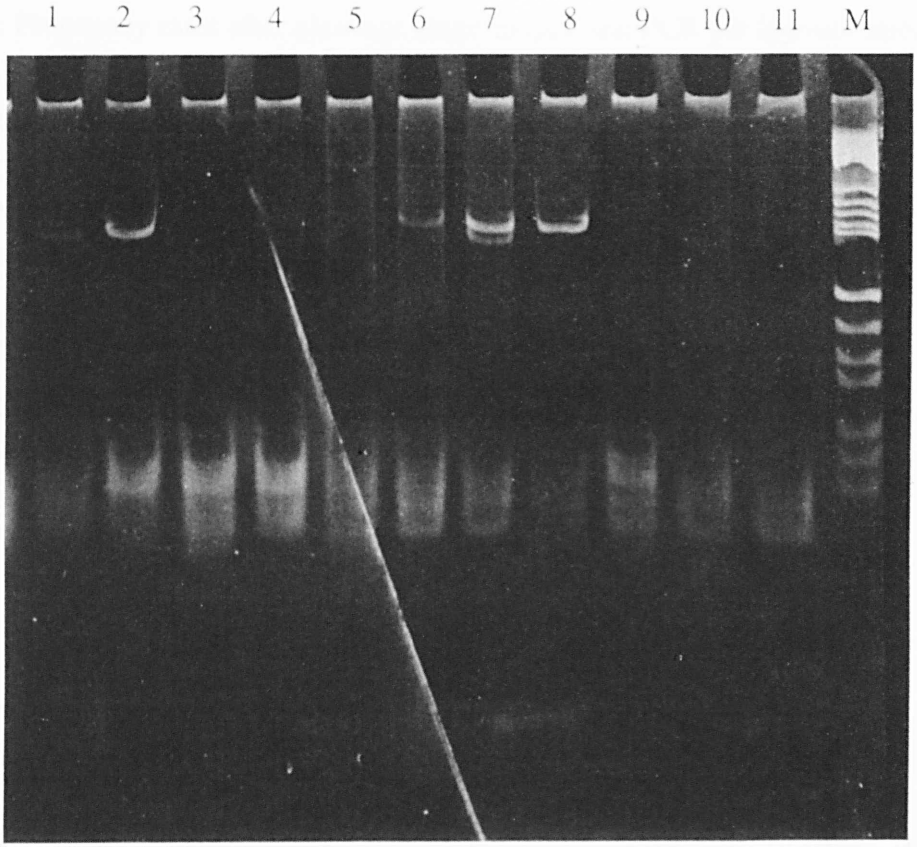


Table 3.7.: Pregnancy rates after cleavage stage biopsy and PCR for identification of sex.

	Pregnancy rates
per egg collection	5 / 21 (23.8%)
per embryo transfer (ET)	5 / 15 (33.3%)
per patient	5 / 13 (38.4%)

	Pregnancy rates
1 embryo transferred / ET	1 / 6 (16.6%)
2 embryos transferred / ET	4 / 9 (44.4%)

3.4. Conclusions

Out of the 13 couples at risk of X-linked conditions that have been treated, six of the women were carriers for Duchenne muscular dystrophy, 3 for X-linked mental retardation (non-fragile X type) and 1 for adrenoleukodystrophy, Lesch-Nyhan syndrome, type II hereditary sensory motor neurone disease and retinitis pigmentosa (Table 3.1.).

The couples had IVF treatment using standard protocols. Of the oocytes collected, 57.2% fertilized. Although the number is small to compare, the fertilization rate was equivalent to the rate after IVF during this period (Hardy, 1993). Nineteen cases went through biopsy. In two cases only one oocyte was fertilized and was arrested at the 4-cell stage on day 3 and in one case no fertilization occurred. In those two cases biopsy was not attempted, due to poor quality or unavailability of the embryos. All normally fertilized embryos that developed to the six to ten-cell stage on day 3 went through biopsy. From 57 embryos, 1 cell has been analysed and from 20 embryos 2 cells. The majority of the blastomeres that have been successfully biopsied were tested by PCR. The high efficiency of the assay is very important, as it ensures that at least two embryos are available for transfer and it was shown that the pregnancy rates after transfer of two embryos is much higher than after a single embryo transfer (Table 3.7.). Out of 78 embryos that were successfully biopsied, 77 were tested by PCR (Table 3.3.). The major loss of single blastomeres happened during biopsy, as 46 out of 147 cells were lysed (Table 3.2.). Out of 86 embryos biopsied, 12 were excluded from analysis because of lysis of the blastomeres during biopsy. In the rest of the unsuccessfully biopsied cells, at least 1 cell was biopsied successfully (Table 3.2.). In one case (F1), 3 blastomeres were biopsied out of 2 embryos. All those 3 lysed and no blastomeres were available for analysis.

Seventeen out of 21(80.98 %) cases reached the stage for analysis of the biopsied blastomeres. In the 10 first cycles, sexing has been done by amplification of the DYZ1 sequence and in the next 7 cases, the Y-alphoid repeat. The numbers of male and female embryos that were identified was similar (Table 3.3.). Analysis of the biopsied blastomere was prevented in 6 cases (Table 3.3.) and these embryos were excluded from transfer. When the embryos from which these cells were biopsied were examined by PCR, the two cells that when tested showed a ladder and the two cells that had faint amplification, were from female embryos. The number of embryos transferred was the same in both groups (1.6 average).

The pregnancy rate per embryo transfer was 33.3% (Table 3.7.). This was similar in comparison with the infertile patients that went through IVF the same period (Hardy, 1993). This is the first time that fertile couples have had IVF and that IVF was used as an aid for prenatal diagnosis instead of infertility. Although the vast majority of the patients were fertile, the success rates are not greatly improved. Possibly this is an indication that the success rates of IVF reflect the natural situation and that the losses after natural conceptions are high, probably due to embryo abnormalities.

With the patients' informed consent, those biopsied embryos in which the sex had been identified by biopsy and amplification of a single cell and were not selected for transfer, were used to confirm the diagnosis. The development of most of the embryos that were not selected for transfer was assessed morphologically each day until they were amplified. No conclusive data on the number of embryos that reached the blastocyst stage from the non-transferred biopsied embryos can be obtained , as the number is very small and most of the embryos were disaggregated at cleavage stages to confirm the sex (Table 3.5.). In one case, the patient was a carrier for Lesch-Nyhan syndrome (D in Table 4.1.) and the embryos were used for measurements of the HPRT activity (see Chapter 6). Overall, out of 41 embryos, that had their sex confirmed by PCR (for the non-transferred ones) or cytogenetic analysis (for the pregnancies) in one

case one male embryo was misdiagnosed as female and in another case a female embryo showed Y-specific amplification in a single biopsied cell. The percentage of misdiagnosis that was found from single blastomere amplification is higher (see Chapter 4). A reason for the smaller misdiagnosis rate found in the preimplantation diagnosis cases is probably that 2 cells have been biopsied from a number of embryos. In two cases (E2 and M1 in Table 3.3.), two cells biopsied from the same embryo gave opposite results. If only one cell was analysed for the assignment of sex, two more misdiagnosis would have occured, and the overall figure would be 4 misdiagnosed embryos out of 41 (10%). Therefore biopsy and analysis of two cells from an individual embryo is preferable to a single blastomere analysis. Also, the sex was confirmed in many cases from amplification of the rest of the biopsied embryo. When the whole embryo was amplified, amplification failure from one blastomere would not be detected.

The misdiagnosis of sex in one of the seven cases (H1) highlights the problems that might arise from the identification of the sex of the embryo on the basis of the presence or absence of an amplified fragment only. In the treatment cycle in which the misidentification occurred, with each of the four remaining biopsied embryos not transferred, the predicted sex was confirmed by amplification from the biopsied embryos at a later stage. This indicates that the problem was an isolated amplification failure rather than a more general technical failure.

The problem of misdiagnosis caused by contamination with male DNA or cells is not as serious in preimplantation diagnosis as it is in prenatal. When prenatal sexing of the fetus is performed for the prevention of X-linked disease during pregnancy, misidentification of a female fetus for a male might lead to an abortion of a healthy fetus. During preimplantation diagnosis, mistaking a female fetus for a male does not cause a clinical misdiagnosis but it would prevent the transfer of a unaffected female fetus. If the number of female embryos during that cycle is less than two or if

the morphology of the remaining embryos does not permit the transfer of two, then the chances of pregnancy can be seriously reduced.

The reasons for amplification failure from single cells are not known. They may include problems with sample preparation, for example, failure to transfer the cell, inefficient cell lysis, degradation or loss of the target sequence and/or problems associated with the polymerase chain reaction (PCR). In addition, with blastomeres isolated from human cleavage stage embryos, sex chromosome mosaicism has been reported (Griffin *et al.*, 1993). Also, Hardy *et al* (1991) report that 5% of cleavage stage blastomeres from embryos with good morphology and 15% with poor morphology have no nucleus. Anucleate cytoplasmic fragments are often large enough to be mistaken for normal blastomeres. For diagnoses that depend on the presence of absence of an amplified fragment, amplification of an anucleate fragment could result in misdiagnosis. For the selection of female embryos for transfer in couples at risk of having children with an X-linked recessive disease, a reliable method for identifying the sex is essential since males have a 50% chance of being affected. Also, in cases where diagnosis is dependent on the detection of an allele after amplification, biopsy and attempted analysis of an anucleate fragment can reduce the number of embryos that are available for transfer. In this study, however, each blastomere was carefully examined and only those with clearly visible nuclei were selected for amplification. Also, the transfer of each blastomere into the distilled water or lysis buffer was confirmed by direct observation.

The molecular basis of X-linked mental retardation and adrenoleukodystrophy had not been identified at the time that these diagnoses were performed. The only option available to the parents at risk of X-linked disease was sexing of the fetus. For adrenoleukodystrophy, an enzyme assay is available for prenatal diagnosis. However, this could not be used at preimplantation stages, as no information about the timing of its expression exists. Although the patients examined here are at risk for mental retardation (the non-fragile-X type), the possibility of offering preimplantation

diagnosis in a fragile-X carrier poses some interesting questions. For the fragile-X syndrome, sexing would not guarantee an unaffected child, as, in general, one third of the female carriers have clinical findings similar to affected hemizygous males. Before the gene was found, prenatal diagnosis was possible using cytogenetic analysis to identify the fragile-X site. However, cytogenetic analysis and identification of the fragile site would not be possible for preimplantation diagnosis, as only a percentage of the cells of the affected individuals show the fragile site and that percentage varies from one individual to the other. More recently, DNA linkage analysis combined with cytogenetic techniques permit a more reliable prenatal diagnosis and carrier detection (Opitz, 1991). Lately, the gene causing fragile-X syndrome has been cloned and sequenced and specific diagnosis is now available for affected and unaffected males and carrier females (Yu *et al*, 1991). The gene contains a number of a CGG repeats, which varies from about 30 in normal individuals to over 200 in affected ones (Fu *et al*, 1991; see also 1.2.5.3. in Chapter 1). Despite the fact that the gene is known and a specific DNA diagnosis is now available, identification of the fragile-X affected preimplantation embryos is impaired by two factors. Firstly, although the detection of the mutation is possible with Southern blot analysis and PCR, it has been very difficult to detect the fully expanded mutation using a PCR assay (Fu *et al*, 1991; Percolizi *et al*, 1993). It would be impossible to detect the mutation when only one or two cells are available using other methods than PCR. Secondly, it has been suggested that the expansion of the repeat happens either during oogenesis or during preimplantation development (Anvret *et al*, 1993). Therefore, the timing of the mutation event has to be established before any attempts to diagnose it during preimplantation stages. Now that a specific diagnosis is available, couples at risk of a fragile-X affected child may be offered the choice between specific prenatal diagnosis, with the risk of termination if the fetus is affected or preimplantation diagnosis and transfer of females, with a possibility that the child could be affected. The likelihood of having an affected child in couples at risk of fragile-X syndrome varies among families and has to be calculated for each case separately by a geneticist. The acceptability of the method depends on the parents; if their reproductive history shows a number of

terminations after specific diagnosis, they might chose preimplantation diagnosis, even if there is a chance to have an affected child. Also, if the disease is very severe, the parents may want to avoid at any cost the risk of having an affected child and in this case it is more likely that they are going to opt for prenatal diagnosis.

In some of the X-linked conditions, for example Lesch-Nyhan syndrome, the biochemical basis of the disease is known and prenatal diagnosis is possible using an enzyme assay. The possibility to perform preimplantation diagnosis for HPRT deficiency by an enzyme assay is examined in Chapter 6 and also by other investigators (Braude *et al*, 1988). Preimplantation diagnosis by measurement of HPRT activity would be feasible in the human only if HPRT can be assayed at a time that the remaining oocyte activity is insignificant compared with the embryo-coded activity. Data presented in Chapter 6 suggests that during cleavage stages, the activity measured is coded by the maternal genome and therefore diagnosis using a biochemical assay is not possible. For the case of HPRT deficiency, not only the biochemical basis of the disease is known, but the HPRT gene is found and characterised. Therefore, preimplantation diagnosis would be possible by DNA amplification. However, developing PCR assays for the specific diagnosis of X-linked disease would be more difficult and quality control studies would have to be undertaken for each individual disease. For these reasons, the initial step for preimplantation diagnosis has been the identification of the sex of the embryos, as this is a method which is applicable to all X-linked recessive diseases, irrespective of the specific genetic defect.

With unique sequences, only two copies of which are present in a diploid cell, loss or damage of the target sequence is a possible cause of amplification failure. With the Y alphoid repeat sequence which is present in about 1000 copies in a single male diploid cell (Wolfe *et al*, 1985), however, this seems unlikely. As amplification failure can cause misdiagnosis, it is essential that the incidence of failures in single blastomeres is assessed. Also further work is necessary to study methods to control amplification failure and to improve efficiency. In Chapter 4, the reliability of

DNA amplification of repeat Y-specific sequences in single blastomeres and methods to control and to improve amplification efficiency are examined.

Chapter 4

Amplification efficiency for identification of the sex of preimplantation embryos by Y-specific DNA amplification

4.1. Introduction

4.1.1. DNA amplification by PCR

The simplest approach to identify the sex of an embryo by PCR is to amplify a sequence specific for the Y chromosome. For most routine applications of PCR for prenatal diagnosis, samples contain DNA that has been purified from about $5x10^5$-$1x10^6$ cells (1-2 μg). In this case, not all of the 1-2 million copies of the target sequence are required to be amplified during every PCR cycle for an accurate diagnosis. However, the analysis by PCR of a single cell is a completely different issue. In this case only 2 copies of the target sequence are present in the cell (only 1 copy for a Y-specific sequence). Therefore very high sensitivity and efficiency of amplification are required for the generation of a specific fragment at detectable levels. Also, if a cell is heterozygous for a gene, both alleles have to be amplified. Unique sequences have been amplified by PCR from single cells including single sperm and fibroblasts (Li *et al,* 1988), unfertilized eggs (Coutelle *et al*, 1989) and single blastomeres (Holding & Monk, 1989). Amplification of unique sequences has two disadvantages; i) nested amplification is needed, to generate enough product to be detected on a gel after electrophoresis and staining and ii) in the unique sequences that have been examined, amplification fails in 10-30% of the cases (Li *et al*, 1991; Boehnke *et al,* 1989; Holding & Monk, 1989).

With Y-specific amplification, an embryo is identified as male if the Y-sequence is present and as female if it is absent. Therefore, amplification failure in a single biopsied blastomere could result in a misidentification of a male embryo as female and in the transfer of a potentially affected embryo. Because of the levels of amplification failure of unique sequences in single cells, amplification of repeated

sequences specific for the sex chromosomes was attempted for preimplantation diagnosis, to increase the number of target sequences present in single or small number of cells. Handyside *et al* (1989) examined this possibility to identify the sex of human preimplantation embryos by DNA amplification of biopsied blastomeres and in 15 embryos the sex (identified in the whole embryo by ISH) was as predicted by single cell PCR. The main problem that has been addressed in this study was that of contamination. In the clinical trial described in Chapter 3, however, another problem became apparent: that of amplification failure. Although PCR failure from single cells was between 15-20% when unique sequences were amplified, it was thought that the selection of a repeated sequence to be amplified would increase the rate of successful amplification at the single cell level. From the data presented in Chapter 3, it was shown that amplification fails even when repeated sequences are amplified. There was no study in the literature at the time about amplification of repeated sequences from single cells. In the work of Handyside *et al* (1989), the number of embryos that was examined was small and among them a few males were identified. Therefore the problem of amplification failure could not have been detected.

The results of the clinical trial suggested that single blastomere PCR of repeated sequences can be used to sex preimplantation human embryos. However, they demonstrate the need for further experiments to assess the reliability and the reproducibility of DNA amplification in single blastomeres.

The PCR technique is so versatile and the range of its applications so wide that no single protocol is appropriate for all situations. Consequently each PCR application requires optimisation. Optimisation of PCR involves testing a number of variables, such as the temperatures and timing for denaturation, annealing of the primers and synthesis of the DNA fragments, the concentration of enzyme, dNTPs, primers, target DNA, the different types of buffers used and the magnesium concentration. Although theoretical models for the calculation of the different

parameters (such as the annealing temperature) exist, practical testing is necessary to determine the ideal conditions.

Multiplex PCR with an additional set of primers has been suggested as an internal control for amplification failure (Chamberlain *et al,* 1990). The possibility of co-amplification of X- and Y-specific sequences in single blastomeres was investigated.

The sequences that have been selected for single cell amplification are DYZ1, Y-alphoid and X-alphoid repeats. The DYZ1 and Y-alphoid repeat have been described in Chapter 3. For co-amplification along with the Y-specific sequences, a repeated DNA family on the pericentromeric region of the X-chromosome was used. The X-alphoid DNA consists of a repeat of 2.0kb BamHI fragments. The 2.0kb repeat consists of subunits of 170-171bp. The individual subunits are 65-80% identical in sequence to each other (also see Chapter 2). There are about 6,000 copies of the 2.0kb repeat in the centromeric region of the X-chromosome, and so the X-alphoid sequences represent 5-10% of the X-chromosome (Waye & Willard, 1985). The monomers of ~170bp are amplified, resulting in ~60,000-70,000 target sequences. The length of the amplified fragment is 130bp.

4.1.2. Aims

To investigate the incidence of amplification failure with the DYZ1 sequence, PCR will be attempted in single blastomeres from disaggregated human embryos. Also the possibility of amplification of a pericentromeric sequence (Y-alphoids) to the single cell level was attempted. The efficiency and reliability of DNA amplification by PCR of the DYZ1 and Y-alphoid sequences in single blastomeres was investigated. To control for amplification failure and to improve the rate of successful amplification, different methods, such as co-amplification of X- and Y-specific sequences and improvement of blastomere lysis will be attempted.

4.2. Methods

4.2.1. Disaggregation of individual blastomeres

Whole embryos were disaggregated after the removal of the zona pellucida with acid Tyrodes by repeated pipetting of the embryo using a pulled and polished pipette until the blastomeres were separated. Blastomeres were washed and prepared as described on 4.2.3.

4.2.2. Y-chromosome specific repeat amplification from DNA dilutions.

The conditions for PCR were optimised using dilutions of male and female DNA at amounts of 200ng, 20ng, 2ng, 200pg, 20pg and 2pg, according to each experiment. For control blanks, distilled water was used instead of DNA. As positive and negative controls, 20ng, 200pg and 2pg male and female DNA were used (DNA was extracted from blood, as described in 2.4.) and diluted in distilled water. The biopsied blastomeres were washed once or twice in distilled water and the washdrop used as a negative control. For experiments using lysis buffer, the DNA controls were diluted in neutralised lysis buffer .

4.2.3. Efficiency of DNA amplification from single blastomeres

To investigate the incidence of amplification failure from single cells, individual embryos were disaggregated and each or several cells were amplified with

the Y-specific primers (DYZ1 or Y-alphoid). The blastomeres were first examined for nuclei using phase contrast optics and were vitally labelled with Hoechst 33342 (Sigma), as described in Chapter 2 (2.2.8.). The blastomeres were put in the tubes for PCR under direct vision using a dissecting microscope. Only cells with one normal nucleus were included in the study.

4.2.5. Co-amplification of X- and Y-specific sequences.

For co-amplification of the X-alphoids with the DYZ1 or the Y-alphoids, a PCR buffer was used that was based on one described by Kogan *et al* (1987) and Chamberlain *et al* (1990) (see Chapter 2). The buffer was modified to omit EDTA (to give greater control over the magnesium concentration) and has different levels of $MgCl_2$. The PCR reaction was as described in Chapter 2 for the amplification of a single sequence, the only difference being the concentration of primers, which was 0.75 μM each.

4.2.6. Preparation and lysis methods for single blastomeres

Two methods were used for preparing samples for PCR :

1) Cells with single nuclei were washed twice in PCR buffer and transferred to 0.5 ml Eppendorf tubes loaded with 10 μl distilled water under a dissecting microscope which allows observation of the transfer of the cell into the tube using drawn out capillary tubing. Tubes loaded with water were kept at 4°C until immediately before use to minimise condensation on the sides of the tubes and help ensure the cell is placed at the bottom of the tube. The caps on the tubes were then closed and left at room temperature for 10 minutes to allow the cells to lyse before freezing and thawing twice to -20°C. The tubes were spun for 20 seconds and then kept frozen at -70°C or transferred to the thermal cycler.

2) The second method was similar to the first one, except that single cells were transferred to 0.5 ml Eppendorf tubes loaded with 5 µl lysis buffer (200 mM KOH, 50 mM DTT). KOH stock was made fresh before each experiment. Each sample in 5 µl of lysis buffer was overlaid with 100 µl of filtered paraffin and incubated at 65°C for 10 min. The tubes were left for 5 min at room temperature and then 5µl of neutralisation buffer (900 mM Tris-HCl pH 8.3, 300 mM KCl, 200 mM HCl) was added to each sample. The tubes were spun for 20 seconds and then kept at -70°C or transferred to the thermal cycler.

4.2.6. Contamination

For precautions against contamination see 2.8 in Chapter 2.

4.3. Results

4.3.1. Y chromosome specific repeat amplification from DNA dilutions and single blastomeres

4.3.1.1. DYZ1 amplification

Initially, the optimal amplification conditions for single blastomeres were determined on male cultured cells in dilutions of 1,000, 100, 10 and 1 cells per sample. Later they were replaced with DNA dilutions (as described in the Methods section in this Chapter), as they are easier to prepare and store. As positive and negative controls, dilutions of male and female DNA were used. Also, for the negative controls distilled water instead of DNA was used.

The optimum number of cycles depends mainly on the number of target sequences that exist in the sample, when the other parameters have been optimised. Using 1000 cells as a starting material, a faint band was visible after 20 cycles. Detectable amplification at the single cell level was possible after 40 cycles (Fig. 4.1.).

The annealing temperature (T_a) that was used for DYZ1 amplification is 65°C, as it was the highest Ta that DYZ1 would amplify without any loss of specificity or lower yield. The yield was reduced when the annealing temperature was over 65°C. The cycling conditions used are 94°C for 30sec, 65°C for 1min 30sec for 40 cycles. The two-step amplification, with a combined annealing and extension temperature at 65°C, gave equally good results with three-step amplification (94°C for 30sec, 65°C for 30sec and 72°C for 1min for 40 cycles) probably because the time that it takes the DNA thermocycler from 65°C to reach 94°C was sufficient for the extension of the specific

fragment. The two-step program was routinely used, as it was faster. An initial denaturation of 10min at 94°C and final extension step at 72°C for 10min was used as the yield of the product was lower when the initial denaturation was omitted. Amplification was male-specific even with a Ta of 45°C, which is lower than the optimal and no difference in yield was observed. Equal yields and specificity of amplification were obtained when the magnesium concentration was ranging from 1.5-3.5mM (Fig. 4.2.) and the amount of Taq polymerase from 0.5-1.0 units per reaction. When single cells were amplified, the yield of the product was increased in 1.5μM each primer (Fig. 4.3.). The amplified fragment size was 149bp. The identity of the amplified fragment was checked by restriction digestion of the 149bp fragment by the enzyme EcoR1, which resulted in two fragments of 54 and 95 bp each.

As a method to avoid contamination, nested PCR was suggested (Jeffreys *et al*, 1988; 1990). A set of nested primers was designed that flank a fragment of 109bp. The amplification conditions for the nested primers were optimised with genomic DNA dilutions. With the outer set of primers, amplification was attempted for 5, 10 and 15 cycles. For the nested amplification, 2μl aliquot of the 100μl of the original reaction were used. The 2μl aliquot was amplified with the nested primers for 40 cycles. Optimal amplification was obtained after 10 cycles of the first PCR reaction and 40 cycles of the nested using a single cell as starting material. After 5 cycles of the first reaction, only faint bands could be visualised and after 15 cycles, contamination was frequent in the blanks. When optimal conditions were used and after 40 cycles, the product from single cell amplification was enough to be visualised in a polyacrylamide gel without the need for reamplification.

Figure 4.1.

Amplification of dilutions of DNA and half embryos after 40 cycles of amplification. Arrow: theY-specific 149 bp DYZ1 fragment. Lanes 1, 2, 3, 4: Amplification product of 1000, 100, 10 and 1 male cell. Lanes 5 & 6, 7 & 8, 9 & 10, 11 & 12, 13 & 14 show the amplification product of 5 8-cell embryos that have been halved and amplified independently. Lanes 5 & 6, 9 & 10 and 13 & 14 show no amplification, indicating a female embryo. Lanes 7, 11 & 12 showed Y-specific amplification. In lane 8, a ladder is present, probably due to multimerization of the primers.

Figure 4.2.

Amplification of a 149 bp fragment (arrow) of the DYZ1 repeat from PV145 male cells with two magnesium concentrations. M: Molecular weight markers. Lane 1 & 2: 1000 PV145 cells amplified (with 3mM magnesium). Lane 4 & 5: 1000 PV145 cells amplified (with 1.5mM magnesium). Lanes 3 & 6: Blanks. In the case of DYZ1 repeat, varying magnesium concentrations (1.5-3.0mM) had no effect on the yield of the specific fragment. The small non-specific bands appear when large amounts of DNA are amplified, but are not apparent at the single cell level.

Sizes of DNA fragments after Hae III digest of pBR 322: (bp) 587, 540, 504, 458, 434, 267, 234, 192, 184, 124, 123, 104, 89, 80, 64, 57, 51, 21, 18, 11, 8.

The same markers have been used through Chapters 3 and 4.

Figure 4.1.

