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## A functional and molecular analysis of parthenogenetic embryonic stem cells

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A detailed analysis of the developmental potential of parthenogenetic embryonic stem cells (PKES) was made *in vivo* and *in vitro*, and has been compared with the development of cells from parthenogenetic embryos (PG). *In vivo*, in chimaeras, PKES cells showed a tissue distribution consistent with that of PG cells suggesting faithful imprinting in the PKES cells with respect to lineage allocation and differentiation. Restricted developmental potential was also observed in teratomas formed by ectopic transfer under the kidney capsule. In contrast, the classic phenotype of growth retardation in PG chimaeras was not observed, suggesting aberrant regulation in PKES cells of genes involved in growth regulation. *In vitro*, following induction of differentiation with retinoic acid or DMSO, the different developmental potentials of PKES cells compared with control and androgenetic ES cells (AKES) was also observed. We also analysed the expression of known imprinted genes after differentiation both *in vivo* and *in vitro*. Igf2, H19 and Igf2r were all appropriately expressed in the PKES derived cells; interestingly, H19 was found to be expressed at high levels following differentiation of the AKES cells.

Our studies show that PKES cells provide a valuable *in vitro* model system to study the effects of imprinting on cell differentiation and they also provide invaluable material for extensive molecular studies on imprinted genes. In addition, the aberrant growth phenotype observed in chimaeras has implications for mechanisms that regulate the somatic establishment and maintenance of some imprints. This is of particular interest as aberrant imprinting has been invoked in the aetiology of some human diseases.

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## Mapping using flow-sorted pig chromosomes

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Efficient flow sorting of individual pig chromosomes by our group (Dixon *et al.* 1992; Langford *et al.* 1993) has opened up the possibility of producing chromosome-specific libraries using flow-sorted chromosomes as the source of DNA. Accordingly, a library of pig chromosome 1 has been produced (Miller *et al.* 1992) using pUC18 as the vector. The average insert size was 405 bp and 210000 independent clones were produced. The specificity of the library was confirmed by chromosome painting. The library is now being used to produce a microsatellite-based map of pig chromosome 1. Using a poly dA-dC. poly dG-dT probe, thirty microsatellites have been isolated from the library. On sequencing, these varied in length from 12 to 25 dinucleotide repeats and included examples of compound, perfect and imperfect tandem dinucleotide repeats. Thirteen of these microsatellites were considered suitable for further study. Flanking primers were designed from their sequences and used in PCR tests on the grandparental DNA from the 5 PigMap reference families. Five of the thirteen microsatellites were sufficiently polymorphic to allow genotyping of all 137 members of the reference panel for their presence. The numbers of alleles present for each microsatellite varied from 2 to 5. The microsatellites along with other markers are being used to produce a map of pig chromosome 1. Isolation of microsatellites from a chromosome-specific library rather than from a genomic library removes the need for chromosome assignment.

## First meiotic division abnormalities in human oocytes; mechanism of trisomy formation?

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Trisomy is the single most frequent type of chromosome abnormality in the human species and has considerable impact on many aspects of human pathology. It arises most commonly through 'nondisjunction' at maternal meiosis I, but the underlying mechanism remains obscure. Many hypotheses imply nondisjunction of whole bivalents at meiosis I but analysis of 140 reject oocytes at second meiotic metaphase from women undergoing *in-vitro* fertilization treatment for their infertility (mean maternal age 34 y) showed that none had an extra whole chromosome. 65% were normal (23,X) and the remainder had different types of single chromatid abnormalities. The most frequent type had 22 whole chromosomes, the missing chromosome being replaced by 2 single chromatids of the same size. Less common were abnormalities with one single chromatid extra (23+1/2), or missing (22+1/2). We hypothesize that the chromatids arise by precocious centromeric division in chromosome univalents, or bivalents functioning as univalents, at first meiotic division. The chromatids would have the potential to form unbalanced gametes at second meiotic division and hence trisomies after fertilization. The results are compatible with many of the factors known about trisomy in man.

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## S-Rex, a gene in which mRNAs are enriched in synaptosomes from rat cerebral cortex encodes a novel channel/receptor

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We have used a modified subtractive cloning procedure for cloning cDNA of 'synaptosome-enriched' mRNA species. A clone corresponding to a gene, named S-Rex, hybridized with three mRNAs whose concentration in synaptosomes is substantially higher than in cytoplasm. The expression of S-Rex gene is restricted to the central and peripheral nervous systems and is regulated in early embryonic development of vertebrates. The gene is highly conserved in evolution. Full length cDNA clones for rat and chicken 1.5 and 3.5 kb S-Rex mRNAs were isolated and sequenced. These transcripts are generated by differential splicing and promoter usage and have a common 3' sequence which includes the C-terminus of a protein with three predicted transmembrane domains. A fourth presumptive transmembrane domain was found in a protein encoded by 3.5 kb mRNA together with a sequence that is believed to be pyruvate-binding site in bacterial enzyme dihydrodipicolinate synthetase. We propose that S-Rex encodes a novel channel/receptor expressed in neurons.

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## Genetic analysis of lethal and subviable deletions on murine chromosome 4

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The murine *brown* locus on chromosome 4 is one of the loci used in the specific locus mutagenesis test. Consequently many new alleles have been isolated at the locus over the years. Radiation mutagenesis experiments generated a panel of over 30 deletions centred on the *b*-locus. Most of these deletions, when homozygous, are embryonic lethals. Combinations of certain deletions are not lethal but reveal a *brown associated fitness* or *baf* gene. When deleted, mice are much smaller than their litter-mates and frequently die at an early age. Analysis of deleted chromosomes when heterozygous with a *M. spretus* chromosome allows mapping of markers to the deleted region. The presence or absence of markers in different deletions positions the marker with regard to other markers and also classifies the deletions into different groups. We have mapped a number of microsatellite probes by PCR to the region. We have sequenced a number of microdissection clones for the region, derived PCR primers which amplify genomic DNA and mapped them to the panel using SSCP.

The PCR primers have been used to screen a YAC library resulting in the isolation of 15 YACs. The physical map at this locus has been improved which will allow isolation of a YAC contig and ultimately the *baf* gene itself.

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## Rat, human and chicken neuro-d4 genes: structure, expression and possible function of encoded proteins

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We have cloned a novel neurospecific, developmentally regulated gene, neuro-d4, that encodes a set of proteins (the products of differentially spliced transcripts) with both 'classic' and new types of zinc-fingers. A rat cDNA probe was used to study the expression of neuro-d4 in the developing vertebrate nervous system. In the mouse CNS, the expression of neuro-d4 dramatically increases after E11 from low basal level and remains high before gradually decreasing during postnatal development. We have cloned and sequenced a human and two chicken neuro-d4 genes from embryonic brain cDNA libraries and have found a high degree of homology, particularly in zinc-finger regions. Some multiple forms of differentially spliced mRNAs were also found to be conserved in vertebrate evolution. Human neuro-d4 gene was localized on chromosome 19. Fused neuro-d4 proteins produced in *E. coli* are capable of binding to GC-rich DNA sequences; that confirms our previous suggestion that neuro-d4 proteins are involved in regulating gene activity in neurons.

