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Co-operative interactions of the human β-globin regulatory elements: implications for gene therapy

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The erythroid specific expression of the human β-globin gene is regulated by several genetic elements. Firstly, using MEL cells, regulatory regions in the promoter have been mapped whose function appears to be mediated via the interaction between erythroid specific and ubiquitous trans-acting transcription factors. Work on both MEL cells and transgenic mice has localized two downstream erythroid specific enhancer elements. More recently, a 'dominant control region' (DCR) located 5′ to the β-globin domain has been characterized. This DCR confers high level, position independent, copy number dependent expression on a linked β-globin gene in both transgenic mice and MEL cells. A large number of deletion and point mutants in both the DCR and β-globin genes have been tested in MEL cells in order to assess the requirements for high level, position independent expression. The results show a marked degree of redundancy in the regulatory elements throughout the regions analysed. Within the context of gene therapy, this information makes it possible to construct a minimal β-globin gene 'domain' within a retroviral vector that should give correctly regulated, high level expression during erythroid development after infection of bone marrow stem cells.

Chemiluminescence: an alternative method for the immunodetection of polymorphic proteins

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Immunoblotting is an important tool in the analysis of protein polymorphism by isoelectric focusing. High sensitivity immunoassay techniques with enzyme labels such as horseradish peroxidase (HRP) conjugated second antibodies can be used to detect filter bound antigen-antibody complexes. The presence of enzyme conjugate, which identifies the position of the protein bands on the filter, is normally detected using an electron transfer dye, such as diaminobenzidine (DAB). We have been investigating (in collaboration with the Home Office CRSE and Amersham International Ltd) an alternative approach for the detection of peroxidase conjugates, using chemiluminescence generated by the oxidation of luminol in the presence of enhancers such as *p*-iodophenol. The method gives rapid results, a permanent record on film and appears to provide an increase in the sensitivity of detection. This makes it very useful for the detection of low-abundance proteins.

Construction of DNA databases and problems associated with the determination of band match probabilities

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Hypervariable loci consist of a large number of different alleles. The number of alleles detected depends not only upon the locus but also upon the size of the population analysed and the resolving power of the electrophoretic

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system. In the construction of population frequency tables, alleles which differ by only a few repeat units may not be distinguished from each other. Before attempting to place an estimate of frequency on any allele, or allelic cluster, an estimate of the precision of data gathering must be made, for example the errors involved in interplate comparison of phenotypes. To assist with these calculations, a scanning instrument was utilized. Bands are assigned to one of 600 possible positions (dependent upon actual position of the band on a blot). The machine stores normalized profiles by reference to control lanes containing bands of known molecular weight. Standard deviations of band position were estimated by analysis of the same fragments in duplicate samples. Three different methods of analysis have been compared: the first simply divides the track on an autoradiograph into sectors of 4 × standard deviation (S.D.) estimates. The frequency of a given band is calculated from the accumulated observations of bands within a given sector. This approach is compared with the use of a sliding window routine which estimates band frequencies based on the assignment of ± 2 S.D. estimates to each of the 600 arbitrary positions in turn. Frequency estimations are then carried out on the absolute position (± 2 S.D.). The third method describes a Bayesian approach using probability density functions.

Changes in *X* chromosome activity in female mouse embryos and *X*-linked gene methylation

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Changes in methylation of CpG sequences 5′ to *X*-linked genes are investigated in different cell lineages of the developing mouse embryo. The approach used is PCR amplification of sequences containing an informative CCGG site, 5′ to the *X*-linked PGK or HPRT genes. The DNA from embryos or embryonic tissues is cut with *Hpa* II before amplification; when the site becomes methylated in female embryonic development, amplification will be resistant to *Hpa* II digestion. Individual preimplantation embryos and dissected regions of postimplantation embryos are analysed. Female and male embryos are distinguished by PCR amplification of *Y* specific sequences and also with a twofold difference in amplification of the *X*-linked sequence (before *Hpa* II digestion). To evaluate the differences in amplification, the PCR must be accurately quantitative. One of the main constraints in obtaining quantitative data from PCR is inherent in the amplification process; because amplification is exponential, small differences in any of the variables which influence the reaction will dramatically affect the yield. Therefore, we evaluated the amount of amplified product by comparison with the amplification of a standard homologous template included in the reaction mix; the standard template uses the same primers as the experimental target but is distinguishable on the basis of size. Our preliminary results suggest that the timing of methylation of a CpG sequence in PGK is closely associated with the timing of *X*-inactivation in female embryonic development.