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14

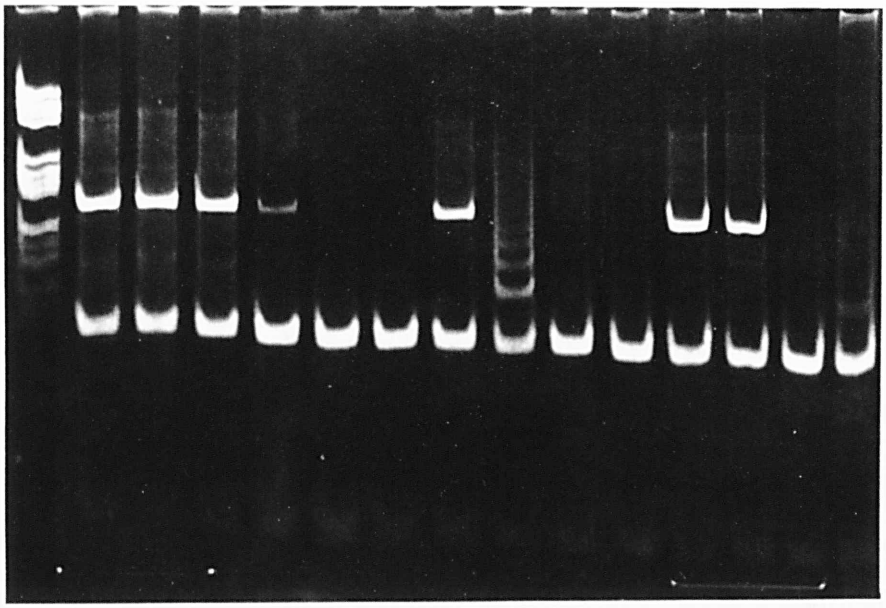
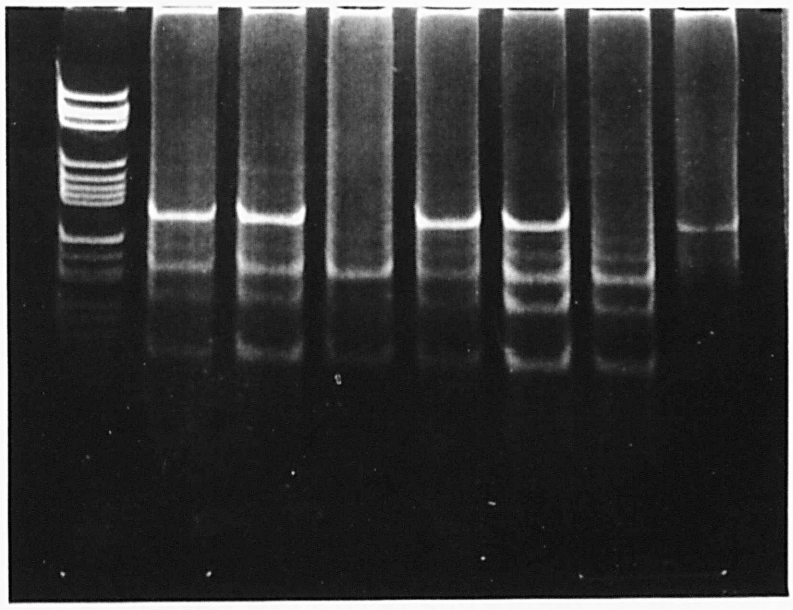


Figure 4.2.

M 1 2 3 4 5 6 7



4.3.1.2. Y alphoid repeat amplification

The optimal amplification conditions for single blastomeres were determined with DNA dilutions (as described in the Methods section). Positive and negative controls were as for the DYZ1 sequence.

The optimal T_a for Y-alphoids amplification is 55°C (Fig. 4.4.). The cycling conditions used were 94°C for 30sec, 55°C for 1min 30sec for 40 cycles. A final extension step at 72°C for 10min was used. 55°C was the highest Ta that the Y-alphoids would amplify without any loss of specificity or lower yield. The yield was reduced when the annealing temperature was over 55°C. At 60°C, weak amplification was observed. At lower temperatures than 55°C, amplification of the female DNA or cells was evident and in Ta≤45°C the amplification yield of the male DNA was equivalent with the female.

The optimal magnesium concentration was 3.6mM (Fig. 4.5.). The yield and specificity of amplification was very sensitive to the magnesium concentration, as 1mM higher or lower would produce non-specific fragments and very low amplification.

The amplified fragment size was 170bp. The identity of the amplified fragment was checked by restriction digestion as described in Chapter 2 (2.7.) (Fig. 4.6.)

4.3.2. Efficiency of DNA amplification from single blastomeres

To investigate the incidence of amplification failure from single cells, each or several cells of individual disaggregeted embryos were amplified with the DYZ1 and the Y-alphoid sequences (Fig. 4.7). Each cell was checked for the presence

Amplification of the DYZ1 repeat.

Figure 4.3.

Optimization of PCR: Effect of primers concentration. Lanes 1, 2, 3: 20 ng, 200pg and 2 pg of male DNA (0.75 μM each primer). Lanes 5, 6, 7 : 20 ng, 200pg and 2 pg of male DNA (1.5 μM each primer). Lanes 9, 10, 11 : 20 ng, 200pg and 2 pg of male DNA (3 μM each primer). Lanes 4, 8, 12: Control blanks. Top arrow: the Y-specific fragment. Middle arrow: primer-dimer. Bottom arrow: Primers. Comparison from amplification yield from 2 pg of male DNA (Lanes 3, 7 & 11) did not show any significant difference. However, primer concentration of 1.5 μM each primer (Lanes 5, 6, 7) was considered optimal.

Figure 4. 4.

Adjustment of the PCR annealing temperature to improve specificity of amplification of the Y-specific alphoid repeat. Left to right: lane 1 molecular weight markers (pBR322 Hae III digest); lanes 2, 3 20pg and lanes 4, 5 2pg male DNA amplified with an annealing temperature of 55°C and 50°C, respectively; similarly, lanes 6, 7 20pg and lanes 8, 9 2pg female DNA. Arrow indicates the 170 bp Y-specific alphoid repeat fragment. Note the incidence of non-specific amplification products (some of which may be due to sequence variation in the monomers of the repeat) with both 20 pg male DNA and especially with female DNA. Also, amplification of the specific product when the lower annealing temperature was used with female DNA.

Figure 4.3.

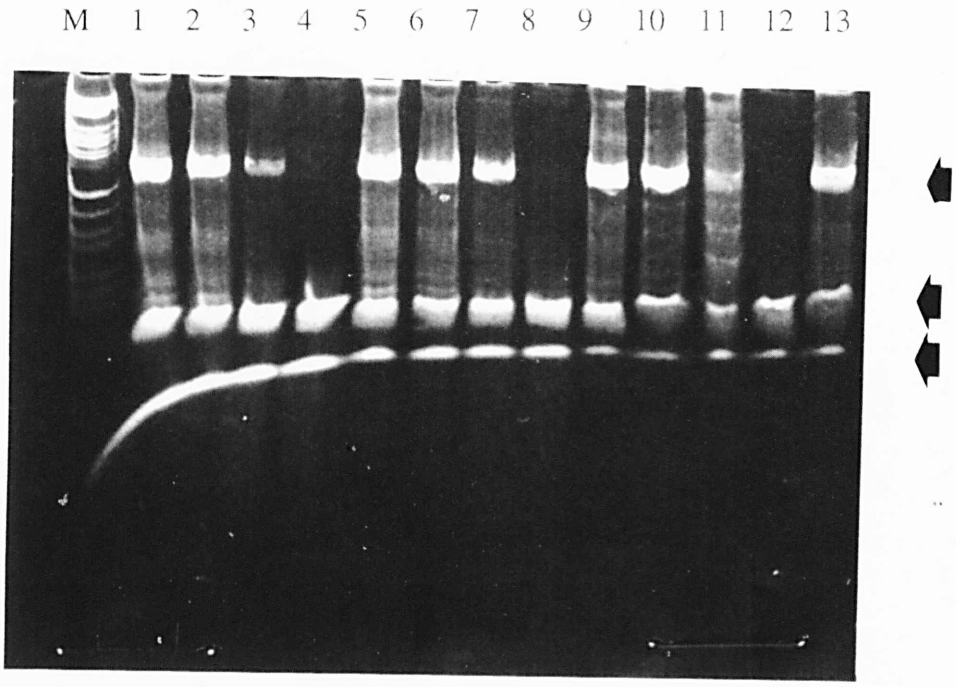


Figure 4.4.

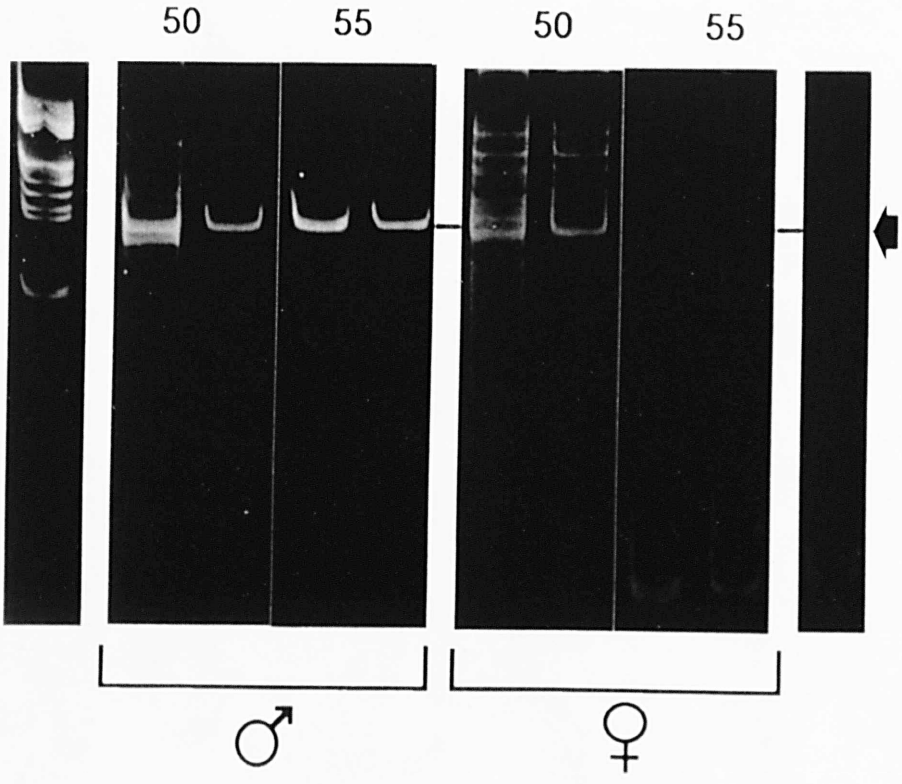


Figure 4.5.

Elimination of non-specific amplification by optimizing the magnesium concentration with 5pg of male DNA. $MgCl_2$ concentration: Lane 1: 10mM. Lanes 2 & 3: 7.2mM. Lanes 4 & 5: 3.6mM. Lanes 6 & 7: 1.8mM. Arrow indicates the 170 bp Y-specific alphoid repeat fragment. The Y-alphoid repeat is very sensitive to magnesium concentration; optimal amplification was acheived with 3.6mM magnesium (Lanes 4 & 5). Higher or lower concentrations cause non-specific amplification and lower the yield of the specific fragment (Lanes 2 &3, 6 & 7).

Figure 4.6.

Confirmation of amplification of the Y-specific alphoid repeat sequence. The amplified fragment was digested with a series of endonucleases at known restriction sites and the size of the digested fragments analysed by gel electrophoresis. Left to right: lane 1 molecular weight markers , lane 2 undigested DNA; lanes 3-7 restriction enzyme digests of the amplified fragment. The expected fragment sizes were as follows EcoR I: 170 bp (no cutting sites); Hinf I: 122, 48 bp (only the larger band visible); Nci I: 87,83 bp; Hpa II: 88, 82 bp; Dde I: 137, 33 bp (only the larger band visible). Arrow indicates the 170 bp Y-specific alphoid repeat fragment.

M: Molecular weight markers (pBR 322 Hae III digest)

Figure 4.5.

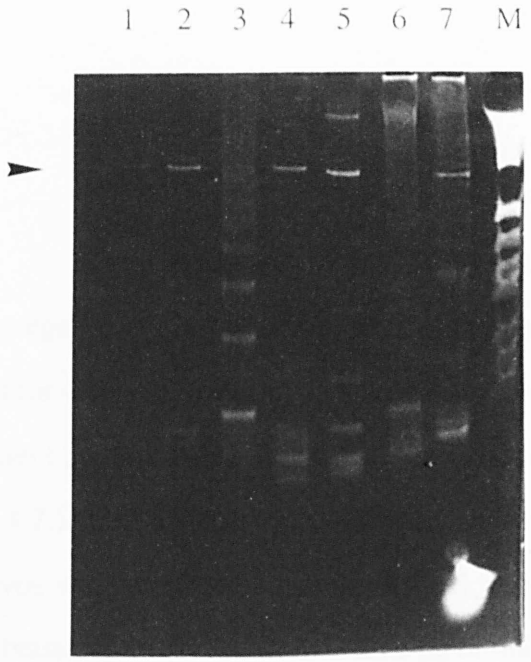
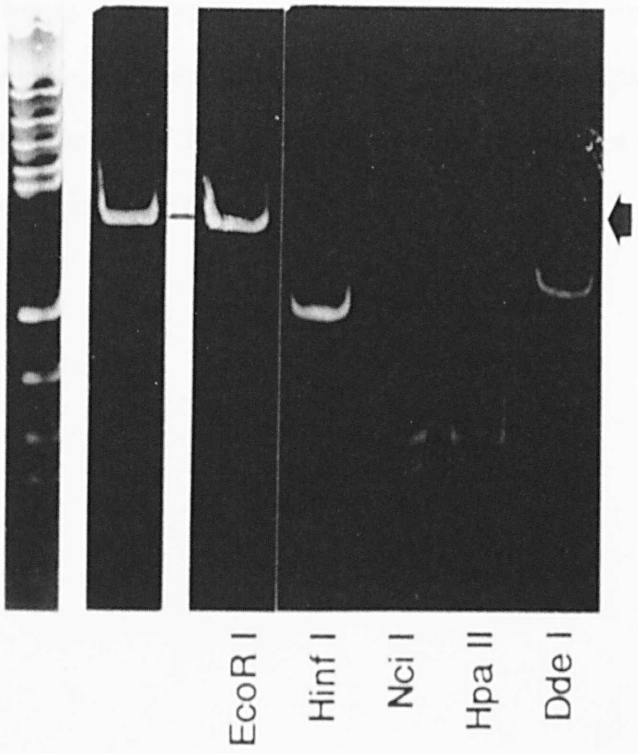


Figure 4.6.



of a single nucleus by phase contrast microscopy or by vital labelling with a DNA fluorochrome before preparation for PCR (Kontogianni *et al*, 1990; Chapter 2).

4.3.2.1. DYZ1

Amplification was attempted from a total of 114 blastomeres disaggregated from 22 normally fertilized human embryos. Amplification of the DYZ1 repeat for 40 cycles from single male blastomeres or 2pg of male genomic DNA yielded sufficient product for detection by ethidium bromide staining after gel electrophoresis (Fig. 4.7.). Fifty one blastomeres from 10 male and 63 blastomeres from 12 female embryos were analysed. Overall, amplification was achieved in 44 out of 51 (86%) male blastomeres (Table 4.1.). With 2 pg of male genomic DNA amplification failed in 1 out of 19 (5%) of cases. Amplification was observed in none of 63 (0%) female blastomeres and 1 out of 39 (2%) control medium blanks. Only 2 samples out of 114 (1.7 %) could not be analysed because of non-specific amplification. However in 10 out of 39 blanks multimerization of the primers prevented analysis (see Table 4.1.). Faint bands were present in a large proportion of female blastomeres (27%) and this prevented assignment of sex (these cells have been classified as +/-). Contamination was at the levels of 10% initially and progressively got worse. The precautions against contamination were not in full force then.

Figure 4.7. Amplification of the Y-alphoid repeat.

Cleavage embryos on day 3 post insemination were partially dissagregated after removal of the zona pellucida with acid tyrodes and 2-4 cells from each embryo were used to identify the sex by PCR. Single cells from male embryos were selected, checked for nuclei and amplified individually. Upper panel: Nine out of eighteen embryos were identified as male by the detection fo the Y-specific fragment. Lower panels: Three out of eleven single cells from six of the male embryos (correspondence indicated by the arrows) failed to amplify. Far left lanes: DNA size markers.

Figure 4.7

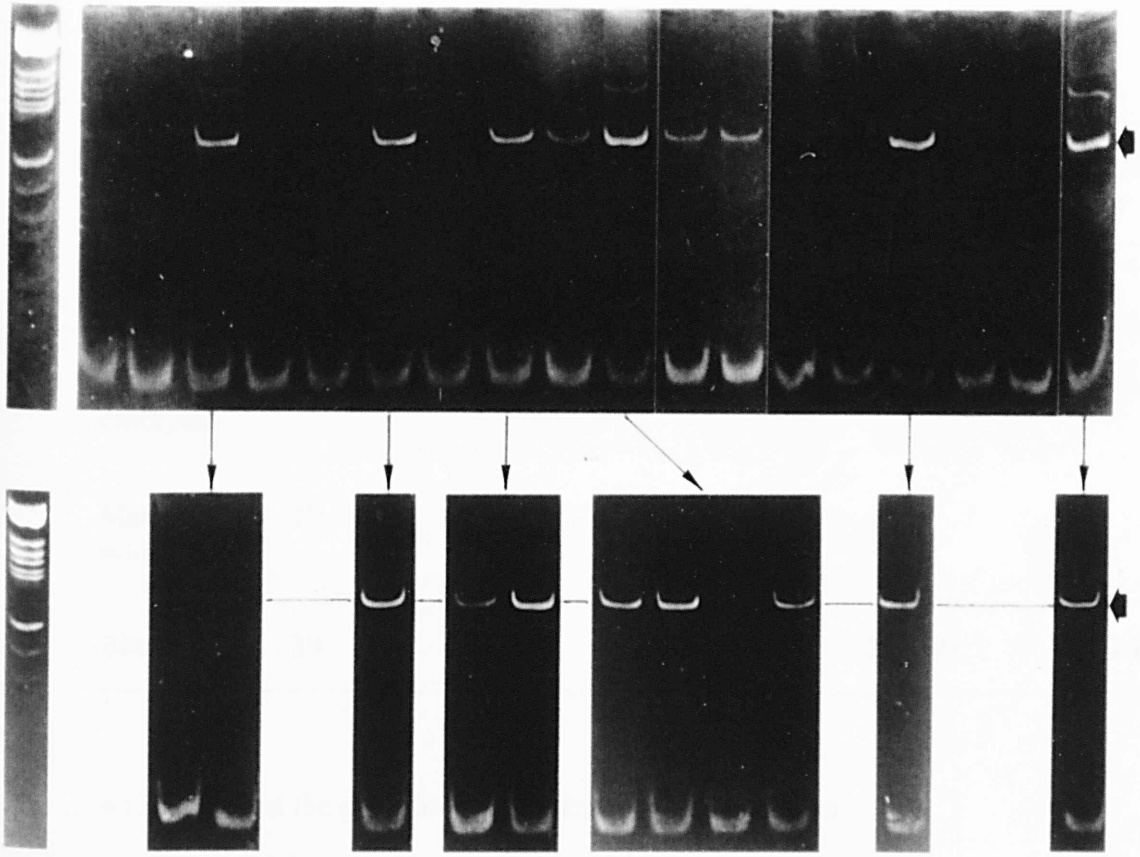


Table 4.1.: DNA amplification of single blastomeres with the DYZ1 repeat

	Single cells	+(%)	+/- (%)	- (%)	N/A(%)
Female embryos	63	0	17 (27)	44 (70)	2 (3)
Male embryos	51	44 (86)	1 (2)	6 (12)	0
Male DNA dilutions	19	17 (90)	1 (5)	1 (5)	0
Blanks	39	1 (2)	2 (5)	26 (67)	10 (26)

+ : Presence of the specific band, indicating a male embryo

- : Absence of the specific band, indicating a female embryo

+/- : Faint band, indication of contamination or not successful amplification

N/A : Non analysed blastomeres because of non-specific amplification

4.3.2.2. Y alphoid repeat

Amplification was attempted from a total of 86 blastomeres disaggregated from 16 normally fertilized human embryos. Fifty blastomeres from 9 male and 36 blastomeres from 7 female embryos were analysed. Overall, amplification was achieved in 43 out of 50 (86%) male blastomeres (Table 4.2.). With 2 pg of male genomic DNA amplification failed in 6 out of 31 (20%) of cases. Amplification was observed in 1 of 36 (3%) female blastomeres and 0 out of 31 (0%) control medium blanks. Only 2 samples out of 86 (2.3 %) could not be analysed because of non-specific amplification. However in 2 out of 60 blanks multimerization of the primers prevented analysis (Table 4.2.). Contamination was at the levels of 3% with single female blastomeres.

4.4. Improvement of amplification efficiency

The reasons for the amplification failure during PCR are not known, although several possibilities have been suggested. Several methods were tested for improvement of amplification efficiency from single cells.

4.4.1. Co-amplification of X and Y-specific repeat sequences

4.4.1.1. DYZ1 and X-alphoid repeat

For the co-amplification of the DYZ1 and X alphoid fragments, a reduced combined annealing and extension temperature at 50°C was used. The optimal Ta for amplification of X-alphoids is 60°C and for the DYZ1 is 55°C. At 65 and 60°C amplification of the DYZ1 was weak and in some cases it was barely detectable. No

amplification of the X-alphoid repeat was detectable at 65 or 60°C. A buffer which was designed for multiplex PCR (Chamberlain *et al*, 1990) was modified and used (see Chapter 2), as it was shown to improve the amplification yield with the Y-alphoid repeat. The sensitivity of amplification, especially of the X alphoid fragment, was significantly reduced. Co-amplification was possible when an 8-cell embryo was amplified, but it was not consistently successful to the level of a single cell, as an interaction between the primers inhibited or eliminated completely in some cases the amplification of the X-specific fragment, even when the ratio of the primers was changed to favor the X-specific sequence (100:1)(data not shown).

4.4.2.2. X- and Y-alphoid repeats

Co-amplification of X- and Y-specific alphoids was attempted at annealing temperatures 45° and 50°C, but at these temperatures non-specific amplification was observed and also amplification of the Y-specific fragment was acheived from female DNA. At 60°C the X-alphoid repeat was amplified but not the Y-alphoid repeat. Co-amplification of X- and Y-specific alphoids at a reduced annealing and extension temperature of 55°C was successful down to the single cell level (Kontogianni *et al,* 1991). Limited analysis of co-amplification from a single blastomere showed that each primer can fail independently in some cases (Fig. 4.9.). However, contamination of the X-alphoid primers prevented the assessment of the reliability of co-amplification. Decontamination by restriction digestion was attempted but was unsuccessful.

4.4.2. Lysis buffer

Amplification was attempted from a total of 173 blastomeres disaggregated from 39 normally fertilized human embryos (Fig. 4.10.). On the basis of

Figure 4.8.

M: molecular weight markers (pBR322 Hae III digest). Lanes 1, 2, 3 and 4: Amplification of the X-alphoid repeat with 20 ng, 200pg, 20pg and 2 pg of male DNA, respectively.

. Lane 9: Amplification of the DYZ1 repeat from 20pg of male DNA. Lanes 5, 6, 7 and 8 : Co-amplification of the X-alphoid repeat with the DYZ1 repeat with 20 ng, 200pg, 20pg and 2 pg of male DNA, respectively. Lanes 12 and 13: Blanks.

Figure 4.9.

Co-amplification of 130 and 170 bp fragments (lower and upper arrows) of X- and Y-specific alphoid repeat sequences with male DNA. Lane 1: molecular weight markers (pBR322 Hae III digest). Lanes 2 & 3: 50 and 5 pg of male DNA. Lane 4: Blank. Note the additional alphoid dimer band (approx. 340 bp) and low level amplification of the X-specific fragment from contaminant DNA in the control blank.

Figure 4.8

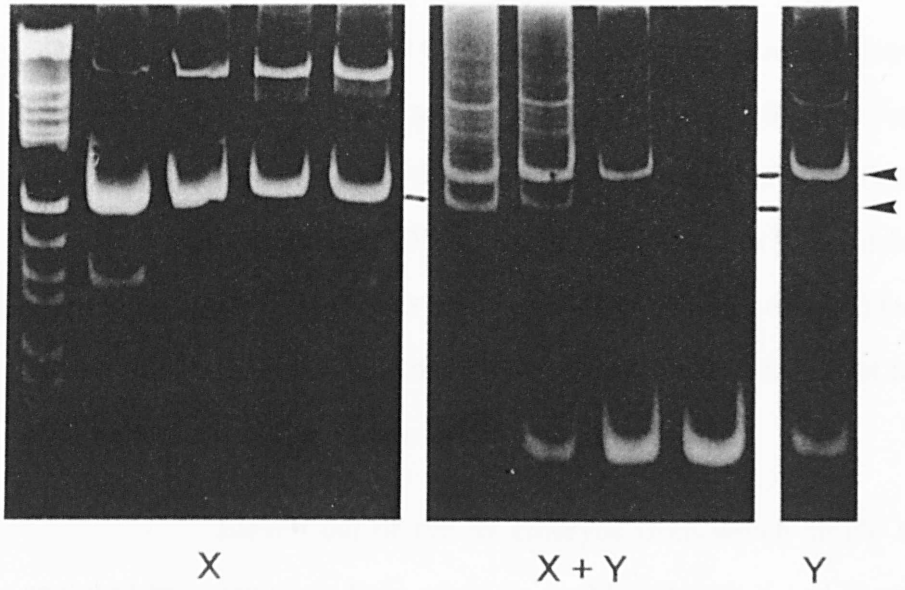
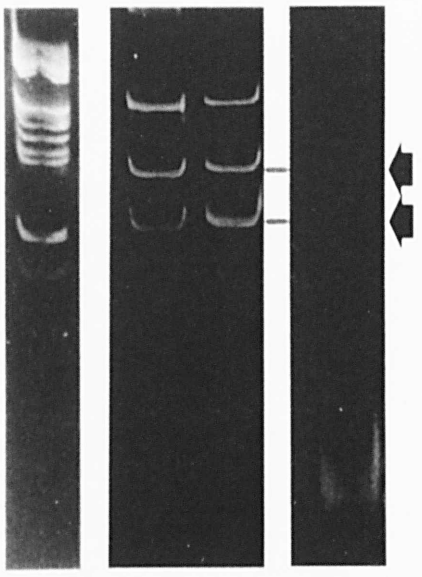


Figure 4.9.



the amplification of the Y-specific repeat fragment in the majority of the blastomeres analysed or prior sexing by biopsy and fluoresent in situ hybridization (FISH), 110 blastomeres from 25 male and 63 blastomeres from 14 female embryos were analysed. Overall, amplification was achieved in 99 out of 110 (90%) male blastomeres (including two cases in which there were only low levels of amplification) (Table 4.2.). With 2 pg of male genomic DNA amplification failed in 9 out of 54 (17%) of cases. Conversely, amplification was only observed in 5 out of 63 (8%) female blastomeres and 5 out of 93 (5%) control medium blanks. Only 3 samples out of 321 (1%) could not be analysed because of non-specific amplification.

