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Parental-origin specific histone H4 acetylation in the imprinted *Igf2-H19* domain

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Parental-origin specific regulation of imprinted genes is controlled by modifications to the DNA and chromatin. In the mouse *Igf2-H19* domain on chromosome 7, many of the key regulatory sites have been identified and parental-specific regions of DNA methylation have been mapped. Indirect evidence has suggested that chromatin modification may play a role in the regulation of imprinting. In this study we have directly assessed whether histone acetylation may be implicated in gene regulation at this domain. Analysis of H4 acetylation on the two parental chromosomes was compared using a panel of antisera generated against the four different acetylated isoforms of histone H4. Chromatin was compared from fibroblasts made from normal embryos and embryos containing maternal duplication/paternal deficiency of the imprinted region on distal chromosome 7. Chromatin was immunoprecipitated and DNA isolated from bound and unbound fractions, then hybridized with probes from key areas in the imprinted domain. The CpG-island H19 promoter, which shows parent-specific methylation and nuclease accessibility, exhibited hyperacetylation on the unmethylated active maternal allele with all four antibodies. The methylated inactive paternal promoter was deacetylated. Interestingly, the *Igf2* promoter, which is also a CpG island but is not methylated on either the maternal or paternal chromosome, also showed hyperacetylation of the active allele. In contrast to the *H19* promoter, the inactive maternal allele did not show hypoacetylation; however, the acetylation level was considerably lower than that on the active allele. These results show a correlation between acetylation and allelic activity and are consistent with a recently described role for methylation in the recruitment of deacetylase activity.

Histone deacetylase complexes in mammals and *Drosophila*

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The steady-state level of histone acetylation in eukaryotes is established and maintained by multiple histone acetyltransferases and deacetylases (HDACs) and affects both the structure and function of chromatin. HDACs form a highly conserved protein family in many eukaryotic species and they can mediate gene inactivation as components of large complexes. HDACs have been shown to interact with sequence-specific DNA-binding transcriptional repressors and the sequence non-specific repressor, methyl-CpG-binding protein 2 (MeCP2). We have investigated the cytological distribution, biochemical properties and composition of these multisubunit complexes, and of the HDAC components, in both mammals and *Drosophila*. In mammals, two separate nuclear complexes can be identified, with either HDAC1/HDAC2 or the smaller HDAC3 as the histone deacetylase components. The minimum size of these complexes, as determined by glycerol gradient sedimentation, is approximately 140 kDa. We have

identified the novel orthologue of mammalian HDAC3 in *Drosophila*. The dHDAC3 protein is predicted to be 84 amino acids shorter than the other known *Drosophila* histone deacetylase, dHDAC1. This additional domain in dHDAC1 is quite repetitive and is rich in charged amino acids, which may comprise a nuclear localization signal. The localization, function and post-translational modification of dHDAC1 are therefore likely to differ from the smaller dHDAC3. We also present evidence that all HDACs are zinc-substituted metalloproteins, on the basis of homology with known metal-substituted hydrolyases, dose-dependent activation of activity by zinc and other biochemical analyses.

H4 acetylation, XIST RNA and replication timing are coincident and define X; autosome boundaries in two human derived X chromosomes

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Dosage compensation in mammals requires the stable genetic silencing, during early embryogenesis, of one X chromosome in each female cell. This process has an absolute requirement for the presence, in cis, of a region defined genetically as the X inactivation centre (*Xic*). Establishment of the inactive X requires rapid dissemination of the silencing signal across an entire chromosome, a process that is often described as spreading and seen as analogous to position effect variegation (PEV) in *Drosophila*. The inactive X (X_i) in mammals differs from its active homologue (X_a) in a number of ways, including increased methylation of CpG islands, replication late in S-phase, underacetylation of histone H4 and association with XIST RNA. The XIST gene lies within the *Xic* and produces a large, non-coding transcript that is, uniquely, expressed exclusively from X_i. Global changes in DNA methylation occur relatively late in development but the other properties all change during or shortly after the establishment of X_i and may play a role in the spreading mechanism. We describe the use of a cytogenetic approach to define the distribution of XIST RNA, deacetylated H4 and late replicating DNA across two human X: autosome translocations. The results show complete coincidence of these three parameters, with all three being excluded from the autosomal component of the derived X chromosome.

The role of protein kinase C (PKC) in mouse neural tube defects

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The curly tail (*ct*) mouse is a model for folate-resistant neural tube defects (NTD). We have previously shown that NTD in *ct* mice can be prevented by exogenous *myo*-inositol which operates by a PKC-dependent pathway. We have now analysed the expression of PKC isoforms during mouse embryo development and investigated the substrates phosphorylated by PKC during TPA and *myo*-inositol treatment of *ct* embryos. The developmental expression of PKC in CD1 embryos was analysed by Western blotting. The individual isoforms of PKC showed distinct patterns of expression, which varied according to distribution between subcellular fractions, onset time, duration, and number of waves of expression. PKC β, δ, ϵ, η, θ, ζ and ι/λ were expressed strongly in E10·5 embryos whilst PKC α and γ were undetectable. Use of PKC inhibitors in whole embryo cultures suggested that PKC β, θ and η are most likely to mediate the preventive effect of *myo*-inositol in *ct* embryos. PKC substrates can be detected by two-dimensional PAGE analysis of ^{32}P-loaded *ct* embryos. The identity of the proteins detected by this method will be discussed and their potential role in neural tube closure outlined.

