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MUTAGENESIS AND ALU SEQUENCE-MEDIATED DNA DISPERSION IN TRANSGENIC MICE MICROINJECTED WITH HUMAN GLOBIN GENES

by

Wendy S. Rubinstein

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1987

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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The City University of New York

I wish to dedicate this thesis to
Dr. Terry Ann Krulwich, for her
commitment to the students at
Mount Sinai.

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The time spent on my Ph.D. is a gift to me; a difficult one, and not unearned, but nevertheless a gift. That is true mostly, but not totally, because of the intellectual joy and discipline of science. I am also the product of much benefaction, not the least of which is my MSTP support. However, these acknowledgements cannot be made lengthy enough to properly express my thanks to the people who have helped me to succeed in bringing this dissertation to fruition, and to develop as a scientist. The greatest proportion of my gratitude is to Jon Gordon, along with my respect and admiration, which need reiteration. The satisfaction I feel in having been challenged to my deepest resources by a remarkable scientist is precious.

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Hardest of all to express is the depth of my appreciation to my parents, whose indefatigable support, even when I choose a more difficult path against their advice, is something I may never fully comprehend unless I become a parent myself. Independent as I like to think myself to be, it is difficult to imagine how I could have prevailed without them.

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INTRODUCTION

During the development of multicellular eukaryotes, the fertilized egg gives rise to all of the differentiated tissues of the adult organism. Determinative events during embryogenesis lead to the establishment and maintenance of a set of genetically determined functions in each differentiated cell type, which correspond to and define each differentiated phenotype. The molecular mechanisms which operate during the developmental regulation of specific genes, and the achievement of differential gene regulation in different cell types remain fundamental unsolved problems of developmental biology. In particular, the relationship of primary DNA sequence information, DNA modification, chromosomal domain, and higher-order genomic organization to the time- and tissue-specific characteristics of gene regulation remain to be elucidated. Furthermore, the mechanisms which cause genomic alterations during development and evolution have been the subject of much speculation but limited experimental analysis.

Attempts to experimentally dissect the complex mechanisms which exert control on the eukaryotic genome have relied heavily on in vitro gene transfer systems which allow the study of the regulation of single genes. However, since the expression of any particular gene depends upon its complete developmental history, the elaboration of technologies for gene transfer into whole intact organisms in order to monitor gene regulation throughout development has been a goal of fundamental importance. This demand has recently been met by the technique of transgenic mouse production. In this system, cloned DNA microinjected into the pronucleus of a fertilized mouse egg can become integrated into a single, random chromosomal site, and be subjected in every cell at every stage of mouse development to the complete array of determinative effects and differentiative transitions. The unique characteristics of the foreign gene, which facilitate its sensitive monitoring, and the practicality of using mice as an experimental mammalian system permit the identification of factors which are relevant to gene control.

Several features of the human ß-like globin genes make these a logical choice for gene transfer into mice. This gene cluster (Fritsch et al., 1980) comprises a set of developmentally

regulated genes (Peschle et al., 1983; Karlsson and Nienhuis, 1985) which are successively turned on and off at specific times and in specific tissues during development. The globins have been the subject of numerous pioneering studies in chemistry, biology and medicine (Weatherall et al., 1976). Due to the accumulation of over a century of research in this field the globins now comprise one of the most extensively characterized eukaryotic gene complexes. The existence of human globin mutations affecting virtually every level of regulation (Collins and Weissman, 1984a) has made the globins a model system for study of gene control during development and genetic change during evolution, and the prevalence of such mutations has provided an impetus for the identification and manipulation of globin regulatory mechanisms. The discovery of repetitive sequences in the globin cluster has spurred interest in the functions they specify in development and evolution. These considerations provide a context for the study of human globin genes in transgenic mice.

The focus of most studies using transgenic mice has been the identification of DNA regulatory sequences which mediate tissue-specificity of gene expression. The finding that a particular gene is expressed in a specific tissue in a transgenic mouse and remains silent in other tissues provides convincing evidence that the foreign gene contains DNA sequence information which mediates its tissue-specific expression. Furthermore, since each independent line of transgenic mice contains the microinjected gene integrated at a different chromosomal location, the phenomenon of tissue-specific expression must be mediated by the foreign gene rather than the chromosomal domain into which it has inserted. Studies on transgenic mice with various microinjected genes (Palmiter and Brinster, 1986) have shown that many of these genes can be regulated in an appropriate and tissue-specific manner which is largely independent of chromosomal position, and this observation has been extended to include the globin genes. Having produced transgenic mice to study these issues, two separate phenomena were observed in mice microinjected with human globin genes, and these experimental opportunities were explored.

In this thesis, data are presented on two lines of transgenic mice which demonstrate mechanisms of genomic variability. The fact that the same DNA clone was microinjected to produce two lines of mice with vastly differing genotypes and phenotypes is in itself an indication of the

readiness of the early mouse embryo to undergo heritable genetic metamorphosis. Evidence that line ß80 carries a mutation of an endogenous mouse gene will be presented along with an analysis of the cloned locus of insertion. Restriction enzyme evidence that microinjected human Alu DNA sequences became dispersed in the genome of line ß19 will be presented in the context of the first in vivo observation of Alu sequence-mediated DNA dispersion.

BACKGROUND I.

Transgenic Mice

The technique of gene transfer into the mammalian embryo by microinjection has been engendered by several other experimental systems (for review, see Gordon and Ruddle, 1985). The relatively recent success of this technique (Gordon et al., 1980) has awaited the realization of several criteria which were met only in part by other approaches. These include a high efficiency of gene transfer, stable integration into mouse chromosomes, genetic transmission of foreign genes via successful germ line integration, and the capability to transfer different DNA clones which vary extensively in size and content. Two methods of germline gene transfer have been successfully applied. Production of transgenic mice by microinjection of cloned DNA satisfies all of the above criteria, and is the method which was used to produce the two lines of mice described in this thesis. Jaenisch developed the technique of infection of cultured embryos with Moloney Murine Leukemia (MoMuLV) retrovirus to establish several lines of mice, designated Mov lines, each carrying one provirus at a unique integration site (Jaenisch, 1976; Jähner and Jaenisch, 1980; Jaenisch et al., 1981). However the generalization of this approach to non-viral genes is hampered by loss of recombinant sequences in the viral clone (Harbers 1981), by a size restriction of the recombinant DNA constructs engineered for these experiments, and by inhibitory and cryptic effects of the viral vector on foreign gene regulation. Recently, the use of retroviral vectors to infect cultured stem cells coupled with the use of these transformed cells in chimeric mouse production (Wagner et al., 1985) has been shown to result in germ line transmission (Robertson et al., 1986), and may lead to greater ease in manipulation of sequences used in the retroviral transfer technique.

The technique of microinjection (Diacumakos, 1973) was first successfully applied to the cultured cell (Graessmann et al., 1979). Microinjection into the fertilized mouse egg, and the subsequent production of transgenic mice (Gordon et al., 1980; Gordon and Ruddle, 1981; Brinster et al., 1981; Costantini and Lacy, 1981) has provided the opportunity to transfer virtually any cloned sequence into the developing embryo. This technology is also applicable to other animals (Hammer

et al., 1985; Wagner and Murray, 1985), and efficient gene transfer techniques are also available for Drosophila (Scholnick et al., 1983; Spradling and Rubin, 1983; Goldberg et al., 1983) and plants (De Block et al., 1984; Horsch et al., 1985, 1986; Abel et al., 1986). If a sequence microinjected into a mouse embryo integrates at the one-cell stage, then each cell in all subsequent cell lineages will contain the gene at the same chromosomal locus. If integration of the microinjected material occurs later in development, which is estimated to occur in about 30% of founder mice (Wilkie et al., 1986), the mouse which develops may be mosaic in its somatic and/or germline cells (Costantini and Lacy, 1981; Wilkie et al., 1986). A decreased blot intensity of adult somatic tissues as compared to positive F1 progeny would indicate mosaicism of somatic tissues, and a germline mosaic would be expected to transmit the foreign gene to fewer than 50% of its offspring. As long as the microinjected sequence gains access to the germline; subsequent transgenic generations contain the foreign gene in every cell. In this case, production of a mosaic would not interfere with the establishment of transgenic lines; in fact, in the case of a harmful phenotypic effect, mosaicism can lead to improved viability (Hanahan, 1985; Small et al., 1986b; Quaife et al., 1987).

Microinjected DNA follows a characteristic mode of processing in transgenic mice. When multiple copies insert into the genome, they are usually present at a single site of integration, arranged in a head-to-tail conformation (Costantini and Lacy, 1981). Strategies for the specific isolation of flanking DNA versus internal copies of the concatemer are useful for study of insertional mutations in transgenic mice. Palmiter and Brinster (1985) have suggested that the formation of concatemers is a consequence of homologous recombination and present models for this theory (Brinster et al., 1981). Two sites of integration have also been observed (Lacy et al., 1983; Storb et al., 1984; Overbeek et al., 1986). When this occurs, either site of integration can be single- or multiple-copy and have a complete or mosaic distribution. In these instances, two independent lines of transgenic mice can be bred from one founder.

The finding that a microinjected vector containing mouse repetitive sequences did not integrate with increased frequency provided an early indication that homologous recombination was not a mechanism of integration (Gordon et al., 1980). Brinster et al. (1981) microinjected a fusion

plasmid consisting of a mouse metallotheinein (MT-1) promoter, homologous to the endogenous MT-1 gene, linked to the Herpes simplex virus thymidine kinase (HSV-tk) structural gene, and found that the endogenous MT-1 gene restriction pattern remained unchanged in transgenic mice containing the fusion gene (Palmiter et al., 1982a). Further evidence for random insertion was provided by in situ hybridization of metaphase chromosomes from 5 lines of mice carrying rabbit ß-globin genes (Lacy et al., 1983). In no case did integration occur into chromosome 7, the site of the endogenous mouse ß-globin genes.

Another characteristic of foreign gene processing in the mouse embryo is methylation. Retroviruses introduced into mouse embryos are initially unmethylated, as are cloned sequences microinjected to produce transgenic mice. The integrated proviruses and microinjected genes, however, are highly methylated (Stuhlmann et al., 1981; Palmiter et al., 1982a). Therefore, a de novo methylase activity must have acted upon the proviral genome either during embryonic development or during transmission through the germ line. Jähner et al. (1982) found that MoMuLV introduced into mouse embryos at the preimplantation stage (1-30 cells) was methylated, whereas virus inserted at postimplantation stages (104-105 cells at day 8) was not. Therefore, an efficient de novo methylation activity was demonstrated in the preimplantation, but not the postimplantation embryo.

Approaches to the Study of Gene Regulation in Transgenic Mice

Studies on the developmental regulation of gene expression employ several kinds of experimental approaches. Expression of a foreign gene can be studied at random sites of insertion, expression characteristics at a specific chromosomal locus can be examined, or altered expression of an endogenous mouse gene can be studied after it sustains an insertional mutation. It has been estimated that 10 to 20% of transgenic mice contain insertional mutations (Palmiter and Brinster, 1985, 1986; Jaenisch et al., 1985).

In the first approach, genes inserted randomly into the genome allow a test of whether the gene contains cis-acting sequences which control the time- and tissue-specific parameters of expression. One of many illustrative cases is mouse immunoglobulin, where in 7 out of 7 lines

carrying microinjected mouse kappa light chain genes, mRNA expression occurred specifically in the B-cells of the spleen, and transgene-encoded light chains were detected in the serum (Brinster et al., 1983; Storb et al., 1984). Clearly, the immunoglobulin genes contain DNA sequence information which directs expression to a specific cell type regardless of chromosomal position. Tissue-specific regulation can also be achieved across widely divergent species (McKnight et al., 1983).

This approach has been most successfully applied to the study of how regulatory elements such as promoters and enhancers influence the tissue-specificity of gene expression. For example, microinjected fusion genes carrying the metallothionein 5' regulatory region (Brinster et al., 1981, 1982; Palmiter et al., 1982a, 1982b) express primarily in liver and kidney, tissues which normally express metallothionein. Tissue-specificity of tumorigenesis in transgenic mice is also programmable by upstream regulatory sequences, as discussed below. In addition, a report on human hypoxanthine phosphoribosyltransferase (HPRT) cDNA in transgenic mice provides evidence that sequences which control tissue-specificity of expression can also reside within or 3' to the structural gene itself (Stout et al., 1985).

Transgenic mice also afford the opportunity to study several other biological phenomena in higher eukaryotes which cannot be addressed using other experimental systems. The transgenic mouse system has shown great applicability in studying the development, regulation and mutability of the immune system (Weaver et al., 1986; Bieberich and Scangos, 1986; Gordon, 1986; O'Brien et al., 1987). Allelic exclusion, which ensures that only a single type of heavy and light chain will be rearranged during B cell differentiation, can be studied by microinjecting functionally rearranged immunoglobulin genes into mouse embryos and studying expression of endogenous immunoglobulin genes (Ritchie et al., 1984; Storb et al., 1985). The process of tumorigenesis, and the ability to relate promoters, enhancers, hormones, and gene products to this process has also been approached in transgenic mice (Brinster et al., 1984; Stewart et al., 1984; Palmiter et al., 1985; Messing et al., 1985; Hanahan, 1985; Adams et al., 1985, 1986; Van Dyke et al., 1985; Lacey et al., 1986; Leder et al., 1986; Small et al., 1986a, 1986b; Rüther et al., 1987; Alexander et al., 1987; Quaife et al., 1987). Furthermore, transgenic mice can serve as experimental models for improving chemotherapeutic

methods (Isola and Gordon, 1986). Transgenic mice have also been used to study aspects of hormonal regulation and physiology. Posttranslational processing of pre-prosomatostatin produced in the anterior pituitary of transgenic mice which contain a metallothionein-somatostatin fusion gene (Low et al., 1985) has revealed that this tissue, which does not normally express somatostatin, nevertheless contains proteases which process this precursor neuropeptide. Norstedt and Palmiter (1984) have used transgenic mice to investigate the physiological effect of growth hormone secretory rhythms on sexually differentiated functions of mouse liver. Mouse models for human disease have been created using transgenic mice (Löhler et al., 1984; Chisari et al., 1985; Small et al., 1986a, 1986b; Sasaki et al., 1986; Hooper et al., 1987; Farza et al., 1987). Conversely, genetic deficiencies have been corrected in mice by microinjection (Hammer et al., 1984; Le Meur et al., 1985; Pinkert et al., 1985; Yamamura et al., 1985). The production of functional clotting factor IX in transgenic mice (Choo et al., 1987) suggests that production of larger amounts of clotting factor IX in transgenic livestock may be feasible. Microinjected sequences are also highly useful as DNA markers. Harbers et al. (1986) have employed mice carrying a proviral genome on the pseudoautosomal region of the sex chromosomes to study unequal recombination, a process with relevance to the evolution of multigene families (Hood et al., 1983; Maeda and Smithies, 1986). The study of cell lineages has also been advanced by using retroviruses as genetic markers (Soriano and Jaenisch, 1986).

Insertional Mutation

An impediment to genetic analysis of development in vertebrates has been the inability to isolate genes, mutations of which cause specific phenotypic abnormalities. Thus, although hundreds of well-characterized mutations have been described in mouse (Green, 1981) and man (McKusick, 1983), isolation of the responsible genes has been elusive. These limitations are being overcome (Orkin, 1986) using such techniques as deletion cloning (Kohne et al., 1977; Kunkel et al., 1985), restriction fragment-length polymorphism (RFLP) probes (Botstein et al., 1980), and chromosome jumping (Collins and Weissman, 1984b).

Retroviruses have been observed to act as insertional mutagens (Varmus et al., 1981; Jenkins et al., 1981; Copeland et al., 1983; Kozak, 1985; Jaenisch et al., 1985), facilitating the isolation of the mutated loci (Hutchison et al., 1984). This approach has been successfully applied in mice infected with retroviral vectors (Jaenisch et al., 1983; Schnieke et al., 1983). Similarly, transgenic mice mutated by integration of microinjected DNA have been useful in the isolation and molecular analysis of genetic loci.

Since most mutations are recessive, it is necessary to cross transgenic mice to homozygosity to uncover the mutant phenotype. (The founder transgenic mouse is hemizygous for the inserted gene.) If such crosses are not routinely performed, the only observable mutations would be those which are dominant and non-lethal.

An important technical difference between mice made by retroviral infection and transgenic mice made by DNA microinjection is that retroviruses integrate as a single provirus, while microinjected DNA typically integrates as a long concatemer. The relative excess of internal copies of foreign DNA in a long concatemer can lessen the fraction of important clones in a library probed with the microinjected DNA. This has prompted the use of cloning shortcuts, where feasible, to ensure isolation of flanking mouse DNA (Woychik et al., 1985; Tarantul et al., 1986).

Wagner et al. (1983) produced 6 lines of transgenic mice carrying human growth hormone sequences. Four of these lines could be bred to homozygosity, as judged by a doubled DNA blot intensity compared to hemizygous parents. Two lines, HUGH/3 and HUGH/4, failed to produce

postnatal homozygotes. The average litter size in hemizygous crosses was reduced in each of these 2 lines, as would be expected for prenatal lethality of homozygotes (although this could not totally account for reduced litter size in the HUGH/3 line). A shift in the ratio of total transgenic offspring from 3/4 would have provided a statistical approach to document the absence of postnatal homozygotes, but not enough offspring were examined to make a statistically valid analysis.

Further studies of these two lines (Covarrubias et al. 1985) characterized the locus of insertion and assessed the time of developmental arrest. Line HUGH/3 contains 3-5 copies of growth hormone gene in tandem array. Cloning of this concatemer indicated that it was interrupted twice by mouse genomic DNA. Comparison to wild-type chromosomes revealed discrepancies indicating translocations or other rearrangements. Line HUGH/4, which also contains 3-5 copies of plasmid DNA, displayed DNA rearrangement at the insertion locus. Alterations of endogenous DNA at the insertion locus is a repeated finding (Woychik et al., 1985; Tarantul et al., 1986.) Both lines involved homozygous lethality in the early postimplantation period.

Palmiter et al. (1984) have described a transgenic mouse pedigree in which males are fertile but never transmit the foreign gene. They concluded that the insert disrupted a gene expressed during haploid stages of spermiogenesis. This event shows the utility of insertional mutations for gaining access to genes which would otherwise be difficult to isolate.

When breeding the 13 substrains of mice that carry exogenous MoMuLV provirus at unique loci, Jaenisch (Jaenisch et al., 1983; Löhler et al., 1984) observed prenatal lethality of homozygotes at the Mov13 locus. Jaenisch cloned the Mov13 locus in an EcoRI fragment and then used genomic flanking (non-viral) DNA from this EcoRI fragment as a probe in Southern blots to assess the Mov13 genotype. Hybridization of this probe for the Mov13 viral insertion site to a 14kb band indicated the presence of the intact locus, and a 23kb band indicated the same locus containing the 9kb Moloney provirus. This approach was used in other lines to reliably assess +/+, +/-, and -/- mice. Fourteen adults and 55 embryos of ages 15 to 19 days of gestation were blotted in this way, and none was homozygous. Reduced litter sizes of +/- x +/- versus -/- x +/- crosses were also consistent with embryonic death of homozygotes. Examination of 13-14 day pregnant females revealed degenerating

fetuses which were arrested between 11.5 to 12 days of gestation. Genotyping of DNA extracted from 35 of these arrested concepti revealed 35 homozygotes, establishing prenatal lethality of homozygotes.

By using mouse DNA of the Mov13 locus as a probe of Northern blots of mRNA in developing mice, Schnieke et al. (1983) showed that the Mov13 gene is activated in normal mice at day 12 of gestation, after which expression sharply increases, and showed that homozygous embryos lacked this mRNA transcript. Northern blots of several cell lines using the Mov13 probe revealed high Mov13 locus expression in diverse fibroblast-like mesodermal tissues. Using the two human cDNA collagen genes as probes, Schnieke et al. showed that homozygous Mov13 embryos lacked the RNA class corresponding to the α1(I) collagen probe. By performing Southern blots using a human genomic probe which hybridized to the 14kb and 23kb fragments, Harbers et al. (1984) showed directly that the Mov13 locus corresponded to the mouse α1(I) collagen gene. Electron microscopy of collagen mRNA S1 nuclease mapping and sequencing showed that the Moloney provirus was integrated into the first intron in a transcription orientation opposite that of the collagen gene.

It is not immediately apparent why the α1(I) collagen gene containing a viral insertion in the first intron should fail to produce mRNA (Weiringa et al., 1984). Interestingly, several other cases of retroviral insertion into an intron resulting in disrupted gene expression have been reported (Varmus et al., 1981; Hawley et al., 1982; Wolf and Rotter, 1984; Jaenisch et al., 1985). In this case, failure to transcribe α1(I) collagen is not due to viral transcription in the opposite direction since Mov13 provirus is not transcriptionally active prior to day 16 (Stuhlmann et al., 1981).

To further investigate this transcriptional arrest, chromatin structure and DNA methylation of the Mov13 locus were analyzed and compared to parental strain DNA. Three prominent DNAase I hypersensitive sites can be detected in the 14kb EcoRI fragment containing the 5' region of the α1(I) collagen gene (Breindl et al., 1984). Two of these hypersensitive sites were found in both collagen and non-collagen producing mouse cells; the third, which is associated with a transcriptionally active α1(I) collagen gene, maps 100-200bp 5' to the RNA transcription start point. This site was absent in the Mov 13 mutant gene, as shown in heterozygous Mov13 cell lines. Thus, integration into the

Mov13 locus was shown to be the direct cause of an altered chromatin structure. Methylation patterns of Mov13 and control parental lines were analyzed at different developmental stages to further study the effect of the provirus on host DNA (Jähner and Jaenisch, 1985). In the Mov13 line, host DNA was unmethylated before proviral insertion, and was methylated at 4 sites within 700bp 5' to the provirus after integration.