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## A functional analysis of MPBF/MGF transcription factor binding sites in the sheep $\beta$ -lactoglobulin gene promoter

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Activity of the mammary transcription factor MPBF/MGF (milk protein binding factor/mammary gland factor) increases in the mammary gland during pregnancy and is maximal during lactation. MPBF activity parallels the induction of many milk protein genes and the presence of potential MPBF binding sites in their promoters suggests that MPBF plays a role in milk protein gene regulation. To test the *in vivo* function of MPBF, site directed mutants of the sheep  $\beta$ -lactoglobulin gene (BLG) have been constructed that abolish MPBF binding at all three sites within a minimal 410 bp BLG promoter. The activities of three constructs carrying different combinations of MPBF site mutations have been tested in the mammary cell line HC11 and in transgenic mice. Mutation of the proximal, high-affinity site, or the two distal sites, has little effect on promoter activity. However, mutation of all three sites reduces expression of the BLG gene both in HC11 cells and in transgenic mice, demonstrating synergism between the sites and the importance of MPBF in regulating milk protein gene expression.

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## XY female sex reversal associated with Yp deletions outside the sex determining region

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Previous studies have shown that Yp-Sxr<sup>a</sup> homologous pairing frequently occurs in X Sxr<sup>a</sup>/Y male mice (Cattanach *et al.* (1990) *Genet. Res.* **56**, 121–128). Furthermore, evidence of asymmetrical exchange was noted and this suggested that duplication and deletion of Yp and Sxr<sup>a</sup> regions might be generated. Analysis of the progeny of X Sxr<sup>a</sup>/Y males carrying Y chromosomes of different origins have identified 7 independently

occurring XY females, these representing 0.5% of the total XY class. Evidence will be presented to show that in each case the testis determining (*Sry*) locus is intact but that *Sry* expression is reduced or absent. The sex reversal is however, associated with deletions involving repeat sequences identified by the pSX1 probe. As it can be deduced from the data that these repeat sequences are located between the *Sry* locus and the Y centromere, long-range position effects disrupting *Sry* action are thought to be the cause of the sex reversal. Further studies utilizing Y chromosomes showing polymorphisms for Yp genes suggest that Yp-*Sxr*<sup>a</sup> meiotic crossing over is mediated by the SX1 repeats and that not all SX1 deletions cause XY female sex reversal. The sex reversal appears to be genetic background dependent.

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## Transgenes of the human minisatellite MS32 (D1S8) which display high rates of mutation in germline and early embryo

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Hypervariable minisatellites have been found in all higher eukaryotic species including mice and humans. Human minisatellites change in length (mutate) via the gain or loss of G/C rich repeat units. Recent evidence from three human minisatellite loci (including MS32) shows that these changes, when occurring in the germline, are restricted to one end of the repeat array. This property of 'polarity' is believed to be due to the presence of a meiotic gene conversion hot-spot, which may be caused by elements located in flanking DNA upstream of the hypervariable end of the minisatellite. By generating transminisatellitic mice it is possible to test models of minisatellite hypervariability. Topics relevant to minisatellite mutation which can readily be addressed using such mice include the effects of flanking DNA, chromatin structure, genotype, age, tissue, genetic background, germline imprinting, and environment (including toxic chemicals and ionizing radiation).

The progress made in generating a mouse model of human minisatellite mutation will be reviewed. Of the six loci currently under study, two are multi-copy and share features of instability, which can occur in either germline or during early embryonic development. The latter is something seen at mouse minisatellites *ms6hm* and *Hm-2*, at human triplet repeat disease loci, but only very rarely at the endogenous MS32 locus in man.

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## The molecular genetics of variation in captive populations of *Macaca fascicularis* and *Equus przewalskii*

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Two parallel pilot studies have been performed investigating the potential use of multilocus minisatellite DNA fingerprinting for the study of genetic variation in captive, inbred populations of two scientifically interesting organisms. The Long-tailed Macaque, *Macaca fascicularis*, is an important primate used in biomedical research. With a life-span and reproductive physiology similar to that of man, *M. fascicularis* provides a very useful model for the study of various aspects of human biology and a number of breeding centres around the world focus upon the captive breeding of Macaques. Przewalski's Wild Horse, *Equus przewalskii*, is thought to be extinct in the wild and around 1200 individuals now remain alive in various centres around the globe. This surviving population is known to be extremely inbred, being founded from only 13 individual animals at the turn of the century, and currently split into two distinct breeding lines. A high frequency of congenital abnormalities, and other manifestations of inbreeding depression, are prevalent. Minisatellite fingerprint analysis shows levels of genetic variation in these populations are lower than those found for outbred populations of comparative species. However, levels of heterozygosity are not as low as those observed in certain island species where the reduction in variation has been very severe indeed.

## TCP-11, a protein encoded within the mouse t-complex, is expressed in elongating spermatids

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The t-complex on mouse chromosome 17 is known to influence male fertility in two major ways. Mice which carry both copies of chromosome 17 in the t-haplotype form are often partly or completely sterile. Mice which are heterozygous and carry one normal chromosome 17 and one in the t-haplotype form display transmission ratio distortion (TRD). We have isolated a gene, *Tcp-11*, which maps to the distal inversion of the t-complex and is a candidate for a gene involved in TRD. The gene is first expressed towards the end of meiosis in secondary spermatocytes and it codes for a 62 kD novel protein of unknown function. Analysis of the predicted amino acid sequence of the protein reveals an RGD sequence and a leucine heptad repeat. We have expressed *Tcp-11* in *E. coli*, purified the protein and used it to raise polyclonal antisera in rabbits. On sections of testis the antisera recognise elongating spermatids with the staining being predominantly cytoplasmic. On Western blots of protein prepared from highly purified populations of germ cells a strong signal is detected in elongating spermatids with a weaker signal in round spermatids.