Eleven out of the 39 embryos from which single blastomeres were analysed had previously been sexed by dual FISH with X and Y-specific primers. In each case (7 males and 4 females) the sex was confirmed by amplification of the Y-specific alphoid repeat. Comparison of the method of lysing the blastomeres prior to PCR demonstrated a marginal improvement with the two-step lysis procedure. Amplification failure from male blastomeres was reduced from 12% to 7% (Table 4.2.).

Figure 4. 10. Amplification of the Y-specific alphoid repeat from single blastomeres disaggregated from individual embryos.

Upper gel Amplified product from male embryos biopsied and sexed by dual fluorescent in situ hybridization (FISH) with X and Y-specific probes. M: Molecular weight markers (pBR322 Hae III digest); lane 1: Control medium blank; lanes 2 - 6: single blastomeres from an individual male embryo; lanes 7 - 12: single blastomeres from a second male embryo (except lane 11: a group of 4 small blastomeres). Amplification is evident in the control blank (lane 1), and conversely, only barely detectable amplification in one case (lane 7) and amplification failure in two cases (lanes 6 and 11). In the second case, it was impossible to confirm the presence of nuclei and these small blastomeres could simply have been cytoplasmic fragments. This result was therefore not included in the overall analysis.

Lower gel Amplified product from embryos of unknown sex. M: as before; lane 1: control medium blank; lanes 2 - 4: single blastomeres from an individual embryo; lane 5: control medium blank; lanes 6 - 11: single blastomeres from a second embryo; lane 12: control medium blank; lanes 13 - 14: single blastomeres from a third embryo. On the basis of the detection of the Y-specific fragment in the majority of single blastomeres, the first two embryos are presumed to be male and the third female. In this case, there was no amplification from any of the three control blanks (lanes 1,5,12) and amplification failure occured with two male blastomeres (lanes 4 and 11). One of these blastomeres (lane 4), however, was not confirmed to have a nucleus.

Figure 4.10.

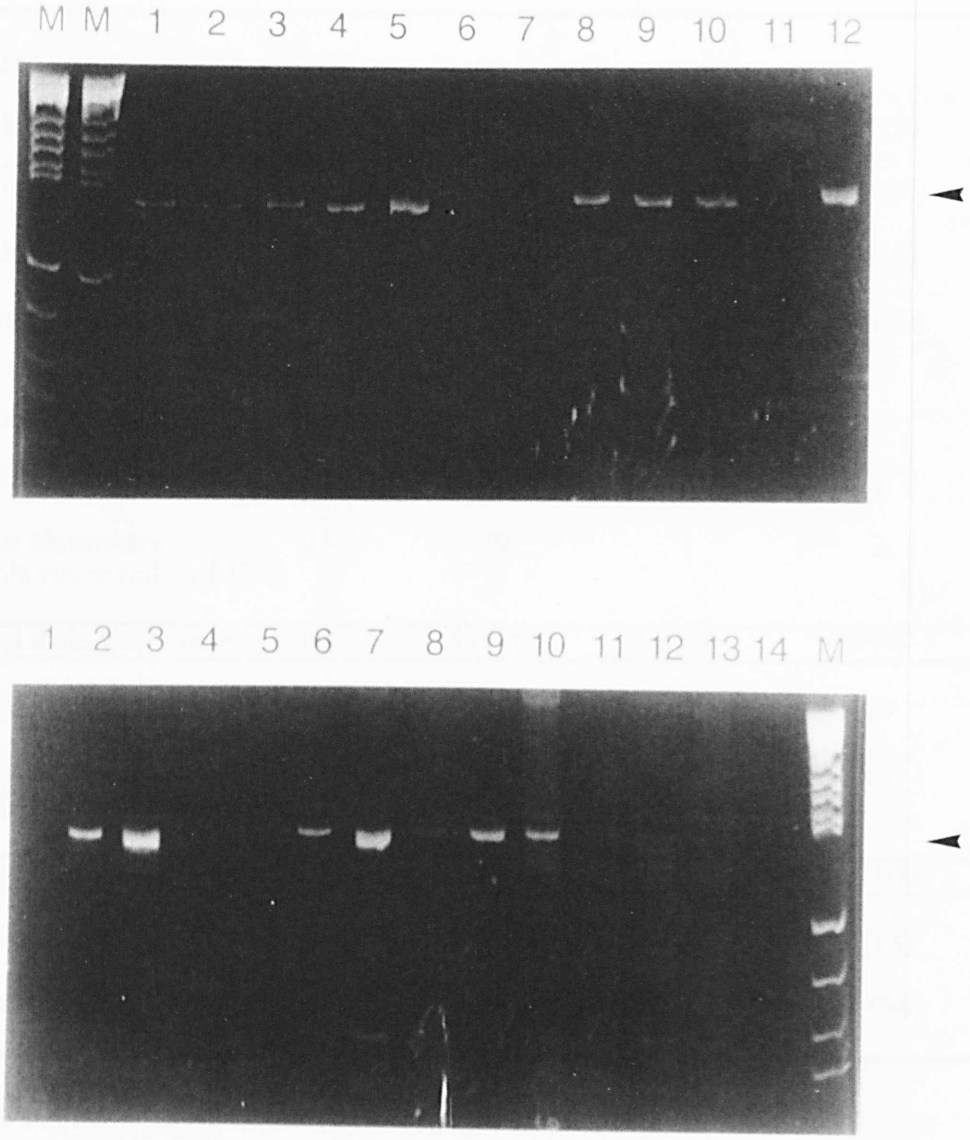


Table 4.2.: Amplification of a Y-specific alphoid repeat from single blastomeres: comparison of two lysis protocols

Type of sample	No. amplified	Amplification of Y-specific fragment (%)			Not analysed
		+	+/-	-	
Sterile water					
Male blastomeres	50	43 (86)	0	6 (12)	1 (2)
Female blastomeres	36	4 (11)	1 (3)	31 (86)	0
Male DNA (2 pg)	31	25 (80)	0	6 (20)	0
Control blanks	60	1 (2)	3 (5)	54 (90)	2 (3)
Two-step lysis (Li and Arnheim, 1990)					
Male blastomeres	17	15	1	1	0
Male blastomeres (embryos sexed by FISH)	43	39	1	3	0
Total male blastomeres	60	54 (90)	2 (3)	4 (7)	0
Female blastomeres	14	0	0	14	0
Female blastomeres (embryos sexed by FISH)	13	0	0	13	0
Total female blastomeres	27	0	0	27 (100)	0
Male DNA (2 pg)	23	20 (87)	0	3 (13)	0
Control blanks	34	2 (6)	0	32 (94)	0

4.5. Conclusions

Amplification of repeated sequences from single blastomeres was demonstrated. PCR is a sensitive technique and requires optimization for many parameters. Cycling conditions were shown to be critical: low annealing temperature allowed non-specific annealing and amplification of other sequences, high annealing temperature resulted in low yield or non specific amplification. The type of sequence is important: with the DYZ1, which is male specific only, amplification was acheived only from male cells even at the Ta of 45°C, which is much lower than the optimal (65°C). Conversely, the Y-alphoids with an annealing temperature just 5°C lower than their optimal temperature showed amplification of the male -specific fragment from female DNA. This is due to the homology of the X- and Y- alphoids which is 89% (Waye & Willard, 1985).

Optimization of the amplification conditions was very important, as it permitted amplification of the Y-alphoid repeat from single cells which was not possible under the original published amplification conditions (Witt & Ericson, 1989)

The amplification signal was variable from blastomere to blastomere. This could be due to the variable number of copies (and therefore amplified fragments) among individuals, as blastomeres from the same embryo or the same family usually show similar amplification levels. Also, as PCR is a very sensitive technique, differences in amplification efficiency during the first cycles would result in different yields.

With the DYZ1 repeat, amplification from the majority of single male cells was easily detected. However, amplification failed in 12% of the cases (Table 4.1.). The false positives were caused by sporadic contamination. Sporadic and carry-over contamination from a previous amplification can cause serious problems and care

Optimum amplification conditions for DYZ1 sequence, Y- and X-alphoids and co-amplification conditions for DYZ1 & X-alphoids and Y- & X-alphoids.

SEQUENCE	DYZ1	Y-ALPHOIDS	X-ALPHOIDS	DYZ1 & X-ALPHOIDS	Y- & X-ALPHOIDS
ANNEALING / EXTENSION TEMPERATURE	65°C	55°C	60°C	50°C	55°C
Mg^{++} CONCENTRATION	1.5-3.5mM	3.6 mM	1.5mM	1.5mM	1.5mM
PRIMERS CONCENTRATION	1.5 µM each	1.5 µM each	1.5 µM each	0.75µM each	0.75µM each
TAQ POLYMERASE CONCENTRATION	0.5 units	0.5 units	0.5 units	0.5 units	0.5 units
NUMBER OF CYCLES	40	40	40	40	40
SENSITIVITY	Single cell level	Single cell level	Single cell level	Whole embryo level (8c)	Single cell level

was taken to avoid them (Table 4.3.). Using the DYZ1 sequence, contamination levels were high (Fig. 4.11.) and decontamination was attempted by restriction digestion of the PCR reaction mix or the primers alone, using the EcoRI restriction site in the amplified fragment. Strom *et al* (1990) reported that, despite techniques of preventing carry-over contamination with the single cell level PCR, decontamination of all the PCR reagents by restriction digestion is the only effective method that can eliminate contamination. Restriction enzyme digestion was not succesful for decontamination in our case, probably because even one single sequence that remains undigested could serve as a template for the next PCR cycle. When carry-over contamination occurred, the anti-contamination precautions were not in full force. Extreme care to avoid carry-over contamination in the first place was the most important step to avoid false positives. When the full precautions against contamination were applied (as described in Chapter 2), there was no contamination problem.

Table 4.3.: Causes of false positives

Sporadic contamination from human DNA from cells from the operator

Sporadic contamination from human DNA preparations

Carry-over contamination of amplified DNA

Non-specific amplification

Primers self amplification

More cells into one tube

Contamination with cumulus cells or sperm

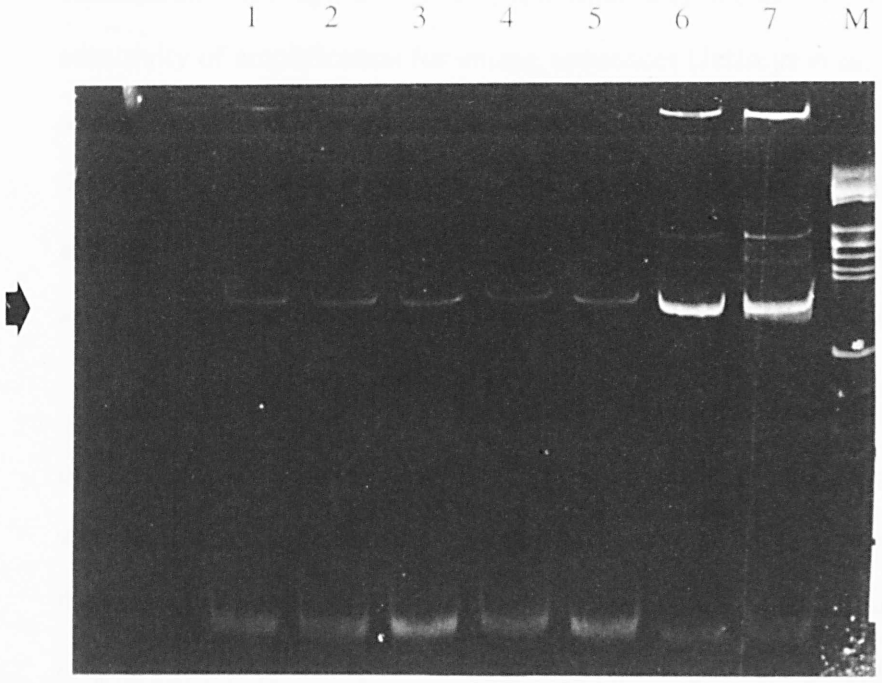
Figure 4.11.

Amplification of 20 and 2 pg of male DNA (lanes 6 and 7). Lanes 1-5: Blanks. Contamination is persistent even after using new buffer, primers, dNTPs, Taq polymerase, water, oil.

Amplification of the DYZ1 repeat.

Full precautions against contamination were not in force.

Figure 4.11.



The conditions for nested amplification were established and were successful. Although nested amplification was shown to increase specificity and sensitivity of amplification for unique sequences (Jeffreys *et al*, 1988), it did not make any difference for amplification of the DYZ1 repeated sequence. Also it was not useful for reducing contamination, as when nested PCR was tested, contamination levels were already high enough. Therefore it was not used routinely for single cell sexing, as the same sensitivity of amplification was achieved with one step amplification.

The DYZ1 sequence is on the long arm of the Y-chromosome and can, in rare cases, be deleted in normal men or translocated to autosomes and inherited by women (Cooke & Noel, 1979), so that each couple had to be screened beforehand for the presence or absence of the sequence. The alphoid sequences have the advantage that they are pericentromeric and therefore screening of the parents was not necessary. Using the Y alphoid repeat, the rate of amplification failure was similar to DYZ1. With unique sequences, only two copies of which are present in a diploid cell, loss or damage of the target sequence is a possible cause of amplification failure. With repeated sequences which are present in over 1000 copies in a single male diploid cell (Wolfe *et al*, 1985; Willard & Waye, 1987), this seems unlikely. The presence of multiple target sequences may explain why it is possible to generate easily detectable amounts from single cells without reamplification. However, the failure of amplification from single cells at the same frequency with unique and repeated sequences, suggests that other factors are more critical than PCR itself.

As single cell PCR is susceptible to amplification failure, identification of the sex of an embryo on the basis of the presence of absence of an amplified fragment alone is not ideal. Multiplex PCR with an additional set of primers has been suggested as an internal control for amplification failure (Chamberlain *et al,* 1990). Co-amplification of two single-copy targets is possible when the starting DNA is 10-100 ng (Gωllensten, 1990). Chamberlain *et al* (1990) suggest 250 ng of DNA for the co-amplification of 9 loci for the diagnosis of DMD and report that smaller amounts of

DNA in the reaction produce weak or undetectable bands. 10-100 ng of DNA is equivalent to 2,000 to 20,000 cells. Co-amplification of repeated sequences has not been described until now. For co-amplification, combination of two repeated sequences on the Y- and one on the X-chromosome were examined. Amplification of these sequences results in amplified fragments of 149 and 130 bp for the DYZ1 and the X-alphoids and in 130 and 170 bp for the X- and Y-alphoids respectively. The difference in the size of the fragments permits resolution on a polyacrylamide gel.

Co-amplification of the DYZ1 fragment and the X-alphoid repeat was possible when an 8-cell embryo was amplified, but it was not consistently successful to the level of a single cell. An interaction between the primers inhibited or eliminated completely in some cases the amplification of the X-specific fragment, even when the ratio of the primers was changed to favor the X-specific sequence (100:1). The sensitivity of amplification, especially of the X alphoid repeat fragment was significantly reduced. Although amplification of DYZ1 alone resulted in high yields of the amplified product at 60 and 65°C, co-amplification with the X-alphoid repeat reduced the sensitivity of amplification of both fragments and especially of the X-alphoid repeat.

Witt & Ericson (1989), who first described a protocol for amplification of the X- and Y-alphoid repeats, attempted co-amplification of the X- and Y-fragments in the same tube using dried blood spots as a source of DNA. Using high annealing temperature (Ta=55°C) they have reported synthesis of the X-specific fragment only, even when the ratio of the primers where X:Y=1:100. Co-amplification was possible when the annealing temperature was reduced at 37°C but under those conditions, non-specific annealing was permitted and numerous non-specific products appeared (Witt & Ericson, 1989).

Co-amplification of several loci from a single cell (2 pg) usually results in very unequal yields of DNA product (Gullensten, 1990 and our observations). Therefore co-amplification from a single cell becomes a very sophisticated and difficult

technique, the main reason being that amplification for two different loci requires compromise between the optimal conditions for the different sets of primers. When the starting material is one cell only, differences in the specificity and efficiency of amplification are greatly enhanced and this results in preferential amplification from one locus. Also as the DNA target is so limited during the first cycles, a large amount of primers is not used to amplify the target sequence. These primers might have a part of their sequence complementary, anneal to each other and amplify. After self-amplification their sequence is altered, because the amplified fragment is a part of the primer. In the next cycles, the number of primers that had been amplified 'unfaithfully' can not anneal to the target sequence, as they contain a small fragment that is not complementary to it. Therefore, the availability of primers for the next cycles is limited.

Co-amplification of the X- and Y-alphoids was demonstrated in whole embryos and in a single blastomere (Fig 4.9.). The reasons for the succesful co-amplification is probably the different buffer and amplification conditions used. The reaction buffer that has been used has been reported to permit co-amplification (Kogan *et al*, 1987; Chamberlain *et al,* 1990). Limited analysis of co-amplification from a single blastomere showed that each primer can fail independently in some cases, but contamination of the X-alphoid primers prevented the assessment of the reliability of co-amplification. Decontamination by restriction digestion was unsuccesful. The reason is that the X-monomers (subunits) are 68-85% identical in sequence to each other and do not have the same restriction sites. Therefore some monomers would escape digestion and could serve as templates for the next reaction.

A possible reason for PCR failure in single cells is insufficient lysis of the cell, which prevents efficient amplification. The incidence of the positive and negative amplification signals varies in relation to the different cell types used (Pickering *et al,* 1991) and to their preparation for single cell PCR (Cui *et al,* 1989), probably because cell types, with their different structure and nature, require different lysis procedures. Although with the majority of unique sequences that have been

examined, amplification fails in 15-30% of single cells (Holding and Monk, 1989; Li *et al,* 1988; Boehnke *et al,* , 1989; Monk *et al*, 1993), signal detection from blastomeres from preimplantation embryos can be even lower.

Holding & Monk (1989) have used a mouse-model for β–thalassaemia (Skow *et al,* 1983) to demonstrate the feasibility of preimplantation diagnosis from a biopsied blastomere. The mutation responsible for β–thalassaemia in the mouse-model is a 3.3kb deletion that includes the entire β–haemoglobin gene sequence. Using nested amplification, they have amplified a fragment of the β–haemoglobin gene. Normal wild-type and homozygous deleted mouse embryos could be accurately diagnosed. Out of 30 blastomeres, biopsied from the control embryos, 25 gave the expected signal. The remaining 5 were false-negatives. From the 26 blastomeres with the deletion, 25 were diagnosed correctly (no signal) and one gave a false-positive result (Holding & Monk, 1989).

Gomez *et al* (1990) used the Shiverer mouse as an experimental system for preimplantation diagnosis. After biopsy of 1-5 trophectoderm cells from mouse blastocysts, the cells have been amplified with two sets of oligonucleotide primers (for the normal and the mutant allele) to produce fragments of different size. In 11 of 36 biopsies no amplification was detected. In 6 cases a cell homozygous for the Shiverer allele was amplified and in 3/6 contamination led to false positive that could be misidentified as heterozygous.

Pickering *et al* (1991) reported very low rates of succesful amplification of the β–globin gene using single blastomeres (45%), in comparison with single cummulus cells and oocytes (83%). In that case, placing the cell in PBI+PVP prior to boiling could account for non-efficient lysis procedures might have accounted for amplification failure. Holding and Monk (1989) found that 5/30 blastomeres failed to amplify. Also Lesko *et al* (1991) reported high efficiency of amplification of the DF_{508} locus for cystic fibrosis using nested primers, but lower efficiency when single blastomeres were used.

Sample preparation is a critical issue, particularly with cleavage stage blastomeres, since we have observed that some of these cells are very resistant to lysis in distilled water. Also, if the cells are rapidly frozen or even snap frozen in liquid nitrogen to speed processing, this can be counterproductive. What seems to occur is that instead of osmotic swelling, cell lysis and dissolution of cell contents, the blastomere remains as a 'ghost' with an intact nucleus which can be visualised by fluorescent staining of the DNA. In this situation, boiling prior to PCR to inactivate endogenous proteinases and nucleases may simply fix the DNA *in situ* which may then be relatively inaccessible to the primers preventing successful amplification in the first critical cycles. This may partly explain the exceptionally low amplification efficiencies (40-45%) reported for a β–globin sequence spanning the site of sickle cell anaemia mutation since the blastomeres were simply freeze-thawed in medium. Also, differences in efficiency between blastomeres compared with unfertilized oocytes and other cell types may have been caused by the different physical characteristics of these cells and the efficiency of lysis under these conditions. For example, the 100% of succesful amplification with unfertilized oocytes (Pickering *et al*, 1992) might be due to the breakdown of the germinal vesicle in oocytes, the easy release and availability of the DNA for PCR.

Initially the lysis method involved freezing and thawing the single cell in distilled water twice and boiling at 95°C for 35 min. This approach was compared with a two-step lysis procedure: Arnheim *et al* (1992) described a lysis method which improves the amplification efficiency with various cell types (from 75% to 95% with single sperm). Although most cells lyse after 10 minutes in distilled water, it was observed that the time required for the lysis varies between different blastomeres and it could be that the method is not optimal for complete lysis, as the DNA of some single nuclei might not be available for amplification by PCR.

A detailed examination of the amplification efficiency of both Y-specific repeat sequences with single blastomeres from male and female embryos has been

reported in this Chapter. Single blastomeres from disaggregated embryos were used to assess the reliability of single blastomere amplification. The sex of the embryo was confirmed either from the amplification of the majority of the cells or by dual (FISH) (Griffin *et al* , 1993) with X- and Y- specific probes. The efficiency of lysing the blastomeres in distilled water prior to PCR is compared with a two-step lysis procedure (Arnheim *et al*, 1992).

The use of a two-step lysis protocol involves an initial incubation in potassium hydroxide and dithiothrieotol followed by a neutralisation step significantly improves amplification efficiencies for a number of unique sequences from single sperm which are otherwise very resistant to amplification probably because of their tightly condensed nuclei and other physical charactersitics (Li *et al.*, 1991). With blastomeres amplification failure was reduced but not eliminated and occurred with 4/60 male blastomeres (7%). However, the small number of blastomeres available does not permit statistical comparison. The cause of these residual failures is not clear but one possibility is the design of the primers. The sequence of the Y-alphoid repeat monomers is variable and partially homologous to the alphoid repeat sequence on the X chromosome. The published sequence of the primers was designed to be complementary to the concensus sequence for the Y alphoid monomers (Witt & Erickson, 1989) and the PCR annealing temperature had to be raised to avoid amplifying the X alphoid repeat. Thus it is possible that by increasing the stringency of annealing, amplification efficiency has been sacrificed to some extent. The use of primers specific for one of the more frequent Y alphoid repeat monomers, while reducing the number of target sequences, was thought to improve amplification efficiency. However, primers that have been synthesized to amplify one of the monomers amplified female DNA as well (data not shown). Also, amplification of another Y-specific repeat, DYZ1, failed with a similar frequency even though the primers in this case match precicely the target sequence (Table 4.1.).

The use of 2-step lysis procedure improves the efficiency of amplification from single blastomeres. In Chapter 3 it was shown that the pregnancy rate is 16.6% and 44.4% when 1 or 2 embryos are transferred. As 4.5+/-0.5 embryos are available for PCR (Chapter 3), it is essential that a very efficient method for sexing is available, to ensure that at least 2 female embryos will be identified and transfered.

One approach is to control for amplification failure by co-amplifying fragments specific for both the X and Y chromosomes using two sets of primers (Kontogianni *et al.*, 1991). Although a recent study has shown that with co-amplification of the DYZ1 and the Y-alphoid repeat, amplification either fails completely from both primer sets or amplification of both the X and Y specific fragments occurs with male cells resulting in an accurate diagnosis (Strom *et al*, 1991), the PCR conditions are necessarily a compromise between the optima for the two primer pairs and amplification efficiency was relatively low (80%). Another PCR based strategy involves target sequences present on both X and Y chromosomes enabling amplification with the same set of primers but which amplify a fragment either of a different size or with differences in restriction sites that allow rapid analysis post amplification. One of these, involves amplifying a region of the amelogenin gene (Nakahori *et al.*, 1991). This method has an advantage over the co-amplification method, as only one set of primers is used, and this helps to avoid artifacts from amplification between the primers. Again, initial experience indicates accurate identification of the sex but relatively low amplification efficiency (Nakahori *et al.*, 1991; Levinson *et al.*, 1992). Amplification of a fragment of the ZFX and ZFY genes on the X and Y chromosomes, respectively, followed by restriction digestion at a site only present in the Y-specific fragment also allows the accurate identification of sex (Chong *et al*, 1993). Also Zhang *et al* (1992) amplified successfully the X-linked steroid sulfatase (STS) locus and the STS pseudogene on the Y-chromosome using a two-round seminested amplification procedure with primers that differ in length, allowing the two products to be identified by electrophoresis. However, in all amplification procedures that amplify more than one locus from a small number of

cells, preferential amplification of one locus could lead to misidentification of the genotype (Walsh *et al,* 1992; Pickering *et al*, 1993). In co-amplification the one amplified fragment is thought as a control for the other and the idea is that the chance for failed or successful amplification is the same for both sequences. However, anticipated amplification of both sequences may be inadequate control, as independent amplification failure of one or the two sequences to be amplified has been demonstrated by us and by other investigators (Grif o *et al*, 1992; Holding *et al*, 1993).

With the detection of the DF_{508} deletion for the diagnosis of cystic fibrosis the alleles present in single cells were accurately amplified and there was no evidence for allelle specific amplification failure (Lesko *et al.,* 1991). The amplification efficiency was lower with blastomeres than fibroblasts. Using the same approach for amplification with one set of primers and the discrimination of the alleles with restriction digestion, Monk *et al* (1993) studied the feasibility of the detection of the sickle cell mutation in single buccal cells. Both alleles were detected in 49/69 cells (71% of the cases). In buccal cells from a heterozygous individual, the normal allele only was detected in 8/69 cells and the mutant in 10/69 cells. In all amplification procedures that amplify more than one locus from a small number of cells, preferential amplification of one locus could lead in misidentification of the genotype. Preferential amplification was reported due to different length of the alleles, different base composition of the specific fragment and/or the primers, which results to different melting temperatures (of the target DNA and the amplified fragment) and selective priming (Walsh *et al,* 1992).