Methylation was also demonstrated 3' to the viral insertion site. Since Mov13 sperm were unmethylated, these sites represent a de novo methylase activity. These de novo methylated sites were present only in the Mov13 allele. The induction of changes in chromatin conformation and DNA methylation patterns in flanking host DNA by provirus, and the association of viral integration with gene inactivity strongly suggest that these structural changes of host DNA are the cause of gene inactivity.

Woychik et al. (1985) reported an insertional mutation discovered in mice created for studies on tumorigenesis. Of thirteen transgenic mouse strains carrying MMTV-myc fusion genes (Stewart et al., 1984), seven were crossed to homozygosity to uncover recessive mutations. Heterozygous crosses in one line resulted in abnormal offspring with bony abnormalities including synostosis of the long bones, oligodactyly, fusion of some carpals, tarsals, metacarpals and metatarsals, and syndactyly, with a normal axial skeleton (Woychik et al., 1985). This mutation resembles the spontaneous recessive mouse mutation limb deformity (ld). Another mutation known as Strong's luxoid (Forsthoefel, 1962) is a dominant mutation which produces the opposite effects; extra bones and digits are present in these mice. All three of these mutations map to the same region on chromosome 2, suggesting that they all affect the same gene.

Matings were constructed to demonstrate cosegregation of the mutant phenotype with the MMTV-myc fusion gene. When the abnormal animals were assessed for the presence of the micro-injected fusion gene, only homozygous offspring demonstrated the abnormalities; all heterozygotes and wild-type mice had normal phenotypes. Therefore, the mutant phenotype co-segregated with the site of insertion of MMTV-myc fusion gene, permitting the locus to be cloned due to its close linkage with the microinjected genes. A novel and efficient cloning approach was used which exploited the

fact that pBR322 sequences had been co-microinjected to produce this line. A cosmid vector was used which had an intact kanamycin resistance gene, permitting selective growth in bacteria; its ampicillin resistance (Amp^r) gene was inactivated. When this vector was ligated to partially digested genomic DNA from transgenic mice, only clones receiving an intact Amp^r gene could grow on plates containing ampicillin. This led to the isolation of 9 kb of genomic DNA linked to the site of insertion. A subclone of this region was then used to isolate the intact locus from a cosmid library constructed from wild-type mouse DNA.

Comparison of these two loci indicated a loss of approximately 1 kb of DNA in the mutant allele, but no gross rearrangements. The use of subcloned DNA as probes against a hamster-mouse hybrid cell panel demonstrated that the ld^{Hd} locus was on chromosome 2, the same chromosome as the phenotypically similar mutations, ld^J and ld^{OR}, observed at Jackson and Oak Ridge Laboratories, respectively (Woychik et al., 1985). A probe spanning one side of the insertion site was used to distinguish the three mutant alleles by assigning unique restriction-length polymorphisms (RFLP's) (Botstein et al., 1980) to each mutation on Southern blots. To find out whether these mutations were allelic, complementation analysis was performed by crossing heterozygotes carrying different mutations. Mice with any two different mutant alleles exhibited the ld phenotype, unequivocally demonstrating allelism of these three mutations.

In this work, as in Wagner's characterization of the HUGH lines, c-myc expression in the limb bud was not formally ruled out as a cause of the limb mutation. Although expression was observed in mammary gland, testis, and salivary gland, it was not evaluated in the developing limb bud; therefore it is not possible to rule out tissue-specific expression of MMTV-myc with a gene dosage effect to account for recessive inheritance of the phenotype. The authors present two arguments against this possibility. First, they point out that the two other limb deformity mutations do not involve aberrant c-myc transcripts from this locus. Second, they reason that since MMTV-myc is dominant in the development of cancer but the ld phenotype is recessive, myc gene expression cannot account for the limb mutation.

Overbeek et al. (1986) microinjected a fusion gene with the RSV LTR linked to the CAT structural gene. Of nine lines produced, two exhibited insertional mutations. One line contained a dominant embryonic lethal mutation which was transmissable by mice with a balanced translocation. Another line had a recessive mutation with a phenotype of fused toes in all four feet.

Mark et al. (1985) reported an insertional mutation caused by microinjection of λRßG2, a Charon 4A phage clone containing adult rabbit ß-globin genes. Line 4 contained 3-4 copies of phage in a head-to-tail tandem array, which were localized to chromosome 3 by in-situ hybridization and shown to exhibit a low level of inappropriate expression of rabbit globin mRNA in mouse skeletal muscle (Lacy et al. 1983). Existence of a prenatal mutation was indicated by failure to obtain homozygous offspring from 64 progeny derived from heterozygous crosses as judged by quantitative dot blot analysis. By culturing embryos derived from heterozygous crosses, developmental arrest was determined to occur at the time of implantation.

Shani (1986) microinjected a chimeric rat skeletal muscle actin/human embryonic globin gene and produced 11 lines of transgenic mice. Line CV4 failed to produce homozygous offspring among 45 progeny derived from heterozygous crosses, as judged by Southern blot analysis, using a single copy control probe. A small average litter size was consistent with prenatal loss of homozygotes. Developmental arrest was determined to occur before midgestation, as no abnormal fetuses were found at this stage.

ß-Globin Transgenic Mice

A remarkable application of transgenic mice has been to study mechanisms of evolution of the ß-globin gene cluster in mouse and man. The demonstration that human fetal genes are expressed in embryonic mouse tissues suggests that the timing of expression of a trans-acting factor was the mechanism for fetal recruitment of simian globin during evolution (Chada et al., 1986). Kollias et al. (1986) confirmed these findings and extended the analysis using a hybrid human 5' fetal gamma-globin/3' adult Beta-globin gene; as predicted by MEL experiments, this hybrid gene was expressed during all stages of mouse development.

Early studies of foreign ß-globin expression in transgenic mice showed a wide disparity of experimental results (Wagner et al., 1981; Costantini and Lacy, 1981; Stewart et al., 1982; Lacy et al., 1983). Later, it became apparent that the inability of a particular construct to express was related to retention of vector sequences, and that tissue- and stage-specific expression of ß-globin were in fact achievable (Chada et al., 1985; Townes et al., 1985). Low levels of ectopic globin mRNA expression without detectable protein, which occurred in some lines, was most likely due to chromosomal position effects (Lacy et al., 1983; Chada et al., 1985).

In one of the earliest publications on transgenic mice, Wagner et al. (1981) reported the production of mice containing adult rabbit globin genes, along with vector sequences, which produced high levels of rabbit globin protein in mouse erythrocytes. However, the lack of appropriate controls or RNA analysis, and the pooling of samples made these results difficult to interpret.

Subsequent reports of foreign globin in transgenic mice revealed quite the opposite results (Costantini and Lacy, 1981; Stewart et al., 1982; Lacy et al., 1983). Stewart et al. (1982) microinjected a plasmid consisting of a 7.6kb HindIII fragment of human ß-globin, the HSV-tk gene and pBR322 sequences and analyzed expression in the resultant transgenic mice. The presence of human ß-globin mRNA in liver, not a major hematopoietic organ at this stage, was tested for by S1 nuclease mapping; no human ß-globin mRNA was found. These experiments therefore did not rule out the possibility of tissue-specific expression.

Costantini and Lacy (1981) microinjected the lambda recombinant vector λRßG2 consisting of the rabbit adult ß-globin gene inserted into a Charon 4A phage. Nine transgenic lines carrying intact adult rabbit ß-globin DNA were identified. Erythrocyte hemolysates from all 9 lines and several offspring were subjected to fractionation by acid-urea-Triton polyacrylamide gel electrophoresis and isoelectric focusing in denaturing gels (Lacy et al., 1983). No rabbit ß-globin protein was detected.

Rabbit ß-globin mRNA was assayed by S1 nuclease analysis using a 3' probe which can distinguish rabbit and mouse ß-globin mRNA's. RNA was isolated from spleens from mice treated with phenylhydrazine to produce anemia, or from bone marrow taken from untreated mice. No rabbit-specific transcripts were detected; using control mouse probes, endogenous mouse globin transcripts were demonstrated. The sensitivity of this assay indicated that rabbit-specific transcripts were at least 10^5 to $5x10^5$ times less abundant than the mouse ß-globin mRNA. These results were in contrast to those of Wagner, who reported such high levels of tissue-specific expression of rabbit ß-globin chains that his mice developed a relative alpha-thalassemia.

To test the possibility of inappropriate expression, mRNA from non-erythroid tissues of mice from 7 lines was similarly tested by 3' S1 nuclease mapping. One mouse expressed rabbit globin in skeletal muscle and at levels about 20 times lower in brain, testis, and lung. Another mouse expressed rabbit globin in testis. Each of these mice expressed foreign globin with correctly terminated 3' ends, at levels representing 10^{-7} of total steady-state mRNA. These results were reproducible among siblings, and in the line which expressed rabbit ß-globin mRNA in skeletal muscle, this pattern of ectopic expression was shown to be a heritable trait across several generations. Correct initiation of 5' termini, an important criterion of correct initiation versus readthrough from upstream mouse promoters, was analyzed by S1 nuclease analysis using a probe spanning the first two exons and extending 99bp upstream of the cap site. Protection of the probe extended to the cap site, indicating correct initiation of rabbit ß-globin mRNA. Finally, this analysis demonstrated correct splicing of the first intron.

To address the issue of species divergence as an explanation for the lack of correct gene regulation, transgenic mice were produced carrying a hybrid mouse/human ß-globin gene (Chada et al., 1985). This construct, which is appropriately regulated in MEL cells (Chao et al., 1983), consists of 5' mouse sequences and 3' human sequences fused at a conserved BamHI site in the second exon. This experiment tests the ability of mouse ß-globin to function in different chromosomal positions (similar to mouse immunoglobulin in transgenic mice); the human sequences provide an experimental means for distinguishing endogenous from exogenous mouse globin mRNA. Placing this hybrid gene into mice is primarily a test of the temporal- and tissue-specific controls of exogenous mouse ß-globin. It also tests whether the presence of human 3' sequences is permissive for these two levels of control of exogenous mouse ß-globin in transgenic mice.

The hybrid gene was injected either as an intact plasmid, from which three positive mice resulted, or as a ClaI-BglII fragment lacking most plasmid sequences, from which seven positive mice resulted. RNA was isolated from blood and bone marrow, from spleen after phenylhydrazine treatment, and from non-erythroid tissues, hybridized against a 3' human probe and subjected to S1 nuclease analysis. Four lines, which all contained the ClaI-BglII insert, specifically expressed the hybrid gene in erythroid cells; however, the three lines retaining vector sequences did not express globin at appreciable levels. The highest level of expression in mice lacking vector sequences was 2-4% of endogenous expression. Primer extension analysis using a labelled primer from the third exon of the human globin gene showed the hybrid transcripts to be correctly initiated. Ectopic expression occurred at very low levels in testis of one line and heart and kidney of another line (contamination with circulating erythrocytes was ruled out by comparison to endogenous ß-globin mRNA).

This was the first study proving that a globin transgene could be expressed in a tissue-specific manner when vector sequences were removed. Inhibitory effects of pBR322 were indicated by a lack of expression in three mice containing the entire construct; these effects were confirmed by Townes et al. (1985).

Magram et al. (1985) analyzed the temporal characteristics of hybrid globin gene regulation in their mice to see if this expression paralleled adult mouse globin. RNA from transgenic embryos was isolated from blood of 10.5 to 11.5 day embryos (primitive erythrocytes originating from the yolk sac), fetal livers of 12.5 to 17.5 day fetuses (primarily fetal erythroblasts), and adult bone marrow. S1 nuclease mapping showed that hybrid mRNA was absent from primitive erythrocytes (which synthesize only embryonic y and z chains at this stage), and was present in fetal liver and adult bone marrow (which synthesize only adult globins). Using adult and embryonic mouse probes, endogenous globin expression was demonstrated at the expected times and in the appropriate cell lineages. Therefore, the developmental regulation of hybrid gene expression was identical to that of the mouse adult ß-globin genes. These results showed that stage-specific regulation can be mediated by globin transgenes which lack extensive flanking sequences; as little as 5kb of adult globin DNA can serve to activate transcription in fetal and adult life specifically in cells which produce adult globins. The data also indicated that the presence of human globin sequences 3' to the fusion site in the second exon are not inconsistent with correct developmental gene regulation in transgenic mice.

Human globin studies in MEL cells have shown that the exogenous human adult α- and ß-globin genes contain the necessary sequence information to be appropriately regulated during terminal erythroid differentiation in mouse cells, indicating that these sequences are not species-specific (Chao et al., 1983). A further specificity of the MEL system is reflected by the finding that epsilon, gamma, and zeta (embryonic alpha) genes are not induced during this differentiative transition. A prediction of correct regulation might tentatively be made for human globin genes in transgenic mice during the analogous differentiative transition of the proerythroblast to the terminally differentiated mouse erythroid cell, notwithstanding such differences as DNA processing between the MEL and transgenic mouse systems. However, the cell-type specific expression of human globin genes in transgenic mice has the additional, more demanding requirements that the developmental mechanisms which mediate both the timing of human globin expression and restriction to erythroid lineages be conserved between species. If these more stringent requirements are fulfilled, as would

be shown by correct temporal- and tissue-specific regulation of human globin genes in transgenic mice, then the developmental changes which correlate with expression of the human globin transgene can be studied to elucidate the mechanisms of developmental gene regulation (Radice and Costantini, 1986).

Townes et al. (1985) carried out a comprehensive analysis of human adult ß-globin genes in transgenic mice. A series of constructs with 5' deletions were made having 4300, 815, 360, 122, or 48 base pairs of 5' flanking sequence. Twenty mice without vector sequences and sixteen mice with vector sequences were produced carrying up to 4300bp of 5' flanking DNA and a constant 1700bp of 3' flanking DNA. Human and mouse ß-globin mRNA's were distinguished using two 21-base oligomers which are complementary to a region in exon 1 which diverges between these species. To quantitate mRNA levels, the assay (S1 nuclease treatment of mRNA/21-mer hybrids) was standardized with known amounts of recombinant single-stranded M13 containing human globin. Results showed erythroid expression of human ß-globin in 15 of 20 transgenic mice lacking vector sequences, but very low or undetectable expression in mice retaining vector sequences. These results confirmed that human globin genes can be appropriately expressed in transgenic mice if vector sequences are removed.

Using the analogous mouse 21-mer, endogenous expression was quantitated to facilitate a comparison of human and mouse globin expression. Expression varied between mouse lines with the same construct, but mice produced from each set of deletions produced correctly initiated human ß-globin mRNA. The highest level seen was in a mouse containing 122bp of 5' sequence (hßG/mßG=0.98, equal to 1900 molecules of human ß-globin per cell). Remarkably, two mice with only 48bp of 5' sequence expressed human ß-globin in blood cells.

Tissue-specificity of expression was analyzed by solution hybridization of mRNA from several tissues of phenylhydrazine-treated animals. In none of the five non-expressing mice (in blood) was human globin detected in ectopic tissues. However all mice which did express human globin in blood also had detectable levels in other tissues. Comparison with endogenous mouse globin levels as well as perfusion experiments suggested that these human signals were due to contamination of

tissues with circulating erythrocytes, and that human globin expression was appropriately restricted to erythroid tissues.

Developmental regulation was assessed by performing solution hybridization of nucleic acids isolated from 11-day fetuses, 14-day fetal livers, and adult reticulocytes, using probes for mouse embryonic eY globin mRNA, mouse adult globin mRNA, and human adult ß-globin mRNA. At 11 days, mouse and human mRNA's were barely within the limits of detection, whereas embryonic globin was abundant. At 14 days, mouse and human adult mRNA's increased to detectable levels in fetal liver and eY dropped significantly. Embryonic mRNA was undetectable in adult reticulocytes, while the mouse and human adult globins rose to high levels. Thus the human adult ß-globin transgene contains the necessary sequence information to be transcriptionally activated in a temporal- and tissue-specific pattern analogous to that of the endogenous mouse adult ß-globin genes.

Both the ability of human globin mRNA to function in the synthesis of protein, and the persistence of human ß-globin chains in red blood cells were demonstrated. ^{35}S-methionine was used to label protein made from reticulocyte lysates, and these proteins were mixed with unlabelled human hemoglobin. After separation on a carboxymethylcellulose column, analysis clearly indicated that the human globin mRNA is functionally translatable, although perhaps not as efficiently as mouse ß-globin mRNA. In addition, isoelectric focusing performed on hemoglobin from mature erythrocytes demonstrated a new band which comigrated with the human ß-chain control, indicating that human globin mRNA is translated in vivo and that human ß-globin chains accumulate in red blood cells in transgenic mice.

A further globin study was performed by Humphries et al. (1984, 1985). λHßG1, the same vector used to produce ß80 and ß19, was used by Humphries for microinjection experiments. Six live animals resulting from microinjected eggs were treated with phenylhydrazine and DNA and RNA were extracted from the erythroid spleen. Genomic DNA was digested with PstI and probed with ^{32}P-cDNA from human reticulocyte RNA. One mouse showed two bands on Southern blot analysis, corresponding to the intact δ-globin and ß-globin genes. The blotting procedure did not determine whether lambda sequences were present in this line, although there is no reason to expect

they were lost. When trying to mate this male, pregnancies were arrested during midgestation and fetuses were resorbed. However at one year of age two offspring were sired, one of which was positive for δ- and ß-globin. Transmission was achieved for second generation offspring, and male subfertility was again observed in this cross. RNA analysis of the original transgenic mouse and the next generation offspring by solution hybridization or S1 nuclease mapping (enabling a detection sensitivity of 5 copies globin mRNA per cell) showed that no human globin message was detectable in erythroid spleen. Offspring of the founder mouse were also assessed for expression in ectopic tissues, but no human globin gene expression was detected in liver, testes, brain, or skeletal muscles. Consistent with a lack of expression was the observation of extensive methylation of the foreign globin genes at HpaII sites in both erythroid and non-erythroid tissues. Thus, the de novo methylation activity decribed in other transgenic mice is operational on genomic sites in this clone.

Taken together, studies of foreign globin genes in transgenic mice indicate that narrow regions of adult globin genes can suffice to confer correct time- and tissue-specific gene regulation, if vector sequences are removed prior to microinjection. In cases where ectopic expression was observed, mRNA expression was detected at very low levels. However the demonstration that globin developmental regulatory mechanisms are conserved between species provides an opportunity to study these mechanisms in transgenic mice (Radice and Costantini, 1986), and to analyze the developmental alterations made during the evolution of the globin gene cluster (Chada et al., 1986; Kollias et al., 1986).

Transgenic Mice and Mechanisms of Oncogenesis

Previous studies on cancer in transgenic mice have involved microinjection of various oncogenes, and the development of cancer has been dependent on the expression of these foreign genes. Tissue-specificity of tumorigenesis was first shown to occur in mice which contain the SV40 enhancer, the MT-1 promoter, and T-antigen genes; these mice developed tumors of the choroid plexus (Brinster et al., 1984). Through a series of deletion experiments, the control region was localized to the SV40 enhancer (Palmiter et al., 1985), and a functional large T-antigen was shown to be essential for tumorigenesis (Messing et al., 1985; Palmiter and Brinster, 1986). The propensity toward and timing of tumor formation was variable between lines of mice, but constant within a particular line, indicating that the site of integration determines the level and timing of T-antigen expression. In mice destined to form tumors, expression was shown to occur early during development (Van Dyke et al., 1985). It is interesting that a late-developing, tumor-producing revertant in a previously normal lineage with no detectable SV40 gene alterations exhibited demethylation in tumor tissue, implicating loss of methylation sites as a cause of derepression of SV40 genes. In other mice, a new pattern of pathology appeared when the SV40 enhancer was deleted but the MT-1 promoter was left intact (Messing et al., 1985). These mice developed peripheral neuropathy, hepatocellular carcinomas and islet cell adenomas. The authors postulate that removal of otherwise dominant SV40 sequences unmasks an enhancing effect of MT sequences on T-antigen genes. When placed under the control of the promoter/enhancer of the rat insulin gene, SV40 T-antigen expression and tumorigenesis were directed to the ß-cells of the endocrine pancreas (Hanahan, 1985). When the SV40 T-antigen was placed under control of the elastase 1 promoter, tumors were produced in the exocrine pancreas (Ornitz et al., 1985).

The direction of T-antigen expression to the lens by the murine alpha A- crystallin promoter resulted in lens tumors (Mahon et al., 1987), which are not produced naturally in vertebrates. Tissue-tropism of tumorigenesis seen in human tumors caused by JC and BK viruses followed a similar tissue distribution in transgenic mice (Small et al., 1986a, 1986b), and bovine papillomavirus directed tumor formation to the skin (Lacey et al., 1986).

In transgenic mice carrying T-antigen under the control of SV40 (Brinster et al., 1984; Van Dyke et al., 1985), the relatively long time which elapsed between the time of T-antigen expression and the formation of ependymomas suggested that a second event was required for tumorigenesis. Similarly, mice carrying bovine papillomavirus developed skin tumors after a latency period (Lacey et al., 1986). In insulin-SV40 transgenic mice (Hanahan, 1985), although T-antigen was expressed in all ß-cells and hyperplasia and tumor cell formation were consistent findings in this line, some mice were shown to express T-antigen before the onset of ß-cell proliferation or tumor production, and only a fraction of islet cells formed tumors in each mouse. This was interpreted to mean that T-antigen expression facilitated islet cell proliferation, and a second event was required for the formation of a clonal tumor (Hanahan, 1985).

Microinjection of the myc oncogene has similarly demontrated that tissue-specificity of gene expression is conferred by the enhancer/promoter region, and has also indicated that oncogenicity requires the occurence of a second mutational event. Stewart et al. (1984) showed that mammary adenocarcinomas develop in mice carrying a myc fusion gene under MMTV control. The requirement for two to three pregnancies to occur before tumors developed, and the absence of bilateral tumor formation suggested that an additional transforming event is required. In a strain which expressed exogenous myc in a wider distribution of tissues (Leder et al., 1986; Pattengale et al., 1986), oncogenicity of myc was extended to tissues other than breast, and it was again shown that myc expression is a necessary but not a sufficient condition for transformation (Leder et al., 1986).