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## Mapping and expression studies on the distal region of mouse chromosome 2 that is subject to imprinting

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Distal Chromosome (Chr) 2 is one of ten regions of the mouse genome which are subject to parental imprinting, the phenomenon whereby paternally and maternally derived genes are differentially expressed during development (Cattanach, pers comm.). Maternal duplication/paternal deficiency of distal Chr 2 leads to hypokinetic mice with long flat sided bodies and arched backs, which die within a few hours of birth, having failed to suckle. Paternal duplication/maternal deficiency leads to the effectively opposite phenotype of hyperkinetic mice with short square bodies and flat backs. These survive for several days but fail to grow normally and display increasingly severe behavioural characteristics (Cattanach & Kirk (1985) *Nature* **315**, 496–498). The region is currently defined as lying distal to the breakpoint of the reciprocal translocation T(2;8)2Wa, (T2Wa), in band H3, and proximal to that of T(2;16)28H, (T28H), in band H4 (Cattanach *et al.* (1992) *Mouse Genome* **90**, 82). The closest known gene markers to the boundaries of the region are *Ada* (adenosine deaminase) proximally and *Ra* (ragged) distally. In order to map markers to the region, two interspecific crosses have been set up incorporating the T2Wa and T28H translocations which define the boundaries. Preliminary data from linkage analysis of ten markers in the T28H cross indicates a gene order of *Ada*-(*Pck-1*, *Gnas*)-*Acra-4* proximal to T28H. These results imply that *Pck-1*, *Gnas* and *Acra-4* probably lie within the region.

Using another more proximal translocation, T(2;8)26H, mice with maternal duplication/paternal deficiency or paternal duplication/maternal deficiency for distal Chr 2 have been generated for the identification of differentially expressed genes within the region. *Gnas* is a particularly good candidate as it has been suggested that its human homologue, GNAS, is subject to imprinting. This suggestion is based on clinical and biochemical studies of patients with Albright's hereditary osteodystrophy (AHO), an autosomal dominant disorder resulting from the possession of a dysfunctional *GNAS* allele (Davies & Hughes (1993) *J. Med. Genet.* **30**, 101–103). *Gnas* expression has been investigated in the kidney, liver and brain of 18.5-day embryos and newborn mice by RT-PCR. Neither the maternal nor the paternal allele is repressed and thus there is no evidence to date that *Gnas* is subject to imprinting in the mouse. *Pck-1* is not differentially expressed in the liver of newborn mice with maternal or paternal duplication for the region. The expression of *Acra-4* is currently being investigated.

## Improved viral thymidine kinase transgenes for conditional ablation in transgenic mice

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Cell specific expression of the herpes simplex virus type 1 thymidine kinase gene (HSV1-tk) in transgenic mice has been used for conditional ablation of various cell types. However, transgenic male mice with HSV1-tk as a reporter gene are often sterile and invariably express truncated versions of HSV1-TK in the testis. These truncated polypeptides arise from translation initiation at downstream ATG codons. Two strategies have been undertaken to characterize a viral TK reporter gene that will alleviate male sterility and be useful in conditional ablation experiments. The first uses herpes simplex virus type 2 thymidine kinase (HSV2-tk) as a reporter gene. The second employs a hybrid HSV1-tk reporter gene in which the two critical ATG codons have been mutated to CTG. Four lines of transgenic mice generated using the HSV2-tk reporter gene coupled to the bovine thyroglobulin promoter had high levels of HSV2-tk activity in the thyroid gland (range: 61.7–95.4 pmoles/min/mg protein). In conditional ablation experiments with these mice, thyroid follicle cells were absent after treatment with the antiherpetic agent Ganciclovir. A majority of the HSV2-tk transgenic males were fertile. Viral TK activity in the testis of HSV2-tk transgenic mice was approximately 10-fold less than in mice harbouring HSV1-tk transgenes. Results from the second strategy indicate that 7 of 8 transgenic male founders are fertile and 3 of 8 transmit the transgene to their offspring. These studies show that the incidence of male infertility decreases with these viral TK reporters and in one case the new transgene has been successfully used for conditional ablation.

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## Molecular and developmental characterisation of allelic methylation in the mouse *Igf2* and *H19* genes

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The Insulin-like growth factor 2 (*Igf2*) and *H19* genes are reciprocally imprinted and about 85 kb apart on distal mouse chromosome 7. *Igf2* encodes a foetal growth factor and is predominantly expressed from the paternal allele, while *H19* is expressed from the maternal allele in the embryo and encodes a transcript which may be involved in down-regulating cellular proliferation.

One of the epigenetic modifications which is thought to be involved in the mechanism of parental imprinting is DNA methylation. We have identified parental allele-specific methylation in a region upstream of the *Igf2* gene, and have carried out a molecular and developmental analysis, including an examination of individual chromosomes using a novel genomic sequencing protocol (Frommer *et al.* (1992) *PNAS USA* **89**, 1827–1831). In mouse embryos and in adult tissues this region is significantly more methylated on the paternal than on the maternal allele.

In choroid plexus, a tissue in the adult brain in which *Igf2* is expressed from both parental alleles, the *Igf2* upstream region and the body of the *H19* gene were found to be highly methylated on both alleles, having adopted a 'paternal type' methylation pattern.

Recently, we have identified a second allele-specifically methylated region in the (3' part of the) *Igf2* gene, which is also more methylated on the paternal allele.

To determine when during development allele-specific methylation arises in the *Igf2* and *H19* genes we have analysed early passage ES cells (normal, androgenetic and parthenogenetic) and ES cell-derived embryoid bodies and teratomas. Our results suggest that allelic methylation in *Igf2* and *H19* arises on differentiation of stem cells in the early embryo and changes progressively during development. Allele-specific methylation may be involved in the somatic maintenance of parental imprinting.

## Linkage disequilibrium across the lactase gene

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Lactase, the enzyme responsible for hydrolysing the milk sugar lactose, is present on the brush-border membrane of the small intestinal enterocytes of all healthy infants. The level of activity declines dramatically at some stage after weaning in some adult humans but persists at high levels in others. Although it is clear that this person to person difference is genetically determined, the molecular basis of this polymorphism is not yet known. Our original hypothesis was that sequence differences within an upstream regulatory region or some other cis-acting element was responsible (Ho *et al.* (1982) *Am. J. Hum. Genet.* **34**, 650–657). Despite the cloning and sequencing of the lactase cDNA and 1 kb in the promoter region the relevant difference has not been found (Lloyd *et al.* (1992) *J. Clin. Invest.* **89**, 524–529; Boll *et al.* (1991) *Am. J. Hum. Genet.* **48**, 889–902), but it may reside at some distance from the known sequence. It is also not known whether the lactase activity shows genetic linkage to the lactase structural gene. In order to answer these questions and to establish whether there is any association between DNA polymorphisms within the lactase gene and with the activity polymorphism, we have analysed up to 7 polymorphic sites spanning a 60 kb region which comprises the whole lactase structural gene. Haplotypes (i.e. combination of alleles on each parental chromosome) were analysed in 50 CEPH families and only 5 of the 128 expected haplotypes were observed, providing evidence of significant linkage disequilibrium. We have also analysed these polymorphisms in a series of unrelated individuals from the same racial and geographic background, who had been characterized with respect to their lactase persistence phenotype. The results of our analyses on the first 32 individuals will be discussed.