The incidence of amplification of contaminating male DNA from female blastomeres and control blanks was 6% (10/156). Other groups, such as Strom et al (1990) have reported that it is impossible to avoid contamination unless they decontaminate the PCR reaction mix with restriction digestion of the target sequence. In our case, when the anti-contamination precedures were in full force, contamination could be kept in low levels, without the need for restriction digestion. When an embryo is sexed at the preimplantation stage, a false positive would not result in a serious

misdiagnosis since the embryo would be identified as male and not selected for transfer but it would have an effect on pregnancy rates by reducing the number of female embryos identified for transfer. Amplification of contaminating DNA could, however, lead to serious errors when attempting specific diagnosis of single gene defects by amplification of the target fragment and restriction digestion. False negatives could not lead to a misdiagnosis but they would eliminate a potentially healthy embryo from transfer. However a false positive, which might have been caused by sporadic or carry-over contamination could let a misdiagnosed embryo be transfered. Therefore, if that method of analysis is preferred, it is essential that the contamination levels are very low.

Amplification of unique sequences from single cells has been reported for specific diagnosis of a variety of single gene defects both in the human and mouse models of human disease (Handyside, 1993). Amplification efficiencies are lower from blastomeres compared with other cell types. The genetic defect is usually detected by a variety of secondary procedures following amplification of the specific fragment of the gene affected. Amplification failure, therefore, would not result in a misdiagnosis but simply prevent any analysis for that particular embryo. Nevertheless, it is still important to achieve a high efficiency to ensure that diagnosis is successful with as many of the biopsied embryos as possible. Pregnancy rates following preimplantation diagnosis are much higher if two unaffected embryos are transferred (Chapter 3). This method is likely to improve amplification efficiencies with any repeat or unique sequences for identification of sex or specific diagnosis of single gene defects.

Chapter 5

Preimplantation diagnosis by a biochemical assay;

Early embryonic gene expression

5.1. Introduction

Many X-linked diseases have been characterized at the molecular level and diagnosis based on DNA analysis is available. However, for the large number of these diseases that have not been mapped or characterized or are very heterogeneous at the DNA level, biochemical diagnosis is possible, if the biochemical basis of a genetic disease is known and an assay is available. To perform preimplantation diagnosis by a biochemical assay, it is essential to understand the timing of gene expression in early human development. Enzyme microassays can be used in preimplantation diagnosis if the embryonic genome is activated, the gene is expressed in early embryos and the embryonic enzymatic activity can be distinguished from the maternal enzyme carried over by the egg.

5.1.1. Maternal versus embryonic expression

In most, if not all, species early development is under the control of the maternal information (Davidson, 1976). Embryo development is dependent on components that are maternally inherited through the oocyte. This dependence probably reflects the embryo's increased needs and inability to synthesize the material and apparatus which is necessary to sustain the developmental processes.

In the mouse, prior to the activation of the embryonic genome at the 2-cell stage, development is supported by components in the egg, such as RNA, protein, ribosomes, mitochondria, etc. The quantities of mRNA, tRNA and ribosomes are high in comparison with those in somatic cells and probably the embryo inherits translational apparatus in an amount that permits immediate protein synthesis, even in the absence of embryonic transcription (reviewed by Johnson, 1981). The majority of RNA is rRNA

and tRNA (Piko & Clegg, 1982; Clegg & Piko, 1983). After fertilization the maternally-inherited mRNA is ready to be used in protein synthesis, as there is virtual absence of RNA synthesis before the 2-cell stage (Piko & Clegg, 1982). As embryonic activity is absent, regulation of developmental events are taking place with differential mRNA activation, differential protein turn over and postranslational modifications, as demonstrated in the mouse (Howlett & Bolton, 1985). The timing of transition from maternal control to embryonic varies between species; for example, in amphibian embryos, the transition happens at the mid-blastula stage (Davidson, 1976); in the mouse the switch happens at the 2-cell stage (Flach *et al*, 1982). The transition is characterized by i) the initiation of mRNA synthesis ii) inhibition of cleavage after treatment with the transcriptional inhibitor α–amanitin iii) a change in the species of mRNA present and iv) a dramatic change in the pattern of newly synthetised polypeptides, which are sensitive to treatment with α–amanitin or physical enucleation (reviewed by Johnson, 1981). The activation of the embryonic genome coincides with the elimination of the maternal mRNA, although the precise timing and the persistence of the maternal products varies between species (Epstein & Smith, 1973; Van Blerkom & Brockway, 1975; Van Blerkom *et al*, 1971; Frei *et al*, 1989; Jarrell *et al*, 1991; Crosby *et al*, 1988; Norris *et al*, 1985; Braude *et al*, 1988; Artley *et al*, 1992; reviewed by Leese, 1993). In the mouse, the maternal mRNA is eliminated by a continuous process; during oocyte meiotic maturation, 60% of the polyadenylated mRNA pool is lost and most of that remaining is degraded between fertilization and the 2-cell stage. Also the elimination of maternal mRNA is a selective process, as the decrease of other classes of RNA as ribosomal RNA is negligible (Piko & Glegg, 1982). Information of the maternal mRNA degradation is more limited in other species than in the mouse.

In human embryos, limited transcription was detected at the 4-cell stage with increased levels observed at the 8-cell stage (Tesaric *et al*, 1986; Tesaric, 1987, 1988). Braude *et al* (1988) studied the protein patterns synthesized by six unfertilized eggs, seven pronucleate eggs, six 2-cells, ten 8-cells and six blastocysts. They showed that a major change in the protein patterns occurs between the 4- and 8-cell stage. Some

of the changes in polypeptides synthesized are dependent on transcription, as they were inhibited in embryos that have been cultured in the presence of α–amanitin. Braude *et al* (1988) have also shown that cleavage in human embryos is not sensitive to α–amanitin until the 4-cell stage. From this data it was concluded that the activation of the human embryonic genome first occurs between the 4- and 8-cell stages. A key-feature in preimplantation diagnosis, which is based in the measurement of the product in question, is the persistence of the maternal products, which have to be sufficiently low so that the embryo's contribution can be detected. However, very limited data exists for the human embryo.

5.1.2. Approaches to demonstrating embryonic expression

The qualitative patterns of proteins synthesized by preimplantation embryos have been studied with one dimensional (mouse: Epstein & Smith, 1974; Van Blerkom & Brockway, 1975; rabbit: Van Blerkom & Manes, 1974; cow: Frei *et al*, 1989; pig: Jarrell *et al*, 1991; sheep: Crosby *et al*, 1988; gerbil: Norris *et al*, 1985; human: Braude *et al*, 1988; Artney *et al*, 1992) and two dimensional (mouse: Johnson *et al*, 1977; Van Blerkom & Manes, 1977; Van Blerkom *et al*, 1976; Handyside & Johnson, 1978; rabbit: Van Blerkom & Manes, 1977; Schultz & Tucker, 1977) polyacrylamide gel electrophoresis.

As a general approach to examine early embryonic gene expression, the pattern of polypeptides synthesized by single human embryos at various times after insemination have been investigated. To detect the newly synthesized proteins, the embryos have been incubated with labelled amino acids. Changes in the patterns of polypeptide synthesis were studied in embryos at the cleavage stage (on day 2) to the expanded blastocyst (day 7) that have been separated by one-dimensional 10% and 7-15% gradient polyacrylamide gel electrophoresis.

Several quantitative studies of uptake and incorporation of a labelled amino acid into protein by preimplantation mouse embryos in vitro showed large increases in uptake and incorporation during late cleavage and blastocyst formation, although from fertilization to cleavage stage no marked increase in incorporation occured (Brinster, 1971; Epstein & Smith, 1973; Braude, 1979; Van Blerkom & Brockway, 1975).

Up to now there has been no data on the levels of protein synthesis from the human embryo. In this study, the quantitative levels of expression throughout preimplantation development to the blastocyst stage have been examined, by measuring the uptake and incorporation of ^{35}S-methionine from single human embryos.

Use of transcriptional inhibitors can distinguish between the embryonic and the maternally coded proteins, so that the persistance of the maternal message and the onset of the embryonic gene expression can be examined. α–Amanitin is derived from the mushroom *Amanita phalloides* and is a specific inhibitor of RNA polymerase II at appropriate concentrations (Levey & Brinster, 1978).

5.1.3. Aim

As a general approach to examine early embryonic gene expression, the pattern of polypeptides synthesized by single human embryos cultured in the presence or absence of the transcriptional inhibitor α–amanitin, was investigated by one-dimensional polyacrylamide gel electrophoresis. Also the quantitative aspects of protein synthesis and the effect of α–amanitin on them was studied by measuring the uptake and the incorporation of ^{35}S-methionine into protein.

5.2. Method

5.2.1. Uptake and incorporation of ^{35}S-methionine

The uptake and incorporation of ^{35}S-methionine have been measured as described in Chapter 2. The morphology of the embryos was assessed before and after treatment. The embryos were described as normally developing if they had reached the 2-4-cell stage on day 2, 8-cells on day 3, morula on day 4, early blastocyst to blastocyst on day 5 and blastocyst to expanded blastocyst on day 6. Arrested embryos had not progressed for 24 hours.

5.2.2. Polypeptide profiles

Embryos were exposed to ^{35}S-methionine in culture for 2 hours and polypeptides from individual embryos were separated in standard and gradient SDS-PAGE, as described in Chapter 2. Gradient gels ensure better resolution of low molecular weight polypeptides, which in a non-gradient system, form diffuse bands at the lower regions of the gel (Van Blerkom & Manes, 1974). Also in a gradient gel, a greater spectrum of molecular weights can be resolved (14-200 kD), while the size of the gel remains small and easy to handle.

The labelled embryos were analysed in two sets of experiments: For the first set, whole individual embryos of different ages were loaded on the same gel. The gels were exposed for between 2-12 weeks. Although the levels of incorporation vary considerably between different developmental stages (see Results, this Chapter) and the patterns obtained from cleavage stage embryos are very faint in comparison with later stages, the intensity of the bands between different embryos is directly comparable. In the second set of experiments, to produce tracks of equal density, equal amount of

radioactivity were loaded on the lanes of each gel and have been exposed for 4-12 weeks.

The gradient gels were made at the same time and run in the same tank. The gels were processed together under identical conditions and then exposed for different amounts of time. To make the comparison for the qualitative differences in individual embryos easier, lanes from the photographs from two gradient gels were cut and pasted together (photographs of the cut and pasted and the original gels are submitted).

The fluorographs were assessed visually and the intensity of each particular protein of the gel was made in relation to the band at 75K, which appears to have a constant intensity in most samples. If in a large part of the track it was possible to define any bands or if they were diffuse, the data was not included.

5.2.3. Uptake and incorporation of ^{35}S-methionine and polypeptide profiles in embryos cultured in the presence or absence of α–amanitin.

The uptake and incorporation of ^{35}S-methionine were measured as described previously for the control embryos. Prior to the labelling, the embryos were incubated individually in 100 µg/ml α–amanitin for 24 hours, were washed twice and labelled (as described in Chapter 2, 2.8.1.).

The significance of the uptake and incorporation results were tested using the non-parametric Mann-Whitney test to analyse for differences on separate days of development.

5.2.4. Lineage-specific polypeptides

The polypeptide patterns of inner cell mass (ICM) cells and the trophectoderm (TE) cells were investigated. The blastocysts were labelled and immediatel y afterwards, the ICM and TE cells were isolated by immunosurgery (Handyside & Barton, 1977). The cells were then placed in lysis buffer (as described in Chapter 2) and kept in -70°C until analysis (for no more than 15 days). They were loaded on a one dimensional gel, and the protein patterns of intact blastocysts, cells from the ICM and TE are compared. The film was exposed for 2 months.

5.3. Results

5.3.1. Uptake and incorporation of ^{35}S-methionine in human preimplantation embryos

The uptake and incorporation of ^{35}S-methionine into protein was measured in 84 embryos (72 normally fertilized and 12 polyspermic embryos). The numbers of normally fertilized embryos examined and the values of uptake and incorporation are presented on Tables 5.1., 5.2. and 5.3. The uptake of ^{35}S-methionine from normally developing human embryos remained unchanged from day 2 to day 4 and showed a 6-fold increase between day 4 and day 7 ($p< 0.05$). A similar dynamic was seen with incorporation of methionine into protein, which was level until day 4 and then significantly increased by a factor of 10 by day 7 ($p<0.05$) (Fig 5.1, Table 5.1.). The morphology of the embryos was assessed before and after labelling and no effect of labelling was apparent.

Normal embryos on day 4 are morulae and on day 5, blastocysts. Arrested embryos have not progressed for 24 hours and are 8 to 10-cells on day 4 and morulae on day 5. The numbers of normally fertilized embryos examined and the values of the uptake and incorporation are presented on Table 5.3. The uptake of arrested embryos was 31% and 59% and the incorporation 19% and 29% of normally developing embryos on days 4 and 5 respectively (Fig 5.3. & 5.4.).

During the initial cleavage divisions (from day 2 to day 4) the uptake and incorporation per blastomere fell 5-fold ($p<0.05$) (Fig 5.2., Table 5.4.) and remained at the same levels from day 4 to day 7. The cell numbers in the cleavage

stages embryos were counted by careful examination. At the blastocyst stage, the cell numbers were estimated from previous studies (Hardy *et al*, 1990).

Table 5.1.: Uptake and incorporation of ^{35}S-methionine by human embryos developing normally from day 2 to 7. (cpm/embryo/2hrs)

Day	Uptake	Incorporation *
2	45550 ± 8375 (n=4)	13680 ± 3064 (n=4)
3	58980 ± 5648 (n=4)	16940 ± 3280 (n=4)
4	48017 ± 13970 (n=8)	13841 ± 7436 (n=8)
5	118185 ± 20731 (n=7)	29708 ± 7455 (n=7)
6	270598 ± 51278 (n=5)	67970 ± 6072 (n=5)
7	374788 ± 65864 (n=8)	128067 ± 12865 (n=8)

*Uptake and incorporation of ^{35}S-methionine were assayed as described in Materials and Methods (Chapter 2). Each value is the mean of a group of embryos +/- standard error ; n is the number of individual embryos assayed.

5.3.2. Uptake and incorporation of ^{35}S-methionine in embryos cultured in the presence or absence of α–amanitin.

The uptake of ^{35}S-methionine by human embryos cultured in the presence of 100 μg/ml of α–amanitin for 24 hours before labelling was not significantly different to untreated embryos on day 3 and 4. In the α–amanitin treated embryos, the uptake levels dropped from day 5 onwards (Fig 5.5., Table 5.2.). A similar pattern was observed with incorporation, which remained similar with the untreated embryos on day 3 and 4 and declined progressively from day 5 onwards (Fig 5.6. & 5.7., Table 5.2.).

The embryos were assessed morphologically before and after incubation with α–amanitin. In early cleavage stages α–amanitin had no effect on the morphology of the embryos. During blastocyst formation and hatching, the effect of α–amanitin was more apparent, as the blastocysts appeared slightly darker. Out of 46 embryos on days 5, 6 and 7, 6 hatched out of the zona, 24 were developing normally and 16 were morphologically compromised. However, out of 54 embryos of the same age, none hatched, 18 were developing normally and 36 were morphologically compromised (Table 5.5.).

The proportion of embryos that were morphologically compromised in a-amanitin in comparison with the control embryos has been tested with the χ^2 test (chi square) and was shown to be statistically significant ($p<0.005$).

Table 5.2.: Uptake and incorporation of ^{35}S-methionine by human embryos from day 3 to 7 after 24-hrs incubation in α–amanitin.

Day	Uptake	Incorporation *
3	43435 ± 7045 (n=4)	6295 ± 2695 (n=4)
4	26838 ± 7592 (n=6)	5250 ± 1611 (n=6)
5	8547 ± 3333 (n=4)	8065 ± 3460 (n=4)
6	6677 ± 1098 (n=4)	5755 ± 2055 (n=4)
7	4556 ± 1213 (n=3)	2876 ± 1240 (n=3)

*Uptake and incorporation of ^{35}S-methionine. Each value is the mean of a group of embryos +/- standard error ; n is the number of individual embryos assayed.

Table 5.3. Uptake and incorporation of ^{35}S-methionine by human embryos developing normally and developmentally delayed or arrested on day 4 and 5.

Day 4

Developmental stage	Number of embryos	Uptake	Incorporation
Normal	n= 8	48017± 13970	13841± 7436
Arrested	n= 5	15090± 4060	2733± 1090

Day 5

Developmental stage	Number of embryos	Uptake	Incorporation
Normal	n= 7	118185± 20731	29708± 7455
Arrested	n= 6	70240± 12622	8660± 1934

The significance of the uptake and incorporation results were tested using the non-parametric Mann-Whitney test. The uptake was significally reduced in arrested embryos in comparison with normals on days 4 ($p<0.05$) and 5 ($p<0.05$). Also the incorporation was significally less in arrested embryos on days 4 ($p<0.05$) and 5 ($p<0.05$).

Table 5.4.: Incorporation and uptake of ^{35}S-methionine per cell.

Day	n	Uptake	Incorp.	No of cells	Uptake per cell	Incorp. per cell
2	4	45550	13680	4.0±0.0	11387	3420
3	4	58980	16940	10±2	5898	1694
4	8	48017	13841	32 @	1500	432
5	7	118185	29708	58.3±8.1	2027	509
6	5	270598	67970	82.2±7.8	3292	826
7	8	374788	128067	126.2±21	2969	1014

*Uptake and incorporation of ^{35}S-methionine. Each value is the mean of a group of embryos ; n is the number of individual embryos assayed. The cell number on days 5, 6 and 7 have been estimated from previous work (see 5.3.1.).

@: The number of cells at that stage can not be counted directly by observation and the number is an estimation.

Table 5.5. Development of human preimplantation embryos from day 5 to 7, after 24 hrs incubation in α–amanitin.

	Normal			α–amanitin		
Day	**Morphol. Normal**	**Hatched**	**Morphol. Comprom.**	**Morphol. Normal**	**Hatched**	**Morphol. Comprom.**
5	8	0	2	8	0	4
6	10	2	12	8	0	28
7	6	4	2	2	0	4

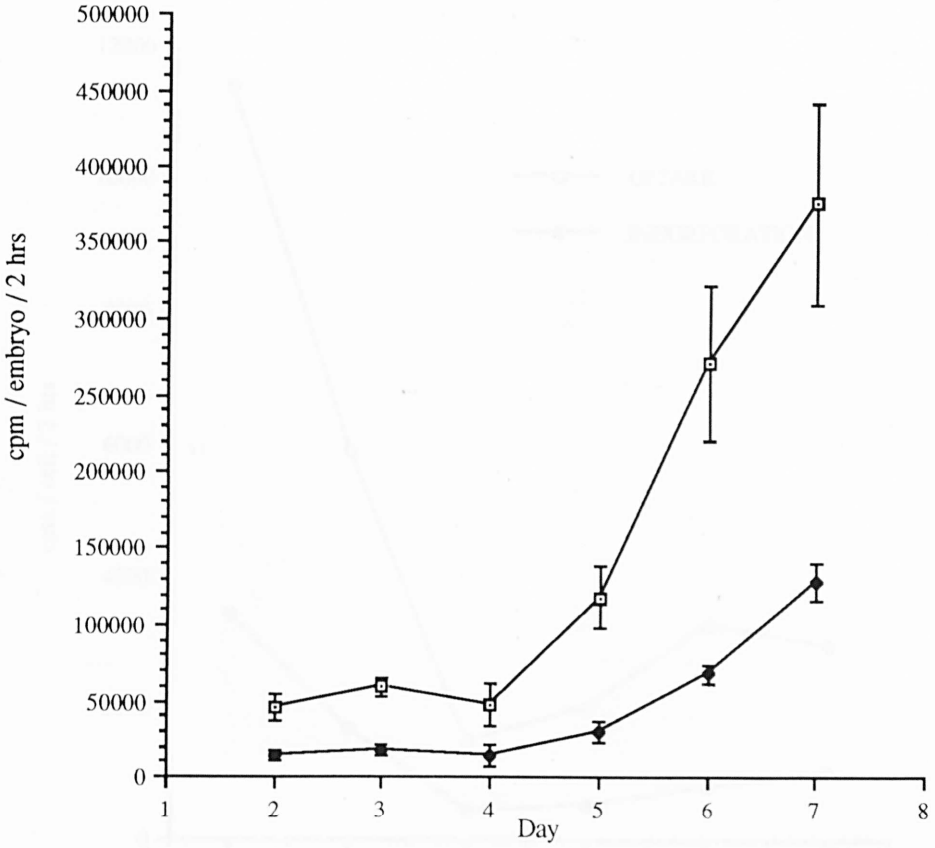


Figure 5.1. Incorporation and uptake of ^{35}S-methionine by human embryos developing normally from day 2 to 7. Each point represents the mean of a group of 4-8 embryos (+/- standard error). White squares: Uptake. Black squares: Incorporation (as for Fig. 5.2.).

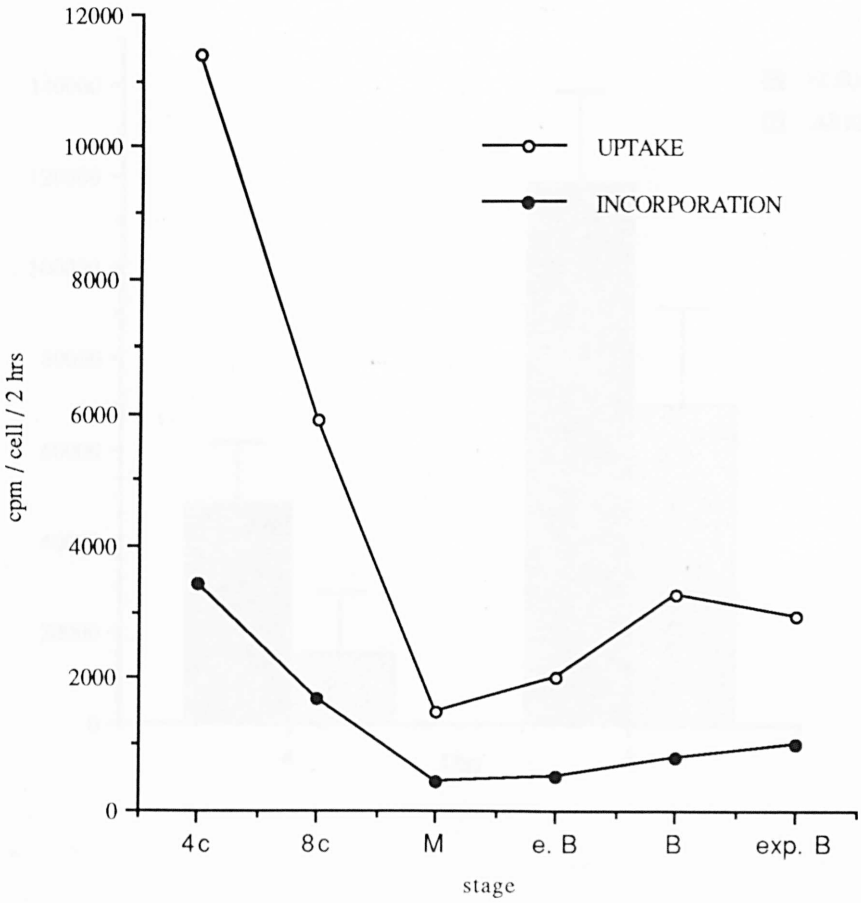


Figure 5.2. Incorporation and uptake of ^{35}S-methionine per cell.(Also see table 5.4.)

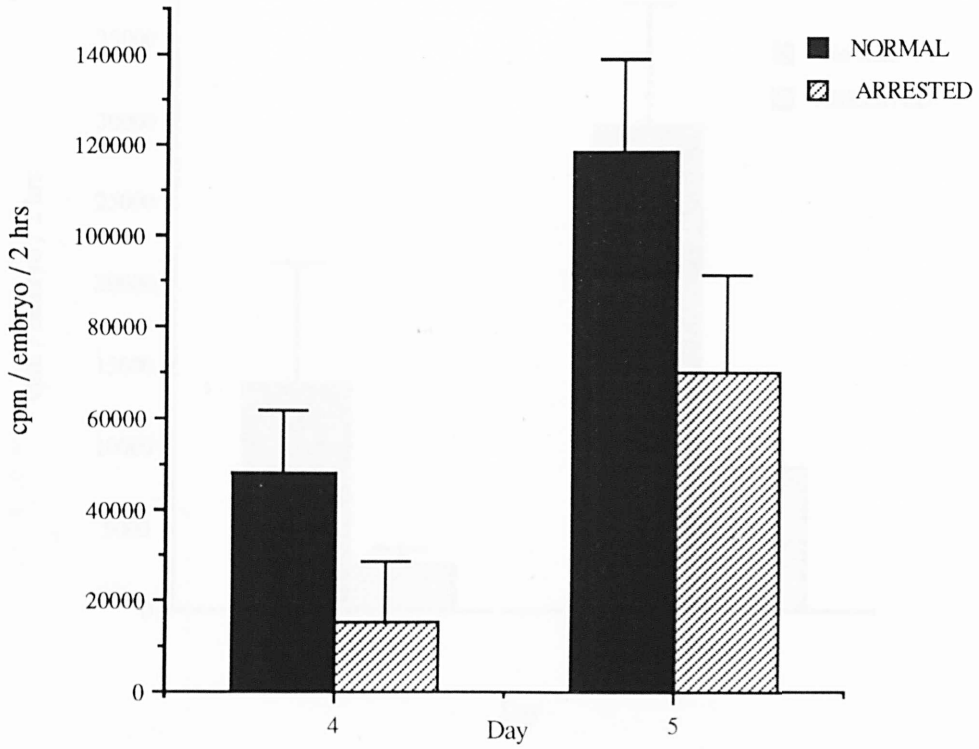


Figure 5.3. Uptake of ^{35}S-methionine by human embryos developing normally or arrested on day 4 and 5. Each point represents the mean of a group of 2-4 embryos (+/- standard error).

