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### **cDNA libraries from single human preimplantation embryos**

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We describe the construction, evaluation and application of cDNA libraries from unfertilized oocytes, and single 2-cell, 4-cell, 7-cell and blastocyst stage embryos. The corresponding parental samples (cumulus cells and sperm) have also been obtained for the construction of genomic libraries. The cDNA libraries display sufficient complexities (between 100000 and 1000000 independent clones) to represent the entire active gene population at these early stages of human development. The ubiquitous cytoskeletal elements, beta-actin, keratin-18 and alpha-tubulin, were detected at the expected frequencies. Sequencing of consecutively picked random clones showed the presence of a variety of sequences, such as the human transposable element, LINE-1 and Alu repeat sequences, the housekeeping gene, HPRT, and tissue-specific genes alpha-globin and FMR-1. A high proportion of novel sequences as well as cDNAs corresponding to known ESTs (expressed sequence tags) in the GenBank and dbEST databases were also detected. Applications of the libraries to several areas of interest, such as expression of CpG island-containing 'tissue-specific' genes, developmental genes expressed in a stage-specific manner, and a search for monoallelic expression of imprinted genes will be discussed. These libraries are an invaluable resource for the unlimited analysis of profiles of gene expression at the onset of human development, at a time when important and irreversible events occur that affect the genetic potential of the individual.

### **Characterization of gene trap integrations expressed in the developing heart**

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We describe the characterization of two gene trap integrations (R124 and R213) that were identified as being retinoic acid (RA)-responsive *in vitro* and expressed in the developing heart *in vivo* [1]. In embryos carrying the R124 integration, reporter gene expression was first detected in the developing heart at day 8.5 post coitum (*p.c.*) and was cardiac-specific throughout gestation. The majority (70%) of animals homozygous for this integration die at birth, possibly due to a right ventricular defect. Surviving homozygotes subsequently develop defects in both the adult heart and kidney, where the reporter has also been shown to be expressed. Molecular analysis of the integration site revealed that the vector had integrated into an exon of a novel gene. In embryos carrying the R213 integration reporter gene expression was first detected in the developing heart at day 8.5 *p.c.* Expression was also detected throughout the yolk sac, detectable in the presumptive yolk sac as early as day 7.0 *p.c.* In late gestation and adulthood, expression broadened to encompass all muscle subtypes, kidney and brain. RACE cloning indicates the gene trap vector has integrated into a gene homologous to the human transcription factor gene, TFEB. Homozygous mice are viable and fertile; however, detailed molecular characterization indicates that the resultant allele is not a complete null.

1. Forrester, L. M. *et al.* (1996). *Proc. Natl. Acad. Sci. USA* **93**, 1677–1682.

### Identification and characterization of a novel mouse gene, G90, which maps to chromosome 6 and encodes a putative tumour suppressor

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By subtractive hybridization we have isolated a cDNA clone designated G90 from the small intestine. Expression analysis of G90 revealed high expression in small intestine, testis, kidney and lung. Lower expression was observed in heart, brain, liver, spleen and skeletal muscle. A transcript size of 1.5 kb is seen in all tissues; however, in testis a second transcript of 1.3 kb was observed. Further analysis provided evidence that in the intestine and testis G90 is expressed only in non-dividing cells. In post-midgestation mouse embryos, G90 is expressed in the cochlea, in the nasal epithelium and in restricted areas of the brain. As assessed by mRNA *in situ* hybridization, G90 is not expressed in proliferating cells of the intestinal epithelium and in intestinal adenomas. Sequencing of G90 cDNA gave no open reading frame, indicating that G90 may be a functional mRNA. Mapping of G90 showed that it is located on mouse chromosome 6, close to the imprinted gene *Mest*. Subsequent analysis of the imprinting status revealed a strain-specific silencing of one allele in interspecific hybrids, but influence of parental sex could not be found.

### Genetic analysis of placental dysplasia in mouse interspecific hybrids

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Placental dysplasia was observed in interspecific hybrids between *Mus musculus* (*mus*) and *M. spretus* (*spr*). When (*M. mus* × *M. spr*) F<sub>1</sub> females were mated with *M. mus* males, placental hyperplasia was observed, whereas placental hypoplasia occurred in backcrosses with *M. spr* males. Both forms of placental dysplasia were more prominent in males than in females. One of the loci contributing to placental dysplasia, *Ihpd* (interspecific hybrid placental dysplasia), was mapped to the central portion of the mouse X chromosome near *DXMit8*. To narrow

down this critical region, several mouse strains were established which contain different *spr*-derived regions of the X chromosome. Using this strategy, *DXMit8* could be confirmed to be a critical marker, although other regions of the X chromosome also seem to be involved in the generation of the phenotype. To explain the divergence of the phenotype between males and females we investigated X-inactivation or X-reactivation in female interspecific hybrids. Further mapping studies will reveal the other X-chromosomal loci that are involved in the development of placental dysplasia.

### Analysis of differentially expressed genes in mouse interspecific hybrid placental dysplasia

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It has previously been shown that abnormal placental development (i.e. hyper- and hypoplasia) occurs in crosses and backcrosses between different mouse (*Mus*) species. The precise genetic basis for these placental malformations has not been determined. However, a locus that contributes to the abnormal development (*Ihpd*: interspecific hybrid placental dysplasia) has been mapped to the X chromosome. We applied subtractive hybridization to isolate cDNAs differentially expressed in normal and hyperplastic placentas. In the first experiment, several cDNAs representing known genes as well as seven novel mouse cDNAs were identified. Further characterization of these cDNAs is in progress. Previous studies in interspecific hybrids of the rodent genus *Peromyscus* ('deer mice') described similar placental phenotypes that are correlated with an altered genomic imprinting of several genes. In our laboratory the expression of imprinted genes such as *Mash2*, *Igf2* and *H19* in placentas of *Mus* interspecific hybrids was determined by RNA *in situ* and Northern analysis. To verify the imprinting status of these genes in *Mus* interspecific hybrids, we are now investigating parental allele-specific expression by RT-PCR.