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## How commonly are intron/exon boundaries conserved?

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We have a long-standing interest in the C6 and C7 genes of the complement system. They, together with C8 $\alpha$ , C8 $\beta$  and C9, are gene duplicates. We found that the intron/exon boundary phase types are completely conserved between C6 and C7<sup>1</sup>, and largely so compared with C8 $\beta$ <sup>2</sup> and C9<sup>3</sup>, despite the gene duplications having occurred some 400 000 000 years ago. By contrast, the contents and lengths of the exons and the lengths of the introns show no more conservation than might be expected. Very similar observations can be made concerning the C3, C4, C5 and  $\alpha_2$ -macroglobulin family. In contrast, the mitochondrial 2-oxoglutarate carrier has different intron/exon boundaries in humans and cattle. It is very easy to make patterns and to believe that they are meaningful, especially when there are only three boundary types. Comparisons therefore require genuinely expert knowledge, and this paper is an appeal for information from the experts – you, the readers!

<sup>1</sup> Hobart, M. J., Fernie, B. & DiScipio, R. G. (1993). *Biochemistry* **32**, 6198–6205 and unpublished observations.

<sup>2</sup> Kaufmann, T., Rittner, C. & Schneider, P. M. (1993). *Hum. Genet.* **92**, 68–75.

<sup>3</sup> Marazziti, D., Eggertsen, G., Fey, G. H. & Stanley, K. K. (1988). *Biochemistry* **27**, 6529–6534.

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## Quality control assessment of techniques for the preimplantation diagnosis of Duchenne muscular dystrophy

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PCR amplification of informative RFLPs within the dystrophin gene together with sequences in the Y chromosome at the single-cell level is now possible. Their respective efficiencies and reliability have been assessed on samples containing one, two and three single buccal cells per tube. Different combinations of PCR reactions of single-copy X and single- or multiple-copy Y sequences have been assayed together in the same sample, to determine the effect of each reaction on the other(s) in the same tube. Results indicate that PCR of each single-copy sequence takes place with an efficiency of about 78% and that each individual PCR proceeds independently

from any other in the same tube. An internal PCR control is therefore not possible at this level of sensitivity. One PCR of a highly polymorphic (CA) repeat region within the dystrophin gene provides a method for coarse fingerprinting of the DNA under test, but this region would only detect a single contaminating cell three out of four times.

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## Gene mapping by analysing 2D-protein patterns of the mouse (*M. m. domesticus* × *M. spretus*)

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In an international collaboration a project has been started to establish a comprehensive genetic map of the mouse genome. The basis of this project is a backcross of the species *M. m. domesticus* (C57BL/6) and *M. spretus* which resulted in nearly 1000 B<sub>1</sub>-animals. While some working groups use anchor DNA markers to set up a tight linkage map of the genome, we use electrophoretic protein polymorphisms to map coding genes. We took 200 animals of the B1 progeny and prepared 5 organs from each of them. In a first approach we separated the soluble brain proteins from 64 females by two-dimensional electrophoresis using large gels (40 × 30 cm) and a highly sensitive silver stain. We started with the evaluation of the acid-half of the gel which reveals about 4100 polypeptide spots. Among these, 706 genetically variant spots were detected. When 'spot families' were taken just as one protein, 485 variant proteins resulted, i.e. 206 qualitative and 279 quantitative variants. So far we have determined the segregation pattern of 128 qualitatively and 105 quantitatively variant proteins in a first series of 20 B<sub>1</sub>-animals. Using this rather small panel of mice in a computer-based linkage analysis of 115 qualitative variants, 79 proteins led to a location on one of the mouse chromosomes. By completing the analysis of the brain proteins (inclusion of the basic-half of the gels and the structure-bound fraction of the brain proteins) and extending the study to the other four mouse organs prepared and to a series of reciprocal B<sub>1</sub>-animals we should be able to map several hundred protein-coding genes in the mouse genome. Partial micro-sequencing and peptide analysis of protein spots offer the possibility to identify the proteins mapped with known proteins and cDNAs. Interesting and unexpected observations may immediately result from such a large-scale study of genetically variant proteins. Our results obtained so far suggest interesting clues on the problem of genomic imprinting and to the pleiotropic effect of genes modifying proteins.

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## Detection of the myotonic dystrophy gene in single sperm and human preimplantation embryos

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Myotonic dystrophy (DM) is caused by amplification of a CTG triplet in the 3' region of the myotonin protein kinase gene on chromosome 19. The gene in its 'premutant' or mutant stage shows meiotic instability in that variation in the number of triplet repeats is observed between parent and child and between sibs. We expect this instability to be expressed during gametogenesis or the preimplantation stages of human embryonic development. Knowledge of the timing of this event is required before we can speculate as to the mechanism of instability. To initiate such a study, we have developed sensitive PCR procedures to detect different alleles of the myotonic dystrophy gene initially in single buccal cells, sperm from normal heterozygous individuals and blastomeres isolated from preimplantation embryos. These procedures will be used to investigate transmission and stability of alleles in the normal range.



## Microsatellite polymorphisms in the mammalian pyruvate kinase gene

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We have analysed a (CT)<sub>n</sub> dinucleotide repetitive sequence in intron 4 of the rat pyruvate kinase (PK) L-type gene for its variability in different inbred rat strains. Altogether the DNA of 30 rats from 18 strains and 3 crossings between them were used. We detected a total of 6 different alleles. Intrastrain variability found at this locus could be confirmed by multilocus DNA-fingerprinting. Thus the polymorphism can be used for linkage analysis in rat as well as for homozygosity testing of inbred rat strains. Although there exists a great homology between the coding regions in the PK-L-gene of several mammals, a (CT)<sub>n</sub> repeat is not present in the relevant intron of monkey, mouse and man. But the latter harbours a polymorphic trinucleotide repeat in intron 11, highly important for diagnostic purposes with respect to PK deficiency.

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## A multiple copy Y-chromosomal 'spermiogenesis gene' in the mouse: genetic evidence and a candidate gene

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By using a deletion mapping approach we have obtained evidence for a multiple copy gene on the mouse Y long arm that is needed for the normal development of the mouse sperm head. A multiple copy Y genomic sequence (Y353/B) has previously been identified that maps to the long arm, and which detects a family of testis-specific transcripts of unknown function. We now show that these transcripts are derived from multiple genomic copies and that they are exclusively expressed in round spermatids, just prior to the shaping of the sperm head. Although no protein product has yet been identified, the transcripts are loaded on polysomes, suggesting that they are actively translated. We are now trying to produce transgenic mice using a cloned Y353/B-related cDNA coupled to a spermatid-specific promoter (from mouse protamine-1). If successful, this transgene will be bred into mice which lack the mouse Y long arm to see if it can 'rescue' the sperm head defect associated with the lack of the long arm.