Characterization of an evolutionarily conserved family exhibiting homology to the methyl-CpG binding domain (MBD), the leukaemia-associated-protein (LAP) zinc finger and the bromodomain

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Vertebrate genomic methylation has been correlated with chromatin alterations and transcriptional inactivity. Normal murine development clearly requires DNA methylation as disruption of the DNA methyltransferase gene (*Dnmt1*) is an embryonic lethal and this phenotype is mirrored following knockout of the gene encoding the methyl-CpG binding protein MeCP2. Given these findings it is curious that the model organisms *Caenorhabditis elegans* and *Drosophila* lack detectable methylation. We have identified a *C. elegans* cDNA, CeZK, that shares homology with the methyl-CpG binding domain of MeCP2 and encodes a protein that contains a bipartite C-terminal

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LAP-type zinc finger (C4HC3) and bromodomain. We have isolated a number of mammalian cDNAs, mZK1 and hZK1, that encode proteins with the same modular organization as CeZK. We have constructed various GFP-fusions of mZK1 with and without the N-terminal MBD-like region to ascertain whether localization is perturbed *in vivo* following deletion of this domain. Transient transfections with GAL4-fusions of mZK1 are being used to determine its effects on transcription. We have mapped hZK1 by FISH to 2q23 – a region found to be deleted in rare forms of leukaemia. A homozygous mutant *C. elegans* line has been isolated and localization studies with microinjected GFP-fusions are being used to assess the functional consequences following loss of this gene.

Accelerated evolution of a gene dependent on chromosomal position

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The X and Y chromosomes of eutherian mammals, although heteromorphic in both size and gene content, are thought to have evolved from a pair of homologous chromosomes. A small region of identity between the X and Y chromosomes, the pseudoautosomal region (PAR), is thought to be a remnant of these ancestral chromosomes. During male meiosis the X and Y chromosomes pair and recombine along this region ensuring correct chromosomal segregation. Genes within the PAR escape X inactivation in females so that the dosage of genes relative to that of XY males is maintained. The human PAR has been well characterized and contains a number of genes, whereas little is known about the equivalent region in the mouse. We undertook a genomic walk from the *Amg* gene on the mouse X chromosome to the pseudoautosomal boundary. We discovered that a gene, *Fxy*, spans the pseudoautosomal boundary in mice such that the 5′ portion of the gene resides only on the X chromosome while the 3′ portion is within the PAR and is therefore present on both the X and Y chromosomes. Analysis of the pseudoautosomal portion of *Fxy* in humans and mice reveals that it is evolving at an accelerated rate perhaps as a result of the high recombination rate observed in the pseudoautosomal region.

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Comparative mapping of human and important vertebrate genomes by fluorescence *in situ* hybridization

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Zoo-FISH has become an important tool for establishing homology links between humans and other species. Using DNA libraries from flow-sorted mouse chromosomes and region-specific clones we have compared the entire mouse genome with rat chromosomes. Seven mouse chromosomes – MMU 2, 4, 9, 12, 16, 18 and X – are conserved as individual chromosomes in the rat. MMU 3, 6, 7 and 19 are conserved as contiguous segments on larger rat chromosomes. MMU 1, 5, 8, 10, 11, 13, 14, 15 and 17 are diverged on different rat chromosomes. Zoo-FISH results were integrated with genetic mapping data of mouse and man. Taking these results together, we have identified at least six large linkage groups in the mouse that are diverged in both rat and humans. There are at least five linkage groups that are conserved between rat and humans, but not in the mouse. In addition, we have established molecular cytogenetics in the pufferfish, *Tetraodon fluvilatilis*. In collaboration with Genoscope (Evry, France) we will map several hundred pufferfish clones that have orthologous loci in the human genome on pufferfish chromosomes. In a pilot study, we are focusing on human X-linked genes.

A large-scale screen for insertional mutations in transgenic mice

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We have screened approximately 40 transgenic lines for insertional mutants by breeding to homozygosity and are currently investigating four phenotypes. All are recessive traits that show statistically significant linkage to the transgene. We have used FISH to tentatively map the transgene in these four lines, and are currently using microsatellite PCR to show concordance or discordance with putative candidate genes selected on the basis of phenotype and map position. This dual approach has benefits for laboratories that are not set up for large-scale genetic mapping studies. One line shows a recessive piebald lethal phenotype, and the transgene insertion maps very close to the *Sox10* locus on chromosome 15. This is interesting, since the only known mutation at this

locus in mice is dominant, with homozygosity being embryonic lethal by E13. The transgene does not lie within 5 kb of the gene and the mutant mice express wild-type levels of *Sox10* transcript in the central nervous system. Another line develops corneal opacity soon after the eyes open around P15, with a progressive anterior lens cataract developing shortly thereafter. Combined corneal/lens phenotypes are rare, and the transgene insertion may map close to the *Tcm* locus on chromosome 4.