### Characterization of a novel mouse gene with homologues on the X and Y chromosomes

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The genes encoding the male-specific minor histocompatibility antigens are located on the short arm of the mouse Y chromosome, in the *Sxrb* deletion interval. Two such genes, *Smcy* and *Uty*, have recently been identified that encode *H-YK<sup>k</sup>* and *H-Yd<sup>b</sup>* epitopes respectively. We have cloned another gene from this region, *Tfy*, which lies between *Smcy* and *Uty* and is homologous to a translation factor. We have also shown that there is an X chromosome homologue of this gene, as well as pseudogene copies on the X and chromosome 15 in some mouse strains. The predicted translation products of the X and Y structural copies differ by only 10 amino acids and both copies are widely transcribed; the Y copy is therefore a candidate for *H-Y*. The X copy maps close to the *Dmd* locus and is expressed from the inactive X chromosome, indicating that the expression of two copies of this gene is required in both males and females.

### Comparative sequencing on the mouse and human X chromosomes

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We aim to sequence 3 Mb from the *Ids-Dmd* region of the mouse X chromosome. This region is homologous to regions Xq28 and Xp21 on the human X chromosome and as such crosses an evolutionary breakpoint. Xq28 is a gene-rich region receiving considerable attention from sequencing groups and there will therefore be abundant opportunity for

sequence comparison between the mouse and human genomes. A minimal tiling path of cosmid and BAC clones covering nearly 600 kb of the *Ids-Dmd* region is available for sequencing plus some existing sequence which has already been analysed. At current throughput, we expect by early 1998 to have completed and analysed 600 kb of sequence. Fingerprinting and generation of cosmid/BAC tiling paths is being carried out at the MGC and Ohio State, USA. Sequencing is being carried out at the HGMP-RC, while further computing analysis, including exon prediction, is performed at the MGC.

### Mutation analysis of mottled mice

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Mottled mice and Menkes disease patients have X-linked conditions caused by aberrant handling of cellular copper. Menkes disease patients classically follow a severe course, dying before 3 years of age. However, there are milder forms of the disease, including cutis laxa. There are over 20 alleles of mottled and these can be grouped into three classes based on the phenotype of the affected males. Males either die before birth, die within a few weeks of birth or survive to adulthood. Mutations in a gene encoding a copper-transporting ATPase (ATP7A) cause Menkes disease. The first mutation found in the homologous gene in mouse (*Atp7a*) was a splice site change in mottled blotchy. This is an allele in which affected males survive to adulthood and may be a good model for cutis laxa. Subsequently, we and others have discovered mutations in mottled alleles of other classes. These range from single base pair substitutions to deletions of several kilobases. Generally, the severity of the genetic lesions correlates well with that of the phenotype. However, our studies on the kinetics of copper processing in cell lines from a number of mottled alleles suggest that there is no significant difference between alleles at this level.

### Genetic and developmental studies of neuronal migration defects in the dreher mouse

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Dreher (*dr*) is an autosomal recessive mutation causing deafness and circling behaviour in homozygotes and,

in some cases, white belly spots. Homozygotes also have misplaced neurons (heterotopia) in the CNS that involve both the cerebral hemispheres and the cerebellum. Genetic mapping of dreher with respect to DNA microsatellite markers led to the positioning of the dreher gene in a 5.78 cM interval on distal mouse chromosome 1, proximal to its previously assigned location. The study also provided flanking markers for dreher that enabled precise genotyping of pre- and post-natal individuals in all subsequent studies. Two genes that map to the dreher region were evaluated as candidates for the mutation: Astrotactin and *Pou2f1*. There were no differences in the mRNA expression pattern of Astrotactin between *dr/dr* and *+/+* littermates between E9.5 and E11.5, making Astrotactin an unlikely candidate, although not definitely ruling it out. Sequencing of the POU-specific domain of *Pou2f1* did not show any mutations in *dr/dr* animals. However, whole-mount *in situ* hybridization of embryos showed a spatially more restricted expression of *Pou2f1* at the hindbrain–midbrain boundary in *dr/dr* compared with wild-type littermates at E11.5, suggesting that this gene may be primarily or secondarily affected in dreher. The neocortex of *dr/dr* mice exhibits a focal or diffuse increase in the neuronal density of layer I. Birthdating studies were performed using bromodeoxyuridine labelling *in vivo*. The heterotopic neurons appear to be generated throughout the period of neocortical histogenesis, not predominantly at the start of neurogenesis, as would be predicted if they were derived from the preplate. Moreover, excessive proliferation of early-born cells (i.e. those destined for layer I) is unlikely to be a contributing factor in the production of heterotopia in dreher. Histological studies reveal abnormalities of the neocortical glial limiting membrane in *dr/dr* mice. This defect may play a central role in the genesis of heterotopic layer I neurons.