When placed under control of immunoglobulin enhancers, myc causes lymphoid tumors (Adams et al., 1985, 1986; Langdon et al., 1986; Alexander et al., 1987). The development of tumors in a tissue which constitutively expresses myc is a finding in line with the known mechanisms of myc deregulation (Varmus, 1984; Cole, 1986): enhancer insertion (Hayward et al., 1981; Neel et al., 1981; Payne et al., 1982), chromosomal translocation (Klein, 1983; Cory, 1986), and gene amplification (Varmus, 1984; Alitalo and Schwab, 1986). Although formation of lymphoid tumors was observed in all mice, the requirement for a second genetic event was indicated by a latency of tumor formation, a pre- neoplastic stage of constitutive myc expression in polyclonal pre-B cells without tumor

progression, a monoclonal origin of tumors, and the inability of transplanted prelymphomatous bone marrow to cause malignancy in syngenic recipients (Langdon et al., 1986). Transgenic mice carrying a c-fos oncogene construct which stabilizes the c-fos transcript and allows high levels of expression in a variety of tissues (Rüther et al., 1987) developed bone tumors of low-grade malignancy. The early onset of tumor formation indicated that a second event was not required, but the lack of tumor progression indicated that bone lesions involved pre-neoplastic changes. Transgenic mice carrying oncogenic c-Ha-ras genes under control of the elastase promoter/enhancer indicate a transforming potential of ras, as pancreatic acinar cells became transformed only a few days after their appearance in fetal development (Quiafe et al., 1987). In contrast, a latency period is seen when an activated human Ha-ras gene is placed under the control of lactogenic hormones (Andres et al., 1987). The difference between these ras lines may reflect an immortalizing potential of a differentiating embryonic tissue, which is absent from a hormone- induced tissue; the latter would require a second mutation, necessitating a latency period. The physiological evidence for a multistep mechanism seen in transgenic mice is consistent with in vitro evidence that progression from a hyperproliferative state to a tumorigenic state (Klein and Klein, 1985) requires the involvement of a second oncogene (Land et al., 1983; Glaichenhaus et al., 1985). The finding that human tumors usually express more than one oncogene (Slamon et al., 1984) is consistent with the hypothesis that cooperativity of oncogenes is necessary for tumorigenesis. In a highly simplistic view of this "two-hit" paradigm, immortalization (Kingston et al., 1985; Nevins, 1986) and transformation (Der et al., 1986) functions would be supplied, respectively, by the nuclear activity of myc and the cytoplasmic activity of ras (Murray et al., 1983).

The hypothesis that abnormal chromosomes are involved with malignancy (Boveri, 1914) is best supported by hemotologic malignancies involving chromosomal translocations (Yunis, 1983; Croce, 1987). Two hematologic malignancies, acute lymphocytic leukemia (ALL) and chronic myelogenous leukemia (CML), have been shown to follow a consistent pattern of chromosomal translocations, and each of these has been associated with an oncogene. In ALL and Burkitt lymphoma, translocations involve exchanges between chromosome 8, the location of c-myc, and other chromo-

somes carrying immunoglobulin genes (Leder et al., 1983; Varmus, 1984; Cory, 1986). Murine plasmocytomas follow the same genetic pattern of chromosomal translocations involving c-myc on chromosome 15. The exploration of this system in transgenic mice can be likened to direct germline insertion of the c-myc/immunoglobulin translocation. Transgenic mouse studies (Adams et al. 1985) have demonstrated an active role of the immunoglobulin enhancer in myc deregulation for which the myc gene alone is insufficient. Langdon et al. (1986) have suggested that the level of myc determines the balance between self-renewal and differentiation of benign polyclonal pre-B cells in transgenic mice. In this model, higher levels of myc promote self-renewal, and a random mutation, such as activation of a second oncogene, is required for tumorigenesis. Consistent with the necessity of an additional mutation, complementation of myc by N-ras is indicated in a Burkitt lymphoma (Murray et al., 1983), and by v-abl (Ohno et al., 1984) and c- mos (Rechavi et al., 1982) in plasmocytomas.

METHODS I.

Transgenic Mouse Production

The procedure of transgenic mouse production has been described in detail (Gordon et al., 1980; Gordon and Ruddle, 1983) and is performed as follows. Three days before microinjection, immature albino females are superovulated with intraperitoneal injections of 5 I.U. of pregnant mares' serum (PMS) followed 48 hours later by 2.5 I.U. of human chorionic gonadotropin (hCG). Ten females are placed to mate with normally pigmented males and examined the following morning (day 0) for the presence of vaginal plugs. Fertilized eggs derived from these crosses can give rise to black or agouti mice, but not albino mice. On the same evening, randomly cycling mature albino females are placed to mate with albino vasectomized males and also examined the following morning for vaginal plugs. These pseudopregnant females are used as foster mothers for the development of microinjected eggs. With this genetic marking system, inadequately vasectomized males sire albino pups, which are distinguished from pigmented pups born from microinjected eggs.

At 2 P.M. of day 0, immature plugged females are sacrificed and their ovaries and oviducts are placed into bicarbonate buffered M16 medium (Quinn et al., 1982). Oviducts are opened at the ampulla under a dissecting microscope in M16 medium supplemented with 1 mg/ml hyaluronidase. Eggs are then washed three times with medium and eggs with prominent pronuclei are loaded into culture dishes containing a drop of M2 medium (Whittingham, 1971) under mineral oil.

Pipets for holding the embryos are made from 1mm glass tubing using a microburner and microforge. Microneedles are pulled on a vertical pipet puller from Omega dot tubing (Glass Co. of America). The holding pipet and microneedle are placed in Leitz micromanipulators fitted to a phase contrast microscope.

The embryos are placed onto the stage. An embryo is fixed to the holding pipet by suction and oriented so that a pronucleus is well positioned for microinjection (figure 1). The microneedle is inserted into the pronucleus and enough DNA solution is injected to cause a visible swelling of the pronucleus (about 1 picoliter). After microinjection, eggs are returned to the incubator and examined

after about one hour. Surviving eggs are then identified and implanted into the oviducts of pseudopregnant females. Pups are born after three weeks, and after about one month of age a DNA sample is extracted from spleen and analyzed by Southern blotting to determine whether microinjected DNA has been retained in adult tissues.

Globin Clones used for Microinjection

The two lines of transgenic mice described in this thesis were created by microinjecting λHßG1, a Charon 4A clone (Blattner et al., 1977) obtained from Tom Maniatis (Fritsch et al., 1980) (figure 2). This recombinant phage clone contains 15.9kb of human genomic insert from the adult ß-globin locus. The genomic insert contains the delta- and beta-globin genes, in addition to two pairs of Alu sequences, and one sequence with Alu-like homology. The recombinant phages λHγG1 and λHεG1 were also microinjected (Fritsch et al., 1980). The ßpst clone containing a 4.4kb PstI fragment spanning the ß-globin gene was microinjected, and was used in a ligation reaction with pFR400, which contains a mutant mouse DHFR (dihydrofolate reductase) gene (Simonsen and Levinson, 1983); this material was also microinjected.

Phage DNA was isolated from liquid lysates (λHεG1) or plate lysates (λHßG1 and λHγG1) as described (Maniatis, Fritsch and Sambrook, 1982). λHßG1 produced insufficient titers to be grown in liquid culture. Conditions for confluent lysis were determined and verified by titering phage obtained from washed plates. Then a plating procedure was performed. Phage DNA was then isolated, analysed, and prepared for microinjection. λHγG1 deleted internal sequences as reported previously (Slightom et al., 1980) and was therefore grown on plates.

Identification of Transgenic Mice and Foreign Gene Analysis

All live offspring and several animals which died shortly after birth were subjected to DNA analysis according to the method of Southern (1975). DNA samples for initial screening of putative transgenic animals were isolated from spleens which were surgically removed under anaesthesia, or from whole animals which died hours or days after birth of unknown causes. (Attempts to screen

animals by Southern analysis of tail DNA were unsuccessful since the relative impurity of these preps did not always permit cutting by restriction enzymes.) Tissues were pulverized in liquid nitrogen (N_2), extracted (Blin and Stafford, 1976) successively with phenol, phenol/chloroform with isoamyl alcohol, and chloroform, dialyzed extensively against 1xTEN (10mM Tris-HCl pH 8.0, 10mM NaCl, 0.1mM EDTA), precipitated with >2x volumes of ethanol (EtOH), dried, and resuspended in 1xTEN. DNA concentration was measured by spectrophotometry at 260/280nm, and 10ug of DNA was digested to completion with an appropriate restriction enzyme. DNA samples were run overnight on 1% agarose gels. After ethidium bromide (EtBr) staining and photographing of gels, DNA was transferred to nitrocellulose filters. These filters were baked either for 2 hours at 80°C in a vacuum oven or at 67°C overnight. Radioactive probes were prepared by nick-translation of the entire microinjected construct. The use of complete recombinant phage probes facilitated analysis of lambda sequences in mouse tissues and made the probes relatively less specific for mouse globin sequences. ^{32}P probes were labelled to a specific activity of $1\text{-}3x10^8$ cpm/ug with best results obtained for approximately 20-75% incorporation of labelled dCTP nucleotide. Overnight hybridization reactions were performed as described (Wahl et al., 1979) either at 65°C without formamide or at 42°C in 50% formamide, using 5-7ng/ml of probe. Filters were washed, dried, and placed against X-ray film for 2-3 days at -70°C. The presence of bands specific to the microinjected human globin sequences indicated successful production of transgenic mice.

Copy number in positive animals was assessed by dilution of digested genomic DNA and comparison to known amounts of standards. Direct assessment of hemizygosity or homozygosity of the transgenic allele was similarly made by comparison of known amounts of DNA on Southern blots. Integration in tandem linear arrays was indicated by the presence of 30kb bands corresponding to left and right phage arms joined in the transgene (Lacy et al., 1983). Mosaicism of original transgenic animals was assessed by germline testing as well as by comparison of hybridization intensity of adult tissues to tissues obtained from F_1 offspring in Southern blots. The number of sites of integration was analyzed by germline testing. Integration was indicated by the presence of junction fragments visible in Southern blots, in addition to germline transmission.

F_1 mice were routinely screened by dot blot analysis of DNA isolated from tails. The dot blot analysis greatly reduces the steps necessary for the identification of positive animals in cases where the foreign gene copy number is high, since the DNA is not restricted or electrophoresed, but analyzed based simply on total hybridization intensity. Initial screening of putative transgenic mice by dot blot procedure, though considerably less arduous than Southern analysis, could miss single-copy transgenic globin mice; therefore all potential founder mice were screened using Southern analysis. All positive mice containing Charon 4A recombinant phage were rescreened by dot blot analysis using lambda phage, a probe specific for the foreign insert.

For extraction of DNA from tails (Davis et al., 1980), approximately the last third of the tail was biopsied. The bone and cartilage were removed with forceps, and the skin sliced into several pieces. This was placed into 500ul tail extraction buffer (50mM Tris pH 8.0, 0.1M EDTA, 0.5% SDS), and incubated overnight at 50°C with 5ul proteinase K (20mg/ml). The next morning the tube was placed at 65°C for 15', vortexed or shaken vigorously, and centrifuged for 5'. The supernatant was removed, and to this was added 500ul $CHCl_3$. This was mixed well, centrifuged for 5', and the supernatant was removed to a new tube. To this was added 200ul 5M potassium acetate. Then the tubes were mixed well and placed on ice for 30', centrifuged for 5', and 900ul of EtOH was added to 450ul of the supernatant. The DNA was centrifuged 2', and the supernatant decanted and discarded. The precipitate was resuspended in 150ul 1xTEN, and its concentration measured using a spectrophotometer. In general, DNA of this quality is not amenable to Southern analysis since it is too impure to be digested by restriction enzymes.

Subclones and Probes used in Various Analyses

The λHßG1 phage was subcloned by digestion with EcoRI followed by ligation to EcoRI-linearized pBR322. HB101 bacterial cells were transformed with this ligated DNA (Maniatis, Fritsch and Sambrook, 1982) and transformants were selected by growth on ampicillin plates. Such bacterial colonies can contain either pBR322 subclones containing globin inserts or recircularized pBR322. Therefore colony hybridizations were performed (Maniatis, Fritsch and Sambrook, 1982) using

whole phage as a probe. Hybridizing colonies were then grown in small cultures under selection and minipreps (Maniatis, Fritsch and Sambrook, 1982) were performed to analyze the insert DNA. Miniprep DNA was digested with EcoRI, which produces a 4.4kb pBR322 band and diagnoses the insert according to size. In this way, all EcoRI fragments comprising the genomic insert were isolated.

A plasmid designated ßpst (courtesy of F. Ruddle, Yale University), containing a 4.4kb genomic clone spanning the adult ß-globin gene and two Alu sequences inserted into the PstI site of pBR322, was used to assess presence of the ß-globin gene, and to analyze the restriction pattern of ß19.

A clone designated ßHindIII was provided by F. Costantini (Columbia University). This clone, which contains a 7.8kb fragment spanning the human adult ß-globin gene, was used in certain cases to screen transgenic offspring, and was used to assess the presence of genomic globin sequences in cosmid clones.

A cosmid clone containing the human fetal and adult globin genes, cosHG28tk, was obtained from F. Grosveld (1981, 1982). This clone served as a control in the hybridization analysis of cosmid clones derived from the ß80 line, and was used as a positive control to assess the presence of genomic sequences in cosmid clones.

Cosmid fragments were subcloned by ligating enzyme-restricted cosmid clones to pGEM3 (Promega Biotec) digested with the same enzyme and treated with Calf intestinal phosphatase (CIP, Boehringer Mannheim) (Maniatis, Fritsch, and Sambrook, 1982) to prevent self-ligation. These could be directly analyzed by plasmid minipreps (Maniatis, Fritsch and Sambrook, 1982), without colony hybridization.

A clone designated λEB was created by subcloning the left arm of lambda into pGEM3. This clone included λ from the BamHI site at 5505bp to the EcoRI site at 21226bp, a 15.7kb insert. The λEB probe contains most of the sequences of the left arm of Charon 4A, but lacks the cos sequence. This clone was used in a pilot experiment (figure 3) to determine if whole nick-translated lambda could be used to probe a cosmid library despite the presence of the cos sequence. In addition, the

electroeluted λEB fragment was used to assess the content of cosmids derived from the cosIII library.

The clone pBLUR8 was used to directly assess the presence of human Alu sequences in the some mice. This clone contains a 300bp fragment comprising a human Alu sequence cloned into pBR322 (Jelinek et al., 1980; Deininger et al., 1981).

ß31A, which contains the 3.1kb and 5.5kb fragments of the λHßG1 locus in pBR322, was used to probe DNA from the ß19 line.

One useful subclone contained the 3.1kb EcoRI fragment upstream of the delta-globin gene. (This fragment was further subcloned into pUC9.) The 3.1kb fragment contains no globin coding sequences, but has two Alu sequences. This fragment was used in the analysis of cosmid clones derived from the cosIII library of the ß80 line, and was also used to probe the cosI library containing ß80 DNA.

Two probes were constructed for future transcriptional analysis of delta- and Beta-globin in this line. Originally intended for studies on tissue-specific expression, these probes were not used for these purposes since we felt it unlikely that these genes were transcriptionally active (see discussion).

Clone pSP64ß (Green et al., 1983) was created in two steps, first, a 3.0kb HincII/PstI fragment spanning the adult ß-globin gene was cloned into pSP64 (Promega Biotec) in an antisense orientation (with 3' sequences of the globin gene closest to the SP6 promoter). Then, a Bal31 reaction was performed on sequences at the 3' end of the gene (closest to the SP6 promoter) to reduce the size of the RNA antisense transcript produced by this probe. This was done because RNA transcripts are difficult to extend beyond approximately 500 nucleotides using RNA transcription vectors, and the original vector would have produced a 767nt transcript using the desired EcoRI site for linearization; see below. (Heterogeneous-sized transcripts, as would most likely be produced by RNA polymerase, would result in heterogeneous, uninterpretable signals using RNAse mapping techniques.)

The 8.8kb ßpst plasmid was linearized with SalI to destroy the HincII site in pBR322 which would have competed in the ligation reaction (and produced an unwanted pBR322 ligation product

indistiguishable by size from the desired fragment). Next, HincII was added and the predicted fragments of complete digestion diagnosed by gel electrophoresis. PstI was used to digest these fragments, producing the desired heterologous-ended fragment. This was directionally cloned into pSP64 digested with PstI and HincII; 2 out of 8 colonies subjected to miniprep analysis contained the target vector. These were diagnosed using PvuII, AccI, BamHI, and PstI plus HincII, and shown to contain the ß-globin gene in the correct, antisense orientation; cloning of the unwanted 3.0kb pBR322 fragment was ruled out.

The 3' end of the globin gene was shortened by using restriction exonuclease Bal31 (Maniatis, Fritsch and Sambrook, 1982) on non-coding genomic sequences. First, the plasmid must be linearized at the desired site of exonuclease digestion. There is a BalI site between the poly(A) addition signal and the PstI site (at the SP6 promoter). Since this site is closer to the PstI site (124bp) than the poly(A) addition signal (450bp), presence of an intact PstI site assured maintenance of an intact promoter after Bal31 digestion. A complicating factor was the presence of a second BalI site at the 5' end of the gene (no BalI sites exist in pSP64). Therefore, a partial BalI reaction was performed on pSP64ß to linearize the plasmid to 6.0kb, using 8 units BalI in a 6.5 hour digest on 10ug of plasmid.

This material was then subjected to a timed Bal31 exonuclease reaction. Using a sample calculation provided (Maniatis, Fritsch and Sambrook, 1982) it was estimated that 0.5 units Bal31, 10ug DNA in a 100ul reaction would remove 27bp/min/end of ds DNA, predicting a 4 minute desired reaction time. The reaction was stopped after 0, 2, 4, and 6 minutes using EGTA, since Bal31 is calcium-dependent. The digestion products were self-ligated overnight, and used to transform HB101 cells. These were grown on ampicillin plates, and 50 colonies were analyzed by minipreps using BamHI and PstI to diagnose the area of deletion. Using this method, the desired clone should have an intact 3.0kb fragment (vector sequences), an intact 1.3kb fragment (5' end of the gene with the complicating BalI site), and a 3' fragment which should be decreased in mobility with respect to the 1.68kb fragment from the intact plasmid control. Clone #39 (derived from a 6 minute reaction) contained the necessary intact 3.0kb and 1.3kb fragments, and a 1.45kb fragment containing the shortened 3' end of the clone.

Due to these manipulations, the predicted size of an RNA transcript starting at the SP6 promoter (near the PstI site), continuing to the EcoRI site at the beginning of the third exon, would be about 500 base pairs. The 3' end of ß-globin is most useful for detecting human globin in transgenic mice since maximal evolutionary divergence between mouse and human globin occurs at the 3' end of the gene; EcoRI is a useful unique site for linearizing the plasmid, used to end RNA transcription by causing the polymerase to fall off the transcription template. When such a probe is hybridized to RNA (containing mouse and potentially human globin), and the RNA is subjected to digestion using RNAses A and T1 (Melton et al., 1984), only precise RNA duplexes will be protected from digestion, which should efficiently differentiate between mouse and human globin RNA.

An 80bp EcoRI/BamHI fragment from the 3' end of delta-globin was directionally cloned into pGEM4 so that RNA transcription from the T7 promoter would produce antisense transcripts (the T7 promoter is more efficient at transcriptional initiation than the SP6 promoter). This fragment was derived from λHßG1 subclone ß30F, which contains a 1.8kb EcoRI insert consisting of the 3' end of delta-globin, along with downstream genomic sequences. To avoid setting up a ligation reaction with competing EcoRI/BamHI fragments, the ß30F Plasmid was first digested with AccI, then with EcoRI, and a 1.2kb AccI/EcoRI fragment was electroeluted. This was digested with BamHI, releasing the small target fragment. The products of this digestion were ligated to pGEM4 which was digested with BamHI and EcoRI and phosphatased. Transformed colonies were analyzed by minipreps using RsaI; a 1.9kb fragment spanning the multiple cloning site in the intact pGEM4 vector was decreased in mobility due to insertion of a new RsaI site in the target fragment. Clone δ21 was selected and grown in large culture.

Analysis of Mouse Globins

Blood samples from transgenic mice were analyzed electrophoretically by Triton gel analysis (Alter et al., 1980) to assess mouse Hbb^d, Hbb^s, and potential human globin proteins. The Hbb^d haplotype (Jahn et al., 1980) produces a "diffuse" band consisting of $ß^{major}$ and $ß^{minor}$ proteins, and the Hbb^s haplotype (Weaver et al., 1980) produces a "single" band. The Triton gel system

separates all human ß-like globins as well as α-globin, and permits mouse and human globins to be distinguished by electrophoretic mobility. Since human ß-globin chains comigrate with the mouse ß^s protein on Triton gels, mouse globin phenotypes in transgenic mice were assessed to unmask human bands which might be present in Hbb^d/Hbb^d mice. The mouse lineage from which transgenic mice are derived, along with corresponding globin haplotypes, shows that transgenic mice can contain the Hbb^d and/or Hbb^s haplotypes (Russell, 1979; Green, 1981). Fertilized eggs used for microinjection are produced from B6D2F$_1$ males and CD-1 females. B6D2F$_1$ mice are progeny of C57Bl/6 mice, which are homozygous for Hbb^s, and DBA/2 mice, which are homozygous for Hbb^d. Therefore B6D2F$_1$ mice are heterozygous for these two alleles at the Hbb locus. CD-1 mice are outbred, and the colony contains, on the average, 23% Hbb^d homozygotes, 17% Hbb^s homozygotes, and 60% Hbb^d/Hbb^s heterozygotes. Controls used for these gels included blood from C57 mice which are homozygous for the Hbb^s haplotype, from B6D2F$_1$ mice which are Hbb^s/Hbb^d heterozygotes, and a mixture of human cord/adult blood.