Mapping recombination hotspots in human phosphoglucomutase (PGM1) using a population-based approach

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Human PGM1 is a highly polymorphic protein. Three mutations and four intragenic recombination events between the three mutation sites generate eight protein variants including four common alleles that occur world-wide (1+, 1−, 2+ and 2−) and four that are polymorphic in Oriental populations (3+, 3−, 7+ and 7−). The mutations 3/7, 2/1 and +/− are in exons 1A, 4 and 8, and are 40 kb and 18 kb apart respectively. Eight polymorphic intron markers were used to investigate the region between exons 4 and 8 (designated site A) in three populations. Allelic association and haplotype-based analyses indicated there is a recombination hotspot in site A in a 750 bp interval including exon 7. Nucleotide sequence analysis provided additional evidence for genomic instability in this region and also the presence of motifs that have been associated with recombination hotspots in other genes. Using two markers in the 5′ end of IVS 1A, allelic association analysis indicated a second region of active recombination between exons 1A and 4 (site B). Segregation analysis in CEPH families identified four instances of intragenic recombination and the recombination frequency in males was estimated to be 2·38–3·17% across the locus.

A cDNA selection approach to isolate Y chromosome genes expressed in testis

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Over the past 50 years the semen quality has declined with an overall reduction in male fertility; 'normal' sperm count was reduced from 60×10^6/ml in the 1940s to the present value of 20×10^6/ml. Male infertility due to defective sperm production is a serious problem affecting 2% of males worldwide. Roughly 13% of these carry *de novo* deletions of the Y chromosome. It is expected that genes expressed in the testis and involved in spermatogenesis will occur on the mammalian Y chromosome. This view has been confirmed with the isolation of the RBM and DAZ gene families. In an effort to identify further Y-linked testis-specific genes, a cDNA selection library was constructed using adult testis cDNA and DNA from 1000 Y-linked cosmid clones. This library was constructed in collaboration between our group, and M. Lovett and R. Del Mastro at the University of Texas. Screening with vector sequences and Y chromosome repeat sequences has led to the elimination of 78·7% of the clones. Six hundred potential cDNA clones remain, and so far 60 have been sequenced. cDNA clones with homology to STSs on the Y chromosome have been identified. Thus far, four of these have been localized on the Y chromosome and preliminary results from RT-PCRs on tissues that have not been sex-determined showed that they are all expressed in testis.

The identification of novel imprinted loci using methylation-sensitive representational difference analysis

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Genomic imprinting is the phenomenon by which a subset of mammalian genes is expressed from only one parental allele. To determine the full extent of the influence of imprinted genes in mammalian development and disease, and to determine the mechanisms by which imprinted genes are regulated, it is important to identify as many imprinted genes as possible. A characteristic of imprinted genes analysed to date is the presence of allele-specific methylation, a feature that may be used in their identification. A new screen has been developed, methylation-sensitive representational difference analysis (Me-RDA), which allows the identification of sites of imprinted methylation. This screen utilizes restriction digest by a methylation-sensitive enzyme, combined with PCR and subtractive hybridization, to isolate fragments from the unmethylated allele. RDA has been applied here to parthenogenetic and fertilized mouse embryos, to isolate restriction fragments with maternal-specific methylation. This screen has, to date, isolated two known

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imprinted genes and has enriched for other known imprinted genes. The imprinting status of a number of candidate genes is currently being determined.

A strategy to identify genes differentially expressed in the growth plate

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Longitudinal bone growth occurs at the epiphyseal growth plates, which are regions of specialized cartilage located at the ends of each bone. Growth plate chondrocytes progress through a proliferative phase and then gradually acquire a terminally differentiated hypertrophic phenotype, which produces a mineralized matrix. Understanding the molecular mechanisms that regulate the key steps in this process is an important scientific goal as its disruption results in growth retardation and skeletal abnormalities. Our laboratory has recently developed a unique strategy for the identification of genes involved in bone growth and development. This strategy is based on the use of agarose gel differential display to compare RNA isolated from chick growth plate chondrocytes separated into maturationally distinct populations by Percoll density gradient centrifugation. Using this approach we have identified a number of genes expressed at different stages of chondrocyte maturation. These genes include beta 7-tubulin, a novel ubiquitin conjugating enzyme, a novel transmembrane protein that contains dileucine motifs at its C-terminus, and a novel member of a recently identified superfamily of phosphotransferases, which may be involved in mineralization.

***Xist* RNA exhibits a banded localization on the inactive X chromosome and is excluded from autosomal material in cis**

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The propagation of X chromosome inactivation is thought to be mediated by the cis-limited spreading of the non-protein-coding *Xist* transcript. In this study we have investigated the localization of *Xist* RNA on rodent metaphase chromosomes. We show that *Xist* RNA exhibits a banded pattern on the inactive X and is excluded from regions of constitutive heterochromatin. The banding pattern suggests a preferential association with gene-rich, G-light regions. Analysis of X: autosome rearrangements in mice revealed that the restricted propagation of X inactivation into cis-linked autosomal material is reflected by a corresponding limited spread of *Xist* RNA. We discuss these results in the context of a model for the function of *Xist* RNA in the propagation of X inactivation.