#### Detection of disturbed folate metabolism in mouse embryos developing neural tube defects

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The role of folate in the prevention of human neural tube defects (NTD) is well established, although little is known about the mechanism by which periconceptional supplementation produces this effect. In order to study the action of folate experimentally, we adapted the deoxyuridine suppression test for use in whole embryo cultures, allowing the efficiency of the folate cycle to be assayed during the period of neurulation. The test uses thymidine incorporation as a measure of the efficacy of the folate/homocysteine

cycle. Thymidine is available from two sources: the kinase pathway uses deoxyuridine monophosphate (dUMP) and folate coenzymes to synthesize deoxythymidine monophosphate (dTMP), whereas the salvage pathway uses thymidine kinase to reutilize thymidine. In the normal condition, dUMP suppresses the uptake of [<sup>3</sup>H]thymidine. If folate metabolism is perturbed, however, the uptake of [<sup>3</sup>H]thymidine is increased, as a greater percentage is used from the salvage pathway. We found that embryos cultured in the presence of folate antagonists such as 5-fluorouracil and cycloleucine have a deranged suppression test, as predicted. The test was then applied to mutant mice which develop NTD. Embryos homozygous for either looptail (*lp*) or curly tail (*ct*) showed normal deoxyuridine suppression tests suggesting that there is no defect in folate metabolism in either mutant. Homozygous splotch (*Sp<sup>2H</sup>*) embryos, which develop exencephaly and/or spina bifida, showed an abnormal suppression test, suggesting that these embryos have an abnormality of folate/homocysteine metabolism. *In vitro* and *in vivo* treatment of homozygous splotch embryos with folic acid or thymidine reduced the incidence of both cranial and caudal NTD whereas treatment with methionine was found to increase the incidence of NTD. These findings suggest that splotch embryos have a defect within the folate cycle *per se*, rather than in the associated homocysteine–methionine cycle, and that the splotch mutant provides a model for analysis of folate-preventable NTD.

#### Genetic and physical mapping around the mouse epilepsy locus, lethargic (*lh*)

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The lethargic trait is an autosomal recessive single locus mouse epilepsy mutant, linked to chromosome 2. Recently the locus has been cloned and identified as a mutant splice variant of the voltage-dependent calcium channel beta 4 subunit (*Cacnb4*). Initially a fine-scale genetic mapping of this locus was created using two crosses as the mapping resource. Marker typing of over 1000 meioses with a range of polymorphic loci on mouse chromosome 2 narrowed the lethargic region to approximately 2.0 cM. A chromosome walk was initiated across the shortest recombinant region using the flanking markers as starting points for isolation of YAC clones. Sequence tagged site and simple sequence length polymorphism capturing techniques have been implemented on existing YAC clones allowing the extension of the two

contigs. Presently a total estimated physical distance of 4 Mb has been mapped. This project initially aimed to isolate and characterize the lethargic locus in mouse. With the elucidation of *lh* via a candidate gene approach, focus has shifted towards assessing this gene's possible role in analogous human disorders. Work will continue to refine these physical and genetic maps, providing a substantial resource for workers concerned with the same region.

#### Differential display analysis of dissected mouse somites

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Conventional techniques of screening for differential gene expression have involved the construction of libraries and subsequent differential or subtractive screening. In the mouse embryo, where tissue availability is limited, such methods are difficult. Differential display provides a rapid alternative approach to this problem. However, conventional display protocols give differential clones that are unsuitable for secondary screening by *in situ* hybridization and have high false positive rates. We have been using our own differential display protocol to isolate novel genes related to somitogenesis. We observe that using the primers of known genes and poly A-enriched RNA from individual dissected mouse somites and presomitic mesoderm, predicted patterns of gene expression can be detected by RT-PCR. Subsequently we have generated reproducible RT-PCR fingerprints using arbitrary primers from which we have been able to clone novel cDNA fragments large enough for rapid secondary screening using *in situ* hybridization. Using this approach, we have isolated a number of novel genes, including a family of transmembrane genes containing leucine-rich repeat motifs related to those of the *Drosophila* genes Slit and Tartan, which have interesting patterns of expression in the somites.

#### Renal agenesis in mice homozygous for a gene trap mutation in the gene encoding heparan sulphate 2-sulphotransferase

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Heparan sulphate proteoglycans (*Hspgs*) are expressed at the surface of most cells in multicellular organisms and have been implicated in the presentation of a number of secreted signalling molecules, including members of the fibroblast growth factor (FGF), Wingless/*Wnt* and transforming growth factor-beta (TGF- $\beta$ ) families to their signal-transducing receptors. The specificity of *Hspg* ligand interactions resides, at least in part, in the structure of the heparan sulphate side chains, which vary in number, length, sequence composition and sulphation pattern between cell type and developmental stage. Thus the production of differentially glycosylated proteoglycans may represent an additional means to regulate cell-cell communication during development. In an attempt to identify molecules important for mouse embryogenesis we have employed a gene trap strategy that identifies, mutates and reports on the embryonic expression pattern of genes expressed in murine embryonic stem cells. We have characterized a mutation in the gene encoding heparan sulphate 2-sulphotransferase. This gene is expressed differentially during embryogenesis, presumably directing changes in proteoglycan side chain structure. Moreover, mice homozygous for the gene trap mutation exhibit bilateral renal agenesis, resulting from a failure of ureteric bud branching, as well as defects of the eye and skeleton. These data provide the first genetic demonstration of HSPG function in vertebrate embryonic development.

### New Wilms' tumour suppressor gene (WT1) functions revealed by YAC transgenic analysis

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We have used YAC transgenic technology to analyse the Wilms' tumour gene (WT1) function and expression. Disruption of WT1 predisposes to childhood kidney neoplasia, leukaemia and gonadal dysgenesis. Homozygous *Wt1* 'knockout' mice die at around 13.5 days of gestation showing no development of kidneys or gonads and disruption of thoracic organs. We isolated a 470 kb YAC carrying the human WT1 locus centrally. This was then truncated 3' of WT1 giving a 280 kb YAC. Expression of the 280 kb YAC transgene on the *Wt1* 'knockout' background rescued the thoracic defects which cause embryonic lethality. However, neonates died within 48 h lacking kidneys, gonads and adrenal glands. Therefore WT1 is required for adrenal, in addition to urogenital, development. A subset of these partially rescued mice develop kidneys which arrest at the S-shaped stage of nephrogenesis; hence WT1 has a role in the maturation of the glomerular podocyte cells. We inserted a lacZ reporter gene into WT1 exon1 of both the 470 and 280 kb YACs. Expression of the reporter gene in transgenic mice carrying either of these constructs mirrors the complex endogenous WT1 expression pattern. Furthermore transgene activity indicates potential new domains of *Wt1* function in epaxial musculature, interdigital limb mesenchyme and the nervous system.