Studies on Cancer-Related Anomalies in the ß80 Line

Cancer in adult mice was analyzed by histopathological examination of affected tissues. These were obtained from animals which died spontaneously or were sacrificed. Hematoxylin and eosin-stained slides were sent to Dr. Albert Jonas (Research Animal Consultants, Boston, MA) for pathological analysis. Blood samples were assessed using a Coulter counter in the hematology lab. Dilutions were made using 44.7 microliters of heparinized blood in 10 mls Isoton.

Cosmid Cloning Methods

Genomic DNA from the ß80 line was cloned using the cosmid vector pHC79 (Hohn and Collins, 1980). Cosmids are hybrids between plasmids and phages and utilize features of both kinds of vectors for the efficient cloning of very large recombinant DNA molecules. The pHC79 cosmid replicon is a 6.4kb plasmid with an ampicillin resistance gene and a plasmid origin of replication, unique cloning sites for the insertion of target DNA, and a cos sequence derived from bacteriophage lambda. The cosmid replicon is digested at a unique cloning site and ligated to genomic DNA digested with a compatible enzyme. This ligated material is then packaged in vitro into phage heads. The cos sequence serves as a signal for this packaging reaction. Only DNA molecules of a specified size range can be packaged with high efficiency (38-51 kb, or 78-105% of the wild-type lambda genome; Feiss et al., 1977) ensuring packaging of large recombinant molecules and selecting against packaging of the cosmid replicon. These phages are then adsorbed to bacterial cells, and the DNA content of the adsorbed phage is injected (transduced) into the cell. Once inside, the DNA replicates as a large plasmid. Ampicillin resistance is used to select for those bacteria which contain cosmids..

Library Construction

Three libraries, designated cosI, II, and III, were constructed from ß80 genomic DNA. Library one, called cosI, was constructed using a double-arm strategy described (Ish-Horowicz and Burke 1981; Maniatis, Fritsch and Sambrook, 1982). A 50ug aliquot of pHC79 vector DNA was digested to completion with SalI, and another 50ug aliquot was digested with HindIII. These were phenol-extracted, ethanol-precipitated, and resuspended in 90ul 10mM TRIS pH8.0. The SalI and HindIII ends were then treated with phosphatase as follows: First, 1ul of each was removed for test-ligations, and stored at -20°C. Then 10ul of 10X CIP buffer (Maniatis, Fritsch and Sambrook, 1982) was added to 89ul vector. 10 mls of 10X CIP buffer was made using 5.0 mls 1.0M TRIS pH 9.0, 100ul

1.0M $MgCl_2$, 10ul 1.0M $ZnCl_2$, and 100ul 1.0M spermidine, plus H_2O. Calf intestinal phosphatase (CIP) was added: 0.3ul at 37°C for 1/2 hr., 0.3ul at 37°C for another 1/2 hr. Calculating from CIP activity (Maniatis, Fritsch and Sambrook, 1982):

1 pmole of 5' ends of a 4 kb molecule = 1.6 ug.
1 pmole of 5' ends of a 6 kb molecule = 2.4 ug.
50ug/2.4ug per pmole 5' ends = 21 pmole of 5' ends.
Need 0.01 units/pmole 5' ends = .21 units CIP
Boehringer-Mannheim CIP = 20 units/ul, .3ul +.3ul = several-fold overdigest.

The reactions were stopped using 75ul H_2O, 20ul 10X STE (TNE) (Maniatis, Fritsch and Sambrook, 1982) and 5ul 20% SDS. These mixtures were heated to 68°C for 15', then extracted twice with phenol/chloroform, twice with chloroform, ethanol-precipitated, and resuspended in 50ul TE (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0) to give a final concentration of 1ug/ul.

Test-ligations (figure 4) were performed to assess the efficiency of 5' terminal phosphate removal. For a reaction which was 100% efficient at removing 5' terminal phosphates, a) self-ligation of phosphatased vector should not be possible, b) ligation of CIP-treated vector to unphosphatased compatible ends should occur, albeit slowly, (simulating the ligation reaction for library construction) and c) self-ligation of unphosphatased vector (the saved aliquot) should proceed efficiently.

10ug of lambda DNA was digested with either SalI or HindIII to provide DNA with unphosphatased, compatible ends, and was extracted, precipitated and resuspended in 20ul TE for a final concentration of 0.5ug/ul.

10ul ligation reactions were performed; prior to adding 1ul of ligase, 2ul of each reaction was removed and stored for comparison to the ligated products. For reaction a, 0.8ul pHC79/SalI or HindIII/phosphatased DNA + 2ul 5X ligase buffer + 7.2ul H_2O were mixed, a 2ul aliquot was removed, 1ul ligase was added and the reaction was ligated at 14°C overnight. For reaction b, 0.8ul pHC79/SalI or HindIII/phosphatased DNA + 1ul lambda/SalI or HindIII + 2ul 5X ligase buffer + 6.2ul H_2O were mixed, aliquoted and ligated. For reaction c, 1.0ul pHC79/SalI or HindIII (unphosphatased) + 2ul 5X ligase buffer + 7ul H_2O were mixed, aliquoted and ligated.

Material which conformed to the criteria outlined above was then digested to completion with BamHI. Complete digestion was verified on a gel by the presence of a discrete 6kb band migrating slightly faster than the linearized vector, and a small 300 bp fragment. The 6kb vector was separated from the small fragment by gel electroelution, and was ethanol-precipitated, resuspended and extensively extracted with phenol and chloroform, ethanol-precipitated, resuspended in 40ul TE, and visualized on a gel. These finished arms were also test-ligated to ensure that the BamHI sites could ligate.

Target (genomic DNA) was prepared as follows: DNA was extracted from the spleen of a heterozygous male mouse (which was a direct descendant of the original founder transgenic mouse); a library constructed from this material should contain both the normal and the interrupted locus of insertion. A crucial requirement for efficient cosmid library construction is that the target DNA be of high molecular weight (>100kb); therefore extreme care was taken not to shear the DNA excessively during isolation.

Target DNA was then partially digested with MboI, which generates ends compatible with those generated by BamHI digestion (and therefore ligatable to the non-phosphatased BamHI sites of the cosmid arms).

Adjusting for the size of the cosmid, the calculated size of target DNA for efficient packaging of ligated material is 31-45 kb. A serial dilution experiment (Maniatis, Fritsch and Sambrook, 1982) was performed to approximate the conditions for the most efficient production of MboI-digested DNA in this size range (this method gave better results than a timed reaction). 10ug of target DNA was dissolved in a final volume of 200ul containing MboI buffer, BSA (100ug/ml), DTT, and aliquoted into ten eppendorf tubes on ice; 30ul was pipetted into tube #1 and 15ul into tubes #2-10. Then 1ul of MboI enzyme diluted in 50% glycerol to 4 units/ul was added to tube #1, and 15ul were serially transferred to tubes #2-10, with careful mixing between transfers. The reactions were allowed to proceed for exactly one hour at 37°C. The samples were analyzed in a 4% agarose gel for the size range of digestion products. Tubes #8 and #9 gave the highest fluorescence intensity in the desired molecular weight range; enzyme activity in tube #8 was calculated to be $4(1/2)^7/30$ units/ul = .0010

units/ul and in tube #9 was $4(1/2)^8/30$ units/ul =.00052 units/ul in a reaction containing 0.75ug/ 15ul=.05ug/ul. These corresponded, respectively, to .02units/ug and .0104units/ug which served as guidelines for scaled-up reactions.

Fluorescence is proportional to mass, not molarity (Maniatis, Fritsch and Sambrook, 1982); therefore, genomic DNA should be underdigested to compensate for the underrepresentation of higher molecular weight fragments as judged by intensity of EtBr fluorescence. In practice, the scaled-up reactions which were chosen to be used in the final ligations were those which gave a higher molecular weight distribution (visually) to satisfy these considerations, rather than strict adherence to calculations of unit activity. In the final reaction conditions chosen, two 100ug target DNA aliquots were digested in a 1000ul reaction volume using .020 and .025 units/ug of MboI. The reactions were stopped by heating at 65°C for 15', cooling to 4°C, and were analyzed in a 0.45% agarose gel using undigested lambda and lambda digested with HindIII (unheated; largest fragment = 27kb annealed arms) as molecular weight standards.

50ug of target DNA (25ug from each MboI digest; 250ul of each) was then treated with CIP using 55ul 10X CIP buffer and 1ul CIP for 1/2 hour, another 1ul for 1/2 hour, the reaction was stopped and extracted as described above, and resuspended in 50ul = 1ug/ul. Successful phosphatase treatment was assessed by the inability of CIP-treated genomic DNA to self-ligate, as compared to the retarded electrophoretic mobility of unphosphatased, self-ligated genomic DNA.

Ligations for the cosI library were performed as follows:

	lig#1	lig#2	lig#3	
target DNA	10.0ul	10.0ul	10.0ul	(=10ug DNA)
HindIII arm	2.5ul	3.5ul	5.0ul	
	=1.0ug	=1.4ug	=2.0ug	
SalI arm	2.5ul	3.5ul	5.0ul	
	=1.0ug	=1.4ug	=2.0ug	
5X lig. buffer	10.0ul	10.0ul	10.0ul	
H_2O	23.0ul	21.0ul	18.0ul	
T4 DNA ligase	2.0ul	2.0ul	2.0ul	
	50.0ul	50.0ul	50.0ul	
	=.24ug/ul	=.252ug/ul	=.28ug/ul	

Ligations were carried out for eight days at 14°C.

Test packaging of ligated products

The packaging procedure is discussed in detail in the following sources: Maniatis, Fritsch and Sambrook, 1982; Grosveld et al., 1981; Hohn, 1979; Earnshaw and Casjens, 1980. The three ligation reactions were each tested for packaging efficiency with 1, 2, or 3 ul of DNA. Reactions were performed in the following order of addition at 25°C:

Buffer A	7ul
DNA	1, 2, or 3ul
Buffer M1	1ul
Sonicated extract (SE)	6ul
Freeze-thaw lysate (FTL)	10ul
	25, 26, or 27ul

Tubes sat undisturbed for 1 hour at 25°C. To each mixture was added 100ul SM, (Maniatis, Fritsch and Sambrook, 1982; per liter: 5.8g NaCl, 2g $MgSO_4$, 50ml 1M Tris pH 8.0, no gelatin added) and 100ul ED8767 cells (see below). Adsorption of phage and transduction of packaged DNA proceeded during a 15-20 minute incubation at 37°C. Then 215ul of NZCYM broth (per liter: 10g NZ amine, 5g NaCl, 5g yeast extract, 1g casamino acids, 2g $MgSO_4$, adjusted to pH 7.5 with concentrated NaOH) was added, and the cells were incubated for 1 hour at 37°C to permit the expression of the ampicillin resistance gene. Finally, cells were plated at 1/10x dilutions on NZCYM plates (15g/liter agar, agarose used for establishing libraries) containing 60ug/ml ampicillin and incubated at 37°C overnight.

Colonies required about 18 hours of growth to become visible (recA- cells grow poorly). Colony size was variable, possibly indicating that cells contained different recombinant clones. The best results were obtained for packaging reactions which contained 2ul of ligated DNA. Ligations #1, #2, and #3 were calculated to yield 47,500, 40,000, and 96,800 colonies/ug, respectively. These were summated to represent 184,000 potential colonies for all three ligations. For a 99% probability of having any sequence represented, 3.45×10^5 colonies need to be plated (Maniatis, Fritsch and Sambrook, 1982); plating 184,000 colonies corresponds to a 95% chance of finding at least one flanking clone.

To ensure representation of the desired clones, two more reactions were performed as in ligation #3. These were carried out for 18 days; this long ligation necessitated a short centrifugation of particulate matter to reverse an inhibition of the packaging reaction (gel electrophoresis verified that DNA remained in the supernatant after centrifugation). However, test packaging of this material showed that no increase in packaging efficiency was obtained from an extended ligation reaction.

For library plating 15,000 colonies were plated on 137mm nitrocellulose filters in 150mm culture dishes. Plating and replication are described in detail below.

Library cosII, utilized cosmid digested with BamHI and treated with phosphatase (figure 4); the double-arm strategy was not employed. In cosII, homozygous genomic DNA was used as the target to double the chances of isolating the genes of interest. The target DNA was partially digested with MboI as before; in this case a timed experiment was used. However, instead of phosphatasing the target DNA as in cosI (which was not feasible, since the vector was phosphatased), the partial digest was size-selected using sucrose density gradient centrifugation.

Timed partial MboI digestion was performed on 320ug DNA in a 600ul reaction containing MboI buffer, BSA, DTT, and 10ul (100 units) MboI. 200ul aliquots were removed after 2, 3, and 4 minutes, and immediately precipitated in 500ul cold ethanol. Samples were pooled, centrifuged, and resuspended.

Sucrose density gradients (Maniatis et al., 1979; Maniatis, Fritsch and Sambrook, 1982) were performed on target DNA. Six 10-40% gradients were layered in 38 ml SW27 tubes using sucrose solutions in the following buffer: 1M NaCl, 20mM Tris-HCl pH8.0, 5mM EDTA pH8.0. No more than 60-70ug target DNA in a volume of under 500ul was loaded onto the gradients. These were centrifuged at 26,000 rpm at 15°C for 24 hours. 1 ml fractions were collected from the top of the tubes using an automated fractionator. Fractions were analyzed by running 70ul of every third fraction with 10ul loading buffer in a 0.5% agarose gel (figure 5). Samples of the desired molecular weight were pooled, dialyzed against TE, causing an appreciable volume increase from 30 mls to 60 mls. This was split into three 20 ml samples, each of which was extracted three times with 10 mls sec-butanol, with 2200 rpm centrifugations for 10 minutes. A final volume of 26 mls was split into two samples,

each of which was extracted with 10 mls of ether to remove the sec-butanol. The ether was vacuum-evaporated, and the samples were precipitated using 1/10 volume NH_4OAc and 3x volumes ethanol. The final yield was 21ug, which was again gel-verified for molecular weight.

Ligations for the cosII library were made using approximately a 5-molar excess of vector to chromosomal (target) DNA. Calculation of molar ratios for a 6kb vector to chromosomal DNA in the molecular weight range of 30-45 kb led to the use of approximately 1ug vector:1-2ug target ratios in the ligation reactions, which were performed as follows:

	lig.A	lig.B	lig.C
target DNA	13.0ul	11.4ul	9.1ul
	=2.3ug	=2.0ug	=1.6ug
vector	4.5ul	5.2ul	5.8ul
	=2.3ug	=2.6ug	=2.9ug
5x lig. buffer	4.4ul	4.4ul	4.0ul
T4 DNA ligase	1.0ul	1.0ul	1.0ul
	21.0ul	22.0ul	19.9ul
	=.21ug/ul	=.21ug/ul	=.23ug/ul

Before adding ligase, 1ul of each reaction was removed to assess the ligation reactions by agarose gel electrophoresis (figure 6). These were compared with 1ul aliquots taken after three days of ligation at 14°C; altogether the ligations were allowed to proceed for five days. Comparison of the ligated and unligated material showed a decrease in intensity of the vector band, as well as retarded migration of the genomic fraction, indicating that the desired products had been achieved in the ligations.

In test packaging reactions for these ligations, a higher rate of colony formation was seen with 1ul than with 2ul of packaged DNA. Packaging of 1ul of ligation A resulted in transformation efficiencies of between 3.6-7.1x10^5 colonies/ug, indicating that just 1ug of ligated material contained enough packageable DNA for a representative library.

CosII plating was performed on a scaled-up packaging reaction; these plates were subsequently found to have fewer colonies than expected from test packaging reactions, presumably due to loss of packaging efficiency in reactions with a larger volume (a 7-fold increase in this case). Therefore, the cosII library consisted of 24 plates with approximately 62400 total colonies, corresponding to a 56% chance representation of any random sequence.

Cosmid cloning: possible effects of cosIII cloning strategy

The cosIII library was constructed in an effort to maximize the number of clones containing the last sequences of the concatemer along with flanking mouse DNA. Partial digestion, which was used to construct cosI and cosII, produces an overlapping library which is useful for walking to isolate adjacent clones, a procedure essential for ordering clones along a chromosome (Lawn et al., 1978; Maniatis et al., 1979; Fritsch et al., 1980). However, a disadvantage of this approach is that it does not select for flanking clones of the concatemer, so that when probing with the microinjected gene, most of the isolated clones will derive from internal portions of the concatemer. This would necessitate further screening of these clones using repeated mouse sequences as a probe in order to isolate those clones containing the locus of insertion. Because of the protocol used in the preparation of target sequences for this library, cosIII is not a representative library, nor can it be used for the purposes of chromosome walking. However, clones isolated from this library may be used as a starting point for expansion of the locus of insertion using the cosI or cosII libraries or a library constructed from wild-type DNA.

The ramifications of the strategy used to construct cosIII involve three aspects favorable toward cloning the desired sequences. First, the clone of interest is designated, by the procedure used, to be bounded on one side by a SalI site from the last copy of the concatemer, and bounded on the other side by either an XhoI or a SalI site deriving from mouse genomic DNA. These meet the precise requirements for a flanking fragment: one restriction site in the concatemer and one site in mouse genomic DNA.

Second, this cloning approach involves the potential to clone genomic sequences which can interrupt concatemeric inserts (Covarrubias et al., 1985; Tarantul et al., 1986). Early analysis of the insert by Southern blotting indicated that processing of sequences had occurred and that the insert was not strictly concatemeric. The analysis of cosIII clones tends to confirm this finding by indicating loss of restriction sites in human globin sequences and rearrangement of mouse DNA.

Third, internal concatemeric copies of phage should be selected against during the packaging procedure due to molecular weight constraints of lambda phage heads. The λHßG1 phage is 46.9kb

in length; to this would be ligated two flanking copies of a 6.4kb vector resulting in molecules which are 53.3kb from one cosmid-derived cos site to the other. This corresponds to 109.9% of the wild-type lambda genome, well above the 105% limit for efficient packaging and therefore too long to be cloned. (If SalI-digested 46.9kb internal phages became self-ligated at SalI sites, creating circular molecules, these would not serve as substrates for packaging since the packaging machinery acts upon concatemeric DNA; see Earnshaw and Casjens, 1980. In addition these would not survive ampicillin selection; see below.) The possibility of packaging part of an internal phage, utilizing a phage-derived cos site and another cosmid- derived cos site is not feasible since these two cos sites are too close, in all possible scenarios, for efficient packaging. For example, if a single copy of phage were released by SalI digestion of the concatemer, and cosmids became ligated to the left and right ends, with all cos sequences in the same orientation, this molecule could be acted upon by the packaging proteins. However calculating the distances between the three cos sites reveals that the two molecules resulting from cos site cleavage would be 35.6kb and 17.7kb, respectively 76.6% and 38.0% of the wild-type lambda genome, both fortuitously too short to be efficiently packaged. (As noted by Feiss and Seigele, 1979, inefficient cleavage of the terminal cos site occurs below 78%, such that the packaging efficiency of a genome even 77% of the length of lambda falls off quite sharply.) Since the longer molecule would have both adult globin genes, and no isolated cosmid clones contained these genes, it is likely that these ligation products, if formed, were indeed not packageable. It is also possible that methylation of concatemeric sequences contributed to the selection against internal clones, since less internal phage copies would be released. In addition, if long tandem sequences of phage were released due to digestion of infrequent unmethylated SalI sites, these might be efficient substrates for packaging proteins and would be of packageable size, 46.9kb. However, since the λHßG1 phage does not contain an ampicillin resistance gene, these would not survive the selection placed upon transduced bacterial cells. In summary, analysis of the clones eventually derived from cosIII strongly indicates that internal copies were indeed selected against. Since the library was screened with sequences pertaining to the phage arms (i.e., lambda DNA), this strategy may have been

the single most important factor in successfully selecting flanking clones from among a large pool of hybridizable, but undesirable internal clones.

All of the clones to be described below were derived from the cosIII library. Therefore its construction will be described in the most detail. The pHC79 vector was digested with SalI and phosphatased. As in cosII, homozygous genomic DNA was used as the target. First, the genomic DNA was digested to completion with XhoI, an enzyme which recognizes no sequences in the λHßG1 phage (figure 7). Since the concatemeric form of this phage in the ß80 transgenic mice consists of 50-100 copies (see figure 14), the concatemeric DNA would be expected to remain undigested by XhoI and therefore be of very high molecular weight after XhoI digestion. All potential XhoI sites must derive from mouse chromosomal DNA; therefore the predicted structure of the concatemer would be an intact human insert bounded by mouse genomic DNA with XhoI ends. XhoI-digested DNA was then fractionated using sucrose density gradient centrifugation as described above, and high molecular weight DNA was retained and pooled (figure 8) for further manipulation.

Fractionated, XhoI-digested high molecular weight genomic DNA was then digested to completion with SalI using 17ug DNA in a 100ul reaction. The λHßG1 phage contains one genomic SalI site 5' to delta-globin, and there are no SalI sites in Charon 4A (figure 9). SalI and XhoI both produce identical ends compatible for cloning into the SalI-digested vector. Since SalI recognizes one site in the genomic insert of the λHßG1 phage, the concatemer would be expected to release the following types of fragments after SalI digestion: 1) internal copies of the concatemer of the original molecular weight of the phage, and 2) the last phage copy on each end would extend from the internal SalI site to either an XhoI site or a newly created SalI site in mouse genomic DNA. (It should be pointed out that both of these enzymes are sensitive to DNA methylation, and therefore not all potential sites would necessarily be digested.)

CosIII plating

Maximal packaging efficiency was achieved using 2ul of DNA per packaging reaction; this yielded $1.5x10^4$ colonies/ug, a result similar to that seen by others (Grosveld et al., 1981). A total of 15 large plates containing 3800 colonies per plate were used, corresponding to 57,000 colonies in cosIII. Ignoring possible enrichment (or depletion) of desired sequences by the method of genomic DNA preparation described, this would predict a 53% chance of representing any random sequence.