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## Mutation analysis at the human phosphoglucosmutase-1 locus

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Phosphoglucosmutase-1 (PGM1) is a highly polymorphic enzyme used extensively in forensic science and as an anchor locus in genetic mapping and population genetics. We have used molecular techniques to establish the structural basis of this classical protein polymorphism. Eight common phenotypes can now be resolved from DNA extracts using a simple PCR and restriction enzyme method, thus increasing the usefulness of PGM1 as a genetic marker. Analysis of the protein polymorphism at the *PGM1* locus is able to resolve a total of ten common phenotypes. We have analysed the DNA sequence of individuals of known PGM1 phenotype, and detected two single-base transitions in the coding region of the gene, resulting in a single amino acid change in each case. The charge changes predicted from the amino acid substitutions were entirely consistent with the known electrophoretic properties of the *PGM1* gene products. These results confirmed the genetic model that the 4-allele *PGM1* polymorphism has been brought about by two separate point mutations, followed by intragenic recombination. We have modified methods used in forensic analysis to detect these mutations in extracts containing as little as 1 ng DNA, and have successfully predicted the PGM1 phenotype of individuals

from blood, saliva and semen stains. This technique should prove extremely useful in forensic science and also in the genetic analysis of large panels of DNA samples.

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## Towards positional cloning of the mouse shaker-1 (*sh-1*) deafness gene

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The Olfactory Marker Protein gene is non-recombinant with *sh-1* in a 1066 progeny backcross (C57BL/10 × *sh-1/sh-1*) × *sh-1/sh-1* segregating the *sh-1* mutation and has been used to construct a 1.4 Mb YAC contig extending over the *sh-1* region. Two approaches to the analysis of the YAC contig in the vicinity of the *sh-1* gene are being followed. Firstly, microsatellites from the YAC contig are being used to identify the closest recombinations breakpoints flanking the *Omp* and *sh-1* loci thus narrowing the search for the *sh-1* gene. Secondly, exon trapping of YACs is being used to uncover expressed sequences for screening against inner ear and cochlear cDNA libraries. Microsatellites isolated from the YAC contig and spanning 450 kb in the vicinity of *Omp* are non-recombinant with *sh-1* and a number of conserved sequences have been derived via exon trapping in this region. Two of the exon trapped sequences are expressed in a mouse cochlear cDNA library. In addition, a potassium channel gene expressed in brain has been identified within the non-recombinant region.

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## Generation of mutations at DIA/LIF receptor gene

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DIA/LIF is a cytokine that regulates ES cell differentiation *in vitro*. The actions of DIA/LIF are mediated through binding to a specific cellular receptor which belongs to the haemopoietin receptor superfamily. DIA/LIF receptor has also been shown to be a component of receptor complexes for oncostatin M (OSM) and ciliary neurotrophic factor (CNTF), which share many biological activities with DIA/LIF. To investigate the role of DIA/LIF and related cytokines in ES cell self-renewal *in vitro* and in early mouse development, we have disrupted the DIA/LIF receptor gene by homologous recombination in ES cells.

We have isolated six overlapping DIA/LIF receptor genomic clones from a strain 129  $\lambda$  library. These clones span 40 kb and contain multiple exons for the two conserved haemopoietin domains which are believed to be responsible for ligand binding. A replacement type targeting vector has been constructed in which 20 kb of genomic sequence containing the major portion of the two haemopoietin domains are replaced by a promoterless Internal Ribosome Entry Site (IRES)-LacZ-*neopolyA* cassette. Homologous recombination will generate a non-reverting null allele. Furthermore, the LacZ expression will be under the control of the DIA/LIF receptor promoter. This will enable visualization of the spatial and temporal pattern of DIA/LIF receptor expression throughout murine development.

Electroporation of the linearized targeting construct into CGR8 ES cells resulted in 11 predicted homologous recombination events out of 58 G418 resistant clones analysed. X-gal staining of the targeted ES clones revealed that  $\beta$ -galactosidase activity is restricted to undifferentiated ES cells, indicating that expression of DIA/LIF receptor is regulated during ES cell differentiation. Chimaeras have been produced from the correctly targeted clones and are being bred for germline transmission of the null allele.

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## Gene interaction during vertebrate hindbrain segmentation

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We have been examining the expression and regulation of Hox genes and the zinc-finger gene Krox20 in vertebrate embryos. Paralogous members of the four Hox clusters have highly similar patterns of expression in the hindbrain, branchial arches and cranial neural crest. The restricted expression patterns of Krox20 and the

Hox genes appear before morphological segmentation and are likely to play an early role in the establishment and identity of rhombomeric segments. Using Hoxb-2/lacZ constructs we have identified an enhancer region upstream of the gene that imposes Krox20-like expression in transgenic mice. This element contains three Krox20 binding sites required *in vitro* for complex formation with Krox20 protein and *in vivo* for rhombomere-restricted expression. Extending this analysis to the Hoxb-2 mouse and chick paralogs – Hoxa-2, Choxa-2 and Choxb-2 we have mapped regulatory elements able to drive the typical Krox20 r3/r5 pattern of expression in early transgenic embryos. In different contexts we have been able to show that Krox20 expressed in ectopic domains can transactivate and upregulate the reporter constructs harbouring the paralog control regions.

Taken together with the results from the mutational analysis these data demonstrate that Krox20 is an essential part of the upstream transcriptional cascade that regulates mouse and chick Hoxb-2 paralogs during the hindbrain segmentation process. The elucidation and comparative analysis of the Krox20 binding site sequences is expected to shed more light on the nature of the remarkable conservation of modules and common molecular mechanisms of gene interaction in early embryogenesis.