### Characterization of a recessive insertional mutation implying a late embryonic lethality

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Several transgenic mouse lines were created by microinjection of a HSP70.1Luc transgene placing the firefly luciferase reporter gene under the control of a murine heat shock promoter (HSP70.1). These lines

were designed to study gene expression and regulation at the onset of embryonic genome activity. Inter-crossing of heterozygotes from the F27 line did not produce any liveborn homozygotes. These observations are typical of a recessive insertional mutation responsible for the *in utero* death of homozygous mutant embryos. Analysis of F27 embryos at various times during gestation shows that homozygous transgenic embryos die between the beginning of embryonic development and the 14th day *post coitum*. They showed extensive external and internal abnormalities (e.g. haemorrhagic regions, misdeveloped organs). Molecular and genetic aspects of this mutation are now under investigation. FISH analysis established that the transgene was located in the proximal region of chromosome 9. Flanking sequence to the transgene has been cloned. Analysis of these sequences failed to identify a candidate gene. However, we were able to identify homology with some new human and mouse DNA gene sequences. (*Acknowledgement*: C.P. is the recipient of Fonds pour la formation la Recherche dans l'Industrie et dans l'Agriculture studentship.)

### Effects of thyroid hormone deficiency on mice selected for increased and decreased body weight and fatness

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A study was undertaken to test whether the elimination of metabolic pathways, which might, *a priori*, be expected to be strongly involved in the selection for growth or fatness, comprising thyroid hormones (TH) and growth hormone (GH), is responsible for a substantial part of the genetic change produced by selection. Mouse lines used in this study have been selected (for *c.* 50 generations) for high and low body weight and for high and low fat content, producing a 3-fold difference in body weight and a 5-fold difference in fat content. Thyroid ablation was achieved by repeated backcrossing into the selection lines a transgene comprising the HSV1-tk gene coupled to the promoter of the thyroglobulin gene. In the absence of TH and GH lines still differ in body weight from 10 days to about 100 days. The effect of the transgene was dependent on the genetic background for almost all body weights and relative gonadal-fat-pad weights, but less for fat content. The data show that TH- and GH-status is not the only cause for line differences in growth and fatness, caused by long-term selection, but both are involved to a significant but nevertheless relatively small extent.

Therefore these results support a polygenic model of selection response.

### Radiation sensitivity in male mouse germ cells

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Experiments used to examine radiation-induced mutation in mice have typically employed the specific locus test and have used tens of thousands of mice due to the low frequency of germline mutation at single copy genes [1]. Minisatellites are tandem repeat arrays that show a very high spontaneous mutation rate, thus providing a very efficient monitoring system for germline mutation. Recent studies have shown that acute doses of ionizing gamma-radiation to male mice cause a significant increase in minisatellite mutation rate [2, 3]. We have extended this analysis, using male CBA/H mice, to ascertain the stage of spermatogenesis that is sensitive to acute gamma-radiation, establish dose–response curves, estimate the doubling dose and compare the effects of chronic versus acute radiation exposure on minisatellite loci. Our current data suggest that mutation induction is only attributable to the spermatogonia stage of spermatogenesis, providing evidence for a meiotic origin of murine minisatellite germline mutation. Frequency of mutations induced by acute gamma-radiation increases linearly with dosage from 0.5 Gy to 1 Gy with the doubling dose between 0.5 Gy and 0.8 Gy. These values are close to those obtained in mice using other monitoring systems [1]. The application of these data for analysis of radiation-induced mutation in humans [4] will be discussed.

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### Identification of H-Y epitopes in Smcy

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The *Smcy* gene maps to the short arm of the mouse Y chromosome into the *Sxrb* deletion interval. This gene

has been shown to encode peptides that express the mouse *H-YK<sup>k</sup>* epitope and two human *H-Y* epitopes that are expressed in association with the HLA B7 and A2 class I molecules. These peptides have been identified. *Smcy* was also found to express the *H-YD<sup>k</sup>* epitope: cells expressing the *Smcy* cosmid (cMEM 14) stimulated an *H-YD<sup>k</sup>*-restricted T cell clone. The expression of this epitope was further localized using subcloned genomic fragments and nested sets of PCR-cDNA products of *Smcy*, to a 310 bp fragment. Comparison of the sequences of *Smcy* and *Smcx*, the X chromosome homologue of this gene, within this fragment identified six amino acid differences between the two genes. Four synthetic peptides that spanned these differences were tested and one was identified that stimulated the *H-YD<sup>k</sup>*-specific T cell clone at concentrations of 1  $\mu$ M. Two shorter peptides were synthesized from this 12mer sequence to identify the optimal peptide that could express the *H-YD<sup>k</sup>* epitope.