Detection of single copy gene sequences in a single cell by PCR amplification

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The study of early mammalian development requires highly sensitive methods to detect gene expression, DNA methylation and gene mutation. We have previously described the development of microassays for the enzymes ADA, PNP, HPRT and APRT, and then their use in the study of gene expression in early development and to develop procedures for preimplantation diagnosis of genetic disease. For preimplantation diagnosis by DNA analysis we have shown that it is possible to amplify single copy gene sequences in single mouse cells taken from the developing embryo. The sensitivity of the PCR reaction depends mainly on the use of nested primers in two sequential amplifications of 30 cycles each. Contamination is prevented even at this level of sensitivity by strict but simple rules of laboratory practice, enabling us to set up PCR amplification of single copy sequences on the open bench. A similar approach is being taken to enable preimplanation diagnosis of beta-thalassaemia in man.

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Tyrosinase-related proteins and the molecular genetics of pigmentation

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Genes regulating pigmentation of the mouse are the longest studied developmental genes of mammals. The origins of some mutations goes back to the mouse fancy stocks. Almost a century of research has produced a rich resource of mutations, making coat-colour genetics an attractive candidate for the application of molecular genetic techniques. The enzyme tyrosinase is the product of the mouse coat-colour locus *albino*. We have identified a cDNA encoding a protein related to tyrosinase (TRP-1) as being the product of the *brown* locus. TRP-1 is not essential for pigment formation, but is required for the development of black, rather than brown eumelanin. We have rescued mutant *brown* melanocytes in culture, by infection with a retrovirus carrying the wild-type TPR-1 gene, which confers a black phenotype on the cells. We have determined the sequence of the *brown* mutant TRP-1 gene and shown there are two amino-acid changes from wild-type. One of these changes a conserved cysteine residue to tyrosine. We have also sequenced the TRP-1 gene of a revertant, from *brown* to wild-type TRP-1 gene, which confers a black phenotype on the cells. We have determined the sequence of the *Harwell*, has a phenotype intermediate between *Black* and *brown*. We have shown that in mice homozygous for this mutation, TRP-1 mRNA is reduced in abundance approximately 100-fold. Two dominant alleles of *brown* appear to exert their effect by killing the melanocytes after initiation of pigment synthesis. One of these, a radiation-induced mutation *White-based brown*, has a gross DNA rearrangement at its 5′ end. We speculate that the cell lethality is due to the TRP-1 promoter being juxtaposed with a foreign sequence, whose transcription exerts the toxic effect. There are a large number of radiation-induced deletions of the *brown* locus, which are homozygous lethal; presumably due to deletions of adjacent essential genes.

Molecular characterization of an ENU induced mutation of the murine β-major globin gene (*Hbb-B1*)

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The molecular characterization of mutations is central to elucidating the mechanisms by which mutagens exert their action. Since haemoglobin is very well characterized, biochemically and genetically, mutations arising in globin genes can be analysed relatively easily. Several mutations have been induced in the mouse β-globin gene complex (*Hbb*) by treatment with the alkylating agent *N*-ethyl-*N*-nitrosourea (ENU), one of which has resulted in a structurally altered β-globin polypeptide (β-diffuse major) which is homologous with haemoglobin Rainier in man (Peters *et al.* 1985, *Genetics* **101**, 709). The murine mutant (Hbb^{d4}) can be detected as a specific restriction site polymorphism, with the loss of an *Rsa* I site within exon III of the *Hbb-b1* gene. Furthermore, primers which differ only in one of their 3′ nucleotides have been used in the Amplification Refractory Mutation System, described by Newton *et al.* 1989 (*Nucleic Acids Res.* **17**, 2514), to establish that ENU has induced a single A/T to G/C base pair substitution within this region. These results confirm the previous biochemical findings and are in keeping with the substitution of β145 tyrosine to cysteine in Hbb^{d4}.

Is *Zfy-1* the mouse sex-determining gene?

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ZFY has received much attention as a candidate for the human sex-determining gene, TDF. Whilst its map location and predicted gene product are as we might expect of TDF, there is still no evidence that ZFY is associated with male development. In the absence of transgenic mouse data, we sought to describe the expression

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of the mouse homologues, *Zfy-1* and *Zfy-2*, during embryogenesis. The male-determining gene ought to be expressed as the bipotential gonad differentiates into a testis, at about 12 days post coitum (d.p.c.). RNA blot analysis and *in situ* hybridization did not detect *Zfy* expression in foetal gonads. PCR analysis of RNA from isolated genital ridges and fetal testes showed that *Zfy-1* transcription is indeed up-regulated in the period 11·5–12·5 d.p.c. *Zfy-2* transcripts were not detected between 9·5 and 14·5 d.p.c. It is important to establish the cell type responsible for *Zfy-1* expression: germ cells, for example, are not required for testis formation. Testes of W^e homozygous embryos, which develop normally despite a lack of germ cells, were found to lack *Zfy-1* expression, providing the strongest evidence to date that *Zfy-1* is not the testis-determining gene.