Parental genome separation and methylation dynamics during mammalian preimplantation development

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The possible roles of separated genomes and dynamic methylation processes in the parental chromatin remodelling of imprinted genes were examined in preimplantation mouse embryos. Male mice were supplied with bromodeoxyuridine (BrdU) in their drinking water in order to label sperm DNA. After mating with females, male chromatin could be detected using an anti-BrdU antibody. In the 1-cell embryos after nuclear envelope breakdown, the paternal genome was completely separated from the maternal genome. Our experiments strongly suggest that parental chromosomes are in separate compartments of interphase nuclei at least up to the 4-cell stage. In a second experiment we analysed the methylation dynamics at the differentially methylated region 2 (DMR2) of the *Igf2* gene during preimplantation development. In the zygote the paternal DMR2 allele becomes demethylated, whereas the maternal allele is methylated *de novo*. Both processes are independent of DNA replication. We propose a model where active and passive processes of demethylation as well as *de novo* methylation and maintenance methylation of imprinted genes are important parts of remodelling of parental chromatin. Our observations suggest an association between separation of parental chromosomes and differential activities of methylation and demethylation during early embryonic development of the mouse.

Maternal DNaseI hypersensitivity and unusual chromatin organization in an insulator/imprinting control element upstream of the imprinted mouse *H19* gene

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The mouse *H19* gene, expressed exclusively from the maternal chromosome, is constitutively methylated on the paternal allele in a 2 kb region, 2–4 kb upstream of *H19*. This element has been shown to be necessary for the imprinted expression of both *H19* and the 5′-neighbouring *Igf2* gene. In particular, on the maternal chromosome it appears to insulate *Igf2* from enhancers located downstream of *H19*. We analysed the chromatin organization of this element by assaying its sensitivity to nucleases in nuclei. Six DNaseI hypersensitive sites (HS sites) were detected exclusively on the unmethylated maternal chromosome, five of which were present in all expressing and non-expressing cell lines and tissues analysed. They were already established in embryonic stem cells and reflect the maternal origin of the chromosome, rather than expression of *H19*. Specifically on the maternal chromosome, micrococcal nuclease assays showed an unusual and strong banding pattern suggestive of a non-nucleosomal organization within an interrupted array, which might be involved in the insulator function of this element. From our studies it appears that unusual chromatin organization with presence of HS sites (on the maternal chromosome) and DNA methylation (on the paternal chromosome) in this element are mutually exclusive and reflect alternate epigenetic states.

***Nesp*, an imprinted gene on distal mouse chromosome 2**

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Distal mouse chromosome (Chr) 2 is subject to genomic imprinting. To date, two paternally expressed genes have been identified on distal Chr 2: *Gnas*, which encodes a G-protein a subunit and is imprinted tissue specifically, and *Nnat*. Using methylation-sensitive representational difference analysis we have detected a new imprinted gene on distal Chr 2 within 150 kb of *Gnas*. The gene has been called *Nesp* because, on sequencing, it is homologous with bovine NESP55 that determines a secreted protein of neuroendocrine tissues whose function is unknown. We have shown that there are multiple sense and antisense *Nesp* transcripts in newborn brain and 12·5 dpc placenta. Furthermore, there is an exclusively maternally expressed sense isoform and also an exclusively paternally expressed isoform of *Nesp*. Both monoallelically expressed *Nesp* isoforms contain an open reading frame homologous to the bovine; however, a 96 bp putative intron present in the paternally expressed isoform is spliced out of the maternally expressed isoform. *Nesp* and *Gnas* are part of the same transcription unit for the maternally expressed isoform is spliced onto exon 2 of *Gnas*. *Nesp* is a candidate for the imprinting phenotypic abnormalities associated with distal Chr 2.

Increased IGFII levels affects *p57kip2* activity, *in vivo* and *in vitro*

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In human and mouse, the *Igf2* gene, localized on chromosome 11 and 7, respectively, is almost exclusively expressed from the paternal chromosome. This insulin-like growth factor plays an important role in embryonic growth. Increased *Igf2* expression has been implicated in several human pathologies, such as the Simpson–Golabi–Behmel (SGBS) and Beckwith–Wiedemann (BWS) overgrowth syndromes. However, IGFII molecular targets have not been identified. Genetic data strongly suggest that the *p57kip2* gene, also located on the human chromosome 11 and also implicated in BWS, may be a likely candidate. P57KIP2 is a tissue-specific inhibitor of cyclin-dependent kinase and is required for normal mouse embryonic development. Here we assess whether a relationship exists between *Igf2* and *p57kip2*. Using both *in vivo* and *in vitro* models, we find that *p57kip2* expression is affected by high levels of IGFII. Our findings have implications for the aetiology of syndromes involving these growth regulators and for understanding biological functions of imprinted genes on chromosome 11.

Epigenetic regulation of the imprinted *U2af1-rs1* gene in the mouse

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Genomic imprinting is an essential mechanism for normal mammalian development whereby certain genes become parental allele-specifically expressed. We have analysed the mouse *U2af1-rs1* gene that encodes a splicing factor and is paternally expressed. We established that a 10 kb domain comprising the entire 3 kb gene is fully methylated on the maternal chromosome throughout development. Analysis of nuclease sensitivity in nuclei established that this 10 kb domain also displays differential 'generalized sensitivity' to DNaseI and *Msp*I, with greater sensitivity on the paternal than on the maternal allele. In addition, two constitutive, paternal DNaseI hypersensitivity sites were detected in the 5′ untranslated region. The biochemical basis of the generalized sensitivity differences is unknown. Perhaps the relative resistance of the maternal allele to *Msp*I digestion and the differential generalized sensitivity to DNaseI reflect interaction of proteins with methylated DNA or, alternatively, are associated with specific histone modifications. We also performed *in vitro* studies on embryonic stem cells that showed that loss of maternal methylation in *U2af1-rs1* is associated with increased maternal sensitivity to nucleases and results in biallelic expression. Our current work aims at determining the interrelationship between DNA methylation and conformational features of chromatin in *U2af1-rs1*, and their roles in gene expression.