### The Y\* rearrangement in mice: new insights into a perplexing pseudoautosomal region

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The Y\* chromosome, in essence, is a Y chromosome hijacked by a non-Y centromere attached to the pseudoautosomal region (PAR). All the Y-specific region appears to be unaltered, but the pairing behaviour of Y\* with the X during male meiosis led to the conclusion that part of the PAR is inverted. Recombination between the X PAR and this inverted PAR region produces two recombinant products; one is an X-attached-Y chromosome and the other is a small chromosome comprising the recombinant PAR attached to the non-Y centromere. Recently, we introduced the X-linked mutation *Paf* into XY\* males and to our surprise found that the X*Paf*Y\* males were phenotypically wild-type. We have subsequently established that this is because the Y\* chromosome carries an X PAR boundary together with the wild-type allele of *Paf*; this X PAR boundary is transferred to the small recombinant product. We hypothesized that the Y\* PAR is flanked by a Y PAR boundary and an X PAR boundary (in the opposite orientation); this predicts that there are two regions of proximal PAR running towards each other and raised the possibility that there is no distal PAR. Steroid sulfatase (*Sts*) is a marker for the distal PAR; by PCR and Southern analysis we have been able to show that *Sts*

is missing from the Y\* chromosome. The recombination event producing the X-attached-Y chromosome recreates the same PAR structure as in Y\*.

### **Meiotic pairing, non-disjunction and germ cell death in wild and laboratory-bred male house mice (*Mus musculus domesticus*) carrying Robertsonian translocations**

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A series of investigations has been carried out on both feral and laboratory-bred male house mice (*Mus musculus domesticus*) to examine the effect of Robertsonian rearrangements on meiotic chromosome pairing and on the frequency of non-disjunction and germ cell death. In mice from a hybrid zone in the north of Scotland prophase pairing was orderly and the level of univalence and germ cell death low. Laboratory-bred animals heterozygous for the Robertsonian translocations Rb(1.3)1Bnr, Rb(11.13)4Bnr and Rb(10.11)-8Bnr had an increased frequency of pachytene pairing abnormalities and germ cell death over homozygotes. The homozygotes had a low level of non-disjunction but substantial germ cell death which could not be attributed to pairing problems as these showed no increase above the level in controls. It was concluded that both genic factors and pairing problems are involved in promoting germ cell death and non-disjunction. Their influence on meiosis is being investigated further.

### **Cytogenetic and molecular genetic techniques for the analysis of single cells**

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An increasing number of genetic analyses are being performed on minute samples of tissue or DNA. Examples include preimplantation genetic diagnosis, analysis of fetal cells isolated from the maternal circulation, forensics, investigation of ancient DNA and analysis of individual tumour cells. Molecular

genetic analysis of such samples is severely limited because there is often insufficient DNA to perform more than one PCR amplification. We have investigated four different methods of whole genome amplification which generate enough DNA for several subsequent PCR amplifications to be performed. Indeed, we have demonstrated that in some instances over 100 independent PCR amplifications can be performed on DNA from a single cell. Cytogenetic analysis is also problematic at the single cell level, mainly because of difficulties in obtaining metaphase chromosomes. Interphase cytogenetics solves some of these problems, but accuracy is reduced when more than five chromosome pairs are analysed due to overlapping signals. However, cytogenetic investigation using comparative genomic hybridization (CGH) overcomes these limitations and can now be performed on single cells following whole genome amplification. Furthermore, CGH can be performed in addition to numerous specific PCR amplifications, allowing simultaneous molecular and cytogenetic analysis of the same cell.

### **Checking the stability of human bone marrow stem cell genome by RAPD-PCR (random amplified polymorphic DNA)**

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To what extent is the genome of human bone marrow stem cells resistant to the treatment regimen of autotransplanted oncology patients? The answer to this question is especially important now, since several retransplantations have been suggested to improve the prognosis of patients. In our study we compared blood samples taken before (A) and approximately 1 month after transplantation (B); occasionally a third sample from the frozen cells used for transplantation was also examined (C). A few patients (5) were studied by classical DNA fingerprinting in a pilot study conducted by P. Nurenberg, but due to the limited amount of DNA (sample B), the method of RAPD with analysis of PCR products by capillary electrophoresis was adopted (30 patients). No differences between samples A and B studied by P. Nurenberg were observed; RAPD analyses also gave similar results. In comparison with classical DNA fingerprinting based on production of different restriction fragments hybridizing with repetitive sequences (GTG,CA) and showing polymorphism, the one-primer RAPD gave nearly identical fragment pattern in all patients. Quantitative differences observed in capillary electrophoretograms most probably resulted from the sensitivity of RAPD to the working conditions, which were never absolutely identical.



### Steady-state levels of subunits of respiratory chain enzymes: clues to the primary genetic defect in cytochrome *c* oxidase deficiencies

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Cytochrome *c* oxidase (COX) is the terminal enzyme complex of the mitochondrial respiratory chain (RC). In common with two of the three other respiratory chain enzymes COX is composed of subunits encoded by mitochondrial DNA (mtDNA) and subunits of nuclear origin. COX deficiencies show a remarkable clinical heterogeneity, with different tissues affected and with variable onset of symptoms. In patients with a childhood or late onset, partial defects of COX are often associated with specific mutations in a sub-population of mtDNA. In paediatric patients, however, little is known about the molecular basis of the disease. Cultured fibroblasts from a group of five infants with COX deficiency have been studied. Southern blot hybridization did not reveal any mtDNA abnormalities. On Western blots, we found reduced steady-state levels of both mtDNA-encoded and nuclear-encoded COX subunits. In addition one patient showed reduced levels of subunits of another RC enzyme of dual genetic origin (cytochrome *c* reductase), often indicative of a mutation in a mtDNA tRNA gene. In another patient, respiratory enzymes of dual genetic origin and the single exclusively nuclear-encoded RC enzyme (succinate dehydrogenase) were affected. Here a mutation in a common nuclear factor, possibly involved in mitochondrial import, is suspected.