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## Molecular characterization of the mouse $B^w$ mutation causing premature melanocyte death – melanocytes and early development

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*White-based brown* ( $B^w$ ) is a dominant mutant allele of the mouse *brown* locus on chromosome 4 which results in hairs being pigmented only at their tips. This phenotype is due to premature melanocyte death. Southern blot analysis revealed a large rearrangement in the first intron of the tyrosinase related protein 1 (TRP-1) gene encoded at the *brown* locus.  $B^w$  specific probes were derived by inverse PCR on  $B^w/B^w$  genomic DNA using TRP-1 primers upstream and downstream of the rearrangement. Molecular analysis showed that the  $B^w$  rearrangement is a large chromosomal inversion. Since the mutation is dominant and its effect seems to be restricted to melanocytes, a likely hypothesis is that the inversion brings a 'toxic' gene under the control of previously characterized melanocyte specific transcription regulating elements (MSE) which direct TRP-1 expression in the wild-type mouse. To identify this gene, we have isolated  $\lambda$  clones from a wild-type mouse genomic library, which map downstream of the TRP-1 MSE in  $B^w$ . A 6.5 kbp plasmid subclone mapping at the 5' end of the inversion was used to probe zoo blots and a strong cross-species hybridization was observed with mammalian genomic DNAs suggesting the presence of a conserved gene downstream of the inversion breakpoint. The normal function of this gene and the mechanism by which it kills melanocytes will be of interest. In order to study the role of melanocytes much earlier in development, we are generating lines of transgenic mice for which the melanocyte lineage is ablated by a toxic construct. Our approach and first results will be presented.

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## Molecular analysis of mottled mutations in the mouse

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Mottled (*Mo*) is an X-linked mutation caused by a defect in copper metabolism which shares many features with Menkes' disease (MNK) in man. Both *Mo* and MNK map close to PGK1 in the same conserved segment of the X chromosome. Mottled mutations, of varying degrees, arise frequently both spontaneously and after mutagenic treatment. These mutations provide tools for studying the structure and function of the gene involved. The recent cloning of human MNK cDNAs has provided probes for most of the coding region of the gene (Chelley *et al.* (1993) *Nature genetics* 3, 14–19). Using these human probes we have positioned the murine homologue of MNK (*Mnk*) immediately proximal to *Pgk-1* using an interspecific backcross; this is consistent with its position on the human X chromosome. We have constructed a 300 kb long-range restriction map around *Mnk* and have also analysed female *Mo/+* mutants for the presence of large deletions by PFGE. Southern blot analysis was also used to look for any rearrangements at *Mnk* in both female and male mice carrying ten independent mottled mutations. So far we have not detected any abnormalities using conventional or pulsed field gel electrophoresis.

Therefore, if *Mnk* is *Mo*, we can conclude that none of the mutations examined is associated with deletions. In addition we have detected an autosomal locus with one of the cDNAs and mapped it to chromosome 18 by linkage to *D18Mit24* using the European Interspecific Backcross. (DNAs kindly provided by Maria Breen and colleagues at the HGMP Resource Centre.)

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## RNA *in situ* hybridization analysis of the dystrophin-related protein gene (UTRN) during mouse embryogenesis

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The UTRN locus is the autosomal homologue of the DMD (Duchenne Muscular Dystrophy) gene and encodes the protein, utrophin. Structural similarity between utrophin and dystrophin may reflect some similarities in cellular function. UTRN is thought to be upregulated in some muscles in the absence of dystrophin and in these conditions utrophin interacts with membrane glycoproteins normally associated with dystrophin.

We have described the spatial and temporal expression of the UTRN gene during mouse embryogenesis and compared it with that of dystrophin. Whilst DMD is expressed largely in mesodermal derivatives such as cardiac and striated muscle, UTRN is expressed in a much larger group of tissues including neural tube, neural crest derivatives and tissues of non-neural origin. In early embryos UTRN transcripts initially accumulate in the mid-neural plate and thereafter in the caudal neural tube. UTRN mRNA then becomes abundant in a subset of neural crest cell derived tissues such as the spinal and facial ganglia and ossifying facial cartilages. Non-neural sites of UTRN expression include the tendon primordia of the developing digits, the thyroid and adrenal glands, cardiac muscle, kidney and lung. The high degree of homology between the UTRN and DMD genes suggests that, as has been shown for DMD, the UTRN locus may also give rise to a family of alternative transcripts. We present preliminary evidence suggesting that at least 2 different transcripts are transcribed from the UTRN locus. The existence of alternative transcripts may explain the widespread and complex pattern of UTRN expression that we have described during mouse embryogenesis.

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## Genetic analysis of the chromosomal region encoding epitopes recognised by H-Y specific T cells

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In the mouse, genes involved in the expression of the male-specific minor H antigen, H-Y (*Hya*), spermatogenesis (*Spy*), primary sex determination (*Sry*), zinc finger containing genes (*Zfy-1*, *Zfy-2*) and a Y-linked ubiquitin activating homologue (*Ube1y-1*), have been mapped to the short arm of the Y chromosome. In particular, *Hya* and *Spy*, *Zfy-2* and *Ube1y-1* have been sub-localized to a deletion interval defined by *Sxr<sup>b</sup>*. H-Y stimulates T cell responses and several class I and class II restricted T cell clones have been raised against different H-Y epitopes. Deletion mapping and chromosomal inactivation data show that these H-Y epitopes can be expressed independently. Here, we have transfected several overlapping cosmid clones spanning a small region of the *Sxr<sup>b</sup>* deletion into antigen presenting cells with the appropriate MHC restriction element. We have examined the transfectants for expression of different H-Y epitopes and have identified cosmids that appear specifically to encode one or more H-Y epitopes. Further results on the identification of specific sequences that encode H-Y epitopes will be presented.

## Mice with DNA repair gene (ERCC-1) deficiency have elevated levels of p53, liver nuclear abnormalities and die before weaning

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Nucleotide excision repair (NER) is one of the systems that has evolved to protect cells from the consequences of DNA damage. Defects in NER are associated with the hereditary human disease Xeroderma Pigmentosum which predisposes to skin cancer. The excision repair cross complementing gene (ERCC-1) was inactivated by gene targeting in the HPRT deficient embryonic stem cell line HM-1. Mice carrying this null allele was generated by blastocyst injection of the targeted ES cell line, followed by breeding from germline chimaeras. Primary cell lines isolated from ERCC-1 null embryos showed extremely low levels of NER. Genotyping of embryos at various stages *post coitum* showed that ERCC-1 deficiency did not compromise viability *in utero*.

Homozygous ERCC-1 mutants were runted at birth and died prior to weaning with liver failure. Examination of organs revealed abnormal liver nuclear size in the perinatal period, progressing to severe aneuploidy by 3 weeks of age. Elevated levels of p53 could be detected by immunohistochemistry in liver, kidney and brain of 3-week-old mice. This supports the hypothesized role for p53 as a monitor of DNA damage. This constitutes the first mouse model with a defined defect in any component of a mammalian NER system.