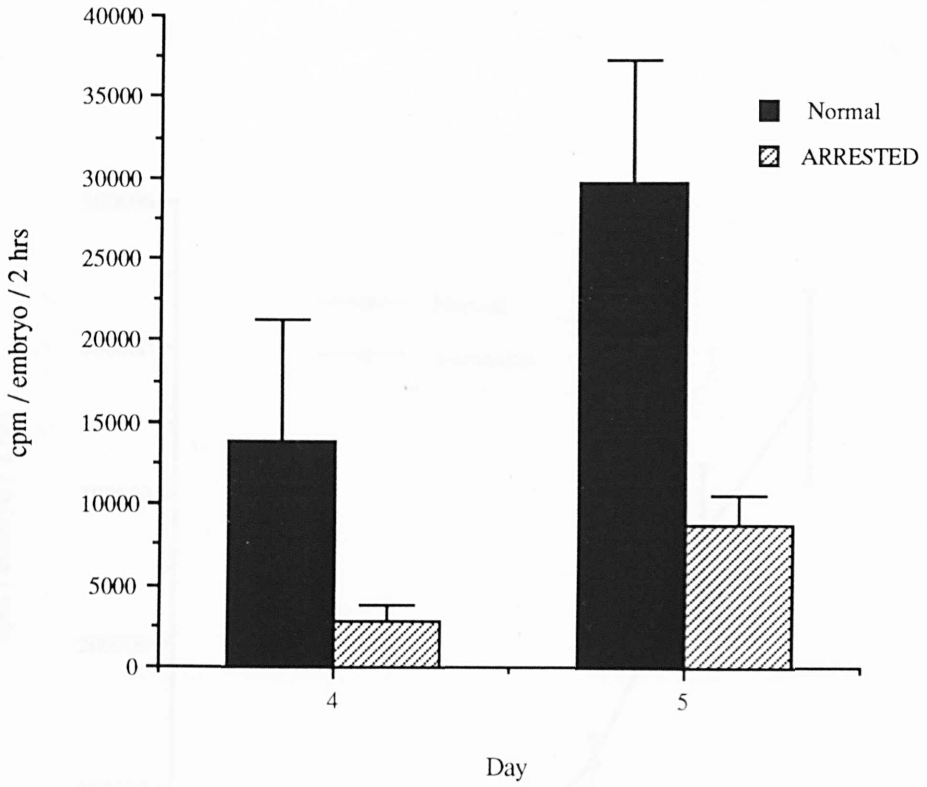


Figure 5.4. Incorporation of ^{35}S-methionine by human embryos developing normally or arrested on day 4 and 5. Each point represents the mean of a group of 2-4 embryos (+/- standard error).

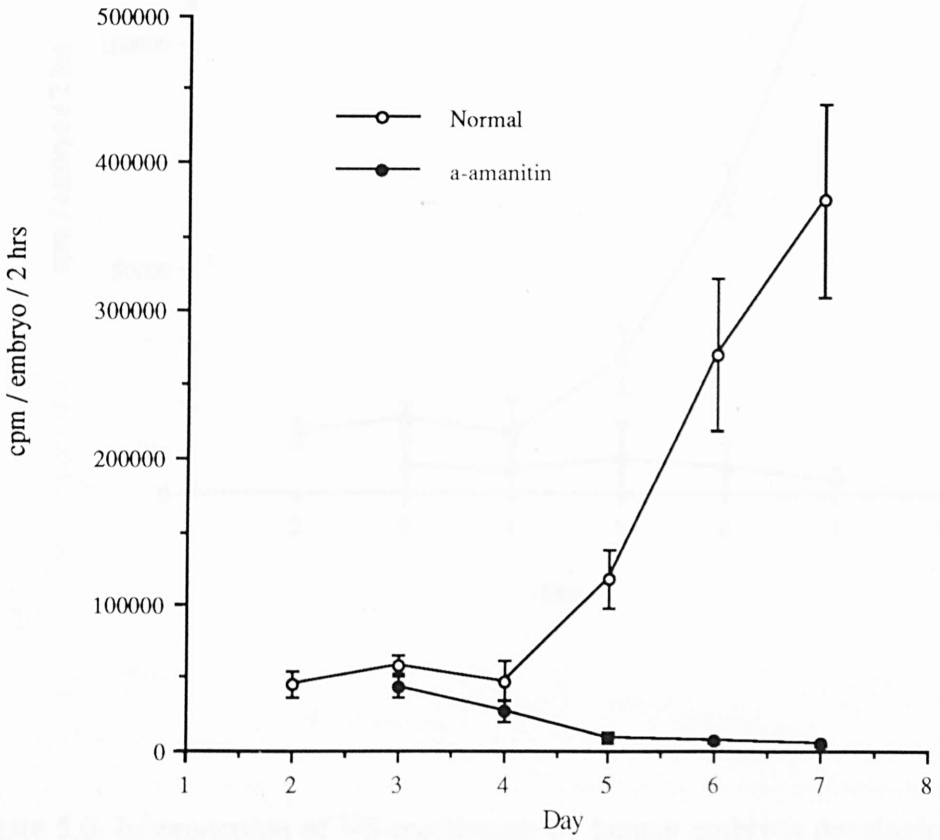


Figure 5.5. Uptake of ^{35}S-methionine by human embryos developing normally from day 2 to 7. The embryos were cultured in the presence or absence of 100 µl/ml of α–amanitin for 24 hrs prior to labelling. Each point represents the mean of a group of 2-4 embryos (+/- standard error)

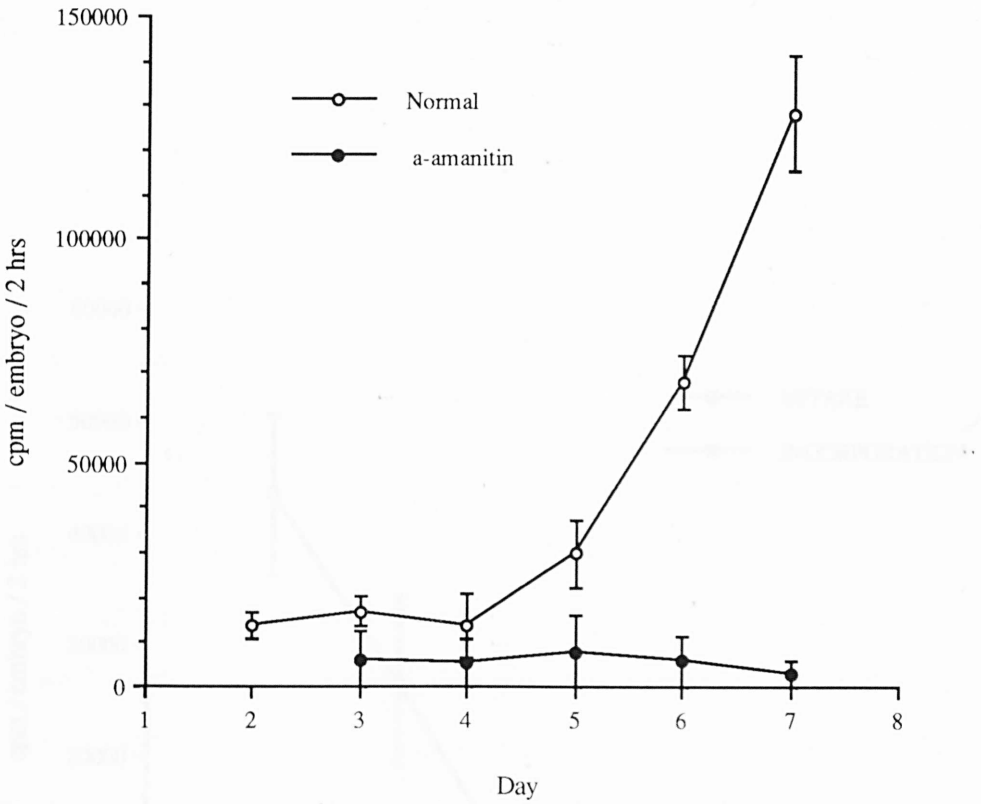


Figure 5.6. Incorporation of ^{35}S-methionine by human embryos developing normally from day 2 to 7. The embryos were cultured in the presence or absence of 100 ul/ml of α–amanitin for 24 hrs prior to labelling. Each point represents the mean of a group of 2-4 embryos (+/- standard error)

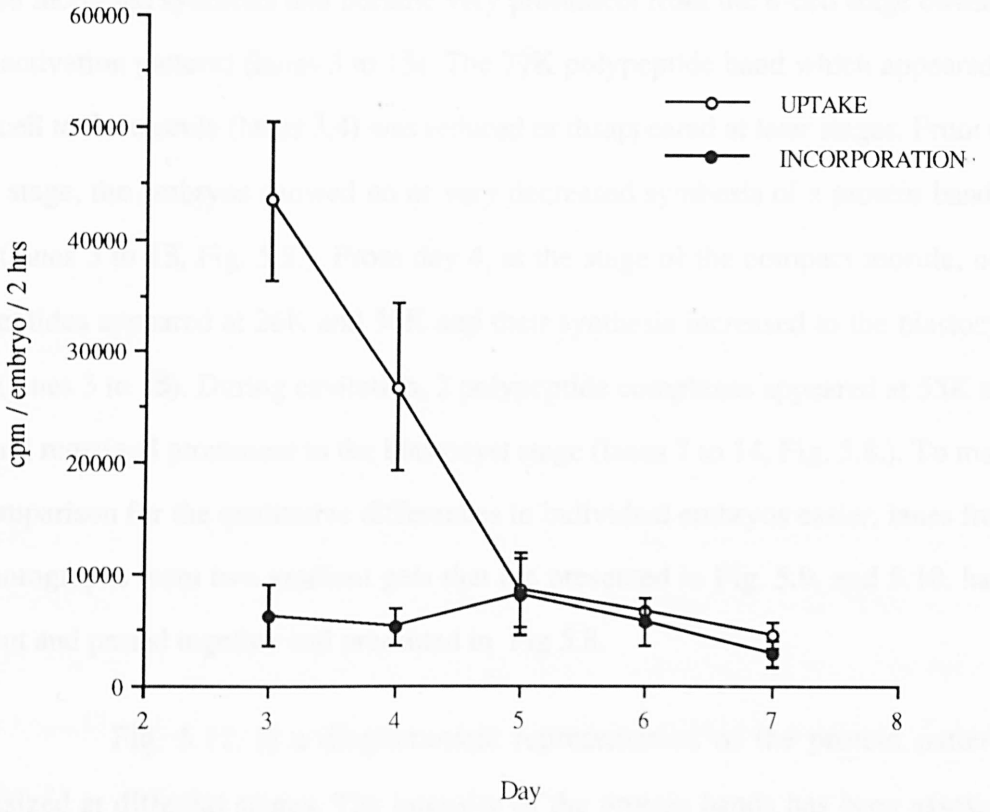


Figure 5.7. Incorporation and uptake of ^{35}S-methionine by human embryos developing normally from day 2 to 7. The embryos were cultured in the presence of 100 ul/ml of α–amanitin for 24 hrs prior to labelling. Each point represents the mean of a group of 2-4 embryos (+/- standard error)

5.3.3. Stage specific polypeptide synthesis profiles

Out of one hundred and thirty five embryos used, a clear pattern was obtained from 91 embryos. A number of qualitative and quantitative changes are evident. The 2-cell and 4-cell embryos are characterized by an intense 40K band and faint 45K, 46K and 95K bands (pre-activation pattern) (lanes 1, 2, Fig. 5.8.) The polypeptides at 45K and 46K that were faint at the 4-cell stage (lanes 1,2, Fig. 5.8.) showed increased synthesis and became very prominent from the 8-cell stage onwards (post-activation pattern) (lanes 3 to 13). The 77K polypeptide band which appeared at the 8-cell to the morula (lanes 3,4) was reduced or disappeared at later stages. From the 8-cell stage, the embryos showed no or very decreased synthesis of a protein band at 40 K (lanes 3 to 13, Fig. 5.8.). From day 4, at the stage of the compact morula, new polypeptides appeared at 26K and 36K and their synthesis increased to the blastocyst stage (lanes 3 to 13). During cavitation, 2 polypeptide complexes appeared at 55K and 60K and remained prominent to the blastocyst stage (lanes 7 to 14, Fig. 5.8.). To make the comparison for the qualitative differences in individual embryos easier, lanes from the photographs from two gradient gels that are presented in Fig. 5.9. and 5.10. have been cut and pasted together and presented in Fig 5.8.

Fig. 5.11. is a diagrammatic representation of the protein patterns synthesized at different stages. The intensity of the protein bands has been assessed visually, using as a comparison the intensity of the band at 75K. The diagram makes it easier to compare protein patterns of embryos that have been run at different times and the gels cannot be compared side to side. It shows that the changes that occured between the 4-cell stage and the blastocyst was consistent in repeated experiments and in a large number of embryos examined.

Embryos cultured in the absence of α–amanitin on day 3 developed normally to the 8-10 cell stage and synthesized polypeptides characteristic of the post-activation pattern (lane 4, Fig. 5.12.). Embryos that were treated with α–amanitin from

day 2 to 3 arrested at the 4-cell stage, failed to synthesize the morula-characteristic pattern and synthesized polypeptides seen in earlier stages, on day 2 (strong synthesis of the 40K band, faint 45 and 46K bands, no detectable 15K and 77K bands) (lane 2,3, Fig. 5.12.). Also the disappearance of the bands at 40K and 95K was blocked. Fig. 5.12. is a composite fluorograph . Lanes 1, 2, 9 and 10 from Fig. 5.9. have been cut and pasted to ease comparison. Fig. 5.13. shows diagrammatically the loss and appearance of the bands shown on Fig. 5.12.

5.3.4. Lineage-specific polypeptides

Five blastocysts were labelled as described at 5.2. After labelling, the inner cell mass (ICM) cells and the trophectoderm (TE) cells were isolated by immunosurgery (Handyside & Barton, 1977). One dimensional protein patterns of intact blastocysts, cells from the ICM and TE were compared. Very faint patterns were obtained from 4 of the blastocysts even after 2 months exposure of the film. In one case however, a very clear image is apparent and permits comparison between the two types of cells and intact blastocysts. Most polypeptides were common to all three types of samples. No bands were found exclusively in intact blastocysts. At least one band at approximately 46 kD was prominent and specific for the ICM (lanes 4&6) and absent from the TE cells (lane 5, Fig 5.14).

Fig 5.8. Composite fluorograph of ^{35}S-methionine-labelled polypeptides from embryos at the 4-cell stage (4c), 8-cell stage (8c), 10-cell stage (10c), morula (M), early blastocyst (eB), blastocyst (B) and expanded blastocyst (EB). The lanes have been cut and pasted together to ease comparison (for further explanation see 5.2.2.). The original photographs are Fig. 5.9. and Fig. 5.10. (Lanes 1-6: from 5.9.; Lanes 7-13: from 5.10.) The polypeptides have been separated by gradient one-dimensional polyacrylamide gel electrophoresis. (Please note that the embryo on lane 4 was at the morula and not at the 10c stage)

Fig. 5.8.

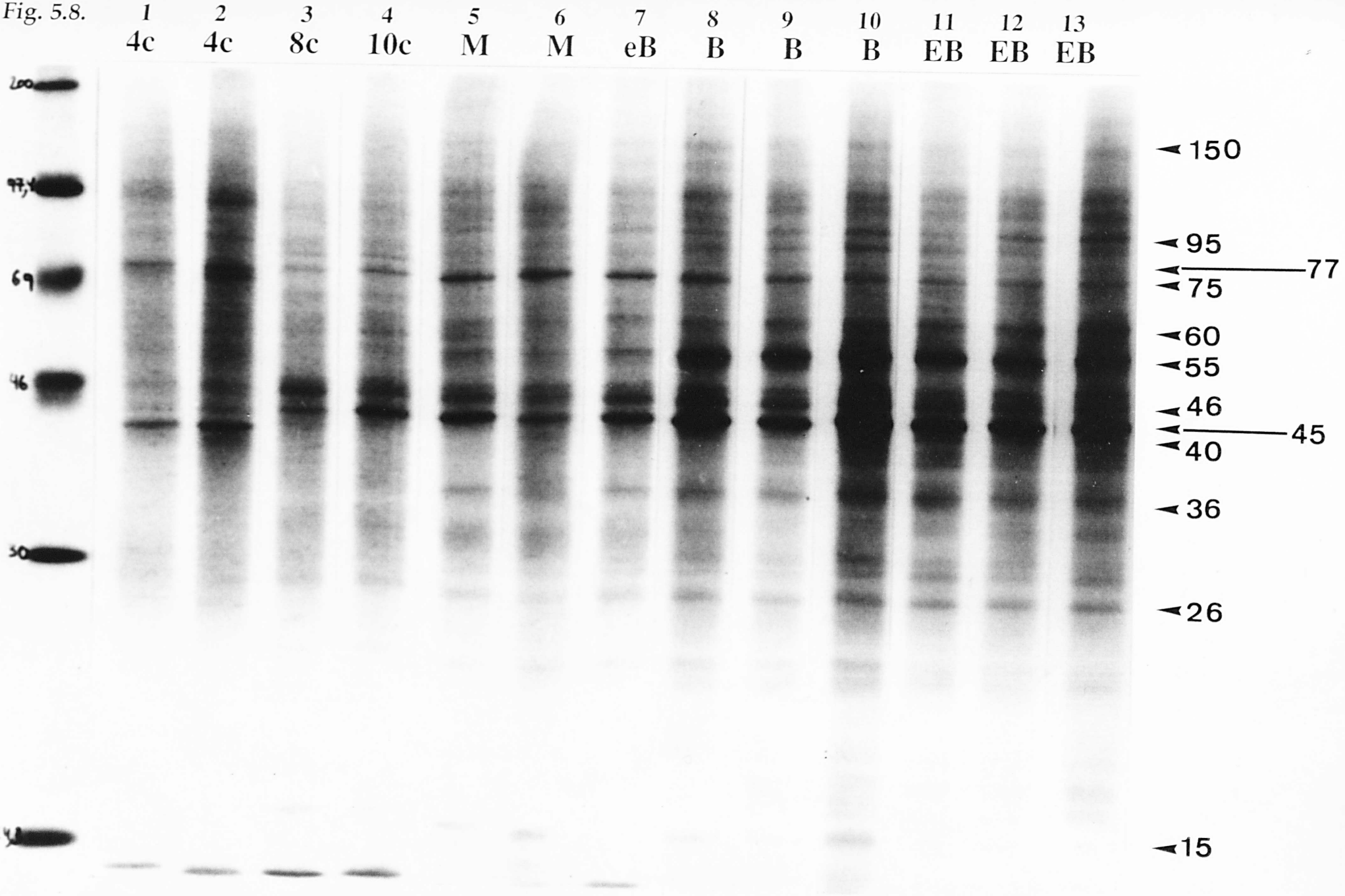

1 4c
2 4c
3 8c
4 10c
5 M
6 M
7 eB
8 B
9 B
10 B
11 EB
12 EB
13 EB
200
97,4
69
46
30
15
150
95
77
75
60
55
46
45
40
36
26

Fig 5.9. Fluorograph of ^{35}S-methionine-labelled polypeptides from embryos at the 4-cell stage (4c), 8-cell stage (8c), 10-cell stage (10c), morula (M), early blastocyst (eB), blastocyst (B) and expanded blastocyst (EB). The polypeptides have been separated by gradient one-dimensional polyacrylamide gel electrophoresis.

Fig. 5.9.

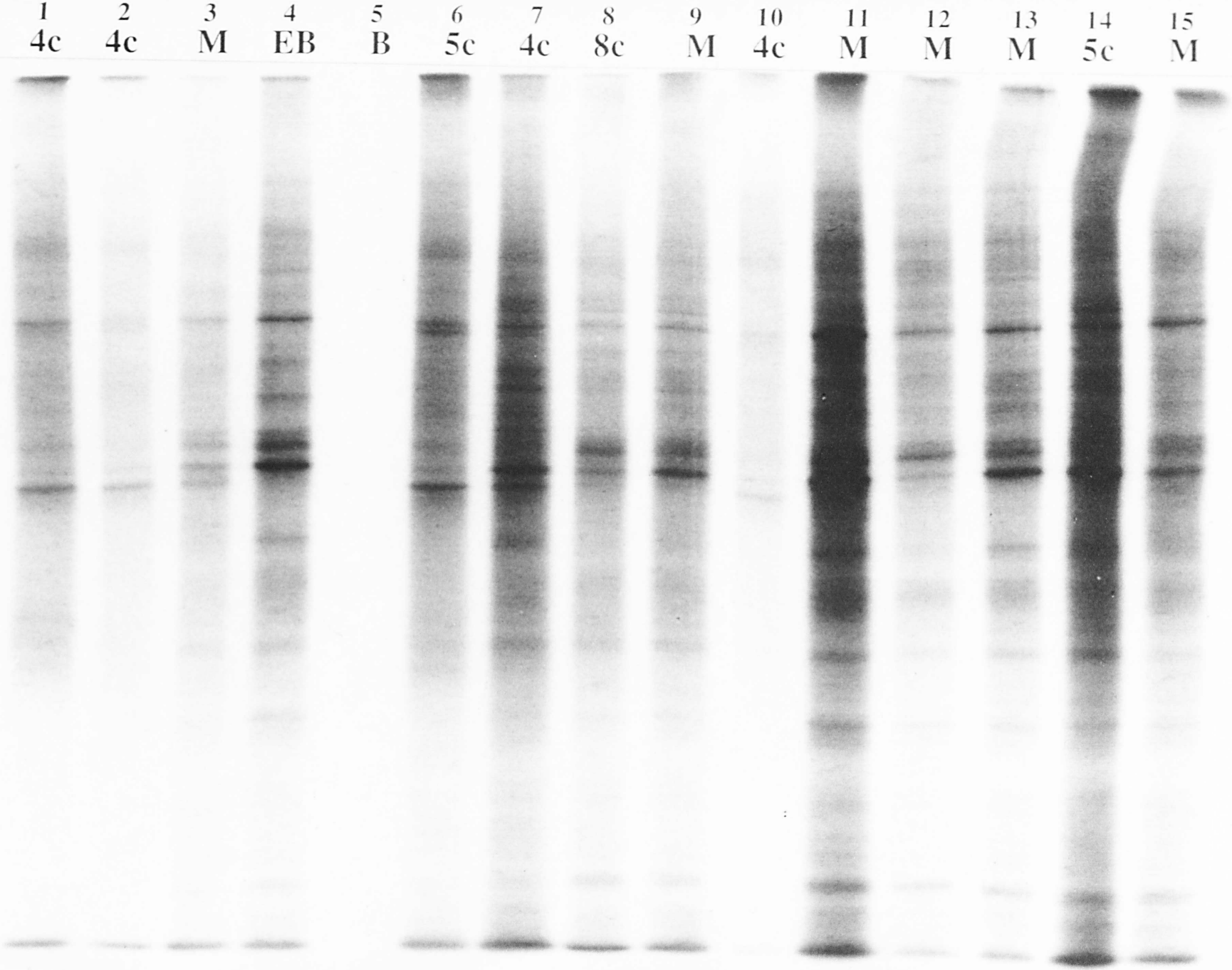


Fig 5.10. Fluorograph of ^{35}S-methionine-labelled polypeptides from embryos at the morula (M), early blastocyst (eB), blastocyst (B) and expanded blastocyst (EB). The polypeptides have been separated by gradient one-dimensional polyacrylamide gel electrophoresis. The developmental stage of the embryo on lane 13 is expanded blastocyst (EB).

Fig. 5.10.

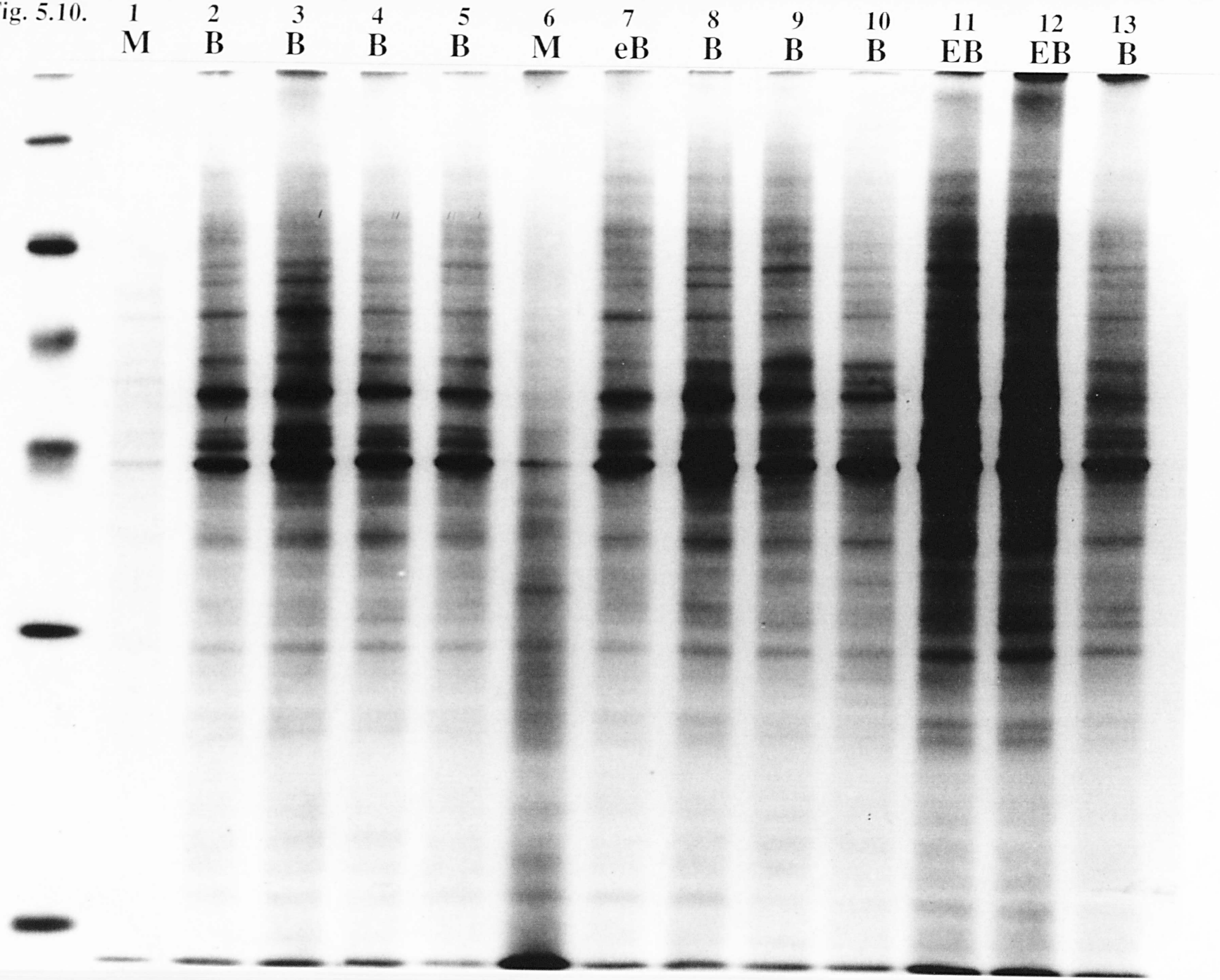


Fig 5.11. Pictorial representation of 17 protein bands of MW 15-150 kD. Each column represents the data obtained from a single embryo. Top row shows the morphological assessmnt of the embryos: 4: 4-cell stage; 2: 2-cell stage; 10: 10-cell stage; M: morula; eB: early blastocyst; B: blastocyst; EB: expanded blastocyst.