### Arylamine *N*-acetyltransferase in human placenta: a role in folate catabolism

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Arylamine *N*-acetyltransferases (NATs) metabolize arylamines and hydrazines and are usually considered as detoxification enzymes. One of two human iso-

enzymes, NAT1, is expressed in many tissues, including first trimester placentas. The other isoenzyme, NAT2, is approximately 1000-fold less active in placentas. Using heterologously expressed human NATs, it has been shown that NAT1, but not NAT2, metabolizes the folate catabolite *p*-aminobenzoyl-glutamate (pabaglu) to the *N*-acetylated form, *N*-acetylpabaglu [1, 2]. The acetylated form of pabaglu (*N*-acetylpabaglu) is the major urinary folate catabolite and its excretion increases during pregnancy. Therefore NAT1 is likely to have a role in endogenous metabolism. Using placental homogenates and pabaglu as substrate, we have demonstrated that *N*-acetylpabaglu is formed. The formation of *N*-acetylpabaglu is inhibited by the NAT inhibitor 5-iodosalicylate. The activity of NAT1 in placenta is sufficient to account for the excess production of *N*-acetylpabaglu during pregnancy in addition to its role in metabolism of environmental arylamines. NAT1 shows functional and structural polymorphism, and the inter-individual variation in NAT1 activity in placentas may affect folate metabolism during pregnancy.

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### MBL gene mutations in a large prospective study of childhood disease

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The Avon Longitudinal Study of Pregnancy and Childhood (ALSPAC or Children of the Nineties) was set up as a multi-faceted study on 14000 children born in the Avon area from 1991 to 1992. The aim of the project is to examine the origins and influences of many different factors affecting the health and development of a geographical cohort from pregnancy onwards. The ALSPAC project is one part of a World Health Organization project taking place in seven countries in Europe. The children are examined at clinics at regular intervals. Additionally, the children and their parents are sent questionnaires to gain

further insight into their lives; all this information is being stored in the ALSPAC database. One aspect of the project is the ALSPAC DNA bank. DNA samples are being made from blood samples from the children and their mothers. This bank will form a unique resource for genetic epidemiology studies and together with the ALSPAC database will allow many nature versus nurture issues to be examined. About 1000 of the children, known as the Children in Focus (CIF), are being studied in greater depth than the rest of the cohort. Some genetic studies on the CIF have been completed. One of these is on mannose-binding lectin (MBL), a protein with a role in innate immunity. Mutations in the MBL gene have a semi-dominant effect and are present in an estimated 40% of the UK population. MBL genotype data, and its relationship to health in childhood in the CIF, will be discussed.

### Fishing upstream of *Myf5*

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We are seeking to understand how skeletal muscle formation is initiated in the mouse by dissecting the regulatory mechanisms that control the first of the myogenic factors, *Myf5*. In the mouse *Myf5* is located 8.5 kb downstream of *Mrf4*. Previous results have shown that regulatory elements controlling dorsal somite expression of *Myf5* are contained in the intergenic region while ventral expression depends on elements in the *Myf5* gene itself. Because of the large size of this region we have isolated the *Mrf4* and *Myf5* genes of the teleost *Fugu rubripes*, with an 8 times smaller genome than that of man. Although synteny is conserved in *Fugu* and the intergenic distance is only 3 kb, non-coding sequence including the introns was poorly conserved. In contrast, sequence comparison between the mouse and human *Myf5* genes was used successfully to eliminate more than 60% of the intron sequence and identify conserved regions in the 3' half of each of the *Myf5* introns, which together with the 3'UTR can activate reporter gene expression in the ventral posterior part of the somites. The results indicate that multiple elements may be required to control particular anatomical subdomains of *Myf5*, adding a further level of complexity. Analysis of the *Fugu Myf5* gene in transgenic mice showed remarkable similarities with the expression pattern of *Myf5* in another teleost, the zebrafish *Danio rerio*. Both are expressed in the presomitic mesoderm as well as the somites, suggesting that the expression of the *Fugu*

transgene is a reflection of its native expression pattern.

### Induction of ectopic cartilage by misexpression of *Sox9* in chicken embryos

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The HMG-box DNA binding protein, SOX9, has been implicated in formation of the embryonic skeleton from mutations in the gene in the human disorder campomelic dysplasia, and from its embryonic expression pattern. We show that misexpression of *Sox9 in vivo* results in ectopic cartilage formation in limbs and *in vitro* is able to change the aggregation properties of limb mesenchymal cells. Ectopic expression of *Sox9* in non-chondrogenic cells also produces ectopic cartilage and results in the induction of markers of terminal cartilage differentiation. In addition, evidence for a role for *Sox9* in ventralization of the somite will be presented.

### Patterns of histone H4 acetylation distinguish structural and functional domains within the human genome

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The pattern of histone H4 acetylation in different genomic regions has been investigated by immunoprecipitating oligonucleosomes from a human lymphoblastoid cell line with antibodies to H4 acetylated at lysines 5, 8, 12 or 16. DNA from antibody-bound or unbound chromatin was assayed by slot blotting. Pol I- and Pol II-transcribed genes located in euchromatin were shown to have levels of H4 acetylation at lysines 5, 8 and 12 equivalent to those in bulk chromatin, but to be slightly enriched in H4 acetylated at lysine 16. In no case did the acetylation level correlate with actual or potential transcriptional activity. All acetylated histone H4 isoforms were depleted in non-coding, simple-repeat DNA in heterochromatin and the CCCTAA repeat at telomeres, although the extent of depletion varied with the type of heterochromatin and with the isoform. Two single-copy genes that map

within or adjacent to blocks of paracentric heterochromatin are depleted in H4 acetylated at lysines 5, 8 and 12, but not 16. Consensus sequences of repetitive elements of the Alu family (SINEs, enriched in R-bands) were associated with H4 that was more highly acetylated at all four lysines than bulk chromatin, while H4 associated with *KpnI* elements (LINEs, enriched in G-bands) was significantly under-acetylated.