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## Genetic mapping and pathology of the myopathy in the kyphoscoliotic (*ky*) mouse

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The *ky* mutation, kyphoscoliosis, exhibits a degenerative muscle disease where regeneration of tonic muscles is arrested so they become weaker and smaller. The earliest abnormality seen in *ky* is muscle fibre necrosis followed by regeneration and the most striking differences between *ky* and the *mdx*, muscular dystrophy mutant, lie in neuromuscular junction (NMJ) morphology with extreme motor axon sprouting in *ky* as well as grossly abnormal distribution of acetylcholine receptor and acetylcholinesterase in affected muscles. As is the case in normal muscles, 43 kD and s-laminin were associated with AChR in *ky* muscles. Using an interspecific backcross segregating the *ky* mutation we have mapped the *ky* locus to a small region of chromosome 9. *ky* is non-recombinant with the microsatellite *D9Mit24* and lies in a conserved linkage group that encompasses human chromosome 3. s-laminin which maps to this region is recombinant with *ky* and, in addition, having identified the map position of *ky*, we have been able to eliminate a number of other NMJ proteins. The *ky* mutation would appear to lie in a gene coding for an as yet unidentified NMJ-associated protein.

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## Positive and negative regulation of the segment-restricted expression of the *Hox-b1* gene in the developing hindbrain

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We are interested in the cellular and molecular mechanisms which govern the establishment and maintenance of rhombomeric segments in the developing vertebrate hindbrain. To address this problem at the molecular level we have identified the *cis*-acting regulatory regions of the *Hox-b1* gene which are involved in modulating its rhombomere-restricted expression in transgenic mice. We have used these mice to perform a detailed investigation on how the cellular patterns of expression are established. There is a broad and diffuse initial domain of expression with no clearly defined cellular boundaries. This pattern sharpens and becomes progressively restricted to the future r4. Deletion analysis was initially performed on a 7.5 kb *Hox-b1/lacZ* construct which reproduces the normal r4 restricted domain of expression and responds to retinoic acid. We have

found that a 1.6 kb fragment on the 5' flanking region is capable of directing both the r4 and retinoic acid response on a heterologous promoter. More extensive deletion analysis has defined two separate regions which interact to generate the r4 restricted expression. One is a 600 bp enhancer which simulates expression in a domain largely confined to r4, but with some expression extending into adjacent rhombomeres. There is a high degree of sequence identity within this region in the chicken *Hox-b1* gene which suggests that regulatory regions may be conserved between these species. To test this we have generated transgenic mice with these chick sequences and shown that they function in a similar manner. Sequence analysis reveals several conserved motifs shared between these species. The second region acts as a negative component and is necessary to restrict the expression of the transgene, stimulated by the 600 bp enhancer, solely to r4. Therefore we believe this region is involved in mediating the normal process which leads to the progressive restriction of *Hox-b1* expression in r4. We conclude that a combination of positive and negative regulation is needed to generate the segment-restricted patterns of *Hox* expression in the hindbrain.

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## MeCP 2: a chromosomal protein that mediates the biological effects of DNA methylation?

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Methylation of CpG dinucleotides within gene promoters is often associated with repression of transcription. Methylation-mediated repression may occur as the result of direct effects on trans-acting factors or be an indirect effect mediated by proteins which bind methylated DNA. MeCP 2 is an abundant chromosomal protein which binds DNA containing a single symmetrically methylated CpG pair in any sequence context. The corresponding gene has been cloned and sequenced and staining with antibodies raised against the protein reveals that the protein distribution on rodent chromosomes mirrors that of 5-methylcytosine.

A null mutation was introduced into the murine MeCP 2 locus by gene targeting in ES cells to test the hypothesis that MeCPs mediate the biological effects of methylation. Using a promoterless vector containing a lac Z reporter gene resulted in 78% of the neo<sup>R</sup> ES clones analysed having the desired mutation. Since the gene is X-linked in the mouse and target cells are of an XY genotype the mutant cells lack all MeCP 2 function. Targeted ES cells grow with the normal vigour of the parental cell line and appear to be capable of differentiating *in vitro* into a number of terminally differentiated cell types. However, injection of targeted cells into mouse blastocysts to produce chimaeric embryos reveals that the MeCP 2 protein may be essential for embryonic viability.

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## Recycling the single cell to detect specific chromosomes and to investigate specific gene sequences

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A major disadvantage of preimplantation diagnosis, compared to prenatal diagnosis during pregnancy, is the relatively low efficiency of pregnancy associated with the IVF treatment. In many cases, pregnancy failure will be due to lethal chromosome abnormalities. It would be a considerable advance if diagnostic procedures could be designed to give both chromosomal and specific diagnostic information for genetic disease in the embryo. Currently, we are developing a method called 'cell recycling', using FISH to study specific chromosomes (e.g. sex chromosomes or common aneuploidies) in a single cell combined with PCR detection of specific gene sequences. The PCR must therefore take place on a fixed cell, i.e. a fixed DNA template. Preliminary experiments so far show that the FISH procedures introduce unacceptable levels of contamination to the PCR analysis. However, we have successfully sexed single mouse blastomeres using FISH immediately after the PCR detection of a mouse beta-globin sequence on the same single cell.

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## The genetic basis of lactase persistence/non-persistence polymorphism in man

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The persistence or non-persistence of lactase into adult life is genetically determined, with persistence being dominant to non-persistence. The molecular basis for this polymorphism is not yet known. It was not even clear whether or not the level of lactase mRNA is lower in lactase non-persistent adults than in persistent individuals (Sebastio *et al.* (1989) *Am. J. Hum. Genet.* **45**, 489–497; Escher *et al.* (1992) *J. Clin. Invest.* **89**, 480–483). We have therefore examined 51 intestinal biopsies and have shown that in 9/10 non-persistent samples both the mRNA and protein are lower than in any of the persistent individuals, suggesting that the control is at the RNA level (C. B. Harvey, Y. Wang, L. Hughes, R. Barton, W. Thurrell, V. R. Sams, D. M. Swallow & M. Sarner (1993) submitted).

In order to test whether the sequence difference(s) are cis-acting to the lactase gene or reside within a trans-acting factor such as a DNA binding protein, we have attempted to identify which homologue of chromosome 2 the mRNA transcripts originate from in individuals who are heterozygous for lactase activity polymorphism. We have exploited an SSCP DNA polymorphism in exon 2 of lactase gene. Primers were designed in exon 1 and exon 3 to amplify the appropriate region from RNA, and the SSCP conditions adapted to detect the same polymorphism as seen in the DNA. Some of the individuals tested express only one of the two alleles at the RNA level. These preliminary results suggest that the sequence differences which lead to the lactase activity polymorphism are cis-acting to the lactase gene.