Developmental consequences of disruption of genomic imprinting on mouse chromosome 12

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Genomic imprinting is a process that makes the maternally inherited genome functionally different from the paternally inherited genome, despite the fact that both genomes carry the same genetic information. This manifests itself at the gene level as parental-origin-specific differences in expression between the two alleles of imprinted genes. Recent studies showed that mouse distal chromosome 12 (Chr 12) is imprinted and its correct imprinting is necessary for viability, because mice have both copies of distal Chr 12 inherited from their father (paternal uniparental disomy, or pUPD), or from their mother (maternal UPD, or mUPD), were not detected during early postnatal life. However, the timing of UPD12 lethality as well as the nature of UPD12 phenotypes are not known. To address these issues, we used a method that generates UPD12 embryos for the entire Chr 12, and a molecular assay to identify these embryos. Results indicate that both mUPD12 and pUPD12 can survive to late gestation. Furthermore, mUPD12 embryos display growth retardation that is evident from at least embryonic day 16 (E16). pUPD12 embryos display growth defects and have skeletal anomalies and approximately half of them die between E16 and E19. We will discuss these findings in relation to the phenotype of human UPD for the long arm of Chr 14, which shows syntenic homology to distal mouse Chr 12.

Characterization of a gene trap integration displaying liver-specific reporter expression

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The I114 gene trap cell line was isolated from an *in vitro* retinoic acid responsive pre-screen that enriches for vector integrations into genes with restricted expression patterns during embryogenesis *in vivo* [1]. The reporter activity profile associated with the I114 integration displays restricted spatio-temporal expression marking the ontogeny of the fetal liver. Reporter activity is observed as early as the 9-somite stage (8·0–8·5 dpc) in endodermal cells of the foregut in the region destined to form the liver diverticulum and is restricted to the hepatic lineage until late gestation. Breeding of the gene trap integration to homozygosity reveals no overt phenotype but its unique pattern of expression prompted us to clone the endogenous sequence. This has proven to be more complex than predicted as insertion of the gene trap vectors produces more than one fusion transcript. The most abundant fusion transcript is a novel ankyrin repeat containing gene which is expressed ubiquitously but does not produce reporter activity. However, the expression pattern of a less abundant fusion transcript correlates exactly with expression of the reporter gene. These two fusion transcripts are linked at the genomic level and work in progress indicates that they could represent tissue-specific isoforms of the same gene.

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Pathways controlling left–right specification and heart development in the mouse

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We are interested in the left–right specification in mammalian embryos. Three TGFβ-related genes, *lefty1*, *lefty 2* and *nodal*, and a transcription factor, *Pitx2*, are expressed asymmetrically in mouse embryos. We have found the hearts of Brachyury *T/T* mice, which fail to form a normal node and notochord, exhibit consistent morphological abnormalities, resulting in ventrally displaced ventricular loops, and a 50% incidence of inverted heart situs. We find that *nodal*, normally expressed around the node and left lateral plate in early somite embryos, is absent in *T/T* embryos. In contrast, *lefty1* and *lefty2*, normally expressed in the left half of prospective floorplate and left lateral plate respectively, are expressed only in a broad patch of ventral cells in, and just rostral to, the node region in *T/T* embryos. These results implicate the node as a signalling source driving left-sided *nodal* and *lefty2*, and subsequently looping and situs of the heart. The expression of the transcription factor *Pitx2* proposed to link *nodal/lefty2* to asymmetrical morphogenesis initially mimics *nodal/lefty2* subsequently remaining in the left of the caudal heart and left lung bud. We expect absence of *Pitx2* expression in *T/T* mice. In *iv/iv* mice (heart looping also randomized) we find predominantly bilateral *Pitx2* expression in the heart. The implications for heart morphogenesis and regulation by *lefty* and *nodal* are discussed.

The role of WNTs in axonal remodelling

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*Wnt-7*a is expressed in cerebellar granule cells as they begin to extend axons and make contacts with their synaptic partners, the mossy fibres. WNT-7a induces axonal spreading and increases the level of synaptic proteins in developing neurons, suggesting a role for WNT-7a in axonal remodelling and synaptogenesis. Lithium has been shown to mimic the effects of WNT-7a in these neurons by the inhibition of glycogen synthase kinase-3β (GSK-3β), a component of the WNT signalling pathway. It is proposed the WNT-7a and lithium cause cytoskeletal reorganization through the inhibition of GSK-3β. We have successfully obtained a soluble, active form of WNT-7a and used it to investigate the effect of GSK-3β inhibition on the neuronal cytoskeleton. Soluble WNT-7a induces shortening of axons, enlargement of growth cones and spreading along the axon shaft. This effect is associated with a loss of stable microtubules from spread areas and down-regulation of the phosphorylated form of MAP-1B, a microtubule-associated protein known to be phosphorylated by GSK-3β. We show here that soluble WNT-7a and lithium affect microtubule stability in both cerebellar granule cells and mossy fibres, causing dramatic changes in cell morphology. Time-lapse recordings of neurons exposed to WNT-7a indicate that these changes occur rapidly. These *in vitro* studies, together with the analysis of the *Wnt-7*a null mutant, support a role for WNTs in axonal remodelling *in vivo*.