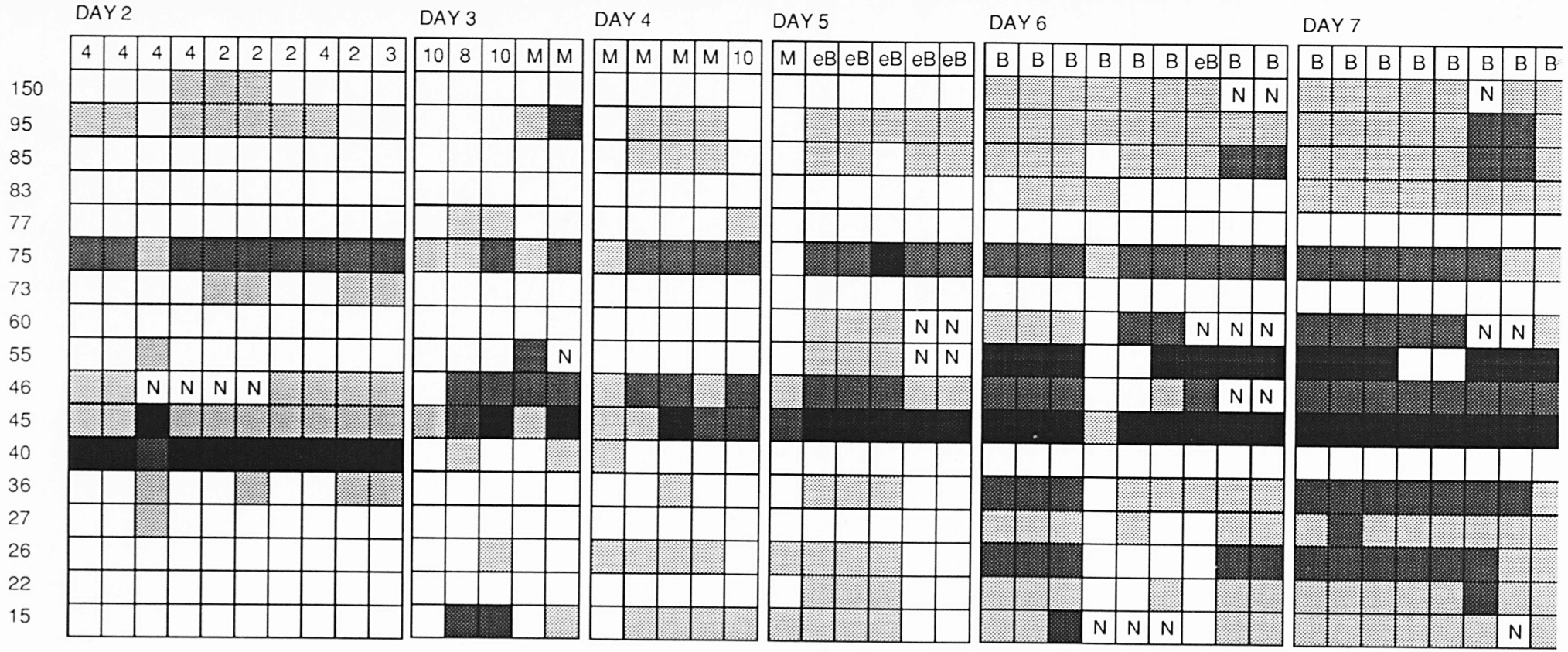
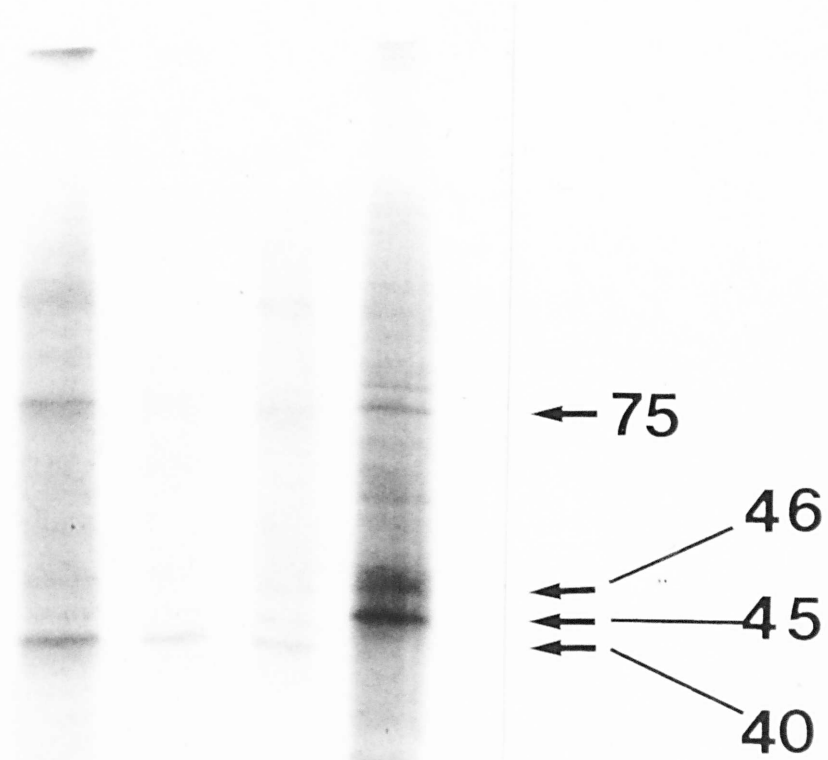

DAY 2
DAY 3
DAY 4
DAY 5
DAY 6
DAY 7
150
95
85
83
77
75
73
60
55
46
45
40
36
27
26
22
15
4 4 4 4 2 2 2 4 2 3
10 8 10 M M
M M M M 10
M eB eB eB eB eB
B B B B B B eB B B
B B B B B B B B
N
Strongly present
Present
weakly present
Absent
Non-detectable
N

Fig. 5.11.

Fig 5.12. Composite fluorograph of ^{35}S-methionine-labelled polypeptides synthesized by individual embryos. Lane 1: 4-cell embryo labelled on day 2. Lanes 2 & 3: 4-cell embryo that have been incubated in α–amanitin for 24 hrs prior to labelling on day 3. Lane 4: 10-cell embryo labelled on day 3.



Day	D. 2	D.3	D. 3	D. 3
a-amanitin	-	+	+	-
stage	4c	4c	4c	10c

Fig. 5.12.

Fig. 5.13. Pictorial representation of the protein patterns of single human preimplantation embryos and the effects of α–amanitin on day 2 & 3. Each column represents the data obtained from a single embryo. The embryos have been preincubated in 100μg/ml of α–amanitin for 24 hours prior to labelling. 4: 4-cell stage; 2: 2-cell stage; 10: 10-cell stage.

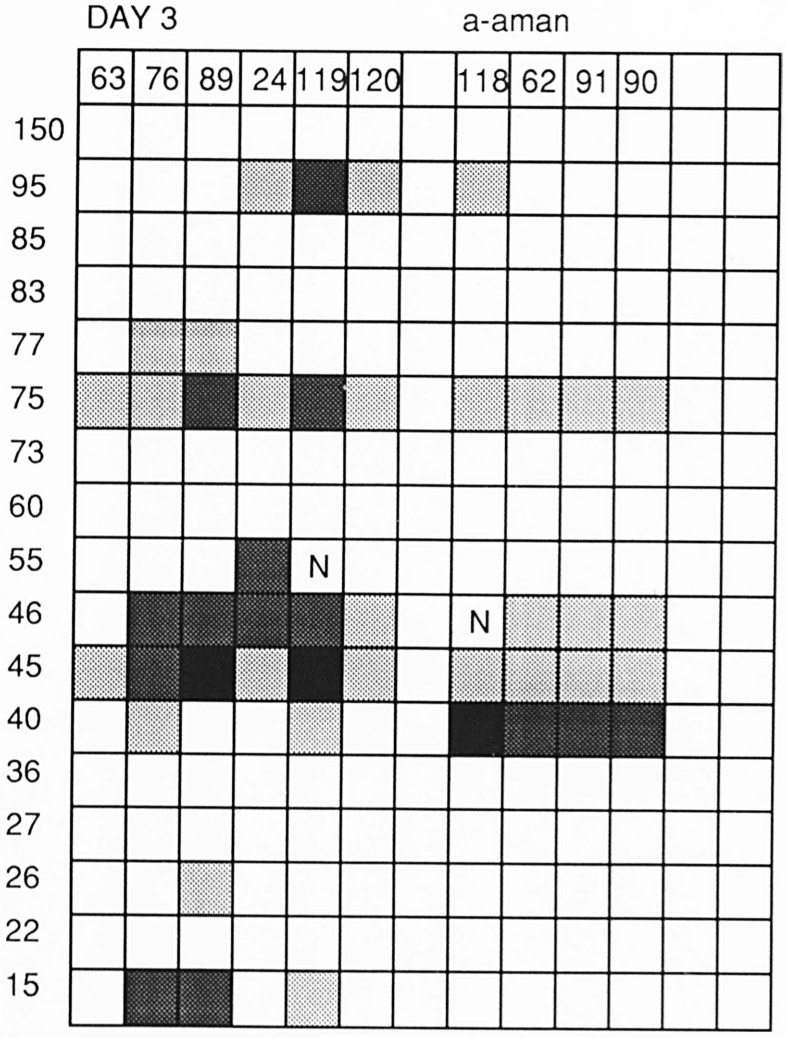


Fig. 5.13.
Day 3

Strongly present

Present

weakly present

Absent

Non-detectable N

Fig 5.14. Fluorograph of ^{35}S-methionine-labelled polypeptides from embryos at the 2-cell stage (2c), 4-cell stage (4c), 5-cell stage (5c), morula (M), blastocyst (B), trophectoderm cells (TE) and inner cell mass cells (I).

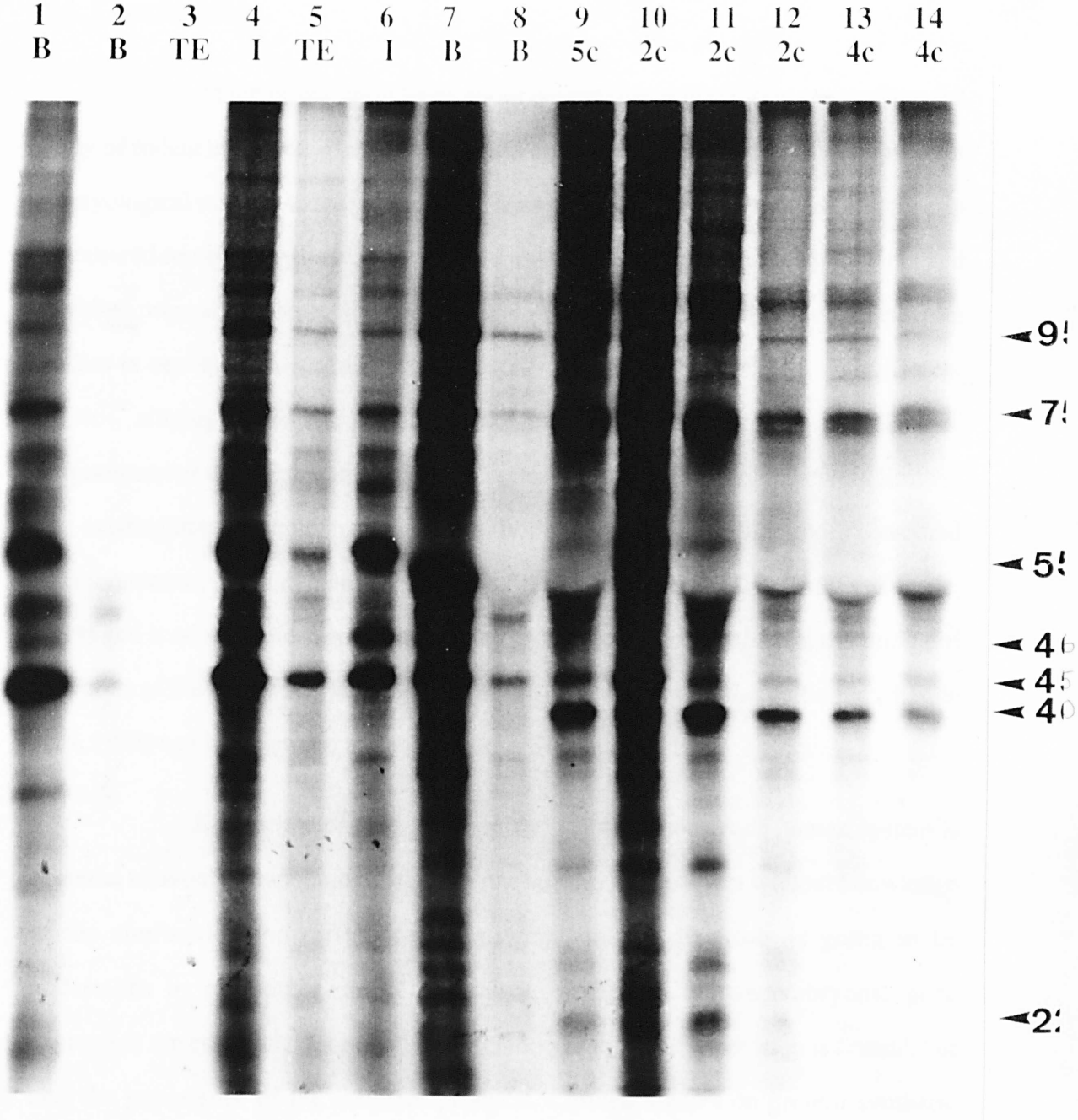


Fig 5.14.

5.4. Conclusions

Much of our knowledge about mammalian embryology comes from the study of rodent and domestic species. Mouse embryos have been extensively used for embryological studies, as the culture conditions have been defined, mouse embryos can be cultured routinely to the blastocyst stage, transfered to pseudopregnant mothers and establish pregnancies with high success rates. However, the information gained from studies in one species is unlikely to be applicable to all. It was shown that different species, although they might share a common general pattern, have individual programmes for development. Data on the preimplantation human embryo is limited. The existing data, although it shows some similarities, has many differences compared with the mouse; in the human embryo, polarization begins at the 2-cell stage (Tesaric, 1989) and it occurs before compaction, unlike the mouse embryo. Also, the timing of activation of the embryonic genome, in the human happens at the 4-8 cell stage (Braude *et al*, 1989) and in the mouse at the 2-cell stage (Flach *et al*, 1982).

Knowledge of the extend of homology between the mouse and human system is essential to avoid extrapolating data from one species to the others without knowledge of the similarities and differences. If preimplantation diagnosis is going to be undertaken by measuring the gene product, studies on human embryonic gene expression are essential. Not only is the initiation of the gene expression is crucial, but also the persistence of the maternal products. Limited studies on protein synthetic patterns of the human embryo have been done (Braude *et al*, 1989; Artley *et al*, 1992) and no studies of the quantitative patterns of protein synthesis.

The rates of uptake and incorporation of ^{35}S-methionine from normally developing human embryos remained constant from day 2 to day 4 and are significantly increased 6-fold and 10-fold respectively between day 4 and day 7. Similarly, in the mouse the uptake and incorporation are constant (Brinster *et al*, 1971) or there is a

slight but reproducible decrease (Van Blerkom & Brockway, 1975) until the early blastocyst stage and increase after blastocyst formation.

In other species that have been investigated, the pattern of amino acid incorporation is very different. In the sheep, the incorporation is constant during the first three cycles, then it drops sharply to very low levels (5% of the initial) during cleavage stages and increases during blastocyst formation, reaching only 25% of the 1-cell rate in the blastocyst (Crosby *et al*, 1988). The steep decline prior to the embryonic gene expression was also reported for the gerbil embryo (Norrris *et al*, 1985). In the cow, the rate of incorporation declined steadily from the zygote to the 8 to 16-cell stage, and rose slightly in the 16 to 32-cell stage, before increasing dramatically in the blastocyst, to reach a level 20 times greater than at the 8-cell stage (Frei *et al*, 1989). Similar results with the cow were reported for the pig embryo (Jarrell *et al*, 1991), although the increase during blastocyst formation is not so dramatic. The drop and rise of protein synthesis was thought to reflect the elimination of the maternal mRNA and the initiation of the embryonic gene activity. The pattern of transmission of protein synthesis from maternal to embryonic control seems to be different for each species studied to date. In some (e.g. rabbit and mouse) the timing of degradation of maternal mRNA coincides with the activation of the embryonic genome and during a period that the embryo is under control of both genomes. In others (sheep, pig, cow and gerbil embryos), the degradation of maternal mRNA is followed by a period of very low translational activity, before the activation of the embryonic genome (Leese, 1991). From our data, the human embryo seems to be more similar to the mouse and the rabbit..

Successful further development and the formation of a blastocyst seemed to be related to increased uptake and incorporation, as expanded and hatched blastocysts incorporate more than non-cavitating embryos. Protein synthesis rates have been used as indications for embryonic viability in the mouse, as embryos that did not develop a blastocyst cavity incorporated less than cavitating embryos (Braude *et al*,

1979) and, as this data shows, the same is true for the human embryo (Table 5.3., Fig 5.3., 5.4.). Probably the presence of high trancriptional activity in the majority of the late morulae cells, which was shown by Tesaric (1989) to be required for blastocyst formation, is followed by an increase in translational activity, which is essential for blastocyst formation.

During the initial cleavage divisions (from day 2 to day 4) the uptake and incorporation levels per cell fell 5-fold respectively (Fig 5.2.) and then remained almost at the same levels from day 4 to day 7. In contrast, in the mouse the incorporation per cell remains constant throughout preimplantation development (Van Blerkom & Broackway, 1975). In the preimplantation embryo, cellular growth does not occur during cleavage; its size remains the same, but it undergoes a series of divisions which reduce the volume of each cell with each cleavage. The ratio of the volume of the nucleus (which remains the same size) to the volume of the cell increases during cleavage and blastulation and, in the mouse, it reaches the somatic value at the blastocyst stage (Smith & Johnson, 1986). Therefore, as the cell size is not the same in every stage of development but is reduced from the 1-cell to the blastocyst, the situation does not correspond precisely to 'cell division' in the sense of producing daugther cells equals in size to the parent cell. The drop in incorporation per cell from day 2 to 4 shows that although the cell number increases, the protein synthesized does not increase at the same rate. Potential explanation could be either that the drop in uptake rates limits the amount of protein synthesized, or that the amount of protein synthetic machinery does not increase with the same rate as cell number from day 2 to 4. This could divide the existing protein synthetic apparatus between daughter cells and limit the overall protein synthetic capacity of a blastomere in comparison with the parent cell. The second hypothesis comes in agreement with the findings of Tesaric (1986), who showed that the total amount of ribosomes decrease during the first three cell cycles. However, both assumptions are probably correct, as it was shown in the mouse that incorporation is dependent on uptake until the incorporation values reach a plateau. After the plateau, even 2.5 higher uptake values do not influence incorporation (Epstein

& Smith, 1973). However, the number of embryos that are available for analysis is limited and therefore no similar measurements for the relationship between uptake and incorporation have been made.

Alpha-amanitin is a specific inhibitor of RNA polymerase II and prevents synthesis of mRNA at appropriate concentrations. This way it blocks the synthesis of proteins which are dependent on newly synthesized message. The uptake and incorporation of ^{35}S-methionine by human embryos cultured in the presence of 100 μg/ml of α–amanitin for 24 hours before labelling was similar to untreated embryos on day 3. In the α–amanitin treated embryos the uptake and incorporation levels drop from day 4 onwards (Fig 5.5. & 5.6.). In the mouse, when the embryonic genome switches on at the 2-cell stage, there appears to be a rapid switch-off or destruction of maternal mRNA and many species of polypeptides are not synthesised any more. The loss of the maternal mRNA is gradual and maternally inherited effects on development persist well beyond the 2-cell stage (review by Johnson, 1981). If the situation is analogous in the human embryo, the activation of the embryonic genome should be synchronous with a progressive decline in the expression of the maternal mRNA. Support for that hypothesis is provided by the fact that the drop in uptake and incorporation that takes place after day 4 in α–amanitin treated embryos (the embryonic genome becomes active on day 2-3) shows that blockade of transcription affects the embryo more severely in later stages, when development and protein synthesis are dependent on embryonic genome only compared with earlier stages, when some of the maternal transcripts are still active.

This study gives information for comparative values of uptake and incorporation. The absolute rate of protein synthesis, which is a function of uptake, endogenous pool size, the type of labelled precursor and protein degradation, cannot be directly calculated from this data. There is some data in the mouse which permits the correlation of incorporation and protein synthesis; in a study for the amino acid uptake and protein synthesis, it was shown that although the uptake of a labelled amino acid

was dependent on the concentration of the amino acid in the culture medium, repeated experiments did not reveal any differences in incorporation levels, even when the uptake was, in the highest concentration, 2.5 times more than in the lowest (Epstein & Smith, 1973). This shows that amino acid incorporation is independent from uptake and can be taken as a measure of protein synthesis. Also the size of the endogenous amino acid pool should be very small in comparison with the amount of the amino acid taken up from the medium. The amino acid used for that study, methionine, was shown in the mouse to have a small endogenous pool (Kaye *et al*, 1982), 0.13% and 1.2 % of the total in the unfertilized egg and the blastocyst respectively, suggesting that this amino acid would be a useful probe for protein synthesis. Although during cleavage the incorporation levels can be taken as a reasonable estimate of protein synthesis, the real incorporation values in blastocysts are anticipated to be higher than the ones reported here, as the labelled methionine is competing with amino acids from a larger endogenous pool (considering the blastocyst cavity as an endogenous pool) (Miller & Schultz, 1983; 1985). Although there is no data in the human embryos about the components of the blastocoel fluid, experiments in the rabbit and mouse resolve the amount of labelled amino acid uptake into cellular and cavity components. It was found that 95% and 70% of the label was accumulated in the cavity, for the rabbit and the mouse respectively (Miller & Schultz, 1983; Miller & Schultz, 1985). As the increase in volume that the rabbit embryo undergoes during blastocyst formation is enormous (Van Blerkom *et al*, 1973), probably the mouse is a system more comparable to the human, as in both species the size of the embryo during blastocyst formation does not increase.

The increase in incorporation differs slightly for the specific amino acid precursor used (Brinster, 1971). The absolute values are variable; however the culture conditions, the incubation times and the specific activity of the labelled precursor are different between investigators. For example, Epstein and Smith (1973) reported 3-fold lower incorporation values in an experiment similar to Brinster's. The uptake values were similar in both experiments. However, the incubation time was 1 hour,

though in the work of Brinster the incubation time in a labelled amino acid was 20-24 hours and only embryos which showed optimal development after labelling were selected . As the labelling time and culture conditions can influence the values of uptake and incorporation, the calculated rates of protein synthesis do not have an absolute value sense but they show the differences between developmental stages.

The overall pattern of protein synthesis does not represent the rate of protein synthesis of individual proteins. As shown in Fig. 5.8. and 5.11., some polypeptides increased in a much greater extent than others and some others decreased. In the mouse there are studies on the absolute rates of synthesis of individual proteins, as for actin and tubulin (Abreau & Brinster, 1978). For actin, the rate of synthesis was found to be low in the unfertilized egg and early embryo, increasing 10-fold at the 8-cell stage and 90-fold at the blastocyst, when ^{3}H-leucine incorporation into total protein increased only 7-fold during preimplantation development (Abreau and Brinster, 1978).

Two patterns of polypeptide synthesis are observed; the first is between the 4- and 8-cell stages and the second from the morula stage onwards, during blastocyst formation (Fig. 5.8. & 5.11.). Between the 4- and 8-cell stages the switch of the maternal to the embryonic genome is characterized by a band at 40K, which is prominent at the 4-cell stage and disappears at the 8-cell stage onwards. The polypeptides at 45 and 46K that were faint at the 4-cell stage showed increased synthesis and became very prominent from the 8-cell stage onwards. Also a new polypeptide band at 15K appears. Braude *et al* (1988) also reported that the major differences in 5 polypeptide bands occur during the 4- to 8-cell stages. In this study, a large number of polypeptides were studied and changes in 17 polypeptide bands are shown in Fig. 5.11. and 5.13. A large number of embryos were studied (91 embryos) and show a consistent pattern between stages from the cleavage stages to the late blastocyst stage.

Braude *et al* (1988) have examined mainly cleavage stages. In this study, most of the embryos examined were between the morula and the blastocyst

stage. Although the major quantitative increase in protein synthesis occur between the 8-cell stage and the blastocyst stage (Fig. 5.1., Table 5.1.), very few qualitative differences occur during morula to blastocyst transition (Fig. 5.8.). This is surprising, as during that period the cells differentiate to produce inner cell mass cells (ICM) and trophectoderm cells (TE), which have different properties. Not only the size of polypeptides synthesized remains unchanged, but even the relative rates of synthesis from one protein band to another remain the same. The reasons for that are that probably the detection system used is not sensitive enough to detect subtle differences in the patterns of synthesis and also both populations of cells from the ICMs and TE are analysed together (the limitations of the detection system are discussed below). The largest qualitative changes in the gel patterns occur between the 4- and 8-cell stage, when the 40K and 95K polypeptides disappear, the polypeptides at 45 and 46K are synthesised at a much higher rate and new polypeptides appear at 76, 26 and 15K (post-activation pattern). From that stage onwards, the rate of synthesis of some of the polypeptides already present increases (at 26, 45, 46, 75K), the rate of some of the polypeptides remains the same (15K) and some new gradually appear (95, 85, 36, 26K on day 4; 60, 55, 22K on day 5; 83, 27K on day 6). No polypeptide present on day 3 disappears, except for a faint band at 77K, which is present in some of the morulae on day 3. The overall pattern from day 3 to blastocyst is similar. An analogous situation has been observed in the mouse, where the largest qualitative change in the gel pattern happens between the 2-4 cell stages and the major quantitative increase between the 8-cell stage and the blastocyst (Epstein & Smith, 1974; Van Blerkom & Brockway, 1975). Both the mouse and the human show increase in protein synthesis 1 division after the activation of the embryonic genome and during blastocyst formation.