ED8767 Cell Preparation, Plating and Packaging controls

ED8767 cells (recA-) were obtained from Jean Lomodoux at Frank Ruddle's laboratory. Single colonies were selected by streaking cells from a stab culture onto a nonselective NZCYM plate. All procedures were performed using cells derived from single colonies. Only small colonies were picked, since small colony size is indicative of retention of the recA- genotype. 99.9% of homologous recombination in E. coli is repressed in recA- cells (Clark, 1973). Phage adsorption occurs via binding to the maltose (LamB) receptor which is induced by growth in maltose. 100cc nonselective NZCYM containing 0.2% maltose was inoculated with a single small ED8767 colony, grown overnight (approximately 16 hours) at 37°C in a shaker, centrifuged the following morning for 10', 4°C, at 2500rpm in an IEC centrifuge, and resuspended in 1/10 the original culture volume using 10mls sterile 0.01M $MgSO_4$. These were streaked on nonselective plates and plates containing 60ug/ml ampicillin; growth occured on the former but not the latter plates. Small colony size was again verified. The day after resuspension of overnight culture (after these criteria were satisfied), packaging reactions were performed for library plating. To control for possible DNA contamination in the packaging reagents, a reaction containing no DNA was performed. To test whether the SM contained ampicillin-resistant colonies (despite autoclaving), SM and ED8767 cells were incubated without the addition of a packaging reaction. Ten-fold more volume was plated on ampicillin plates than on nonselective plates in these two controls to ensure there was no contamination. No colonies resulted from these procedures when tested on Amp plates, but colonies grew on nonselective plates.

Filter preparation

Millipore HATF 137mm nitrocellulose filters were used for plating and replicating libraries. Filters were wet in distilled H_2O and submerged for 5', blotted dry on Whatman #1 paper for 5', and sandwiched between Whatman #1 paper cut to about 15x19cm in stacks of 50 filters or less. Extra sheets were placed on top and on bottom of the stack. Several sheets of wet Whatman #1 paper were then placed on top and on bottom of this stack to maintain a water reservoir during sterilization. The stacks were then wrapped in aluminum foil, placed flat in the autoclave, and sterilized 40 minutes on liquid cycle. When removed from the autoclave, filters were cooled (flat), and then wrapped in a plastic bag using a heat sealer to prevent evaporation. Thus prepared, filters were usable for weeks to months; once packages were opened fungus could contaminate the stacks.

Plating and Replication

The advantages of using 150mm plates are 1) less plating and filter replication work is involved, since fewer filters are used, and 2) the stack of filters in the hybridization reaction is much smaller, leading to more efficient hybridization. The latter consideration is thought to be highly important. Optimally, plates are used when they are one day old. Ampicillin plates were poured at 60ug/ml (Amp_{60}). Thickly poured plates were used to establish colonies. NZCYM plating broth was autoclaved for 50'.

For master plates, filters were marked on the colony side with a ballpoint pen, placed colony side down on an Amp_{60} wetting plate, lifted and placed colony side up on an Amp_{60} plate containing 5% glycerol for freezing purposes. Cosmid-containing cells in volumes of 200-350ul, containing the desired numbers of colonies, were plated directly onto the nitrocellulose filters. Spreading, using an autoclaved and ethanol-flamed glass plating bar, was performed extensively, to ensure an even distribution of cells on the filters, while avoiding the outer 2-3mm along the edges. It is essential that colonies on the master plate be approximately 1mm in diameter and not exceed 2mm, to avoid smearing of colonies during replication; therefore plating was done in the early evening and colony size was watched carefully during incubation in the 37°C room the following day. At this and later

steps, plates were put into the cold room (4°C) for a few hours when necessary to avoid overgrowth.

Replication of filters was performed three times. Replication number and plate number were marked on the filters using a ballpoint pen. The first set of replicas was used for eventual picking of colonies, after results of the hybridization were obtained. Master plates (with filters) could thus be immediately stored frozen, before hybridizations were performed. Also, since master plates and replicas are mirror images, replicas all have the same orientation, avoiding confusion when picking colonies. The second and third replicas were each taken through the same hybridization. This was useful in ruling out hybridization artifacts and making choices in eventual colony selection for the second screen (figure 10).

A replication device (Hanahan and Meselson, 1980) was made in the following way: a heavy glass plate was put on the lab bench, and a piece of cardboard without corrugations was taped to it. Next, two sheets of Whatman #1 paper were put down. Three sheets of Whatman #1 (about 15x19cm) were placed on this; these were continually replaced as each filter was replicated. The master filter was then placed on this stack, colony side up. A replication filter, with its colony side already marked and moistened on a plate, was carefully placed upon the master filter, always bending the replica filter so that the concave middle touched the master first. Any sliding movement of the replica, or bubbling due to incorrect placement, defeats the replication procedure and may ruin the master filter. One or two sheets of Whatman #1 paper (discarded after each use), then a round glass plate (about 200mm diameter), then two sheets of Whatman #1 paper, then a heavy glass plate were placed on top. Pressure was exerted for a few seconds, then the layers above the two pressed filters were removed. The use of extra pieces of Whatman #1 paper in the upper layers keeps the filters from being dislodged when the two glass plates are lifted.

It is essential that filters be keyed before they are peeled apart, or the process is again defeated. If this step is forgotten, any master can be replicated again, but it may be necessary to let the colonies grow back for a few hours. One, two and three holes were made in characteristic positions along the edges of the filters, (e.g., at 12:00, 4:00 and 8:00,) using india ink and a 10cc, 20-gauge needle (figure 10). Other types of dye are not reliable, and smaller needles do not adequately mark the filters. Then

the filters were peeled apart; a characteristic tearing sound indicated that the filters had been pressed together tightly enough to achieve replication.

Master filters can be replicated not more than 2-3 times at one sitting for efficient transfer of all colonies; therefore the master plates were placed in the 37°C room for 90' before making the second and third replicas. These were both made at one time, without allowing growth of colonies in between. In all replications subsequent to the first, the holes now present in the master filter were made in the replica filters.

Filters were treated as follows: Master filters were frozen on plates containing Amp_{60} and 5% glycerol. (Concentrations of glycerol above 5% inhibited colony growth.) The plates were sealed with parafilm, wrapped in plastic sleeves, and Master plates for all three libraries were stored at -20°C. Replicas in the R_1 series were grown for three hours to a size of not more than 2mm, then placed into the cold room until colony selection could be made. (To avoid fungal contamination of these plates, hybridization should not be delayed too long.) Replicas in the R_2 and R_3 series were amplified overnight on NZCYM plates containing 500ug/ml chloramphenicol (Hanahan and Meselson, 1980). A pilot experiment performed with cosI indicated that chloramphenicol treatment did cause an amplification of cosmid resulting in a better hybridization signal.

Colony Lysis

A procedure similar to that described (Maniatis, Fritsch and Sambrook, 1982) was used to lyse DNA from colonies on the second and third replicas. Whatman #1 sheets were cut to fit Pyrex baking dishes, or placed on beds of aluminum foil when larger reservoirs were desired (however aluminum should not be used with Denat since NaOH oxidizes the aluminum). Filters were taken through the following steps using Whatman #1 paper saturated with the following solutions: 10% SDS for 3'; Denat (0.5M NaOH, 1.5M NaCl) 5'; blot dry and repeat Denat 5'; Neutralizing Solution (Renat, 1M Tris pH 8.0, 1.5M NaCl) 5'; repeat Neutr. 5'; 2X SSPE (.36M NaCl, 20mM NaH_2PO_4, 2mM EDTA, pH 7.4) 5'; let dry on Whatman #1 at room temperature 30-60'. Filters were then baked at 70°C overnight.

Hybridization

To remove excess cell debris, filters were floated on 6X SSC until wet and then submerged for five minutes. Filters were transferred to a circular pyrex dish fitted to large filters (150mm dishes, with glass covers). Filter stacks contained 35 or (preferably) fewer filters. These were prewashed at 42°C using 50mM Tris pH 8.0, 1M NaCl, 1mM EDTA, 0.1% SDS. The prewash proceeded for at least one hour, fresh prewash solution was added, and filters were washed for another hour or longer.

Prehybridization was performed at 65°C for three hours in the following solution: 6X SSC, 50mM NaP_i, 10X Denhardt's solution, 1% glycine, 500ug/ml sonicated salmon sperm DNA, using at least 2.5 mls per filter.

Hybridization was performed at 65°C overnight in: 6X SSC, 20mM NaP_i, 2X Denhardt's solution, 100ug/ml sonicated salmon sperm DNA, and 7ng/ml of radiolabelled probe, using the same volume as the prehybridization.

It is useful to keep the following considerations in mind: Filters should be restacked one at a time between the preceeding steps since they tend to stick to each other; solutions must be allowed access to all filters at all steps (especially during hybridization). Since much water evaporates during this hot overnight incubation, care should be taken to fill the bath as much as possible; weighing down the filter dishes may be necessary due to buoyancy. The bath must be shaken vigorously enough to allow access of the solution to reach all filters (especially the bottom ones). It is also important to maintain a tight seal between the dish and cover to avoid evaporation of the hybridization solution.

Filters were taken through three washes ending in a third stringent wash: 1) 0.1% SDS, 2X SSC at room temperature; 2) 0.1% SDS, 0.2X SSC 65-68°C; 3) 0.1% SDS, 0.1%SSC 65-68°C; filters were inverted between washes and continually separated. Filters were air dried and taped to Whatman #1 paper cut to fit large film cassettes, and wrapped with saran wrap to protect the filters. Duplicate filters were arranged side-by-side for comparison. It is important that the filters remain permanently attached to the backing so these can later be keyed to the film. For later alignment of the film to the filters, slightly radioactive ink is used to mark the Whatman #1 backing, before applying saran wrap.

If the ink is too hot, it will produce imprecise marks on the film, and accurate alignment will not be possible.

Colony Selection for second screen

It is very important that selection not be applied at this point, or efficiently growing clones can outgrow other colonies in mixed populations of cells. A light box is useful in the following procedures. First, films are placed against the filters, using the asymmetric dots made by radioactive ink to align them precisely. The ink spots made on the filters during filter replication are marked onto the film. Then, using the films alone, hybridization signals are compared between replica filters, and a judgement is made as to which are likely to represent true signals. (It is highly recommended that duplicate filters be used.) Then, R_1 plates are aligned with the films, using the assymetrical keying holes. Colonies on the plate corresponding to areas of hybridization are picked using a sterile toothpick and placed in an eppendorf tube containing 500ul NCZCYM with 20% glycerol and Amp_{60}. The tube is immediately vortexed to disperse the cells. The glycerol prevents selective growth of clones in the mixed population, and is useful for storing colonies for months at 4°C. To ensure that the colony of interest is selected at this step, it is advisable to pick areas for screening which are in the immediate vicinity of the hybridization signal; alignment is never accurate enough to pinpoint a target colony.

All second screens are replated on small, 82mm filters, which fit 85mm plates. Colonies are titered so they can be plated at a low enough density to select single, pure colonies after the second hybridization. To titer colonies, 2ul are added to 198ul NZCYM with 20% glycerol and Amp_{60}; this is again diluted 2ul into 198ul. Then 50ul of the second (10^4) dilution is plated onto small selective plates, colonies counted after overnight growth, and multiplied by 200 to calculate the final titer (colonies/ul) in the undiluted tube. To plate colonies for the second screen, a calculated 200 colonies are taken from the undiluted or 10^2 diluted tubes, added to NCZYM broth for a total plating volume of 100ul, and plated directly onto nitrocellulose filters as before. Filters are replicated and hybridized as described for the first screen, using small pyrex dishes to fit these filters. For the second screen,

1 master and 2 replicas are made; it is not necessary to perform the hybridization in duplicate since positive colonies provide an internal control. On the second screen, several or all colonies on a given plate should give a positive hybridization signal, or, all colonies should be negative; this was the pattern seen. Due to the low density plating used in this screen, single colonies can be selected, and the hybridization pattern on the plates should be consistent with the sucessful isolation of positive cosmid clones. Single colonies are selected with toothpicks as before and stored in 500ul NZCYM with 20% glycerol and Amp_{60}. Positive clones were also stored frozen at -70°C by vortexing 700ul of an overnight culture with 300ul 50% sterile glycerol.

Isolation of Cosmid DNA from bacterial cells

Due to the large size of cosmids, they are not amenable to most isolation procedures used for plasmid DNA. Therefore, a modified Birnboim-Doly method (Birnboim and Doly, 1979) was used. Chromosomal DNA is selectively denatured in alkaline solution and is precipitated upon neutralization. After centrifugation, the cosmid DNA, which remains in solution during this treatment, is precipitated and isolated.

Solution I

lysozyme 2mg/ml
50mM glucose (180.2g/mole), made from 1M filter sterilized solution
10mM CDTA, made from 500mM solution (CDTA is more soluble in EtOH than EDTA, and chelates metal more efficiently)
25mM Tris pH 8.0, made from a 1M solution

Solution II

0.2N NaOH (40g/mole)
1% SDS

This solution is stable at room temperature for 1 week.

Solution III

3M NaAcetate, pH 4.8
0.3 moles of NaAc (82.04g/mole) is dissolved in a minimal volume of water, glacial acetic acid is used to bring the pH to 4.8, and the volume is adjusted to 100mls.

This solution can be stored cold for weeks but precipitates after time.

Overnight cultures were inoculated in 50cc capped tubes using 40 mls NZCYM broth containing $Amp_{100\text{-}125}$ and 25ul of inoculum. These were shaken overnight and no chloramphenicol was used to amplify the cultures. The following morning, cultures were spun at 2500rpm for 10' in an IEC centrifuge, the supernatant was decanted, and the pellet was vortexed to suspend the cells in

a thick slurry. These were resuspended in 2 mls solution I and placed on ice for 10'. The tubes were then brought to room temperature to avoid precipitation of SDS in the next step due to the cold temperature. Next, 4 mls of solution II were added for alkaline lysis. Tubes were mixed gently at room temperature, then placed on ice for 15'. Next, 3 mls of solution III (precooled) were added, and the tubes were gently mixed, and then placed on ice for 30-60'. At this step, chromosomal DNA renatures and aggregates to form an insoluble network, and protein-SDS complexes and high molecular weight RNA also precipitate. The tubes were then spun for 10' at 2500rpm in an IEC centrifuge, and the clear supernatant was pipetted into 2 volumes (18 mls) of ice-cold EtOH, and placed at -70°C for 10'. The nucleic acid precipitate was resuspended in 1.5 mls 0.1M NaAc/0.05M Tris pH 8.0, reprecipitated with 3 mls cold EtOH for 10' at -70°C, and spun 10' at 2500rpm. The precipitate was washed with a 70% EtOH solution in 0.1M Tris pH 8.0, resuspended in 1ml TE, and stored at 4°C. The RNA can be removed by RNAse, the DNAse activity of which has been removed by boiling. However, this was not done since the contaminating DNAse activity was difficult to remove and the RNA did not interfere with any of the procedures. In practice, approximately 25ul of the resuspended cosmid contained enough DNA to digest with a restriction enzyme and visualize on a gel.

Hybridization Analysis of Cosmid Clones

Cosmid clones were digested with different restriction enzymes, and analyzed sequentially with several different probes to ascertain the DNA content of the clones.

Twenty to 40ul of cosmid DNA (sometimes adjusted for the yield of each clone) was digested in an 80ul volume reaction for 1-2 hours using up to 7ul restriction enzyme. These were electrophoresed overnight in agarose gels, using 0.85% agarose for EcoRI, BamHI, HindIII, SalI, and KpnI digests, and 1.7% agarose for BalI, EcoRV, and BglII to resolve low molecular weight fragments.

DNA was transferred to rehybridizable GeneScreen Plus membranes (DuPont) with certain modifications made from the standard Southern protocol, as specified by the supplier. Each side of the GeneScreen Plus filter is utilized for a different application; for this procedure the B side (concave side) was used for DNA binding. This side was marked with a pen, and a filter cut to a size 2mm larger than the gel in length and width was wetted in distilled water. The gel was stained with EtBr and photographed, then incubated in Denat (0.5M NaOH, 1.5M NaCl) for 30', then neutralized in Renat (1M Tris pH 8.0, 1.5M NaCl) for 30' (in contrast to 90' for Southerns). The filter was placed in 10X SSC for 15', and the blotting was set up in the usual way overnight. However, it is essential to place the B side against the gel during blotting. The next day, the filter was immersed in 0.4N NaOH for 30-60 seconds to denature the DNA, then placed in 0.2M Tris pH 7.5, 2X SSC. The filter was dried, DNA side up, at room temperature. DNA need not be baked onto GeneScreen Plus, saving a day before hybridization.

Hybridization is simplified by using a short 15' prehybridization at 65°C using 1% SDS and 1M NaCl, with no dextran sulfate. For the hybridization, boiled DNA was added to the prehybridization solution to a final concentration of 100ug/ml, and boiled probe was added at 7ng/ml and hybridized at 65-68°C overnight.

Filters were washed using: 1) 2X SSC, room temperature, 5', 2) 2X SSC/1% SDS, 65°C, 3) 0.1X SSC, 65°C. A one-night exposure at -70°C with a screen was usually sufficient for achieving the appropriate intensity.

To rehybridize filters with a different nick-translated probe, filters were stripped of probe using 0.4N NaOH in 42°C, or 65°C when necessary. It was sometimes necessary to strip the filters for at least an hour to remove the probe. The filters were neutralized in 0.1X SSC, 0.1% SDS, 0.2M Tris pH 7.5, incubated at 42°C. This procedure was assessed by using a counter, and/or by autoradiographing stripped filters. In this way, the blots were sequentially hybridized and the identity of the fragments comprising the clones was determined.

Chromosomal Mapping of ß80 locus

EcoRI fragments from cos 5-1 were subcloned into pGEM3 (figure 11) and used for mapping the ß80 locus of insertion using a mouse/hamster hybrid panel provided by Frank Ruddle.

Restriction Fragment-Length Polymorphisms

Subclones from cos 5-1 were used to search for RFLP's associated with the insertion locus. DNA from a heterozygous transgenic mouse, a wild-type mouse, from hamster, and from human placenta were digested with EcoRI, BamHI, or PstI and probed with cos 5-1 subclones A9 and A12.

Figure 1
DNA Microinjection

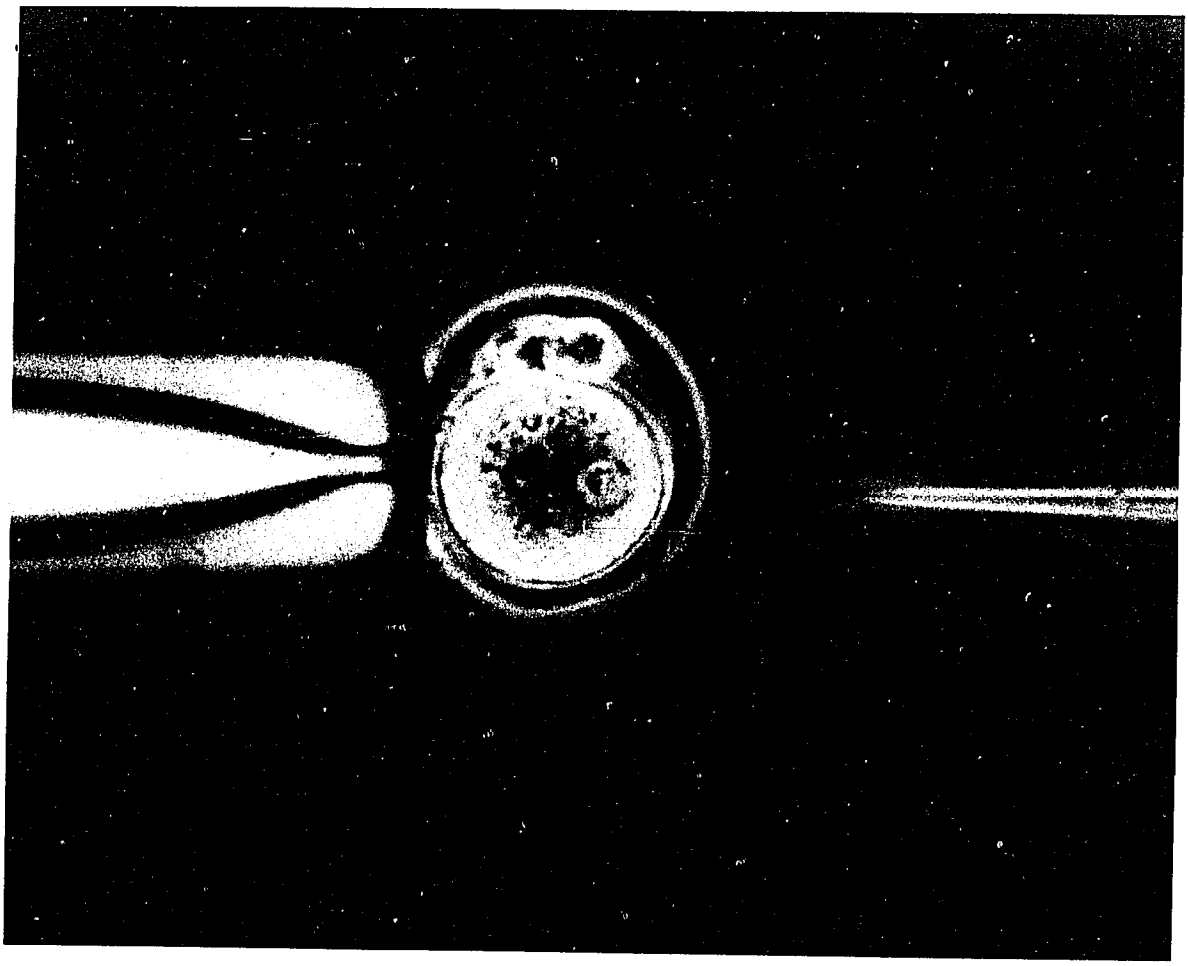
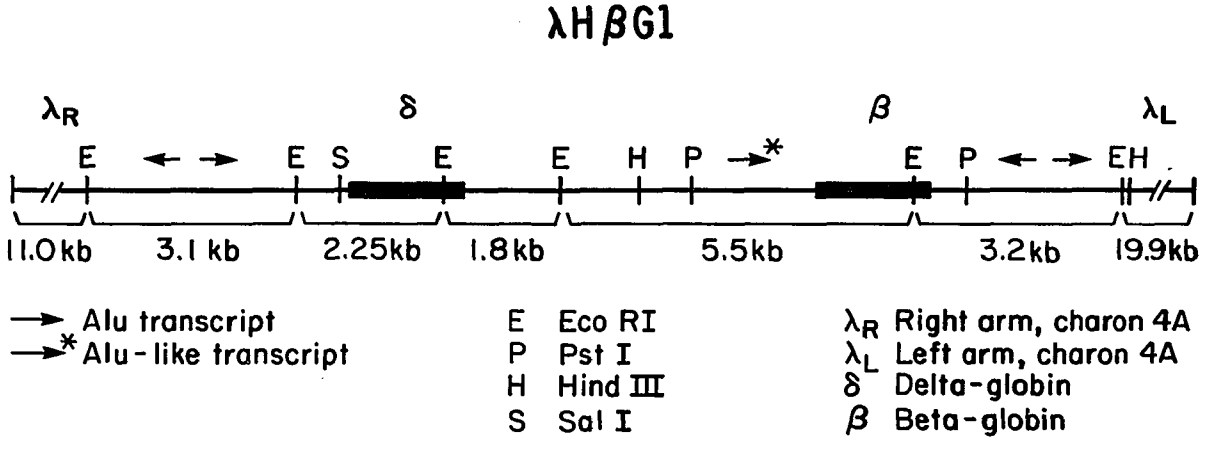
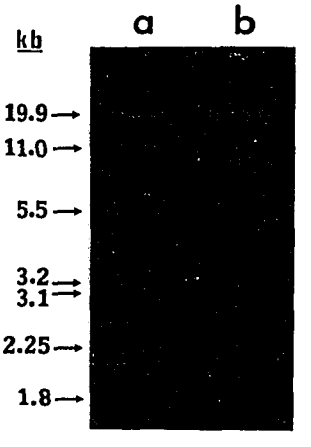