Amino-terminal deletion in a desmosomal cadherin causes the autosomal dominant skin disease striate palmoplantar keratoderma

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The N-terminal extracellular domain of the cadherins, calcium-dependent cell adhesion molecules, has been shown by X-ray crystallography to be involved in two types of interaction: lateral strand dimers and adhesive dimers. We have found the first human mutation in a cadherin present in desmosome cell junctions, which removes a portion of this highly conserved first extracellular domain. The mutation, in the DSG1 gene coding for a desmoglein (*Dsg1*), results in the deletion of the first and much of the second beta-strand of the first cadherin repeat and part of the first Ca^{2+}-binding site, and would be expected to compromise strand dimer formation. It causes a dominantly inherited skin disease, strait palmoplantar keratoderma (SPPK), mapping to chromosome 18q12.1, in which affected individuals have marked hyperkeratotic bands on the palms and soles. In a three-generation Dutch family with SPPK we have found a G-to-A transition in the 3′ splice acceptor site of intron 2 of

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the DSG1 gene which segregated with the disease phenotype. This causes aberrant splicing of exon 2 to exon 4, which are in-frame, with the consequent removal of exon 3 encoding part of the prosequence, the mature protein cleavage site, and part of the first extracellular domain. This mutation emphasizes the importance of this part of the molecule to cadherin function, and of the *Dsg1* protein and hence desmosomes in epidermal function.

Gv1, a gene controlling endogenous proviral expression, maps to mouse chromosome 13

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The genomes of inbred strains of mice harbour multiple integrated endogenous retroviruses and many of these sequences are homologous to murine leukaemia virus (MLV). *Gv1* is a single locus that coordinately regulates the expression of proviral sequences, although its mode of action remains unclear. A positional cloning strategy has therefore begun with attempts to map *Gv1* in a genetic cross. A reliable quantitative nuclease protection assay has been established to type backcross mice of known *Gv1* genotype. Use of this assay demonstrates that the segregation of a single gene is responsible for the endogenous MLV expression phenotypes observed and that not all classes of provirus are responsive to *Gv1*. Results from REVEAL PCR have identified two markers that show linkage to the *Gv1* locus. Using somatic cell and radiation hybrid mapping panels, both have been mapped to chromosome 13. Microsatellite markers have been analysed to refine the map around *Gv1*, which lies at approximately 37 cM from the centromeric end, although a more detailed position will be required for the isolation of positional candidates.

Identification of candidate genes for X-linked cleft palate and ankylglossia in an Icelandic kindred

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Facial clefting disorders are among the most common dysmorphologies seen in man. Isolated cleft palate (CP) occurs with a frequency of 1 in 1500 and is inherited most commonly as the result of interaction between genetic and environmental factors. An Icelandic kindred displaying an X-linked mode of inheritance of non-syndromic cleft palate and/or ankyloglossia (CPX) has been used in a positional cloning approach to identify the gene responsible. The CPX critical region is estimated to be 2·0 Mb, and is spanned by a contig of yeast artificial chromosomes (YACs) between Xq21·31 and Xq21·33. To facilitate gene isolation strategies, a contig of P1 artificial chromosomes (PACs) has also been completed across the CPX interval. Using this resource, the interval is currently being DNA sequenced in collaboration with the Sanger Centre. An ACEdb database has been set up to display the results of genomic sequence analysis. In an effort to further define coding sequences, exon-trapping has been performed on the majority of CPX PAC clones. Identification of the defect responsible for CPX will allow a better understanding of the mechanisms involved during craniofacial development.

Genetic analysis of placental dysplasia in mouse interspecific hybrids

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Placental dysplasia, caused mainly by overgrowth of the spongiotrophoblast, was observed in interspecific hybrids between *Mus musculus* (mus) and *Mus spretus* (spr). Genetic mapping analysis resulted in a significant linkage for the whole proximal and central part of the X chromosome. Congenic mouse strains were generated to reveal critical intervals within this large chromosomal region. One of these strains retained a spr-derived part on the X chromosome between 11·5 and 31·7 cM. This line clearly developed enlarged placentas, although placental hyperplasia was less pronounced compared with F1 females. Further dissection of this portion resulted in the loss of weight increase in each of the subcongenic strains. However, it could be shown that at least in two of these subcongenic lines the spongiotrophoblast layer was still enlarged. Interestingly, the spr-derived regions of these subcongenics did not overlap. Further investigations will have to show which genetic mechanism causes placental dysplasia in mouse interspecific crosses.