Although 5 out of 6 4-cell embryos showed the characteristic pattern of day 2 embryos (lanes 1, 2, 3, 7, Fig. 5.1 1.), in one case one embryo showed a pattern of protein synthesis which was more similar to the 8-cell embryos (third lane from left to right, Fig. 5.1 1.); interestingly, that embryo also displayed increased uptake and incorporation values (1.5 times more than the average), at the same level as 8-cell

embryos. Out of 32 embryos that developed to the late morula or blastocyst stage, none exhibited the pre-activation pattern, suggesting that the activation of the embryonic genome is necessary for blastocyst formation.

A great proportion of embryos fails to develop to the expanded blastocyst stage. The rate of embryo failure increases around the 4- to 8- cell stage (Bolton *et al*, 1989). As the timing of failure coincides with the activation of the embryonic genome, it has been suggested that failure of gene activaton is a potential cause of developmental arrest (Bolton *et al*, 1989). However of 4 embryos that have been arrested at the 4-8-cell stage, 3 show the post-activation protein pattern (data not shown). Also one embryo which is arrested shows an intermediate pattern, indicating that either the activation of the embryonic genome is progressing or that the embryonic genome is activated in some of the blastomeres only. Although the data indicates that failure of the embryonic expression is not, in these cases, the cause for arrest, the number of arrested embryos examined is very small for conclusions. Recent work by Artley *et al* (1992) demonstrates in a large number of embryos (54) that the embryonic genome is activated in over 50% of arrested embryos, suggesting that cleavage arrest is not always accompanied by failure of activation of the embryonic genome and that other factors, like suboptimal culture conditions and chromosomal abnormalities, might be the cause for developmental arrest.

Alpha-amanitin prevents the production of mRNA and consequently the production of proteins that are coded by newly synthesized messages. Therefore the presence of a protein band from an embryo that has been treated with α–amanitin shows that its synthesis was dependent on pre-existing mRNA. Similarly, the absence of a protein band from an α–amanitin-treated embryo indicates that newly synthesized transcripts (coded from the embryonic genome) code for this protein.

It was demonstrated that transcription inhibition during the 4-8 cell stage blocks the appearance of new proteins and also blocks the disappearance of the old ones (Fig. 5.12.). This is in contrast to the findings in the mouse, where α–amanitin does not block the elimination of the old proteins during the transition from the embryonic to the

maternal genetic control (Flach *et al*, 1982). From the mouse data it was concluded that degradation of the maternal mRNA was independent from the activation of the embryonic genome. However, our data suggests that the degradation of maternal mRNA in the human preimplantation embryo is dependent, at least partially, on the newly synthesized embryonic messages. A potential explanation is that the enzymes that are necessary for the selective degradation of the maternal information are coded by embryonic genes. Another possibility is that the half life of the maternal messages is prolonged after the non-appearance of the new transcripts, as a mechanism to compensate for the lack of new proteins.

The correlation of a polypeptide band and a specific cell or stage of development could be misleading in some cases; for example, the earliest time a protein is detected in a gel is not necessarily the earliest stage at which it is synthesized, as it could have been synthesized in a previous stage to a lesser degree than detectable by the sensitivity of the system. Also, in some cases, post-translational modifications of proteins at a certain stage may alter their electrophoretic mobilities and create the impression that they are stage specific.

I have attempted to examine the fluorographs using scanning densitometry to obtain more information about the quantitative changes between individual proteins. The information that was obtained was not much more helpful than the visual assessment. Also, those results are only semiquantitative, as the embryos have been incubated in labelled methionine and the proportion of methionine in certain proteins varies.

There are limitations to the number of proteins that can be resolved on one-demensional SDS-PAGE gels. Under the denaturing conditions of sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and the presence of 2-mercaptoethanol in the system, proteins are dissociated to component polypeptides and each band on the gel may represent a large number of polypeptides that have similar molecular weights. Any qualitative changes that occur within the population of

polypeptides that compose one band might not be detected. Only those proteins that are present in high concentrations can be visualized by electrophoresis and autoradiography. Proteins that are present in the embryo in amounts insufficient to be detected individually probably produce the "gray" backround between the autoradiographic bands (Van Blerkom & Manes, 1974). Autoradiography requires a long exposure time when the amount of incorporated radioactivity is low, as with preimplantation embryos. Fluorographic amplification of the radioactivity contained in the gel reduces the time of exposing the film. Also, if differential protein synthesis occurs between cells, for example the ICM and the TE, would not be detected and although it could be that an intact embryo does not show any qualitative changes in protein synthesis, differential synthesis might take place in different cells or regions of the embryo.

The different patterns synthesised by isolated cells from the inner cell mass and the trophectoderm cells have also been investigated. Cells from the ICM and TE are examined separately and it was shown that at least one band was specific for the inner cell mass (lanes 4&6) and absent from the trophectoderm cells (lane 5, Fig 5.14). This is a very interesting result, as in the mouse, differentiation at the protein synthetic level is only detected at the blastocyst stage by 2-dimensional electrophoresis (VanBlerkom, 1982). However, our data can only be treated as an interesting suggestion, as the number of embryos used is very small. Immunosurgery, which followed labelling, lyses the TE cells and leaves the ICM intact. Therefore, most of the labelled protein in the TE cells would be lost during immunosurgery. The main reason for not repeating the procedure with a larger number of embryos is that immunosurgery is not succesful with human embryos and the ICM was not recovered in most cases due to insufficient lysis of the TE cells. This data suggests that further experiments should be done, possibly by using micromanipulation to isolate the ICM and the TE cells.

Chapter 6

Preimplantation diagnosis by a biochemical assay:

HPRT activity in preimplantation human embryos

6.1. Introduction

6.1.1. Biochemical detection of X-linked disease in prenatal diagnosis

About 350 genetic disorders are known to be caused by an inherited enzyme defect (Besley, 1992). They result in impaired production or function of an enzyme or other type of protein. As many of those defects have not been characterized at the DNA level, diagnosis by DNA analysis is not always possible. As an alternative, around 100 can be diagnosed reliably by enzyme assays (Ben-Yosef, 1992) and their number is increasing every year, as the biochemical basis of the diseases is further investigated. Before attempting prenatal diagnosis by an enzyme assay, some important factors have to be considered. First, the biochemical defect has to be known and an assay to be available. Second, the defect has to be expressed in tissues that can be safely obtained from the fetus, early enough to permit termination of the pregnancy in case the fetus is affected. Thirdly it is desirable to test the enzyme in the parents or other members of the family, to ensure that they have detectable enzyme activity. Several cases of pseudodeficiency have been described, in which heterozygotes for the disease appear to be deficient. In these cases, an individual can have an allele which is active *in vivo* but not *in vitro* (Ben-Yosef, 1992).

X-linked recessive disorders present some difficulties in the biochemical detection of the heterozygotes. Male embryos have only one X chromosome and they are either deficient for the enzyme (affected) or normal (Lyon, 1971). Random X-inactivation means that all females are mosaics for the X-chromosome activity. Therefore the proportions of active mutant and normal X chromosomes in the tissues that are involved in the pathogenesis of the disease will lead to a great variety of phenotypes and determine if a female heterozygous will be normal or have mild manifestations of the disease. For prenatal diagnosis, the proportions of active mutant

and normal chromosomes in the fetal biopsy sample may affect the levels of activity measured.

6.1.2. Possibilites for preimplantation diagnosis

Diagnosis of a genetic disease by a biochemical assay at the preimplantation stage is only possible if the protein or enzyme measured is coded by the embryonic genome at this stage of development. Also the levels of the maternal protein or enzyme, inherited in the oocyte cytoplasm, should be sufficiently low to permit detection of the embryonic gene expression.

6.1.3. Methods for biochemical detection of protein

Application of routine enzyme assays in preimplantation diagnosis is not possible, as the amount of enzyme activity in embryos is minute. However, several microassays have been developed, that are sensitive enough to be applied to a single embryo or a single cell. Monk & Kathuria (1977) developed a double biochemical microassay for the X-linked HPRT and the autosomal APRT enzymes. This microassay was used for the first preimplantation diagnosis of Lesch-Nyhan syndrome in a mouse model (Monk *et al*, 1987). Monk *et al* (1987) detected the presence or absence of enzyme activity in single cells removed from embryos at the 8-cell stage while the embryos remained in culture. Normal and HPRT-deficient embryos were then transfered to separate foster mothers and the diagnoses were confirmed by measuring enzyme activity in the resulting fetuses later in gestation.Also the microassay was used successfully to sex mouse embryos by measurement of the ratio of X-linked HPRT and autosomal APRT levels, at the morula stage, when both X-chromosomes are active. Microassays have been developed for adenosine deaminase (ADA), which is the enzyme which is lacking in severe combined immunodeficiency disease (SCID)

(Benson & Monk, 1988); for glucose phosphate isomerase (GPI) (West & Green, 1983); and for hexosaminidase (Serman *et al*, 1990). Also microassays based on detection by high performance liquid chromatography (HPLC) have been developed (Leese *et al*, 1991; Martin *et al*, 1993)

6.1.4. X-chromosome inactivation

An important aspect of X-linked gene expression is that in females with two X chromosomes there is a double dose of these genes compared with males, which have one X and one Y chromosome. However, the levels of gene products between the sexes are equal, due to the inactivation of one of the X chromosomes in the somatic cells of the female. Sex chromosome inactivation was first suggested by Lyon (1971) and occurs as a mechanism for compensating the difference in gene product dose (Gartler & Riggs, 1983). Genes on the inactive X-chromosome are not expressed in somatic tissues during the lifetime of the individual, but are reactivated in germ cells shortly after meiosis. X-inactivation in somatic cells is very stable and clonally inherited, so that each daughter-cell has the same inactivated X-chromosome as the parent cell (Monk, 1992). Therefore, a proportion of cells have the paternal X chromosome active, while in the other cells, the maternal X chromosome is active. The proportion is variable, because the inactivation mechanism is random and occurs during early embryonic development. Theories of X-inactivation support the idea that an inactivation centre exists and inactivation is spread from that centre to the chromosome. Recently, a gene has been identified on the X-chromosome in humans (XIST; Brown *et al*, 1991) and in mouse (Xist; Borsani *et al*, 1991) which is expressed only by the inactive chromosome and it maps in the area where the centre for X-inactivation is located.

The mouse has been used extensively for studies on X-inactivation. X-chromosome activity can be monitored using very sensitive assays for X-linked enzymes. In female embryos prior to X-inactivation, both X chromosomes are active at the morula stage and the dosage of an X-linked enzyme in female embryos is double that in males. In the mouse it was possible to detect a two fold difference in the distribution of HPRT and autosomal APRT in single biopsied blastomeres and identify

male and female embryos (Monk *et al*, 1987). The timing of X-inactivation in the mouse has been studied in detail. During oocyte growth and maturation, both X-chromosomes are active. In spermatogenesis, the X-chromosome is inactive. After fertilization, the paternal X-chromosome becomes active at the morula stage, but then it is preferentially inactivated again in the trophectoderm (at the blastocyst stage), as extraembryonic tissues are formed. In the cells that the fetus will be derived from (ICM of the blastocyst), either the maternal or the paternal X-chromosome is randomly inactivated. X-inactivation is very stable in somatic cells, but in the female germ line, the X-chromosome is reactivated around the time of meiosis (reviewed by Monk, 1981). Information about the timing of the X-inactivation in the human embryo is lacking.

6.1.5. Animal work for preimplantation diagnosis

The first successful preimplantation diagnosis of genetically defective embryos has been established in a mouse model for Lesch-Nyhan syndrome (Monk *et al*, 1987; Monk & Handyside, 1988; Hooper *et al*, 1987). A sensitive microassay was used (Monk *et al*, 1987) to determine the levels of HPRT and APRT in single blastomeres or 5-10 cells biopsied from blastocysts. During the assay procedure, the biopsied embryos remained in culture. Samples from affected male embryos had very low HPRT activity and low HPRT:APRT ratio and were easily distinguished. Embryos were then transferred separately back to different pseudopregnant mothers. In all cases the embryos were correctly diagnosed (Monk *et al*, 1987). Using this sensitive microassay to measure the relative activity of X-linked HPRT activity and an autosomal enzyme identification of sex of biopsied embryos was possible (Monk & Handyside, 1988). In the mouse, both X chromosomes are active during the morula stage of preimplantation development. Female embryos with two chromosomes, therefore, have twice the dosage of X-linked enzymes (and show bimodal distribution) compared with male embryos with one X and one Y chromosome. At the blastocyst stage the bimodal

distribution is lost, probably due to the X-inactivation in the majority of the cells. Monk and Handyside used the 2:1 difference in the amount of X-linked gene products before X-inactivation occurs in females. This approach can not be used for conventional prenatal diagnosis, because X-inactivation is already complete before cells are sampled by CVS or amniocentecis.

6.1.5.1. Effect of transcriptional inhibitors on HPRT activity

If an enzyme assay is used for preimplantation diagnosis, as an alternative to DNA analysis, the timing of the onset of the embryonic gene expression after fertilization and the persistence of maternal gene products inherited in the oocyte cytoplasm must be established. In the mouse, HPRT activity increase at the 4- to 8- cell stage of development and the increase in activity is dependent on transcription from the embryonic genome (Monk & Harper, 1978). Preimplantation diagnosis of HPRT deficiency by an enzyme microassay was successful in a mouse model (Monk *et al*, 1987; 1988). However, in the mouse it has been established that embryo-coded enzyme predominates over maternally-coded enzyme inherited from the oocyte at these stages (Monk & Harper, 1978; Monk 1987) and therefore measurements of the embryo-coded enzyme permitted preimplantation diagnosis by an enzyme assay.

In contrast with the mouse model, the levels of HPRT activity measured in 46 individual human oocytes and embryos from 4-cell stage to the blastocyst, using the same microassay as in the mouse, were low and variable at all stages (Braude *et al*, 1988). The average levels of activity for morula and blastocyst stages was almost at the same levels with the unfertilized oocytes and instead of a rise in activity, a depression was observed. Inhibition of transcription failed to produce any decrease in enzymatic activity, indicating that the measured HPRT activity is maternal in origin. Also, levels of enzymatic activity were found to be substantially variable between batches of

embryos from the same family, individual embryos and blastomeres biopsied from the same embryo (Braude *et al*, 1989).

Using a more sensitive HPLC microassay and higher substrate concentrations, however, higher levels in activity were measured (Leese *et al*, 1991). Leese *et al* (1991) have examined the profiles of HPRT activity in human embryos developing *in vitro* between day 2 and day 6 after insemination. The activity of HPRT increased between day 2 and day 4, but declined sharply by day 6 . This was thought to reflect a combination of decreasing levels of maternally inherited enzyme and the initiation of embryonic gene expression followed by X-inactivation at the blastocyst stage on day 6. To demonstrate that preimplantation diagnosis for HPRT deficiency (Lesch-Nyhan syndrome) is feasible in the human embryo, it is essential that the HPRT activity is predominantly embryo-derived at the stage that the embryo is going to be diagnosed. For that reason, the effects of α–amanitin (a transcription inhibitor) at various stages of development was investigated .

In the mouse, both X chromosomes are active during the morula stage of preimplantation development (Epstein *et al*, 1978). Female embryos with two chromosomes, therefore, have twice the dosage of X-linked enzymes compared with male embryos with one X and one Y chromosome. Using a sensitive microassay to measure the relative activity of X-linked HPRT activity and the autosomal enzyme APRT biopsied embryos have been sexed successfully (Monk & Handyside, 1988). In order to sex by quantitative assays of X-linked products, the assay must be performed at a time when the residual oocyte-coded activity is insignificant compared to the embryo-coded activity but before X inactivation. Though this idea has the advantage of being applicable to all X-linked recessive conditions, even if the genetic defect is not characterized, it cannot be attempted in human embryos, as nothing is known about the timing of X-linked gene expression and inactivation and very little is known about human gene expression during preimplantation development.

6.1.6. Aims

The profiles of HPRT activity have been examined in normally fertilised human embryos developing *in vitro* between the morula stage on day 4 and blastocyst stage on day 6. In order to investigate the persistence of the maternal mRNA, the effect of transcriptional inhibition on a group of embryos was also studied.

To sex by quantitative assays of X-linked products, the assay must be performed at a time when the residual oocyte-coded activity is insignificant compared to the embryo-coded activity, but before X inactivation. The profile of HPRT activity in male and female embryos are also informative about the timing of X-inactivation.

6.2. Method

6.2.1. HPRT activity and trancriptional inhibitors

The profiles of HPRT activities were examined in normally fertilised human embryos developing in a normal rate *in vitro* between the 4-cell stage on day 2 and blastocyst stage on day 6 after insemination. A group of embryos were randomly allocated between a control group and a group exposed to α–amanitin, to block transcription of mRNA from the embryonic genome. The activity of HPRT was assayed in extract of the reaction product, inosine monophosphate (IMP) separated by HPLC.

Alpha-amanitin was diluted in Earles medium in concentration of 1mg/ml and stored at -20°C. Before the incubations, an aliquot was diluted with Earles medium, for final concentration of α–amanitin of 10 or 100 µg/ml. Drops of the diluted α–amanitin were prepared on a petri culture dish under oil and the petri dish was incubated for at least an hour before the addition of the embryos.

6.2.2. HPRT activity and sexing

The profile of HPRT activity was measured as described previously (Chapter 2). While the extract of the embryo was analysed, the lysed embryonic ghost was transfered in a tube loaded with 10µl of distilled water. The embryo was subsequently sexed by PCR as described in Chapter 2 (2.14.3.).

6.3. Results

6.3 1. Trancriptional inhibitors and HPRT activity

HPRT activity was assayed in 68 single normally fertilized human embryos on days 4, 5 and 6. HPRT activity declined from 20.32 ± 3.1 pmol / embryo / hr on day 4, to 17.31 ± 3.9 pmol / embryo / hr on day 5 and 13.21 ± 3.7 pmol / embryo / hr on day 6. The results show that there is some variation among individual embryos at the same developmental stage (16.66-35.03 pmol / embryo / hr on day 4, 11.32-25.01 pmol / embryo / hr on day 5 and 4.23-27.09 pmol / embryo / hr on day 6).

HPRT activity after incubation in 10μg/ml α–amanitin for 24 hours declined from 28.04 ± 4.9 pmol / embryo / hr on day 4, to 11.44 ± 4.3 pmol / embryo / hr on day 5 and 11.16 ± 3.9 pmol / embryo / hr on day 6. HPRT activity after incubation in 100 μg/ml was 20.33 ± 3.9 pmol / embryo / hr on day 4, to 6.2± 0.5 pmol / embryo / hr on day 5 and 6.0± 0.6 pmol / embryo / hr on day 6. The results show that at 10μg/ml α–amanitin there is a partial depression in HPRT activity on days 5 & 6 and a greater reduction at 100μg/ml α–amanitin (Fig. 6.1.).

The results that have been reported in the previous paragraphs were obtained when all embryos on days 4, 5 and 6 were assayed. A group of 47 embryos of good grade was selected, that were at the most advanced developmental stage for their age. However, the range of activities was not different from the overall group. HPRT activity in control embryos declined from 24.67± 2.8 pmol / embryo / hr on day 4, to 20.10± 3.6 pmol / embryo / hr on day 5 and 10.59± 3.3 pmol / embryo / hr on day 6. Transcriptional inhibition showed similar effects in this group (Fig. 6.2.). After incubation in 10μg/ml α–amanitin for 24 hours, HPRT activity declined from 31.50±

4.6 pmol / embryo / hr on day 4, to 15.77± 3.4 pmol / embryo / hr on day 5 and 12.37± 2.9 pmol / embryo / hr on day 6. HPRT activity after incubation in 100µg/ml was 25.40± 4.1 pmol / embryo / hr on day 4, to 6.2± 0.5 pmol / embryo / hr on day 5 and 6.0± 0.6 pmol / embryo / hr on day 6.

6.3.2. HPRT activity in male and female embryos

The activity of HPRT and APRT during morula and blastocyst stages has been measured in 34 embryos and subsequently the lysed embryo was sexed by PCR. As the assay conditions can vary between experiments, the embryos have been prepared and stored at -70°C and assayed at two batches and reported separately (Series 1 and Series 2, Fig. 6.3. & 6.4.).

The profiles of HPRT and APRT activity have been measured as described previously. While the extract of the embryo was analysed, the lysed embryonic ghost was transferred in a tube loaded with 10µl of distilled water. The embryo was subsequently sexed by PCR. In the first set of experiments (Fig 6.3., Series 1), HPRT activity on day 4 of male embryos was 20.94 ± 5.6 pmol / embryo / hr and of female embryos 8.3 ± 2.8 pmol / embryo / hr. on day 6, the activity of male embryos was 7.73 ± 3.6 pmol / embryo / hr and of female embryos 11.57 ± 5.4 pmol / embryo / hr. In the second set of experiments (Fig 6.4., Series 2), on day 4, HPRT activity of male embryos was 23.26 ± 5.04 pmol / embryo / hr and of female embryos 21.78 ± 1.4 pmol / embryo / hr. on day 6, the activity of male embryos was 12.37 ± 0.45 pmol / embryo / hr and of female embryos 20.00 ± 0.05 pmol / embryo / hr.

In the first series, the activity levels were very variable and females on day 4 had less activity than males. On day 6, the pattern was reversed and female embryos had higher activity than males. In the second series, the variability was less, but the number of embryos examined was smaller (3-4 embryos at each stage). In that series, on day 4 the activity of male and female embryos is the same and then on day 6, the activity

of female embryos is almost double than in males. The difference appears higher, but the number of embryos used is very small to permit conclu sions.

Although there is a correlation of HPRT activity with the sex of the embryo, probably a more clear effect is masked. A possible reason could be contamination in sexing by PCR: the zonas surrounding the embryos contain enough sperm to be amplified and the embryo could be mistaken for male. Amplification of empty zonas was positive in 1/4 cases. To overcome the problem of sperm contamination from the zona, a series of embryos have been assayed after the removal of the zona. However, most of the embryos were dissolved in the buffer after the assay. Few of them were clearly visible after extraction of the buffer for the assay, but even then, the embryos were very sticky and could not be transferred to tubes for PCR.

The significance of the HPRT activity results were tested using student's t-test. HPRT activity levels were not significantly different between the three groups (normal embryos, 10μg / ml α-amanitin and 100μg / ml α-amanitin) on day 4. On day 5, there was no significant difference between normal embryos and embryos treated with 10μg / ml α-amanitin, but both groups were significantly different from the embryos treated with 100μg / ml α-amanitin ($p<0.05$ & $p<0.05$, respectivelly). On day 6, there was no difference between normal embryos and embryos treated with 10μg / ml α-amanitin. Both groups were significantly different from the embryos treated with 100μg / ml α-amanitin ($p<0.05$ & $p<0.05$).