TCP-1 is one component of a heteromeric protein complex present in somatic and spermatogenic cells

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A set of seven monoclonal antibodies specific for the *t*-complex polypeptide-1 (TCP-1) have recently been described (*Cell* **57**, 621, 1989). The subcellular distribution of TCP-1 is associated with the cytoplasmic face of the *trans*-Golgi network (TGN) as well as numerous vesicles distributed throughout the cell periphery. The anti-TCP-1 antibodies have been used to immuno-isolate a particle containing the 57 kDa TCP-1 protein. The particle sedimented at 19·5–20·5% sucrose and, in addition to TCP-1, consisted of approximately 7–10 polypeptides (depending on the cell type of origin) which could be resolved by one-dimensional and two-dimensional PAGE. We have called the other polypeptide components 'TCP-1 associated polypeptides' or TAPs. The TAPs isolated from tissue culture cells are very similar to those isolated from spermatocytes, but are significantly different to those isolated from haploid spermatids. The alternative TAP distribution may be significant in understanding the role of the increased expression of TCP-1 mRNA and protein levels during the haploid spermatid stage of spermatogenesis.

Mucins: a family of glycoproteins whose genes exhibit extensive length polymorphisms

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Mucins are high-molecular-weight glycoproteins glycosylated on their serine and threonine residues, which form a protective barrier on the surface of epithelial cells. The first human mucin gene to be identified was *PUM* (now renamed *MUC1*), which is located on the long arm of chromosome 1, at 1q21. *MUC1* is expressed in lung, breast and kidney tissue and the protein can also be detected in urine. The *MUC1* gene is highly polymorphic and both the protein and the DNA alleles show the same length polymorphism which appears to be due to varying numbers of tandem repeat units within the gene. A second human mucin gene (*MUC2*) has recently been identified. Gum *et al.* (*J. Biol. Chem.* **264**, 6480, 1989) have isolated a cDNA coding for an intestinal mucin. In collaboration with J. Gum and Y. S. Kim we have mapped this gene to chromosome 11p15 and we have also been able to demonstrate length polymorphisms at this locus. However, *MUC2* shows additional variation which appears to be due to restriction fragment site polymorphisms. Our investigations are directed towards understanding the origin and significance of the high level of polymorphism at these loci. Studies in progress on the mutation rates and variation in methylation will be reported.

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Molecular genetic analyses used to identify cattle carrying specific alleles of the milk protein genes

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The major milk proteins, the four caseins and the two whey proteins, all exist in a number of polymorphic forms. The particular forms expressed in milk can have a pronounced effect on the processing quality of the milk; particular alleles have also been observed to be linked to important production characteristics. This information has not been incorporated into routine selection of dairy bulls because of the complex progeny testing required. We have used available information on the structure of these genes to design molecular genetic tests (based on routine RFLP analysis or, more recently, PCR technology) to identify the presence of specific alleles at these loci in cattle DNA samples (obtained from blood, milk or semen). We can routinely identify both the alleles of κ-casein and one of the alleles at the β-casein locus. A survey of dairy cattle has shown that selection for favoured alleles at the milk protein loci is now possible but its effectiveness may be limited by genetic and developmental factors.

A contig map of the human *Y* chromosome

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It has been proposed that the entire human genome be sequenced. A desirable starting point would be to have available a set of ordered clones. Various methods have been suggested. The method of Coulson *et al.* involves analysis by restriction enzyme digestion of random cosmid clones to produce a 'fingerprint'. The fingerprint consists of 20–30 radiolabelled restriction fragments separated on an acrylamide gel and visualized by autoradiography, the pattern of which should be unique to each clone. Different clones can then be analysed to detect those which share a number of restriction fragments and overlap to form a contiguous stretch of DNA or 'contig'. Various other methods such as the use of RNA probes requiring less of an overlap to detect matches can then be used to link small contigs to form larger contigs and ultimately a complete contig map. Such a map of *Caenorhabditis elegans* has been defined and correlates well with already known genetic markers. We are using this method to analyse 1600 random cosmid clones from the human *Y* chromosome with a view to producing mapped clones useful both as a starting point for sequencing and in the identification and study of coding sequences.

Characterization of DNA from carcinogen-treated human cell lines and tumours after heterotransplantation into nude mice

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Hae III-digested DNA from the human glioblastoma cell lines HeRo and HeRo-SV (the latter was experimentally transformed with SV40 virus) was probed with the synthetic oligonucleotide $(GTG)_5$ and the minisatellite probe 33.15. Using this fingerprint analysis we could show that changes in the hybridization pattern occurred with increasing passage number. DNA analysis before and after heterotransplantation of tumour cells and solid tumours into nude mice also revealed differences in the fingerprint pattern. DNA from colorectal carcinomas, adenomas, and metastases and non neoplastic tissue (mucosa) from the same patient were digested with *Hae* III and subsequently hybridized with the synthetic probe $(GTG)_5$. In 5 out of 10 carcinomas differences in the hybridization patterns were observed. In the case of the kidney carcinomas 1 out of 6 tumours showed differences in the fingerprint pattern with either of the two probes, $(GTG)_5$ and 33.15. Furthermore, DNA from carcinogen-treated HeRo cells was characterized using the gene probes $(GTG)_5$ and 33.15.

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