Figure 2
λHβG1 Vector



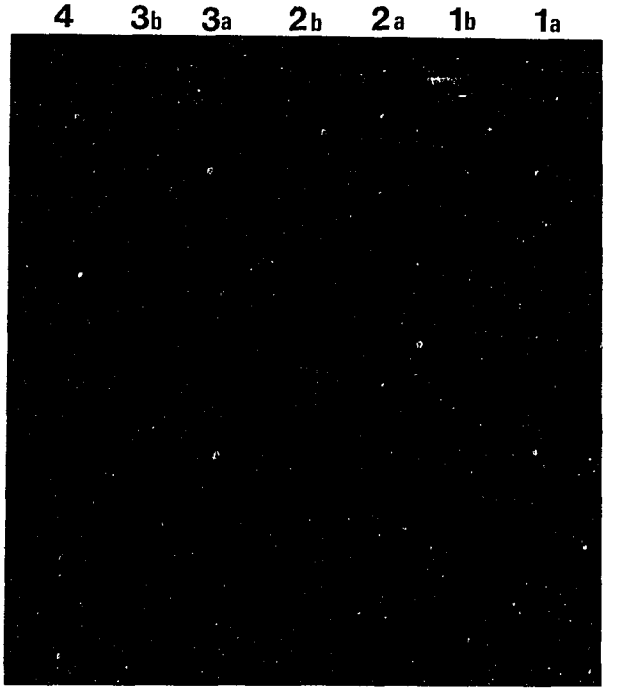


a: EcoRI-digested
λHβG1 phage
b: HindIII-digested
λ phage

Figure 3
λEB pilot experiment

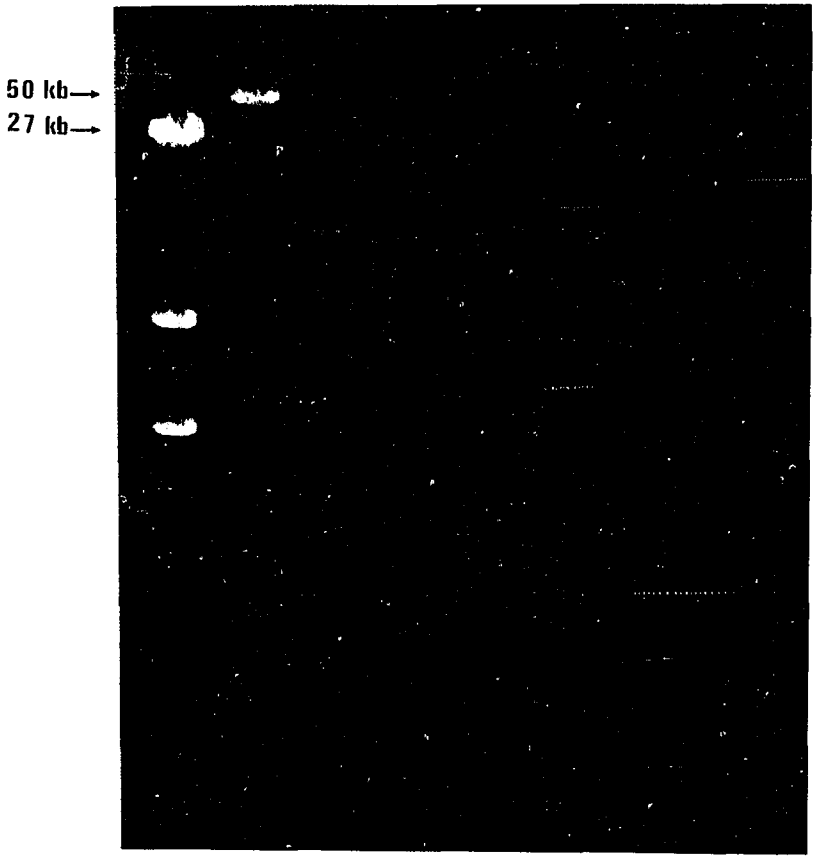
Agar plates were streaked with ED8767 cells (a), HB101 containing pHC79 (b), or HB101 containing λEB vector (c), and colony hybridization was performed using whole nick-translated λ phage as a probe. A large differential in hybridization of λ to pHC79 vector vs. λEB vector indicated a low enough specificity of λ for the cos sequence in pHC79 to use λ to probe a cosmid library.

Figure 4
Test-ligation: BamHI-digested pHC79 cosII vector



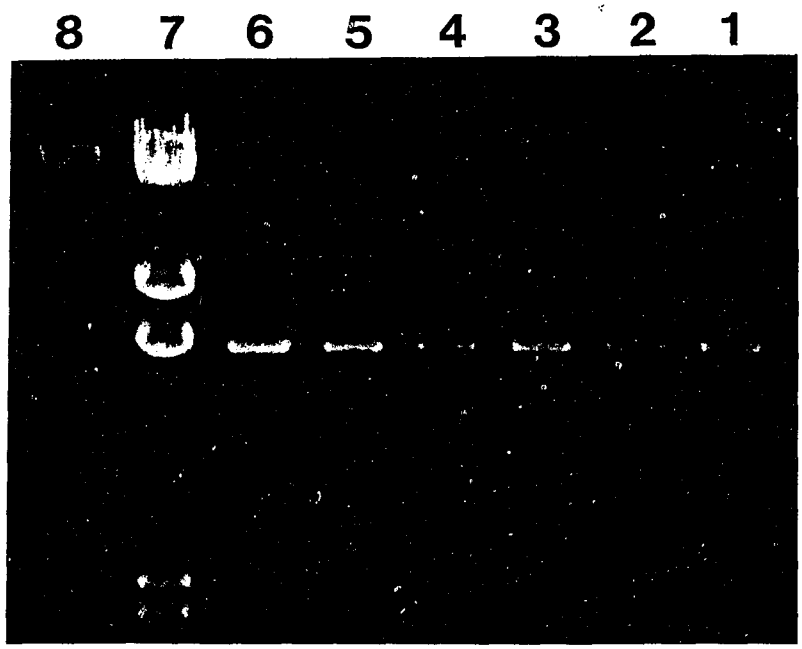
Lane	Sample
1a	pHC79 phosphatased, unligated
1b	pHC79 phosphatased, ligated
2a	pHC79 phosphatased + BamHI-digested pBR322, unligated
2b	pHC79 phosphatased + BamHI-digested pBR322, ligated
3a	pHC79 unphosphatased, unligated
3b	pHC79 unphosphatased, ligated
4	λ/HindIII

Figure 5
Agarose gel analysis of sucrose density gradient-fractionated target DNA for cosII



Loading buffer contained 0.25% bromophenol blue, 0.25% xylene cyanol, and 40% w/v sucrose. Molecular weight standards were undigested lambda, and lambda digested with HindIII and unheated (largest fragment corresponds to annealed ends, 27kb); these were matched to the samples for salt and sucrose concentrations by using a comparable buffer in order to ensure comparable gel entry and electrophoretic migration.

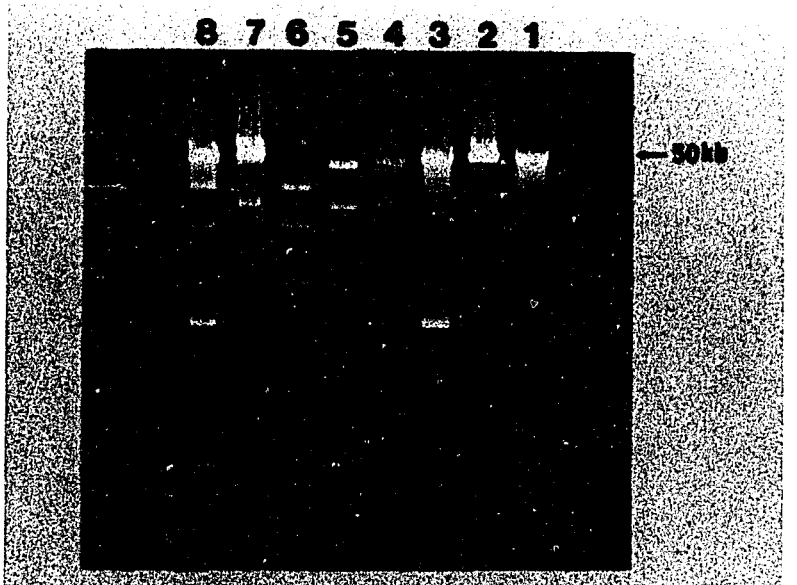
Figure 6
Assessment of cosII ligations A-C



Lane	Sample
1	cosIIA unligated
2	cosIIA ligated
3	cosIIB unligated
4	cosIIB ligated
5	cosIIC unligated
6	cosIIC ligated
7	λ/HindIII unheated
8	λ undigested

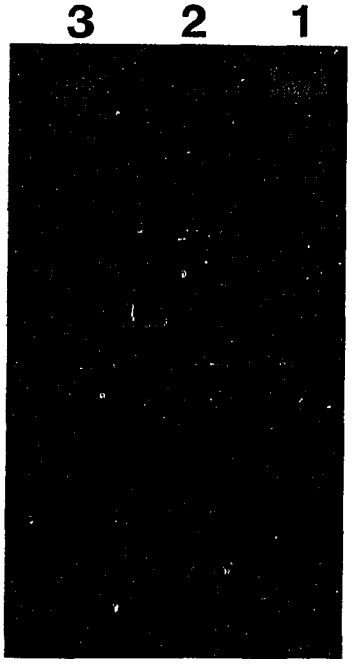
1ul aliquots of three cosII ligation reactions assessed before (lanes 1,3,5) and after (lanes 2,4,6) three days of ligation at 14°C.

Figure 7
Test XhoI digestion of λHβG1 phage DNA



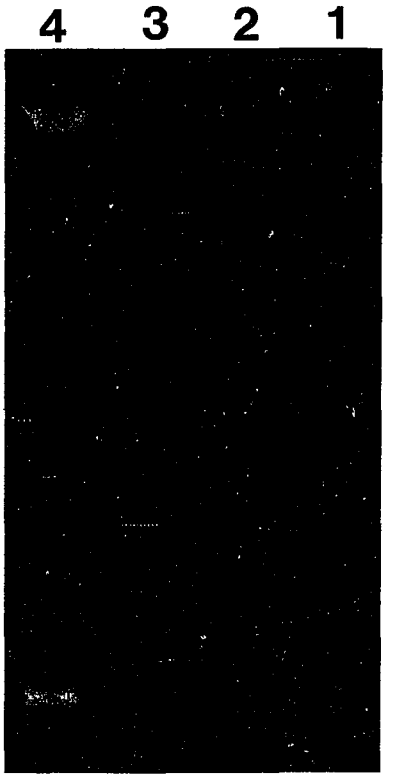
Lane	DNA	Enzyme
1	λHβG1	undigested
2	λHβG1	Xho I
3	λHβG1	Sma I
4	λ	undigested
5	λ	Xho I
6	λ	Sma I
7	λHβG1 + λ	Xho I
8	λHβG1 + λ	Sma I

Figure 8
Pooled, XhoI-digested, sucrose density gradient-fractionated target DNA for cosIII



Lane	Sample
1	λ/HindIII
2	λ undigested
3	XhoI-digested genomic DNA; fractionated and pooled

Figure 9
Test SalI digestion of λHεG1 phage DNA



Lane	Sample(s)	Enzyme
1	λ	SalI
2	λ + λHεG1	SalI
3	λHεG1	SalI
4	λHεG1	EcoRI

Figure 10
Replicate filters used in the cosIII first screen

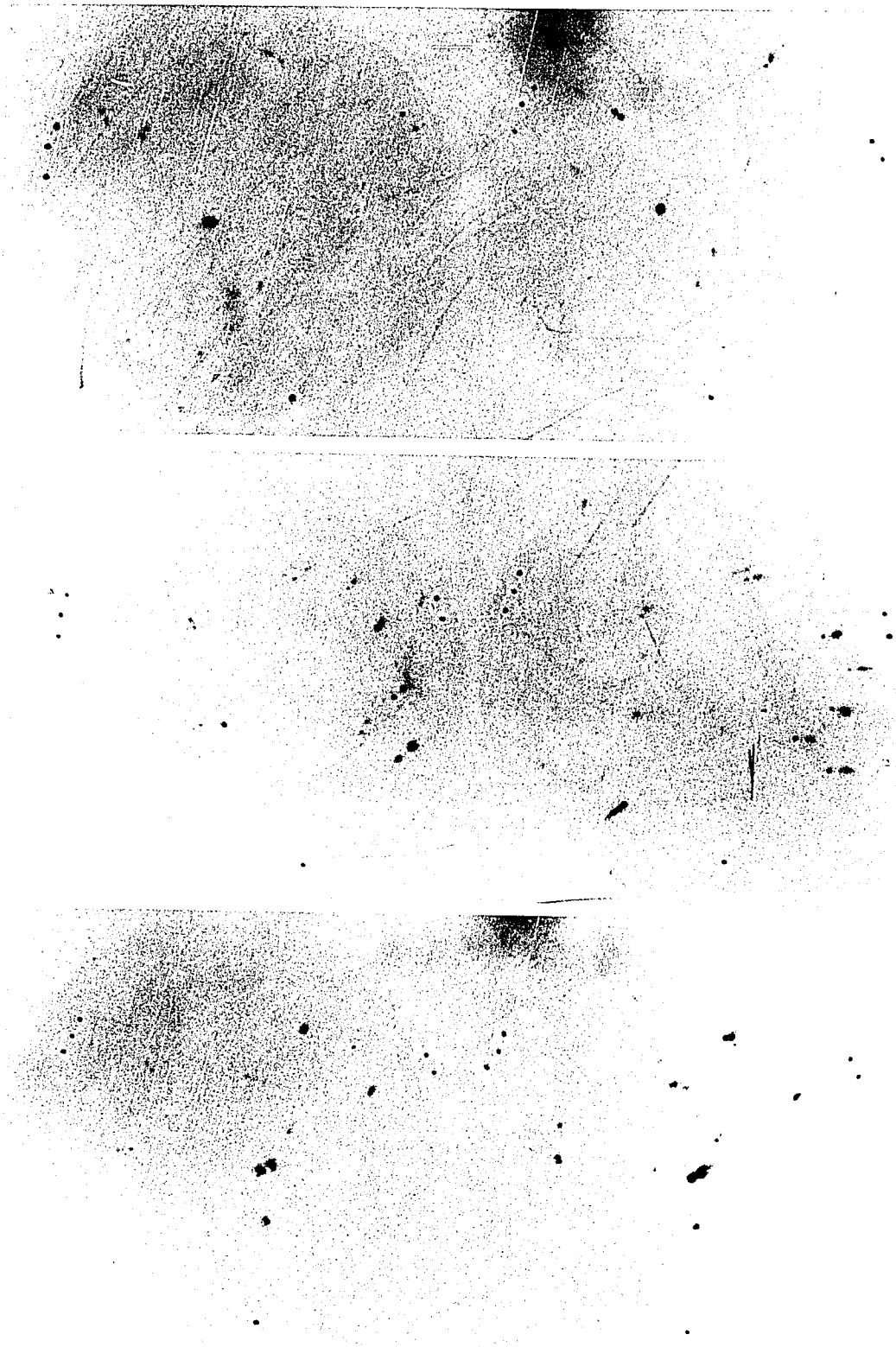
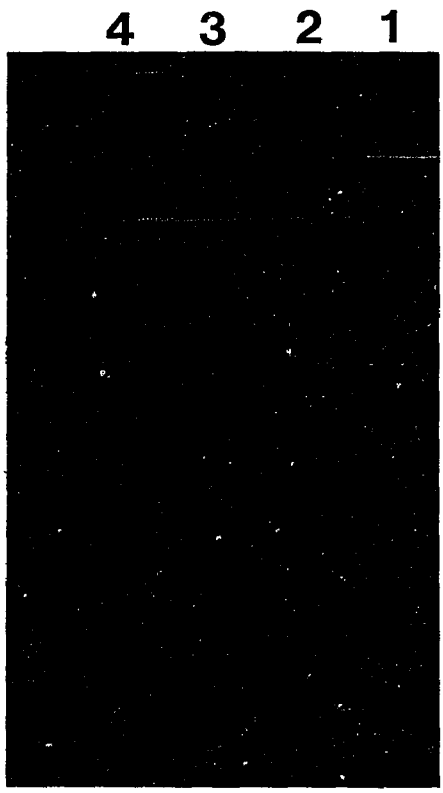


Figure 11
Cos 5-1 subclones



Lane	Sample	Enzyme	insert
1	A2	EcoRI	5.0kb
2	A9	EcoRI	5.5kb
3	A12	EcoRI	6.3kb
4	λ	HindIII	

RESULTS I.

Microinjection of Clones and Characterization of ß80

Table 1 summarizes the data obtained from all microinjection experiments. Three positive mice were produced. ß80 and ß19 contain λHßG1 sequences. E2 contains λHεG1 sequences, including the epsilon globin coding region and lambda phage.

Figure 12 is a Southern blot of EcoRI-digested spleen DNA from the ß80 founder mouse, which was probed with whole nick-translated λHßG1 phage. All of the expected bands derived from EcoRI digestion of λHßG1 are seen, indicating integration of intact λHßG1. In addition, this blot contains fragments which do not correspond to internal phage-derived EcoRI fragments, at intensities less than internal EcoRI fragments. These indicate either junctional fragments, or partial rearrangement of λHßG1 sequences. A 30kb band is also seen (figure 15), consistent with covalently linked left and right phage arms in the head-to-tail concatemer (Lacy et al., 1983).

A dilution strategy was used to assess copy number of phage in ß80 (figure 13). Since the phage lane is closest in intensity to the 50- and 100-fold dilutions, it is estimated that there are 50 to 100 copies per cell of λHßG1 in ß80.

Genetic methods and Southern analyses were first performed to determine if the ß80 line contains an insertional mutation. These data indicated genetic linkage between foreign gene insertion and the mutant phenotype .

Characterization of the ß80 mutation

The first indication of a mutant phenotype in the ß80 line was that there were subviable mice born in crosses between first-generation heterozygous carriers of the human insert. These mice were smaller than their littermates, held their forearms bent downward, and they usually died within a few days after birth. Mice which survived a few weeks had an abnormal gait. Figure 14 shows a growth chart for a litter of mice derived from two heterozygotes. The smallest mouse was approximately one-third the size of its littermates, and was shown by Southern analysis to be homozygous for the foreign

insert (figure 15). Altogether 35 abnormal mice were observed among 473 pups derived from two heterozygous parents (table 2).

Three strategies were undertaken to determine if this line carries an insertional mutation. First, heterozygotes were crossed to normal animals and offspring were analyzed for evidence of mutation. Second, a statistical analysis of genotype was performed on the population of mature animals derived from crosses between two heterozygotes to assess the ratio of positive to total offspring. Third, blotting intensities of abnormal animals were compared with the blotting intensity of a known heterozygote.

In heterozygous vs. wild-type matings the foreign sequences were transmitted through the germlines of 5 males and 5 females. No abnormal offspring were seen among the 328 animals born. This provides evidence that the ß80 mutation seen in newborn pups is recessive. This approach was also used to determine whether normal positive adults were actually homozygotes which had been missed by dot blot analysis. Three randomly selected F2 generation transgenic males were mated to wild-type females, and male and female offspring were blotted to see whether any animals transmitted the ß80 sequences 100% of the time. None of these males produced 100% positive offspring, indicating that the adult ß80 transgenic population is not comprised of homozygous, phenotypically normal mice.

In the second strategy, 99 mature mice derived from crosses between two heterozygotes were blotted (table 3). If abnormal mice do not survive, they would not be expected to contribute to these blotting data. Therefore a 2/3 ratio of positive to total offspring would be expected if the homozygous class is missing, or a 3/4 ratio if it is not missing. When the data were subjected to a Chi-square analysis, the hypothesis that the data fit a 3/4 ratio was rejected with greater than a 99% level of confidence. However, the blotting data fit a 2/3 ratio. This analysis provides statistical evidence that the homozygous class does not survive to become part of the population of mature animals.