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## Enhanced growth of mice bearing bovine keratin VI/insulin-like growth factor II transgenes

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Using the bovine keratin VI promoter, expression of insulin-like growth factor II (IGF-II) was directed to the suprabasal layer of skin in four lines of transgenic mice. In each line mice had wrinkled skin, which could be scored from 11 to 14 days after birth, and in the two lines examined measurements of wet weight and total DNA content showed that the growth of skin was increased. The skin in one of these lines exhibited an obvious hyperplasia and hyperkeratosis, which was confined to a slight local thickening of the epidermis in the other. Animals from both lines displayed gross enlargement of certain other organs which included the appendix, colon and uterus. The disproportionate growth phenotype partly correlates with sites of transgene expression, suggesting this results from autocrine or paracrine modes of IGF-2 action. Another striking feature of the phenotype was a greatly reduced fat content at all sites examined so far. This character, at least, might represent an endocrine effect of increased IGF-2 levels.

These transgenic mice demonstrate that extra IGF-2 can enhance growth *in vivo* and provide a model for studying raised IGF-2 levels in adults which have, for instance, been associated with tumour hypoglycaemia.

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## Chimaeras demonstrate defective T/T cell movements during gastrulation

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To investigate *T (Brachyury)* gene function, a chimaeric analysis of midgestation (8.0–11.5 days *post coitum*) embryos has been performed. Embryonic stem (ES) cell lines homozygous or heterozygous for the *T* gene have been introduced into wild-type host embryos by blastocyst injection, and the resulting chimaeras scored for morphological abnormality and extent of colonization by *T/T* cells. Chimaerism has been assessed both by

glucose phosphate isomerase electrophoresis and by direct visualization of ES cell progeny by virtue of a *lac Z* reporter gene marker. 8.0–11.5 dpc *T/T* $\langle - \rangle$  $+/+$  chimaeras exhibit many morphological features of intact *T/T* mutants. In addition, a dramatic bias of *T/T* cells towards caudal regions (such as tail and allantois) was observed in all chimaeras tested and ectodermal derivatives contained a higher proportion of mutant cells than did mesodermal ones. Examination of chimaeras containing ES cells marked with a *lac Z* transgene demonstrates that mutant cells accumulate beneath the primitive streak during gastrulation. Since the anterior part of the streak becomes incorporated into the tailbud, this would explain the preponderance of *T/T* cells in the caudal aspect of the tail. It also indicates that one function of T may be to activate alterations in cell autonomous molecules affecting adhesion or motility which normally occurs as cells emerge from the streak and differentiate into mesoderm.

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## Role of the receptor tyrosine kinase *Sek* in segmental patterning in the vertebrate embryo

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The subdivision of developing tissues into segments is a widespread mechanism of pattern formation during animal embryogenesis. In vertebrates, segmentation occurs in paraxial mesoderm, leading to the formation of somites, and the hindbrain, where the rhombomeres underlie the segmental organization of nerves. A number of transcription factors have been implicated in the segmental patterning of the hindbrain, but little is known of genes involved in cell–cell interactions that must also underlie segmentation. A candidate is the receptor tyrosine kinase, *Sek*, that is expressed in domains that presage the formation of rhombomeres and somites. In the hindbrain, *Sek* RNA and protein is expressed in alternating domains that correspond to prospective rhombomeres r3 and r5. In mesoderm, *Sek* RNA is expressed transiently in each prospective somite prior to its condensation into a definitive epithelium, but *Sek* protein persists for longer and becomes localized in the newly formed somite. These expression data suggest that *Sek* may be involved in the segmentation of the hindbrain and mesoderm. We have tested this hypothesis by expressing dominant negative mutants of *Sek* in order to interfere with the signal transduction function of *Sek* protein. Results indicate a critical requirement for *Sek* function in the segmentation of the hindbrain.

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## Creating somatic cell hybrids which respond to the morphogen retinoic acid

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Our aim is to create a novel cell hybrid for the isolation of tissue-specific expressed sequences from human chromosome 21. Many abnormalities (such as Alzheimer's disease, Down syndrome) have been associated with this chromosome. Monochromosomal hybrid cell lines provide a powerful resource for isolating and characterizing genes from defined chromosomal regions. We created cell hybrids containing human chromosome 21 by performing whole cell fusion between a cell hybrid, RA21, which carried human chromosome 21 on a mouse background and the P19 mouse embryonic carcinoma (EC) cell line. We also performed microcell mediated chromosome transfer (MMCT) to obtain a cell line which has only human chromosome 21 on a P19 EC background.

P19 EC cells have been widely used as a model system for studying early murine development. These cells are normally undifferentiated but on treatment with retinoic acid (RA) they go through a process which appears to mimic neurogenesis *in vivo*. Since the retinoic acid receptor (RAR) is believed to play an essential role in mediating RA action and the differentiation of our hybrid cells depends on the number of alpha receptors expressed, it is essential to examine the expression of RAR alpha mRNA in our hybrid cells. The hybrid cell line can give us access to a stage-specific neural set of expressed sequences (or expressed sequences due to the effect of RA treatment) from human chromosome 21.



## Targeted mutagenesis of the Oct-4 gene

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The pluripotent nature of early embryo cells is likely to be governed by specific gene transcription. A candidate transcription factor is the POU-domain protein Oct-4 (also known as Oct-3). Expression of Oct-4 is confined to pluripotent embryo cells *in vivo* and to undifferentiated embryonic stem (ES) cells in culture where it constitutes the major octamer-binding protein. In order to assess the function of Oct-4, we have used homologous recombination in ES cells to disrupt the Oct-4 gene.

Isogenic DNA was employed to construct a promoterless targeting vector in which the DNA-binding domain was replaced by a *lacZ-neo* ( $\beta$ geo) fusion gene containing its own polyadenylation site. In the targeting event, the  $\beta$ geo fusion gene is spliced to exon one of the Oct-4 gene via a splice acceptor sequence situated 5' of the  $\beta$ geo gene. Functional  $\beta$ geo fusion protein is produced independent of the Oct-4 reading frame by employing the EMC virus internal ribosomal entry site (IRES). The integrated lacZ reporter enables visualization at the cellular level of Oct-4 expression sites, whilst the deletion of the entire POU-domain will allow definitive determination of the requirements for Oct-4 in embryogenesis.

Electroporation of the targeting vector into ES cells resulted in a high frequency (> 80%) of targeted clones, as determined by Southern hybridization using 5' and 3' flanking probes. RNA analysis reveals the presence of the predicted Oct-4-IRES- $\beta$ geo fusion transcript in Oct-4<sup>+/-</sup> ES cells. Expression of lacZ is confined to undifferentiated cells *in vitro* and to pluripotent early embryo cells *in vivo*, reflecting the normal pattern of Oct-4 expression. Chimaeras have been generated which give rise to ES cell derived progeny.

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