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Progress towards the positional cloning of the murine deafness gene bronx waltzer (*bv*)

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Bronx waltzer (*bv*) is an autosomal recessive mouse mutation with a unique pathology of the inner ear sensory epithelia [1]. In the cochlea, the inner hair cells of the organ of Corti are specifically affected and degenerate between gestational day 17·5 and birth, which accounts for the hearing impairment in bronx waltzer. In the vestibular system, the hair cells of the maculae and cristae are affected, resulting in bronx waltzer hyperactivity, head-bobbing and circling behaviour. Previous analysis of a large intraspecific backcross generated a high-resolution genetic map around the bronx waltzer locus on mouse chromosome 5 [2]. In the current backcross, a total of 1073 animals have been analysed and we have positioned *bv* in a 1·86 cM region, lying between the markers *D5Mit25* and *D5Mit209*. We have also constructed a physical map of bronx waltzer using yeast artificial chromosomes (YACs) that forms a contiguous array across the region. We are currently using smaller bacterial artificial chromosomes (BACs) to further map the *bv* mutation to a single BAC clone. Two publically available interspecific backcross mapping panels are also being used to locate the positions of our markers from the intraspecific backcross. This is allowing us to define the order and position of other markers and genes in the *bv* region, which will help with the physical mapping.

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The role of the POU domain transcription factor Oct4 in development of the mouse blastocyst

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Oct4 is expressed exclusively in the pluripotent stem cells of the mouse embryo. Embryos lacking *Oct4* fail to develop after implantation, resulting from failure to establish a true inner cell mass (ICM). Cell numbers in mutant blastocysts are normal and appropriately partitioned into putative ICM and trophectoderm. However, mature ICM or epiblast derivatives do not develop, either *in vivo* or *in vitro*. In culture the inside cells consistently differentiate into trophoblast giant cells. The prior commitment of these mutant inside cells to a trophectodermal lineage is evidenced by their expression of the epithelial marker Troma-1. *Fgf4* is a candidate target gene of *Oct4* and its expression is greatly reduced in *Oct4* mutant embryos. Embryos lacking *Fgf4* also fail to develop beyond implantation. Addition of *Fgf4* to *Oct4* mutant embryos does not rescue the ICM. Instead *Fgf4* causes proliferation of cells expressing markers for extra-embryonic ectoderm. Extra-embryonic ectoderms from normal embryos also respond to *Fgf4*. This suggests that *Fgf4* is the signal from the ICM that sustains proliferation of the polar trophectoderm. *Oct4* therefore controls pluripotent identity and paracrine growth signalling in the blastocyst.

Investigation of the role of arylamine *N*-acetyltransferase, an enzyme of folate metabolism, in development

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Arylamine *N*-acetyltransferases (NATs) have been extensively studied as enzymes metabolizing arylamines and hydrazines. More recently, a folate catabolite, *p*-aminobenzoylglutamate (pABGlu) has been proposed as a potential endogenous NAT1 substrate in humans. We have previously demonstrated a good correlation between NAT1 activity and pABGlu *N*-acetylation activity in human placentae of different gestational ages. Here, we report evidence that mouse NAT2 (the equivalent of human NAT1) can also metabolize pABGlu in a variety of tissues. pABGlu *N*-acetylation activity was much higher in the 'fast' than in the 'slow' *N*-acetylator strains and correlated well with the *N*-acetylation activity of *p*-aminobenzoic acid (pABA), which is a known mouse NAT2 (and human NAT1) substrate. The implication of NAT in folate metabolism may further suggest a possible role in the folate-dependent protection against neural tube defects. If this is the case, it should be

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expressed very early in development, before the onset of neurulation. We were recently able to detect human NAT1 expression as early as the blastocyst stage, by screening a cDNA library constructed from human preimplantation embryos. Mouse NAT2 expression has also been demonstrated in mouse embryonic stem cells, by RT-PCR. Interest has now focused on the production of NAT2 knock-out mice. This is assisted by a mapping project in the vicinity of the mouse NAT loci, which has provided a detailed restriction map of the area, as well as significant information about the relative position of the three NAT genes. Phenotypic analysis of the knock-outs will provide very useful information about the role of NAT2 in mouse development.

Generation and propagation of neural precursors derived from embryonic stem cells

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Mouse embryonic stem (ES) cells are pluripotent cells derived from preimplantation embryos. In culture these cells can differentiate into a broad variety of cell types, including neural cells. This capacity for multi-lineage differentiation is retained during genetic manipulation and *in vitro* propagation. Therefore, ES cells provide an attractive system for the molecular and genetic dissection of developmental pathways *in vitro*. They are also a potential source of cells for transplantation studies. These prospects have been frustrated, however, by the disorganized and heterogeneous nature of development in culture. We have therefore developed a genetic selection strategy which enables the isolation of purified neural precursor from differentiated ES cell progeny. This approach is based on targeted integration of a selection marker βgeo into the *Sox2* gene, expression of which is restricted to the proliferating neuroepithelial cells during development. *Sox*-selected cells differentiate efficiently into neuronal networks in the absence of other cell types. A fraction of *Sox*-selected cells can be expanded as a morphologically homogeneous population capable of giving rise to both neurons and astrocytes.

Transcription of homeobox genes in human oocytes and preimplantation embryos

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In most animal species the regulation of early embryonic development is under the control of maternal mRNA and protein until the embryonic genome is activated. Despite the fundamental importance of this transition in the developmental programme, very little is known about the molecular basis of embryonic gene activation in mammals (especially humans) and the repertoire of genes involved. Therefore, identifying maternal and embryonic genes whose structures would imply a role in transcriptional regulation, such as *Oct3* and *Oct4*, which are maternally expressed homeobox genes essential for mouse embryogenesis [1, 2] might lead to a greater understanding of early human development. We describe a highly sensitive method for mRNA purification and solid-phase cDNA synthesis on oligo-(dT) magnetic beads followed by PCR amplification in a single tube to generate cDNAs and corresponding libraries with complexities ranging from 10^5–10^6 clones from four unfertilized oocytes, single 2-cell, 4-cell, 8-cell and blastocyst stage embryos, and 10-week-old whole fetus. Using these libraries, we also present data showing that PCR using primers that amplify the homeodomain of homeobox genes [3] can be used rapidly and efficiently to survey for the presence of homeobox sequences. Preliminary analyses have revealed the presence of HOX, POU, HEX and novel homeobox genes.