Blotting intensity data provide more direct evidence for the genetics of this line (figure 15). In the ß80 line, viable but abnormal mice were born, which permitted this strategy to be explored. Abnormal animals were followed closely, animals which died were saved, and phenotypes were

recorded. Lanes 1, 2, 3, and 7 are approximately twice as intense as lane 10, indicating these abnormal mice contain twice the number of copies of the human insert than the obligate heterozygote. In this experiment, the mutant phenotype was correlated with a predicted homozygous blot intensity in 100% of the abnormal animals from which DNA could be retrieved.

An estimate of penetrance can be made by accepting two assumptions, which are supported by these data: 1) Prenatal lethality and abnormal forelimb development mutations are expressed only in homozygotes, and 2) all homozygotes show one of these mutations. Using these assumptions, the data can be extrapolated (table 2) to provide an estimate of 76% penetrance of homozygous prenatal lethality and 24% of homozygotes surviving to display abnormal forelimb development.

An abnormal mouse was identified which was an apparent hemizygote for the human sequence (figures 16a-f). This mouse had an abnormal left forelimb, but a normal right forelimb (hence it was named AbnL), and was slightly growth retarded compared to its littermates (figure 16b). Attempts were made to breed this mouse, a male, but it never sired any offspring. At six months of age, the animal became ill, and was sacrificed. Figure 16e shows an enlarged abdomen at the time of sacrifice, and indicates the left forelimb anomaly. Necropsy revealed a massively enlarged liver and spleen (figure 16f). The thymus was also enlarged.

Southern blotting demonstrated that the animal was transgenic. Gene dosage studies, though not unequivocal, were most consistent with hemizygosity for the foreign insert.

Phenotypic anomalies in adult ß80 transgenic mice

The AbnL mouse served as an alert to the possible development of cancer in this line, and at least six more cases were subsequently noted. These are tabulated with other phenotypic abnormalities in table 4. Histopathological analysis of affected tissues is presented in figures 17a-t. Excluding AbnL, the average age of development of leukemia (as noted in the terminal stages) was 14 months, with a range of 13-16 months. Pancytopenia was observed in mouse F3.31; in fact this mouse died from hemorrhage when obtaining a blood sample, and anemia was immediately

apparent from examination of the retina and blood sample. The presence of pancytopenia as a primary effect is difficult to assess, since leukemic infiltration of the bone marrow can crowd out other blood components (myelophthisis), causing pancytopenia secondary to leukemia. For this reason, mice are now being followed throughout development to detect preleukemic alterations in blood components.

The development of leukemia was seen in obligate hemizygotes. Mouse F2.12-3 was derived from a cross between a transgenic male and a CD-1 female, and mouse F3.32 produced less than 100% positive progeny.

In addition, other anomalies were noted in certain mice (table 4). Mouse F3.22 continually twirled counterclockwise; its brain was saved for future analysis. This mouse did not have any offspring although it lived well past the age of sexual maturity.

Failure to reproduce after reaching the age of sexual maturity was observed in several male and female mice from this line. Mouse 80.23, a first- generation female offspring of the ß80 founder mouse, showed twirling behavoir similar to F3.22, and it also failed to reproduce. Mouse F3.20, a female, successfully mated (as shown by the presence of vaginal plugs), but rarely sustained pregnancy (one delivery of one mouse occured). Bleeding from the vagina was seen in this mouse. Male infertility was investigated in F2.12-1, an obligate heterozygote, which is a sibling of F2.12-3. This mouse successfully produced vaginal plugs in female mice, but of 12 such females, none became visibly pregnant or delivered progeny. Nine of these females were sacrificed to search for resorption sites in the uterus, and none were found. One female which mated with F2.12-1 was sacrificed so that the ability of this male to achieve fertilization could be assessed. No pronuclei were seen in eggs derived from this female indicating a lack of fertilization, and no sperm were seen in the medium surrounding the eggs, indicating inability of sperm production. To pursue a possible cause for male infertility, the animal was anaesthetized and its testes were examined visually. Sperm formation was not seen except in a small region in one testis. However the size was comparable to that of normal, mature, testes. Since testicular development is dependent on sperm and testosterone production, testes may not reach full size if there is a primary defect in fertility. The anatomy observed

indicates a post- maturational infertility in this mouse. The liver and spleen appeared normal. Blood counts were assessed twice during this time and were comparable to normal controls.

Sex transmission distortion

Analysis of the sex ratios of offspring derived from crosses between two heterozygotes revealed a sex distortion (table 5). Among 99 total mice derived from heterozygous parents, 38% were male and 62% were female. A Chi-square analysis of whether these data are consistent with 50% transmission of the X or Y chromosome leads to:

$$5.024 < X^2 = 5.34 < 6.635$$
$$.025 > p > .01$$

Therefore, H_0 is rejected with about a 98% level of confidence; i.e., sex transmission distortion is observed. The data were analyzed to determine whether the ß80 allele is preferentially transmitted to a particular sex by testing the ratios of positive mice over total mice for each sex. In heterozygous crosses, 63% of the males were positive, and 62% of the females were positive for the ß80 allele. Therefore, the ß80 allele is not preferentially transmitted to either sex, but its presence is deleterious to Y chromosome transmission, regardless of the resulting ß80 genotype. These effects are in contrast to the transmission distortion mutation described by Palmiter et al. (1984), which resulted in preferential transmission of the non- transgenic chromosome due to interference with meiosis in sperm carrying the transgenic allele.

This effect was further investigated to assess whether the sex transmission distortion is observed in heterozygous vs. wild-type matings, and whether the effect is seen for positive parents of a particular sex. The founder mouse, a female, was bred to produce 40 offspring; 15 were males and 25 were females. In this cross, equal transmission frequencies of the ß80 allele are seen for males and females, with 33% of males and 33% of females transgenic. The low transmission frequency indicates that the ß80 founder mouse was a germline mosaic.

For crosses between transgenic females and wild-type males, the data are consistent with a sex tranmission distortion (9 males and 13 females); not enough animals were blotted to assess

preferential ß80 transmission. If these data are added to data from the founder mouse (which should not be relevant to sex transmission, regardless of whether the germline is mosaic), a 95% level of confidence is achieved for rejection of the H_0 hypothesis:

$$2.71 < X^2 = 3.16 < 3.84$$
$$.05 > p > .025$$

Since transgenic females are capable of exerting sex distortion, this shows directly that Y-carrying transgenic sperm are not necessarily affected.

Analysis of crosses between transgenic males and wild-type females indicated a lack of sex transmission distortion. 32 males and 25 females were produced (not enough were blotted to assess preferential ß80 transmission):

$$0.455 < X^2 = 0.860 < 1.323$$
$$.50 > p > .25$$

Therefore, H_0 is not rejected.

These data indicate that sex distortion results from an effect on female transgenics, and implies that the sex distortion seen in heterozygous crosses was due to the female transgenic component in these crosses. This would indicate a postfertilization effect on the development of Y-carrying embryos, or a deleterious effect on fertilization of transgenic mouse-derived eggs by Y chromosome-carrying sperm. The data show that the presence at this point of the ß80 insert does not affect Y chromosome transmission, since it is transmitted at the same frequency in males and females. This indicates a female meiotic effect, where eggs derived from ß80 female heterozygotes exert a deleterious effect on the fertilization and/or development of Y-carrying embryos, which is independent of how the ß80 allele assorts in the egg or the sperm.

Absence of human globin expression

Protein analysis was performed prior to RNA studies since the ß80 line displayed a visible phenotype which might have been due to the production of human globin protein during development and was potentially visible on Coomassie-stained gels. This analysis is also useful for the production

of ß80 substrains which contain only one type of mouse globin mRNA, which may simplify Northern analyses in future globin mRNA studies. An interesting result of performing these gels was a fortuitous discovery of a new alpha-globin variant in the mouse colony (B. Alter).

In Triton gel analysis, absence of mouse $ß^s$ chains unmasks potential human ß-globin chains. Hemoglobin lysates were prepared from positive mice and analyzed by Triton gel analysis for the existence of bands comigrating with human adult ß-globin (figures 18a-c). The absence of human globin bands was established for thirteen mice which were both hemizygous at the ß80 locus and homozygous at the mouse Hbb^d locus. Most notably, a confirmed homozygote (z) which died at day 17 of age had the mouse $ß^d$ homozygous genotype and had no visible bands comigrating with human globin on Triton gel analysis.

Analysis of Cosmid Clones

Nine unique cosmid clones were derived from the cosIII library. Clone 5-1 was isolated from a single colony in the first screen (and was rescreened), and the other eight clones (labelled α) were isolated from the second screen. Ethidium bromide staining of digested cosmids indicated the isolation of clones which were in the size range of cosmids (35-50kb). Clone 4-4α was about 21kb, indicating deletion of sequences after DNA packaging.

For Southern analyses (table 6), cosmids were digested with eight enzymes, EcoRI, BamHI, HindIII, SalI, KpnI, BalI, EcoRV, and BglII. The control used was cosHG28tk, a cosmid which contains the fetal and adult human ß-globin genes. These digests were Southern blotted and hybridized sequentially with nick-translated probes. Cosmids digested with the first five enzymes (EcoRI, BamHI, HindIII, SalI, and KpnI) were all successively hybridized with lambda phage, wild-type mouse genomic DNA, pBR322, ßHindIII fragment (gel- electroeluted human insert of the plasmid), and λEB fragment (gel-electroeluted phage insert). Cosmids digested with BalI, EcoRV, and BglII were probed with the Charon 4A vector λHεG1. Cosmids digested with EcoRI were also probed with the 3.1kb EcoRI fragment (containing genomic sequences upstream of delta- globin).

Hybridization to a lambda DNA probe verified the presence of lambda sequences in all cosmids (figures 19-21b). The intense hybridization seen in comparison to the control, and the presence of several migrating bands in certain clones using enzymes with only one recognition site in the cosmid vector, were initial indications that the ß80 locus was successfully cloned. Hybridization of lambda to cosHG28tk demonstrated that cos homology was detected by this probe on Southern analysis (this clone contains no other lambda DNA), and hybridization of pBR322 (from which the pHC79 vector is derived) (figures 19-21d) to the same fragment verified this interpretation. Since hybridization might have resulted from homology to the cos sequence present in all clones regardless of their content, three experiments were performed to assess the presence of non-cos sequence-derived lambda. First, blots were reprobed with pBR322 to determine whether fragments existed which hybridized with lambda but not vector. Second, blots were reprobed with λEB (figures 19-21c), a fragment of the left arm that does not contain the cos sequence, but is homologous to the left

arm of Charon 4A. Third, clones were digested with BglII, which releases the 1.8kb BglII fragment spanning the cos sequence (and cohesive ends), and were probed with a Charon 4A vector capable of hybridizing to any λHßG1-derived lambda sequences (figures 24a,h). This last method ensures that transgenic mouse-derived Charon 4A vector sequences, if present, will be detected in non-cos sequence-associated fragments.

A high molecular weight fragment was observed which hybridized to lambda but not pBR322 for EcoRI-digested 10-5α (figures 19b,d). This indicated that at least part of the lambda homology detected was not related to the presence of cos sequences associated with the cosmid vector. In general, clones digested with EcoRI, BamHI, HindIII, SalI, and KpnI showed a close association between lambda and vector sequences, indicating that these enzymes did not release fragments associated only with lambda. Since most of the right arm of Charon 4A is replaced with sequences not homologous to lambda, these fragments would not be detected by this method.

Comparison of blots probed with λ or λEB assessed the presence of λ DNA- derived fragments from the left arm of Charon 4A which were separated from cos sequences by digestion. Left arm-derived DNA was seen for EcoRI-digested 14-7α (figures 19b,c) and BamHI-digested 14-2α, 14-7α, 15-4α, and 5-1 (figures 20b,c). A negative result in this experiment may mean that unique left arm- derived DNA is not released by these particular digests. Not detectable by this experiment are right-arm derived Charon 4A sequences.

Hybridization of BglII-digested cosmids to λHεG1 (figure 24h) demonstrated the presence of non-cos sequence-derived Charon 4A in all cosmids. A 1.8kb fragment representing the cos sequence was seen in all clones, and in the two positive controls. Additional hybridizing fragments were seen in all other cosmid clones, which must derive from non-cos related Charon 4A sequences of the transgenic λHßG1 insert. This demonstrates that all nine cosmids contain DNA from the ß80 locus of insertion.

The observation of cosmid fragments (other than the 1.8kb fragment) which comigrate with λHεG1 fragments on the BglII blot provides the strongest evidence that Charon 4A-derived sequences are present in all cosmids. In addition, BglII digestion of cosmid clones 4-4α and 10-5α

and the λHεG1 control release a 4.9kb fragment, which derives from the right arm of Charon 4A. The BalI (figure 22h) and EcoRV (figure 23h) blots may also contain comigrating fragments.

To determine whether cosmid clones contain mouse DNA flanking the ß80 locus of insertion, blots were probed with nick-translated mouse genomic DNA. Cosmid clones 7-6α, 10-5α, 12-1α, 13-3α, 14-2α, 14-7α, and 15-4α hybridized to mouse DNA in all five digests analyzed (figures 19-21e), indicating that they contain repetitive mouse DNA. Absence of hybridization to cosHG28tk showed that human repetitive sequences were not detected by this procedure, reconfirming the presence of flanking mouse DNA in hybridizing clones. In addition, hybridization to single-copy sequences, such as globin, was ruled out. Lack of hybridization of clones 4-4α and 5-1 indicated that these clones do not contain repetitive mouse sequences. Clone 4-4α is composed of lambda sequences, as all ethidium bromide fragments hybridize to lambda DNA (figures 19-21a-c, 24h). However, clone 5-1 contains several fragments which do not hybridize to lambda and which do not comigrate with or hybridize to known λHßG1- derived fragments. Since these were thought likely to be derived from mouse DNA, cosmid 5-1 was further subcloned for chromosome mapping and RFLP analysis.

Hybridization analysis using the ßHindIII fragment was performed to assess the human genomic content of the cosmid clones (figures 19-21f). Specificity of the probe for globin was demonstrated by hybridization to cosHG28tk, and cross-hybridization to delta-globin was also shown. However, no new bands were seen for any ß80 cosmid clones. (The bands seen are derived from previous hybridization to pBR322, for which the stripping procedure was incomplete.) The absence of hybridizing internal fragments of human genomic DNA, the failure to observe bands with increased intensity, and the use of cosHG28tk as a positive control showed that no ß80 clones contain delta- or beta-globin.

A lack of hybridization of the 3' end of the human globin clone is consistent with absence of nearby sequences of the left vector arm. A 1.5kb KpnI fragment present in the λEB clone and in Charon 4A was not detected (figures 21a-c), indicating that this region was not cloned.

These two results indicated that genomic globin sequences downstream of the SalI site were not cloned in these cosmids. Since ß80 genomic DNA was digested with SalI to produce the cosIII library, the 3.1kb EcoRI fragment corresponding to genomic DNA upstream of the SalI site was used to assess the presence of these sequences using EcoRI-digested cosmid clones (figure 19g). Specificity of this probe for the 3.1kb EcoRI fragment was demonstrated by intense hybridization of cosHG28tk, which contains this fragment; other fragments did not hybridize. Specificity for 3.1kb fragment-homologous DNA was further indicated by comparison to the pBR322 hybridization (19d). pBR322 hybridized to low molecular weight bands in clones 7-6α, 12-1α, 13-3α, 14-2α, 15-4α, and 5-1 which were not detected by the 3.1kb probe, ruling out artifactual hybridization due to possible vector contamination of electroeluted probe. All cosIII clones hybridized to the 3.1kb EcoRI fragment, demonstrating the presence of human genomic DNA in every ß80 cosmid clone. However, none of these fragments migrated at 3.1kb, indicating loss of EcoRI recognition sites and/or rearrangement of DNA at the ß80 locus. Close association of homologies between vector and 3.1kb fragment DNA for most bands also indicated cloning of 5' genomic sequences upstream of the SalI site, and loss of the adjacent EcoRI site.

Overlapping fragments seen among cosmid clones (table 7) are a further indication that the ß80 locus of insertion was successfully cloned. The occurrence of comigrating fragments which hybridize to vector in digests which only recognize one site in pBR322 indicate extension into homologous cloned DNA fragments. This places clones 7-6α, 12-1α, and 14-2α into one group, and 14-7α and 15-4α into another group of overlapping clones. Hybridization to lambda DNA (λ, λEB or λHεG1) is consistent with this grouping, but cloning of different loci containing lambda homology might occur in a rearranged locus. The BglII digest indicates that lambda DNA in clone 5-1 may come from the same locus as 14-2α, which would link 5-1 to the locus defined by the other clones; clone 4-4α may also follow this pattern through linkage of Charon 4A sequences. These data are consistent with the cloning of a single mouse locus, but leave open the possibility of having cloned two mouse loci associated with the ß80 insert.

Chromosomal Mapping of the ß80 locus

The 5-1 subclone A12 was used to probe a mouse-hamster hybrid panel (figure 25). In this experiment, the ß80 allele was ruled out as being on all chromosomes except 1, 3, 8, and 9. Additional data are required to determine on which of these chromosomes the ß80 insert resides. The question of whether a translocation is involved in this mutation is not addressed by this experiment, since the A12 clone is not presumed to span a potential translocation breakpoint; therefore other chromosomes than that identified by A12 may be involved. However, this question will by addressed by cytogenetic experiments in progress.

Restriction Fragment-Length Polymorphisms

A search for RFLP's using 5-1 EcoRI subclones A9 and A12 showed that the subcloned fragments were derived from mouse genomic DNA, since comigrating EcoRI fragments were seen in mouse DNA and the plasmid control (figures 26a,b). Homologous DNA was not detected in human or hamster DNA. Therefore, the clones probably lacked a conserved coding region, but were useful for somatic hybrid analysis described above. A comparison of wild-type and ß80 heterozygote DNA shows a similarity of restriction pattern for each clone in all three digests. However, a band which is seen in EcoRI-digested heterozygote DNA probed with A12 is not seen in the wild-type DNA. This may identify an RFLP in a probe derived from the insertion locus. If so, the A12 clone may contain sequences at the site of insertion which were rearranged, creating a high molecular weight EcoRI fragment homologous to A12.

Table 1
Microinjection Experiments

Clone	copies/pl	embr. impl.	embr. u-inj.	mice born	total expts.	total deliv.	%born/ impl.	rate of preg.
λHγG5	75-1000	660	1100	32	30	9	4.8%	30%
λHßG1	250-400	735	1296	100	36	19	14%	53%
λHεG1	200-500	66	126	7	3	2	11%	67%
ßpst	500,1000	30	48	1	2	1	3%	50%
ßpst/ DHFR	500	78	120	4	3	3	5%	100%
		1569	2650	144	74	34	9.2%	46%

%impl/u-inj. = 60%

Table 2
Mating Data for ß80 Line

Parental lineage:	+/- x +/-		+/- x -/-		-/- x +/-	
	M	F	M	F	M	F
# of different parents used:	18	20	5	12	4	5
# of litters:	73		31		5	
Total # of pups:	473		283		45	
# of pups displaying phenotype:	35		0		0	
% abnormal phenotype:	7.4		0		0	

473-35 = 438 normal mice

438/3 = 146 = expected # of homozygotes

35/146 = 24% of homozygotes surviving
to display abnormal phenotype
= 76% penetrance of homozygous prenatal lethality

Table 3
Chi-square analysis of heterozygous matings in the ß80 line

Blotting results:	+	-	total
observed:	62	37	99
3/4 expected:	(74.25)	(24.75)	
2/3 expected:	(66)	(33)	

3/4 X^2 analysis: $X^2 = (62-74.25)2/74.25 + (37-24.75)2/24.75$
$= 2.02 + 6.06 = 8.08$

$7.88 < X^2 < 10.83$
$.005 > p > .001$

Reject H_o with >99% level of confidence.

2/3 X^2 analysis: $X^2 = (62-66)2/66 + (37-33)2/33$
$= .24 + .48 = .727$

$.455 < X^2 < 1.323$
$.50 > p > .25$

Do not Reject H_o.