### **The T transcription factor functions as a dimer and exhibits a common polymorphism in the conserved DNA binding domain of the human protein**

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Using *in vitro* synthesized T protein we have demonstrated that human T binds to its target DNA, 5'-AGGTGTGAAATT-3', as a homodimer and that truncated protein containing only the N-terminal 233 amino acid residues, which comprise the DNA binding domain, can form a dimer. We also report a common human polymorphism, Gly177Asp, within the DNA binding domain at a position which is a conserved glycine residue in T homologues from other vertebrates. This amino acid change appears to decrease the stability of the dimer. It seems possible that T forms heterodimers with other members of the T-box transcription factor family. This idea has been investigated in preliminary studies using Tbx6 protein. We have shown that Tbx6 binds to the same DNA target as T as a dimer, but thus far there is no evidence for the formation of heterodimers between T and Tbx6.

### **Characterization of a new protein family which resembles E2 enzymes but lacks the catalytic site**

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In a search by RNA differential display for genes highly expressed in proliferating, as compared with differentiated, HT29 M6 cells, a cDNA was identified which encodes a protein similar to the E2, ubiquitin conjugating enzymes. However, this protein differs from the E2 enzymes in that it does not possess a critical cysteine in the active site. Analysis by Northern blotting and RT-PCRs show that this gene is present

in at least four alternatively spliced forms. These are deduced to form proteins with two distinct N-terminal regions and the presence or absence of a 50 amino acid stretch. These four forms are expressed ubiquitously. The gene (UBE2V) encoding these proteins was localized to 20q13. There is at least one pseudogene. Two PACs containing the coding sequence were identified. Analysis of these by PCR showed that the sites of possible alternative splicing were coincident with intron-exon boundaries. The exons containing the two first codons and their 5' UTRs were subcloned and initial sequencing results show that they are both TATA-less. During this time two other proteins similar to E2 enzymes have been described: DDVit1 as a gene whose transcription is induced by vitamin D and Tsg101 as a gene implicated in breast cancer.

### **Polymorphism of the lactase gene in different populations**

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Expression of the small-intestinal enzyme lactase-phlorizin hydrolase (lactase) declines after weaning in most mammals. In humans lactase persists into adult life in some people but not others, and this difference in developmental down-regulation is genetically determined. Allele frequencies vary in different populations: persistence of lactase into adulthood is common in Northern European populations and pastoral nomadic tribes – both groups that drink milk as part of their diet. It has been shown that the relevant sequences controlling lactase persistence are *cis*-acting, although the sequence differences have not been identified. However, seven polymorphisms have been described and in European populations these associate to form just four common haplotypes. We will describe recent results involving polymorphisms and haplotype analysis of various non-European populations, and concentrate on one small highly variable region upstream from the promoter. This region contains seven variants, four of which have not been published; all were characterized by Denaturing Gradient Gel Electrophoresis (DGGE) and sequencing. Each DGGE variant corresponds to a small haplotype spanning 84 bases. A model for the evolution of these haplotypes will be proposed. The region containing variation is otherwise highly conserved in the pig as

well as primates, which suggests a possible functional significance.

### **The evolution of the murine retroviral restriction gene *Fv1***

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*Fv1* is a single autosomal gene in mice which determines the resistance or susceptibility to a certain class of mouse retroviruses, the Murine Leukaemia Viruses (MLVs). There are two main alleles: the *Fv1<sup>a</sup>* allele allows infection by N-tropic MLV but restricts B-tropic virus, and the *Fv1<sup>b</sup>* allele allows infection by B-tropic MLV but restricts N-tropic virus. There are also several other alleles which give rise to slightly modified phenotypes. *Fv1* is unique in structure with a product which effectively immunizes mice against certain retroviral infections. It is retrovirally derived, a remnant of an integration event over 10 million years ago which has undergone extreme change. How *Fv1* has changed during the course of evolution has been the focus of this work. In order to address this question, *Fv1* genes from many diverse mice, both recently- and distantly-diverged members of the genus *Mus*, were cloned and sequenced. The analysis of this sequence data has provided considerable information about the events that have led to the evolution of the alleles we see today.

### **Identification and characterization of a family of mammalian methyl CpG-binding proteins**

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CpG methylation is usually associated with local transcriptional silencing in eukaryotes. This silencing appears to be mediated through repressors which specifically bind to methylated DNA. MeCP1 and MeCP2 are two proteins identified due to their ability to specifically bind methylated CpGs. Both are capable of repressing transcription *in vitro*. While MeCP2 consists of a single polypeptide, MeCP1 is a large protein complex. A component of human MeCP1 which binds methylated DNA and is capable of repressing transcription has been identified by its sequence similarity to the methyl CpG-binding domain (MBD) of MeCP2. This protein was named PCM1 for Protein Containing an MBD. We report

the identification and characterization of the murine PCM1 gene as well as three additional novel genes (PCMs 2–4) in humans and mice containing MBD-like domains. No further sequence similarity exists between any PCM outside of the MBDs with the exception of PCMs 2 and 3, which show 78% overall identity at both DNA and amino acid levels. PCMs 2 and 3 have been found to bind specifically to methylated CpGs and are thus candidates for components of MeCP1. Characterization of this protein family may provide insights into the mechanism of transcriptional repression by DNA methylation.

### **The role of *Xist* in the regulation of X chromosome inactivation**

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The onset of X chromosome inactivation *in vitro* and *in vivo* is preceded by a marked increase in the steady-state level of *Xist* RNA. We present data to demonstrate that this process is not regulated at the level of transcription. We show that upregulation can be accounted for by increased stability of *Xist* RNA. Using RNA FISH we demonstrate that unstable *Xist* transcript is produced by alleles both in XX ES cells and in XX embryos prior to X inactivation, and that following differentiation, transcription from the active X chromosome allele continues for a period following stabilization and accumulation of *Xist* transcript on the inactive X allele.