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The identification and characterization of genes active in early human development

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The aim of our research is to identify and characterize genes involved in human preimplantation development. Such studies provide a molecular framework for further research into early human development and clinical programmes associated with infertility and disease diagnosis. Conventional techniques of screening for genes expressed in different tissues or stages of development involve the construction of libraries and subsequent differential or subtractive hybridization. In the human embryo, where tissue availability is limited, such methods are difficult. The recent creation of PCR-generated libraries from human unfertilized oocytes and single preimplantation embryos allows the study of gene expression by new techniques such as differential display and subtractive hybridization. We present preliminary data on the

identification and isolation of a number of genes participating in the early developmental programme using an adaptation of the differential display protocol. Sequence comparisons have identified a number of novel clones and some that show homology to ESTs. Of particular interest is an embryonic clone which shows homology to the DNA-binding domain of a human methyl-CpG binding protein thought to be involved in transcriptional repression. We have demonstrated that differential display using human oocyte and embryo cDNA libraries is an effective method for identifying genes expressed at specific stages of preimplantation development. However, further work needs to be undertaken to confirm the stage-specific nature of these genes and to determine their function in early human development.

Mice with a targeted disruption of the Batten disease gene (CLN3) display a progressive storage disorder

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The neuronal ceroid lipofuscinoses (NCLs) are the most common neurodegenerative disorders of childhood. Batten disease, the juvenile-onset form of NCL, is caused by mutations in the *CLN3* gene which encodes a predicted transmembrane protein of unknown function. NCLs occur naturally in a wide range of animal species and resemble the human NCLs to varying degrees in terms of clinical symptoms and accumulation of characteristic storage material. However, there are no specific models for Batten disease (*CLN3*) that mimic the disease both phenotypically and genetically. Therefore, a murine model for Batten disease was generated by gene targeting in mouse ES cells. Exons 1–6 of mouse *Cln3* were replaced by a Neo cassette, resulting in a null allele that demonstrates loss of *Cln3* mRNA expression. Crosses between heterozygous mice yield offspring in the expected 1:2:1 genotype ratio. Homozygous null mice are viable and fertile and do not exhibit obvious clinical symptoms at 7 months of age. However, there is progressive accumulation of autofluorescent storage material that parallels the pathology of Batten disease in humans. More rigorous studies of retinal function and behaviour are in progress to assess disease progression.

Molecular interactions during limb initiation and development

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The limb buds initiate at distinct lateral positions as opposing pairs and subsequently undergo a number of precisely controlled events to ensure normal development. We have used four mouse mutations – dominant hemimelia (*Dh*), luxate (*lx*), sasquatch (*Ssq*) and extra toes (*Xt*) – to investigate some of these events. All four mutations belong to the luxoid group, and are characterized by having pre-axial polydactyly that correlates with ectopic anterior expression of *Shh*. However, it appears that ectopic expression of *Shh* can be induced through at least two different mechanisms. In *Xt* and *Ssq* ectopic expression of *Shh* at E11·5 is the first sign of abnormal development, whereas in *Dh* and *lx* ectopic expression of *Fgf4* and *Fgf8* in the AER precedes that of *Shh* by at least 24 h. Thus our data suggest that *Dh* and *lx* disrupt limb development at an early stage prior to induction. In *Dh* the phenotypic characteristics also include a rostral shift of the hindlimbs. We have found that in *Dh* embryos the anterior boundary of *Hoxc10* is positioned two segments more anterior than observed in the wild-type. This shift of expression correlates with the shift of the limbs, suggesting a role for *Hoxc10* in positioning the hindlimbs.

Lined mice are *Rsk2* deficient but are they a good model for Coffin–Lowry syndrome?

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Coffin–Lowry syndrome (CLS) is a rare X-linked semi-dominant disorder where affected males have severe mental retardation, characteristic face and hands, and progressive skeletal problems. Heterozygous females with CLS have also been reported; they are mildly affected and are usually identified as they are related to CLS males. Two years ago mutations in the gene encoding *Rsk2*, a member of a family growth-factor-regulated serine/threonine kinases, were identified in CLS patients. Although these mutations were predicted to affect the function of *Rsk2*, it is difficult to correlate them with the different aspects of the CLS phenotype. We have discovered that the lined (*Li*) mouse phenotype results from an X chromosome deletion that removes the *Rsk2* coding

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region. Lined males and some lined heterozygous females die prenatally whereas surviving females are unaffected except for fine coat stripes. Despite having skewed X-inactivation, these females still have a proportion of cells with an active deleted X chromosome, and it is interesting to study the distribution of these *Rsk2*-deficient cells in various cells and tissues. We are currently characterizing the deleted region and investigating the phenotype of *Li* mice to provide insights into the function of *Rsk2*.

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