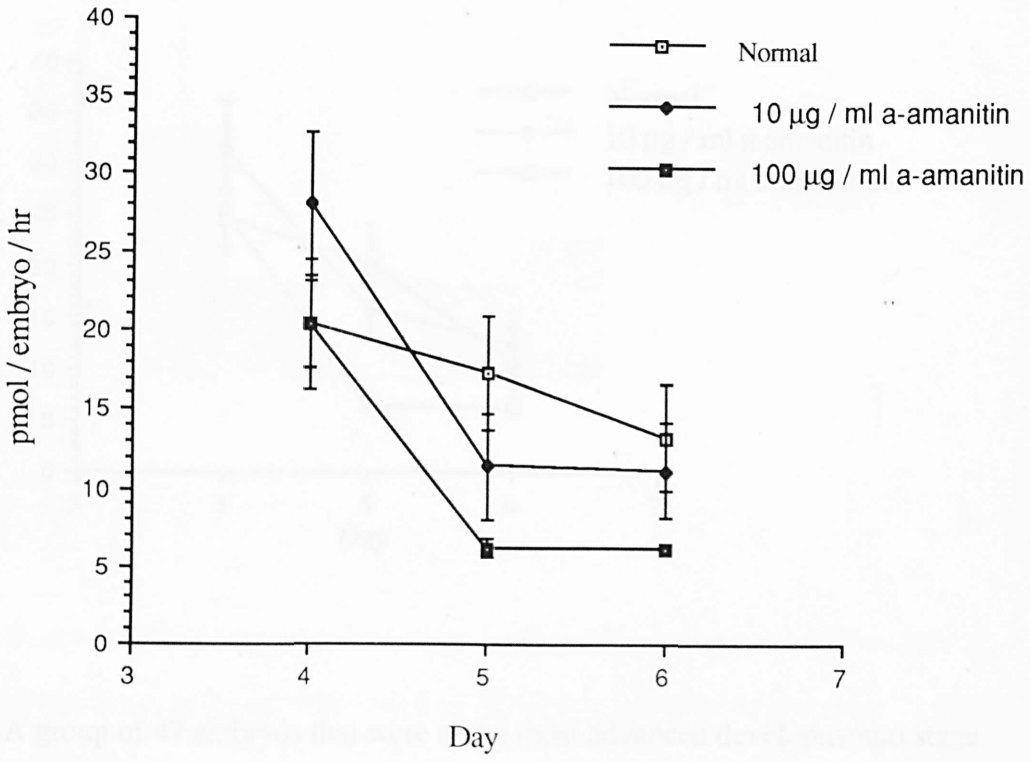


Figure 6.1. Measurements of HPRT activity from 68 human preimplantation embryos cultured in absence or presence of 10 or 100 µl/ml of α–amanitin for 24 hours prior to collection (+/- standard error)

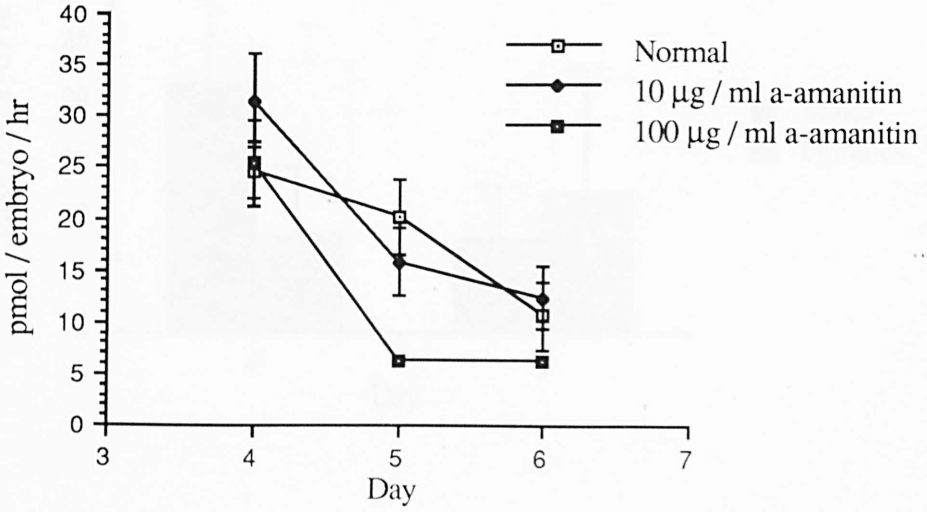


Figure 6.2. A group of 47 embryos that were at the most advanced developmental stage for their age and of optimal morphology was selected from the group presented on Fig. 6.1. HPRT activity was assayed from embryos cultured in absence or presence of 10 or 100 µl/ml of α–amanitin for 24 hours prior to collection (+/- standard error)

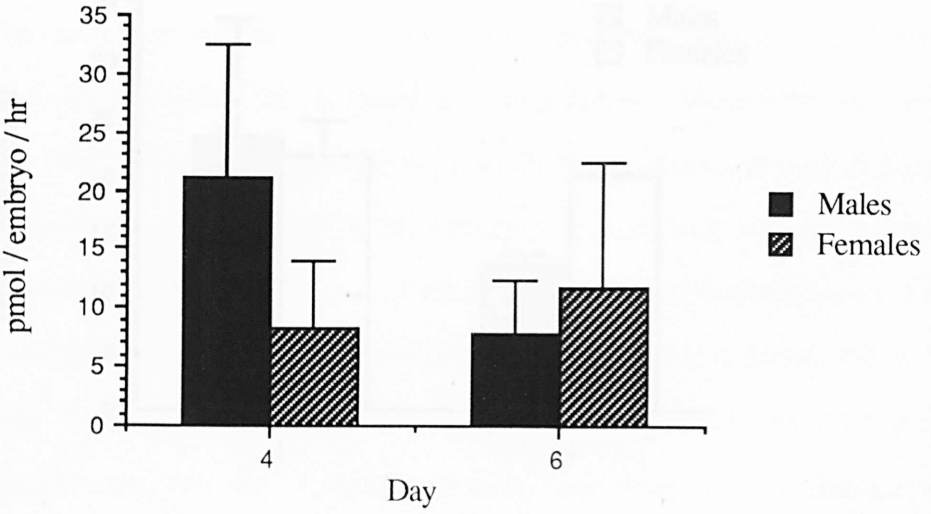


Figure 6.3. Series 1. HPRT activity of normally fertilized human embryos. After HPRT measurements the lysed embryos were sexed by PCR. Total number of embryos: 21 (+/- standard error)

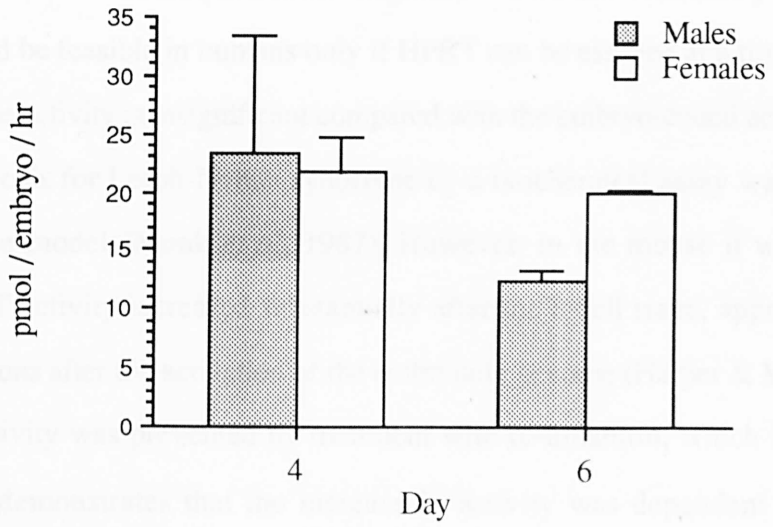


Figure 6.4. Series 2. HPRT activity of normally fertilized human embryos. After HPRT measurements the lysed embryos were sexed by PCR. Total number of embryos: 13 (+/- standard error)

6.4. Discussion

Preimplantation diagnosis for HPRT deficiency by an enzyme assay would be feasible in humans only if HPRT can be assayed at a time when the remaining oocyte activity is insignificant compared with the embryo-coded activity. Preimplantation diagnosis for Lesch-Nyhan syndrome by a biochemical assay was very successful in a mouse model (Monk *et al*, 1987). However, in the mouse it was demonstrated that HPRT activity increased substantially after the 8-cell stage, approximately 2 cleavage divisions after the activation of the embryonic genome (Harper & Monk, 1983). That rise in activity was prevented by treatment with α–amanitin, which inhibits transcription. This demonstrates that the increase in activity was dependent on transcription and synthesis of new enzyme. Therefore, in the mouse from the 8-cell onwards, the measured activity would be embryonic-coded rather than maternal. Two laboratories reported experiments on measuring the HPRT activity in human embryos. The first group (Braude *et al,* 1989) measured HPRT activity in 46 individual human unfertilized oocytes and embryos up to the blastocyst stage, using the double microassay that has been developed in the mouse by Monk (1987). HPRT levels were in the range of 750-5781 fmol / embryo / hr. No rise in activity was observed and the levels of HPRT at the blastocyst stage were similar to those found in unfertilized oocytes. Although this could reflect the elimination of the maternally coded and the rise of the embryo-coded enzyme, transcriptional inhibition with α–amanitin did not produce an affect on enzymatic activity, indicating that the activity measured was due to the maternal genome. The second group also examined HPRT activity between days 2 (2-4 cell stage) and 6 (blastocyst stage) in 118 embryos (Leese *et al*, 1991), by measuring the rate of production of the reaction products inosine monophosphate (IMP) and adenine monophosphate (AMP), separated by high performance liquid chromatography (HPLC). The activity of HPRT increased between days 2 and 4 and declined sharply by day 6. The autosomally coded APRT

remained low between days 2 and 5 and increased on day 6. Also the enzyme activity levels were higher than the ones reported by Braude *et al* (1989). The enzyme levels increased from 12.1 pmol / embryo / hr on day 2 to 17.3 on day 4 and declined to 8.0 on day 6.

The difference between the two assays that may account for the different values obtained, is probably due to the concentration of the substrates used. The method used by Braude *et al* (1989) uses concentration of hypoxanthine and adenine of 10 μM, instead of 50 μM used by Leese *et al* (1991). The Michaelis constants for the two enzymes are in the range of 8 μM for HGPRT and 1.4.-2.7 μM for APRT (Banholzer & Grobner, 1983; Loffler & Grobner, 1983). This indicates that the substrate concentration used by Braude *et al* (1989), might not allow the activity of HPRT to proceed to its maximal rate. Also the long incubation times used (up to 20 hrs), might have depleted the substrate and led to underestimation of enzymatic activity.

Leese *et al* (1991) showed an increase in HPRT activity between days 2 and 4. It was reported previously that the activation of the embryonic genome occurs at the 4-8 cell stage (days 2 to 3). Therefore, it was suggested that the rise in enzymatic activity might reflect the initiation of the expression of the embryonic enzyme and that the rise in activity could not be detected by Braude *et al* (1989), as the overall activities reported were lower. Also the decline of enzymatic activity on day 6 could be due to X-inactivation in female embryos. To test the possibility that the changes in activity levels are due to newly transcribed embryonic message, the effects of transcriptional inhibition on day 4 (when the activity peaks), day 5 and day 6 (when the activity drops) were investigated. Also, to examine the timing of X-inactivation and its potential correlation with the enzymatic levels in male and female embryos, the profiles of HPRT and APRT activity were measured and the lysed embryonic ghost was subsequently sexed by PCR. For the detection of the enzymatic levels, the HPLC assay was used, as described by Leese *et al* (1991). The enzymatic activities reported here on days 4, 5 and 6 are similar to the ones reported by Leese *et al* (1991). There is considerable variation in the levels of

activity. The variation might be due to small technical differences from one experiment to another and also due to differences between individuals. The second hypothesis is supported by the fact that variation in activities was less in groups of eggs or embryos recovered from a single ovulatory cycle. The variability among individual embryos poses an important problem for the clinical application of preimplantation diagnosis by an enzyme assay. We have reported that the mean number of embryos available for analysis after biopsy is 4.5±0.5 (Chapter 3). With such a small number of embryos, the variability in activity levels, might not permit a reliable diagnosis. Also, in our experiments, the whole embryo has been assayed. Although the activity is high enough to permit detection at the single cell level, the variability in activity between single blastomeres has not be examined and might be even higher.

Overall results show that there is an effect of transcription inhibition on HPRT activity during blastocyst formation. It was thought that maybe the enzymatic activities of embryos that were arrested or developmentaly delayed were lower and these could mask a more clear difference between the α–amanitin-treated and the control group to show. A group of 47 embryos that were at the most advanced developmental stage for their age and of optimal morphology was selected. However, the range of activities was not very different from the overall group. Transcriptional inhibition showed a similar effect in this group. Our results suggest that the activity measured on day 4 is not dependent on transcription. On days 5 and 6, at the blastocyst stage, the embryo-coded HPRT activity predominates over maternally-coded enzyme .

The activity of HPRT and APRT during morula and blastocyst stages was measured and subsequently the lysed embryo was sexed by PCR to investigate the potential 2:1 difference in the amount of X-linked gene product before X-inactivation occurs in females. In the first series of experiments (Fig. 6.3.), no clear correlation of the activity levels with the sex of the embryo was found; on day 4, the activity of male embryos was almost double in comparison with females; on day 6, the situation is reversed, with female embryos showing higher activity than males. The second series

of experiments, however, seems more promising, as male and female embryos have similar levels of activity on day 4 and on day 6, the activity in female embryos is almost double in comparison to the males (Fig. 6.4.). Contamination from the sperm in the zona was shown to occur in 1/4 cases and could cause the embryo to be mistaken for male. However, measurements of HPRT activity after the removal of the zona pellucida was succesful, but subsequent sexing of the embryo was impossible, because of technical problems. Data with transcriptional inhibition with α–amanitin, are very interesting, as it suggests that the embryonic HPRT activity at the blastocyst stage predominates over the maternal and that there are possibilities for preimplantation diagnosis of HPRT deficiency at the blastocyst stage. Also, the results with HPRT measurements and sexing could suggest that both X-chromosomes are active on day 6, as the activity of the X-linked HPRT is almost double in female embryos than in males. However, although these suggestions are interesting, the number of embryos studied is very small. Therefore, it would be important that the results are treated with caution andthat these experiments are repeated with a large number of embryos, before any conclusions are drawn.

Another enzyme that has been analysed at different stages of human preimplantation development is glucose phosphate isomerase (GPI). The activity in human embryos, the most advanced of which was an early blastocyst, was similar to that in unfertilized eggs (West *et al,* 1989), showing that the levels of GPI activity are maintained throughout human preimplantation development. However, for that enzyme, it is not known if the activity measured is due to activation of the embryonic genome and decline of the maternally-coded enzyme or just prolonged persistence of the maternally-coded product. Also the levels of β–N-acetylhexosaminidase activity in human oocytes and embryos has been examined (Sermon *et al*, 1991). Mutations in the α–chain of β–N-acetylhexosaminidase can lead to Tay-Sachs disease. The activity that was found in human embryos was >500 times of the activity that it was found in the mouse and that the activity found in human oocytes and embryos was virtually constant during preimplantation development. Recent work of Martin *et al* (1993) shows that

the activity of 5 enzymes remains the same during human preimplantation development and the activity of 2 decreases during the same time.

The timing of the activation of the human embryonic genome at the 4-8 cell stage and the persistence of the maternal products, have important concequences for the time during which preimplantation diagnosis can be performed. If preimplantation diagnosis is based on the measurement of an embryonic gene product, it certainly cannot be performed before the 8 cell stage, as between the 4-8-cell stage embryonic gene activity begins. Even after the activation of the embryonic genome there are some complications that may restrict the use of preimplantation diagnosis by an enzyme assay:

a) If an embryonic gene is transcribed at a certain stage of embryonic development it does not mean that it is expressed immediately. For example, in the human embryo, although mRNA for the β–hCG can be detected at the 6-8 cell stage, the corresponding polypeptide is secreted in the culture medium by the blastocyst on day 7 (Fishel, 1984; Hay & Lopata, 1988).

b) Maternally inherited proteins and probably mRNA persist further after the activation of the embryonic genome. Consequently the detection of a gene product in an embryo at later stages does not prove that it is coded by the embryonic genome. Therefore any gene product which could be diagnostically interesting, should be assessed for the timing of its specific expression and the persistence of the corresponding maternally coded product.

c) Also activation of the embryonic genome does not equal activation of all embryonic genes.

Chapter 7

Conclusions

7.1. Genetic analysis from single cells

Initial attempts for preimplantation diagnosis have been focused on sexing embryos from couples at risk of an X-linked inherited disorder. Using sexing instead of diagnosing the specific defect has many advantages: first, sexing is possible by amplification of a repeated sequence present on the Y-chromosome. This increases the number of target sequences present in a single cell and therefore it was thought to reduce the possibility of amplification failure. Secondly, sexing can be applied for all the X-linked recessive diseases, even if their molecular basis is heterogeneous or is not known. For example, 4 out of 15 patients that have been treated were carriers for genetic diseases for which no specific diagnosis was available at the time. Those patients had sexing as the only means of prevention of the genetic disease .

The approach to sexing that has been described here is amplification of Y-specific repeat sequences. Despite the fact that repeated sequences are present in approximately a few thousand copies in a single male diploid cell, amplification failure with both the Y-alphoid and the DYZ1 was at levels of 12%. The result obtained from a single blastomere can not be confirmed, as the only material available is used for the test and a false result can cause misdiagnosis. One of seven fetuses that resulted from the first application of preimplantation diagnosis was a male misidentified as female and this highlighted the problem of misdiagnosis when one cell only is available. Potential causes of misdiagnosis are technical problems, such as amplification failure, or inherent abnormalities of the embryo, like mosaicism. As all the precautions to avoid technical problems have been taken, the only factor that has not been ruled out is that of amplification failure and methods to improve amplification efficiency were explored. A strategy to control for amplification failure is the co-amplification of fragments specific both for the X- and Y-chromosomes. Co-amplification of X- and Y-specific alphoid sequences is possible down to the single cell level. However if the optimal conditions

for amplification of the two sequences are different, it is likely that the amplification conditions would have to be compromised between the two optima and that one of the two sequences could be more susceptible to amplification failure than the other. Therefore further work is necessary to examine that this method is an effective control, since there is evidence that amplification from different sets of primers can fail independently from each other.

Although in the case of unique sequences the possible damage or loss of the target sequence are potential explanations for amplification failure, this cannot be the reason for the repeated sequences. This indicates that maybe sample preparation is more important than PCR itself and suggests that amplification efficiencies can be increased by improving procedures for the lysis of blastomeres and releasing the DNA from the nucleus in preparation for PCR. The use of a 2-step lysis procedure was demonstrated to increase the percentage of succesful amplification, indicating that inadequate lysis of cells was in some cases responsible for amplification failure and that amplification rates can be improved.

For the specific diagnosis of single gene defects, in which the detection of the defects is secondary to the initial amplification of the target sequence, amplification failure can not cause a misdiagnosis, but may exclude a potentially unaffected embryo from transfer. As the number of embryos that are available after IVF and after successful biopsy is very limited, high amplification efficiency is very important as it maximises the possibility of identifying unaffected embryos and having at least two available for transfer. However, when both alleles are amplified in a single cell from a heterozygote embryo, due to amplification failure of one of the two alleles, an unaffected carrier embryo can be mistaken for affected. Also, if the disease in question is dominant, amplification failure of the mutated allele can lead in the misdiagnosis and transfer of an affected embryo. In DNA amplification by PCR, preferential amplification of one allele is possible, the reason being that the amount of target DNA in single cell PCR is minute and differences in the efficiency of

amplification during the first cycles tend to become maximised, which can result in higher amplification of one allele.

Another method to identify male and female embryos is to quantify the X-chromosome dosage. In the mouse, sexing was successful in the mouse by quantifying the X-linked enzyme HPRT using a biochemical assay and the feasibility of that approach to the human embryo has been examined in Chapter 6. However, quantification is much more difficult with DNA amplification by PCR. PCR can be a quantitative method, but only under certain conditions (Ferre, 1992). The most important factors affecting quantitative PCR are optimization of the process, simultaneous denaturation of all molecules to be amplified, detection of the product during the linear phase of the reaction and the sensitivity of the detection system (Ferre, 1992). For preimplantation diagnosis, speed is very important and a way to reduce the time for the diagnosis is to generate enough product so that it can be visualized on a stained gel. To make sure that the maximum product is produced, the reaction is permitted to continue beyond the linear phase and reach the plateau phase, making quantification impossible. Also, sensitive detection methods that can be used for quantitation, e.g. radiolabelling of the primers and exposure of the film, take more time than gel electrophoresis and staining.

The identification of females in couples at risk of transmitting X-linked conditions based on the presence or absence of amplified Y-specific sequences is susceptible to misdiagnosis because of amplification failure of single cells. As two cells can be biopsied from each embryo without adversely affecting the development of human embryos in vitro (Hardy *et al*, 1990), the preparation of the samples using the two-step lysis procedure combined with independent amplification from two cells should reduce errors to a minimum.

The role in preimplantation diagnosis of a method which is used for prenatal diagnosis has been studied: karyotype analysis is the most common procedure used for routine prenatal diagnosis and it is very reliable for sexing the fetus.

Karyotyping is dependent on having good quality banded metaphase chromosomes and this has been proven very difficult to achieve with only a small number of cells (Handyside & Delhanty, 1991) (see also 1.4.5.3.)

An alternative way to sex embryos is by in situ hybridization (ISH) with sex-chromosome specific DNA probes. Using ISH, interphase as well as metaphase chromosomes can be analysed and this is an advantage when only a small number of cells is available. Although ISH was applied in the identification of sex in human preimplantation embryos (West *et al,* 1987; 1988) using radiolabelled Y-chromosome specific probes, detection of the labelled probe by autoradiography takes several days (see also .1.4.5.4.). The time of detection was reduced to one day with the use of a biotinylated probe and a streptavidin-linked alkaline phosphatase detections system (Penketh *et al,* 1989). The disadvantage of that method was that the efficiency of hybridization was relatively low and it was estimated that at least three cells had to be obtained for a reliable result. Fluorescent methods for ISH (FISH) show increased specificity and sensitivity, can be performed in a day and have the additional advantage of simultaneous detection of more than one probes using different fluorochromes (Handyside & Delhanty, 1993). FISH has been demonstrated for X- and Y-specific probes (Griffin *et al*, 1991) and for a chromosome 18 specific probe (Shrurs *et al*, 1992). Using the two probes indepentently, although the hybridization efficiency was high, hybridization failure and artifactual signals with an incidence of tetraploid nuclei demonstrated that a large number of cells would be needed from each embryo for a reliable result (Griffin *et al,* 1991). Recently a dual fluorescent in situ hybridization method for the X and the Y chromosomes has been developed witch allows accurate sex determination from the nuclei of single cells (Griffin *et al,* 1992). However, the problems with the preparation of single nuclei have resulted in a relatively high incidence of embryos which can not be analysed. To identify the sex of a high proportion of biopsied embryos it is critical, since out of an average of 5-6 normal fertilized embryos following IVF, approximately 50% are expected to be female and the pregnancy rates after transfer of 2 embryos are likely to be in excess of twice that with

single embryo transfers. Therefore for that reason again at least two cells need to be biopsied.

Another advantage of PCR for preimplantation diagnosis, is that development of PCR methods for single cell amplification can be applied for the detection of many single gene defects, though the development of techniques like ISH, can be applied for a more limited range of defects.

A method that would permit the analysis of more than one cell would be the biopsy of the embryo during the blastocyst stage, when up to 10 cells can be biopsied and analysed. Blastocyst biopsy for preimplantation diagnosis has been halted from initial reports that embryo transfers at the blastocyst stage were not as successful as conventional transfers on day 2 (Dawson *et al*, 1988). Recent reports in which a large number of blastocysts have been transfered, has shown that the success rates after blastocyst and cleavage stage transfers are similar (Bolton *et al*, 1991). However, only around 50% of the embryos reach the blastocyst stage (Hardy, 1993). The main advantage from blastocyst biopsy is the greater number of cells that can be analysed, which improves the rate of successful amplification.

7.2. Embryonic gene expression

Investigation of gene expression in the human embryo gives primarly basic information. Many improvements in IVF have been based on extrapolating data obtained from mammalian and other species, especially the mouse, to the human embryo, without the knowledge of the similarities and the differences between the mechanisms and the timing of basic events among the human and other mammalian embryos. Although there seems to be a general common strategy for development, each mammalian species has its own specific mode. Therefore basic knowledge for the mechanisms that are governing the early development of the human embryo is essential to avoid unjustified use of methods established in other species. Information on early developmental mechanisms is essential not only for the development of techniques for preimplantation diagnosis, but for successful IVF and the expansion of related fields like embryo micromanipulation, freezing etc.

In Chapter 5, the quantitative and qualitative patterns of protein synthesis during preimplantation development were examined. The rates of protein synthesis in normally developing human embryos remain constant from day 2 to day 4 and are increased between day 4 and day 7. From day 2 to day 4, protein synthesis per cell fell 5-fold and then remained at the same levels from day 4 to day 7. The initial drop in protein synthetic levels per cell reflects the subdivision of the pre-existing protein synthetic machinery between cells in the first cleavage divisions. Protein synthesis in arrested embryos was significantly reduced in comparison with the normally developing embryos on days 4 and 5. The uptake and the incorporation of ^{35}S-methionine by human embryos cultured in the presence of 100 μg/ml of α–amanitin for 24 hours before labelling was not significantly different to untreated embryos on day 3. In the α–amanitin treated embryos both levels drop from day 4

onwards. From the data it is suggested that the maternal mRNA is only gradually eliminated and that its effects persist beyond the stage of the activation of the embryonic genome.

Although the major quantitative increase in protein synthesis occur between the 8-cell stage and the blastocyst stage, very few qualitative differences occur during morula to blastocyst transition. Transcriptional inhibition after treatment with α–amanitin during the activation of the embryonic genome blocks the appearence of new proteins and also blocks the di sappearance of the old ones. From this data it is suggested that either the degradation of maternal mRNA is dependent on the newly synthesized embryonic messages or that the half life of the maternal messages is prolonged after the non-appearance of the new transcripts, as a mechanism to compensate for the lack of new proteins.

Preimplantation diagnosis for HPRT deficiency by an enzyme assay would be feasible in humans only if HPRT can be assayed at a time when the remaining oocyte activity is insignificant compared with the embryo-coded activity. The levels of enzymatic activity in human embryos varied greatly and that variability among individual embryos poses an important problem for the clinical application of preimplantation diagnosis by enzyme assay. The mean number of embryos available for analysis after biopsy is 4.5 (Chapter 3). With such a small number of embryos, the variability in activity levels, might not permit a reliable diagnosis. Also, in our experiments, the whole embryo has been assayed. Although the activity is high enough to permit detection at the single cell level, the variability in activity between single blastomeres has not be examined and might be even higher.

Transcriptional inhibition has an effect on HPRT activity during blastocyst formation (days 5 & 6), after the period that gene activation is known to start in humans. The activity measured is coded by the maternal genome and probably embryonic HPRT activity is not higher than the maternal until day 4. On days 5 and 6, transcriptional

inhibition depresses the levels of HPRT activity, suggesting that the embryo-coded HPRT activity predominates over maternally-coded enzyme .

In one series of experiments, male and female embryos have similar levels of activity on day 4; on day 6, the activity in female embryos is almost double than in males. These results are very interesting, as they could suggest that both X-chromosomes are active on day 6. However, the number of embryos studied is very small and therefore more extensive studies have to be done before any conclusions are drawn.

7.3. Prospects for preimplantation diagnosis of X-linked disease

Preimplantation diagnosis of genetic diseases involves detecting the genetic defect either directly at the DNA level or by measuring the absence or abnormal expression of the gene product responsible for the condition. The latter approach has the additional advantage that the specific gene defect, which might differ between individuals, does not have to be characterized. However, although embryonic gene expression first occurs at the 4- to 8-cell stage, the timing is likely to vary for individual genes. Also the persistence of maternal gene products goes far beyond the 8-cell stage. In the case of HPRT, as the embryonic enzyme is predominant over the maternal on days 5 & 6, it might be feasible to perform preimplantation diagnosis of the Lesch-Nyhan syndrome by an enzyme assay, after more extended work is completed. Only genetic defects that are expressed compared with the maternally-derived activity would be potential candidates for preimplantation diagnosis. Although preimplantation diagnosis by an enzyme assay remains a possibility for the future in human embryos, preimplantation diagnosis by DNA amplification is a reality and several children have already been born. Initially preimplantation diagnosis was focused on the identification of sex for the prevention of X-linked diseases. Recently it was extended to the detection of a single gene defect, cystic fibrosis (Handyside *et al*, 1992).

Preimplantation diagnosis is most likely to benefit couples that are at high risk for single gene defects and need to avoid the possibility of a therapeautic abortion. Although most of the couples that participated in the clinical trial were fertile, it can be anticipated that few fertile couples would choose preimplantation diagnosis as an alternative to prenatal diagnosis in the future, mainly because of the low pregnancy success rate and the cost of IVF. This is the first time that fertile couples are going

through IVF and that IVF is used as an aid for prenatal diagnosis instead of infertility. If uterine lavage is sufficiently developed to be safe and efficient, it could provide an alternative method to IVF. However, the use of IVF becomes more and more widespread and it is the main option that is offered to couples that face unexplained infertility. As one in ten couples have an infertility problem, it is probable that a proportion of them would also require prenatal diagnosis for the detection of a genetic defect. In those cases, preimplantation diagnosis is a viable alternative, which could spare the risk of a termination of a much wanted pregnancy. Also in situ hybridization can be used for the detection of numerical chromosomal abnormalities. As the age of women undergoing IVF is increased in comparison with the general population, ISH that could be used for the detection of chromosomal abnormalities whose frequency increases with maternal age (for example Down's syndrome).

Future use of preimplantation diagnosis is also dependent on the reliability of the diagnosis.Any diagnosis that is based on a small number of cells is unlikely to be as accurate as diagnosis following amniocentesis or CVS, when thousands of cells can be analysed. Further studies need to be undertaken to assess the accuracy of the diagnosis according to the technique used and the particular defect to be identified.

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