### **Imprinted expression of SNRPN in human pre-implantation embryos**

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The SNRPN gene encodes the small nuclear ribonucleoprotein polypeptide SmN, a 29 kDa spliceosomal protein expressed predominantly in brain and heart. The SNRPN/*Snrpn* gene is parentally imprinted in human and mouse, being expressed from the paternal allele only. The human SNRPN gene maps to the smallest deletion region involved in Prader–Willi syndrome (PWS), a neurogenetic dis-

order, thus implicating the involvement of the gene in the disease. Imprinting results in a lack of SNRPN expression in PWS cases with paternal deletions or with maternal uniparental disomy (UPD). Further cases are caused by disruption of the imprinting centre (IC), resulting in a failure to establish appropriate epigenetic information on the parental SNRPN alleles. It is expected that the phenotype induced in disease states involving imprinted genes will manifest itself from the time of onset of monoallelic expression, when no compensatory gene dosage is available from the silent allele. Using single-cell sensitive RT-PCR and a previously reported polymorphism, we have established that SNRPN expression is monoallelic and expressed from the paternal allele only during human pre-implantation development. We conclude that the epigenetic information necessary for monoallelic expression of SNRPN is already present at this early developmental stage. We will discuss the effect of imprinted SNRPN expression in PWS with respect to data obtained from murine models, in which a compensatory elevation of the highly homologous snRNP protein SmB is observed. There are further implications from observations of the distribution of these two proteins within small nuclear ribonucleoprotein (snRNP) particles. Thus despite the recent evidence excluding SNRPN as a major factor in PWS, it remains possible that aberrations in SNRPN expression experienced in PWS are likely to contribute to some of the features of the disease, and that the effect will be initiated early in development.

#### Search for imprinted genes involved in Silver Russell syndrome

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Silver Russell syndrome (SRS) is a dysmorphic syndrome associated with intra-uterine growth retardation, short stature, triangular faces and in some cases skeletal asymmetry. Maternal uniparental disomy for chromosome 7 (mUPD 7) has been described in approximately 10% of cases of SRS. Five cases of mUPD 7 have been found out of 55 SRS patients in our study. All five cases are heterodisomic, and using multiple markers across these samples, no areas of common isodisomy have been found. This suggests that the exposure of recessive gene is not the likely cause of the observed phenotype. It is more likely that there are one or more gene(s) on chromo-

some 7 which are imprinted and play a part in the aetiology of SRS. We are investigating three growth-related genes on 7p12–13, a region homologous to a known imprinted region on proximal mouse chromosome 11. The candidates under study are the epidermal growth factor receptor (EGFR) and the insulin-like growth factor binding proteins 1 and 3 (IGFBP 1 and IGFBP 3). These are all important in growth and are proposed as candidate genes for SRS. We have shown that EGFR is not imprinted in humans using an RFLP polymorphism, and are using a sequencing polymorphism to determine the imprinting status of IGFBP 1. We are currently searching for a polymorphism in IGFBP 3. Further investigation is planned of genes on chromosome 7 found to be imprinted.

#### Developmental effects of genomic imprinting on mouse chromosome 12

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The term 'genomic imprinting' refers to the fact that the two genomes present in an embryo (one inherited from the mother and the other from the father) are not functionally equivalent, even though they carry the same genetic information. This functional inequivalence between the two genomes manifests itself as parental-origin-specific differences in expression between the two alleles of certain genes, known as imprinted genes. Thus, some imprinted genes are expressed from the paternally inherited chromosome and others from the maternally inherited chromosome. Genetic studies in mice designed to produce offspring in which the parental-origin-specific inheritance of distal chromosome 12 (chr12) was disrupted by having both copies of distal chr12 inherited from the same parent (uniparental disomies or UPDs), strongly suggested that distal chr12 is imprinted and that its correct imprinting is required for normal development. However, the phenotype and the cause of death of these embryos is not yet known and none of the published imprinted genes map to chr12. To begin to investigate the embryonic role of imprinted genes on chr12, we are carrying out an embryological study to isolate and characterize in detail embryos UPD for chr12 (UPD12). The phenotype of such embryos will be discussed.

**Expression of polymorphic murine *N*-acetyltransferase (NAT2) in CD-1 embryos**

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*N*-acetyltransferases (NATs) are phase II xenobiotic metabolizing enzymes. Mice have three *Nat* genes (*Nat1*, *Nat2* and *Nat3*), of which *Nat2* is known to be polymorphic. In addition to its role in xenobiotic metabolism, NAT2 is involved in the clearance of folate, a vitamin which protects women at risk of giving birth to offspring with neural tube defects. The rate of folate breakdown increases during pregnancy,

and recent studies reported at this meeting suggest that this could be due to changes in the placental expression of NAT. In order to determine whether the embryo itself expresses NAT2, polyclonal anti-NAT2 antisera [1] were used for immunohistochemical detection in CD-1 mouse embryos. At 9.5 days *post coitum*, NAT2 was expressed in the neural tube and yolk sac. By 11.5 days, NAT2 expression was reduced in the neural tube but still strongly evident in the yolk sac, and by 13.5 days segmental expression of NAT2 was apparent in the developing spinal column. In addition, NAT2 was observed in the brain, eye and heart of 13.5 day embryos. These results imply that both the embryo and its mother must be taken into account in elucidating the role of NAT in the aetiology of neural tube defects.

1. Stanley, L. A., Mills, I. G. & Sim, E. (1997). *Pharmacogenetics* 7, 121–130.