Table 4
Phenotypic anomalies in the ß80 line

Mouse:	1	2	3	4	5	6	7	8	9	10	11
Sex	M	M	F	F	M	F	F	F	M	F	F
genotype	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
oblig. +/-			*				*		*		*
Lymphoma	+	+	+	+	+	+	+	?	-	-	?
Age of onset	6mo.	15mo.	16mo.	13mo.	13mo.	14mo.	13mo.				
	see appendix 1										
Hepato-splenomegaly	+	+	+	+			+		-		
Enlarged thymus	+		+								
Ascites			+			+			-		
Pancytopenia			+						-		
Skeletal defect	+	-	-	-	-	-	-	-	-	-	-
Neurologic (twirling)	-	-	-	-	-	+	-	-	-	-	+
Growth retardation	+	-	-	-	-	-	-	-	-	-	-
Hypogonadism	+					+	-		+	+	+

Number	Mouse	Comments
1	AbnL	F3.31 blood values: value and (normal)
2	F2.43	WBC $1.0x10^3$ ($8.3x10^2 \pm 700$); RBC $1.26x10^6$
3	F3.31	($8-10x10^6$); Hgb 3.5 (14); Hct 7.7 (45);
4	F2.12-3	MCV 61.2 (45 ± 2); platelet $1.68x10^5$
5	F3.17	($1.0x10^6$)
6	F3.22	
7	F3.32	
8	F2.67	Died of sepsis (leukopenia?)
9	F2.12-1	
10	F3.20	
11	80.23	

Table 5
Sex Ratio Distortion in the ß80 Line

parental lineage:	+/- x +/-		+/- x -/-		-/- x +/-	
	M	F	M	F	M	F
Male offspring	38		32		24	
% males	38%		56%		39%	
Female offspring	61		25		38	
% females	62%		44%		61%	
Total mice	99		57		62	

Figure 12
Southern analysis of EcoRI-digested spleen DNA:
Initial identification of β80 founder mouse using λHβG1 probe

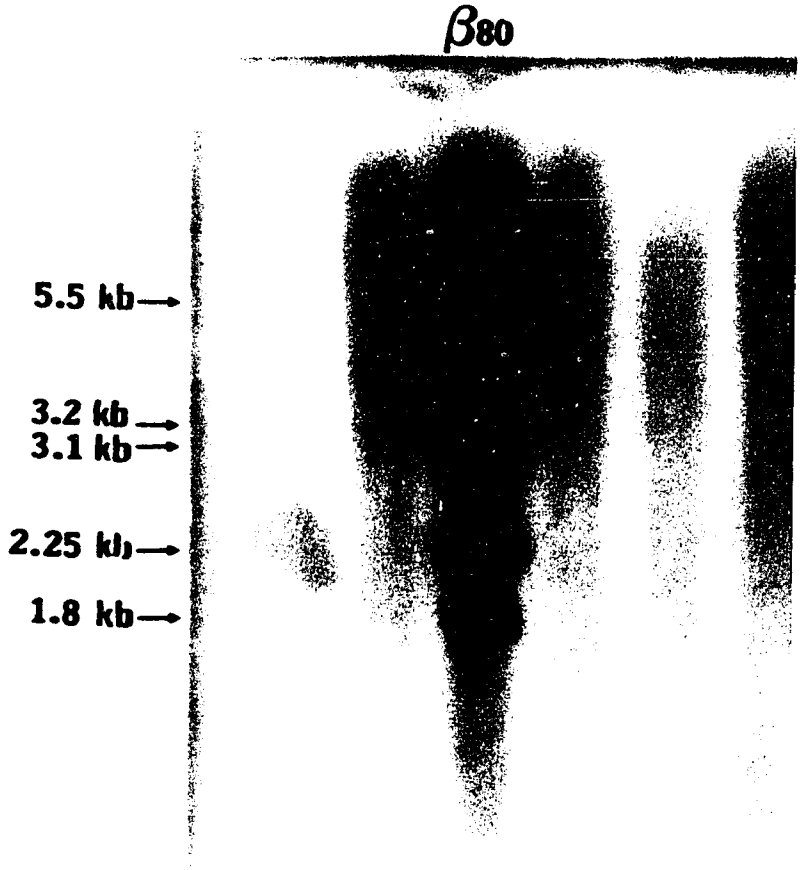
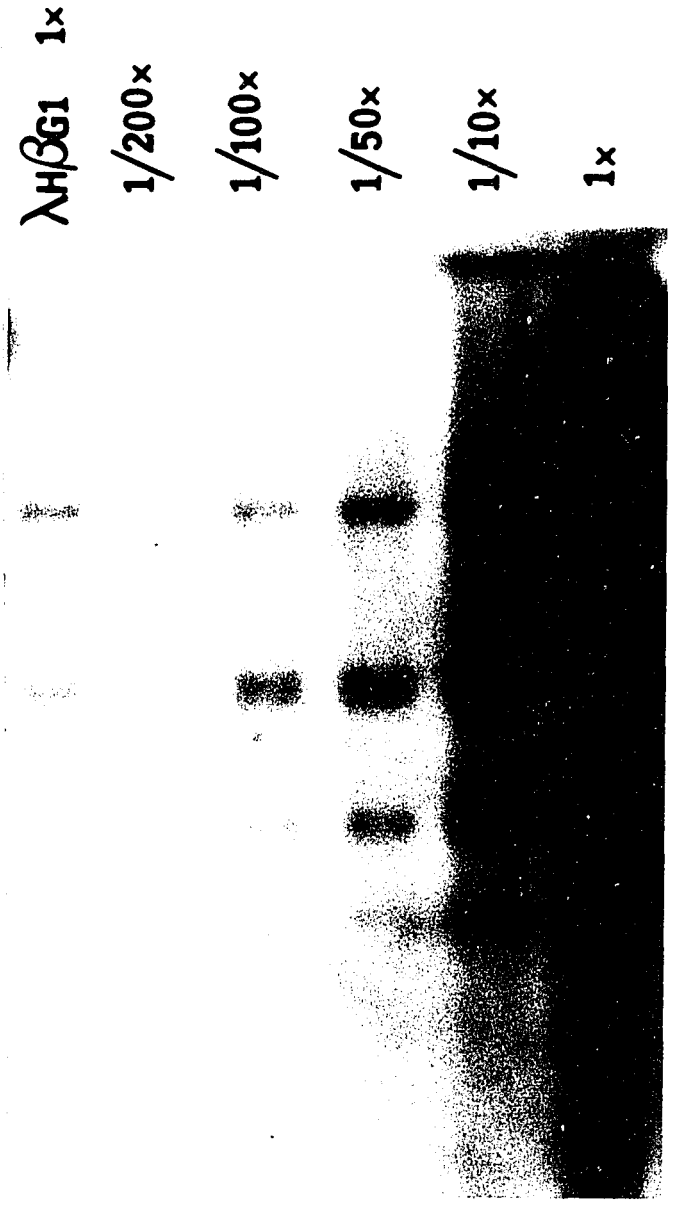


Figure 13
Copy number analysis of β80



Dilution Blot of β80: EcoRI-digested phage DNA was loaded at the equivalent of one copy per cell in the left-most lane. EcoRI-digested genomic DNA was diluted from right to left as indicated.

Figure 14
Growth chart of +/- vs. +/- litter

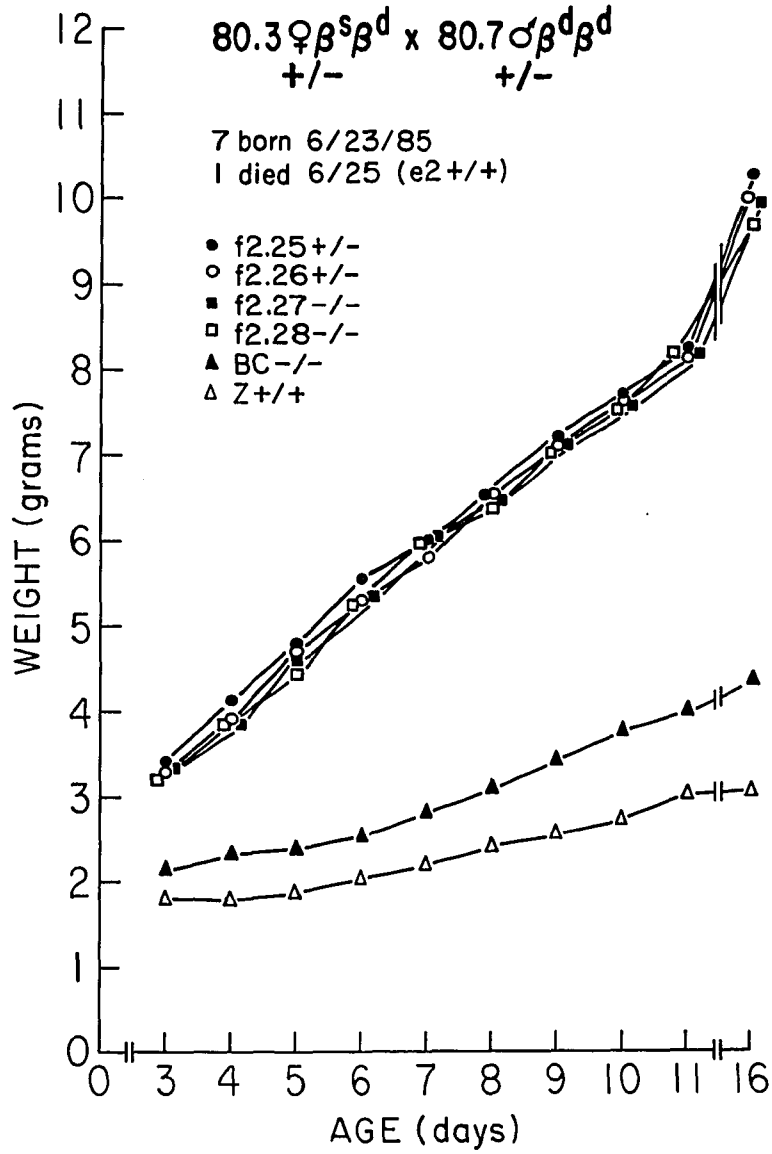
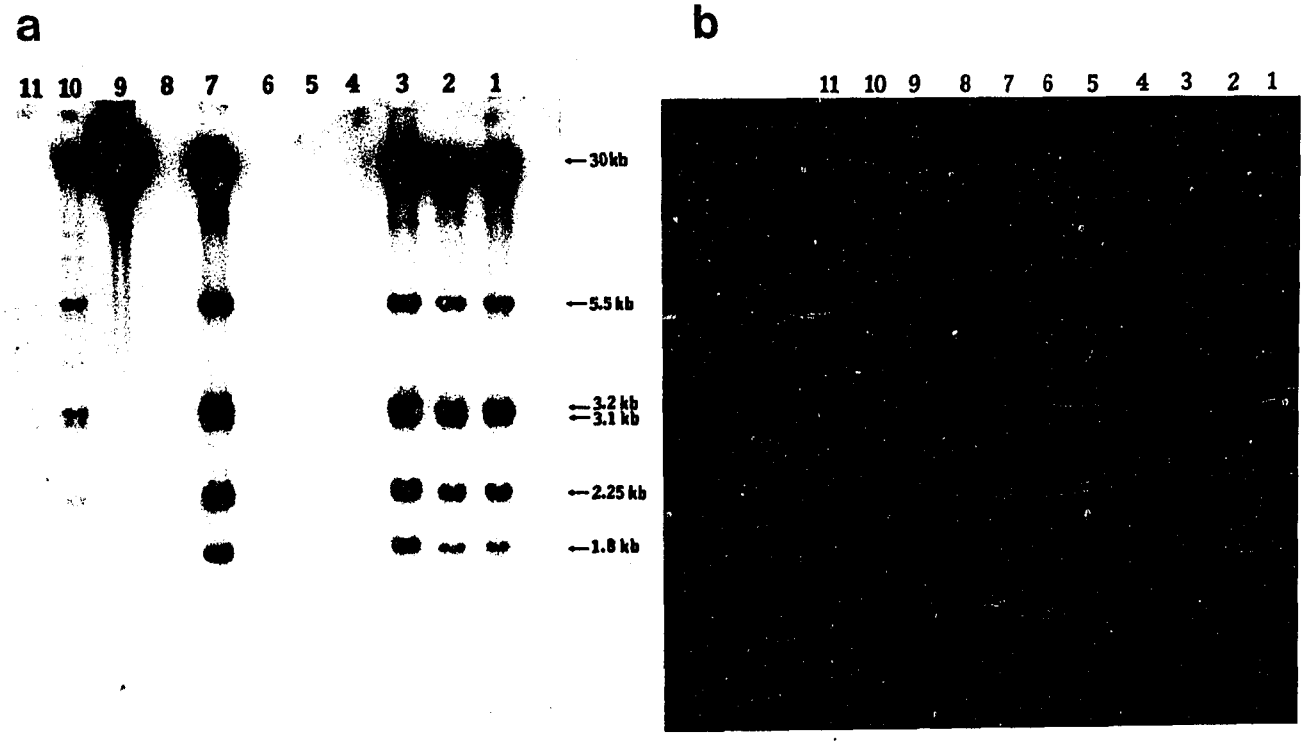


Figure 15
Genotypic analysis of mutant β80 mice



Lane	Sample	Mutant phenotype
1	e1	++
2	z	++
3	e2	++
4	e3	no
5	x1	no
6	e4	no
7	e5	++
8	e6	++ (sample degraded)
9	e7	++ (sample undigested)
10	80.3	positive control
11	80.1	negative control

Figure a; Southern blot probed with λHβG1. Figure b; ethidium bromide stain. Lane 10 contains DNA from a direct descendant of the founder mouse; therefore it is an obligate heterozygote. Lane 11 contains a negative control. Lanes 1,2,3, and 7 contain DNA from abnormal mice whose DNA was rescued for analysis before the samples became degraded. All samples were loaded at the same DNA concentration, as indicated by ethidium bromide staining.

Figure 16
AbnL mouse

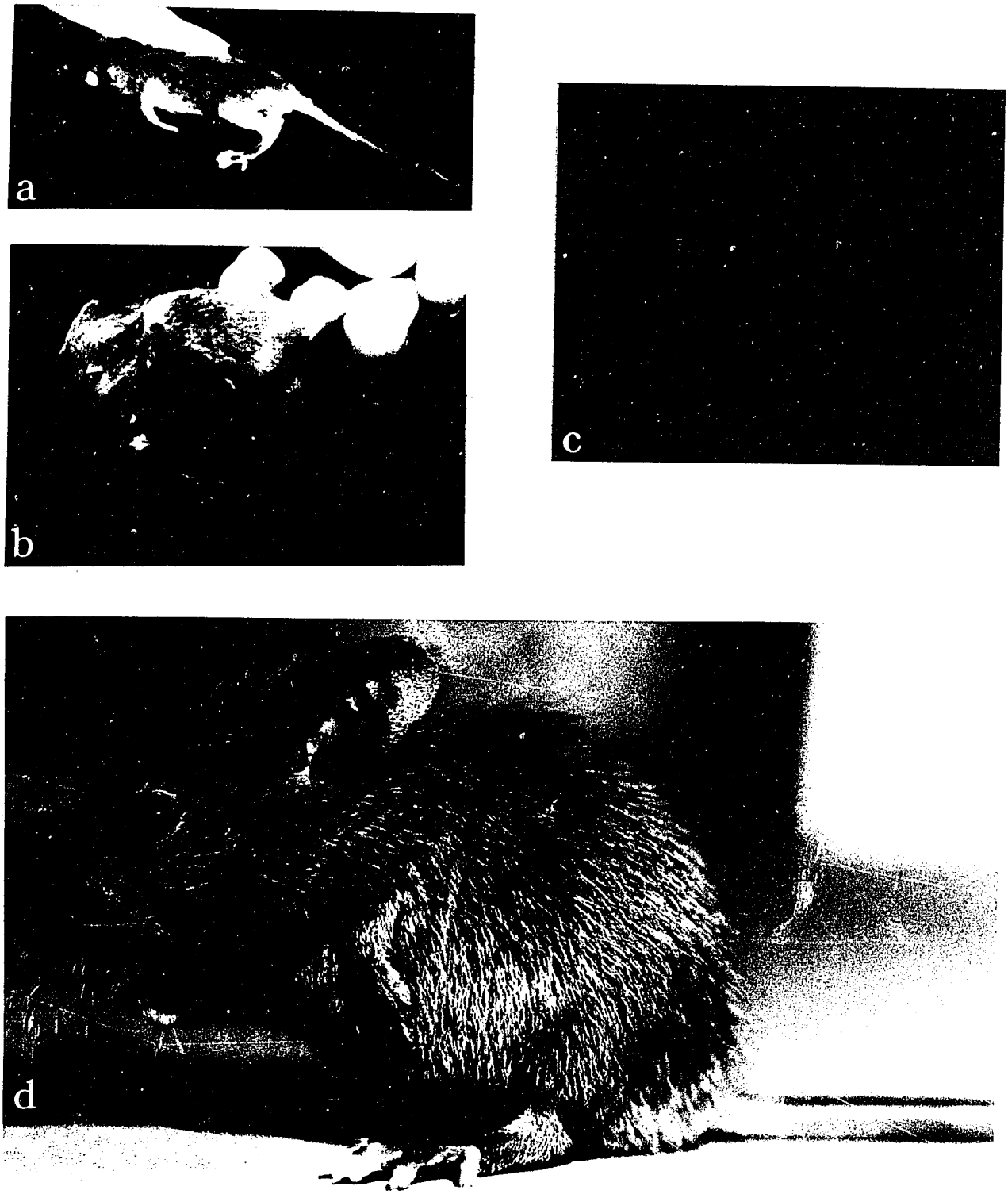


Figure 16
AbnL mouse

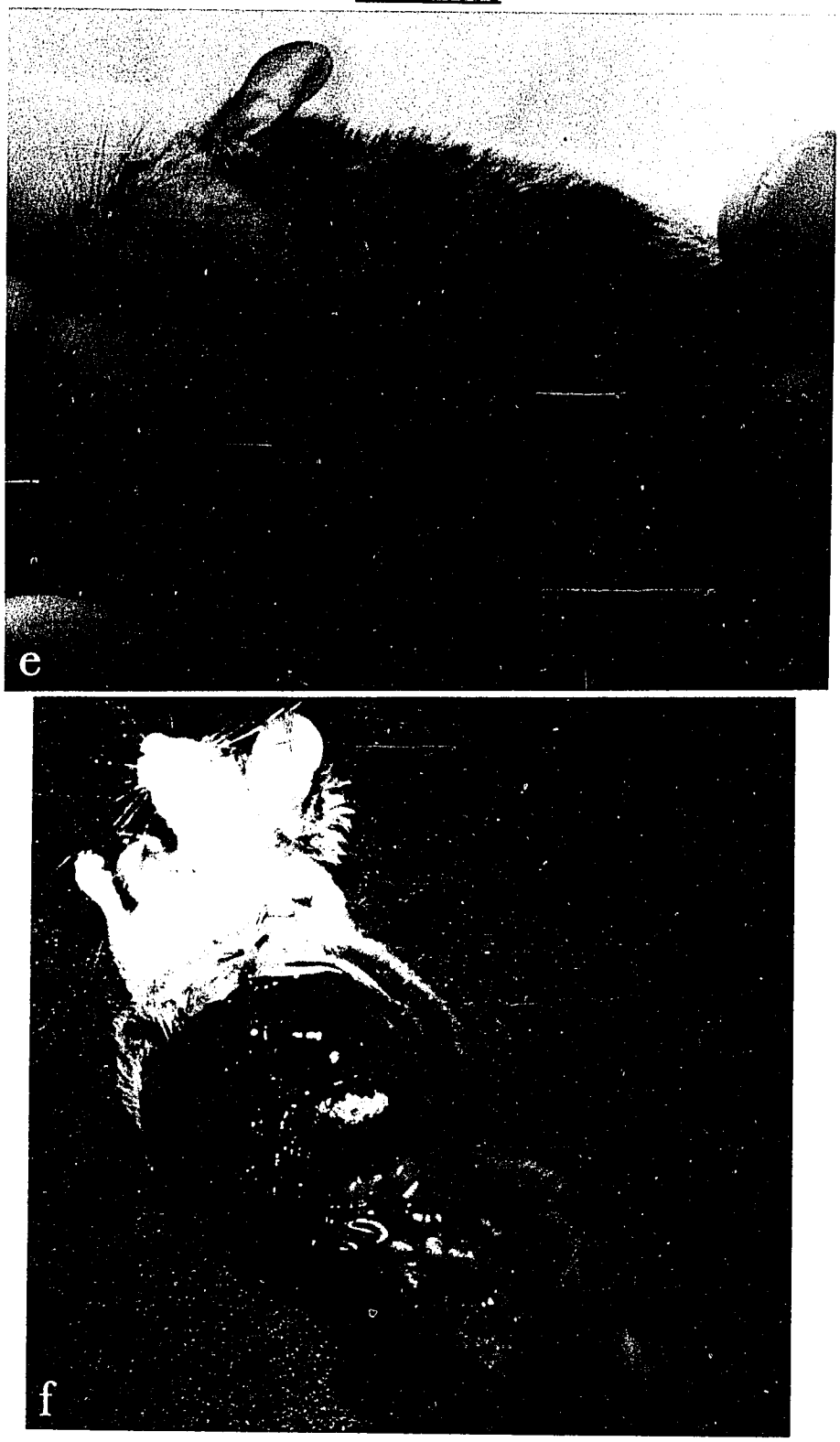
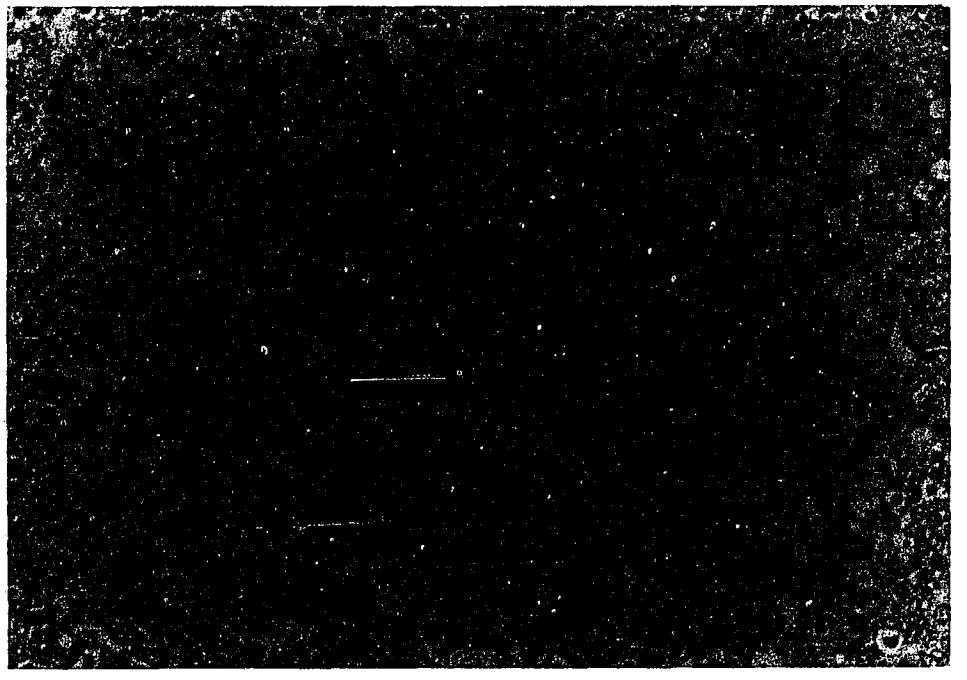
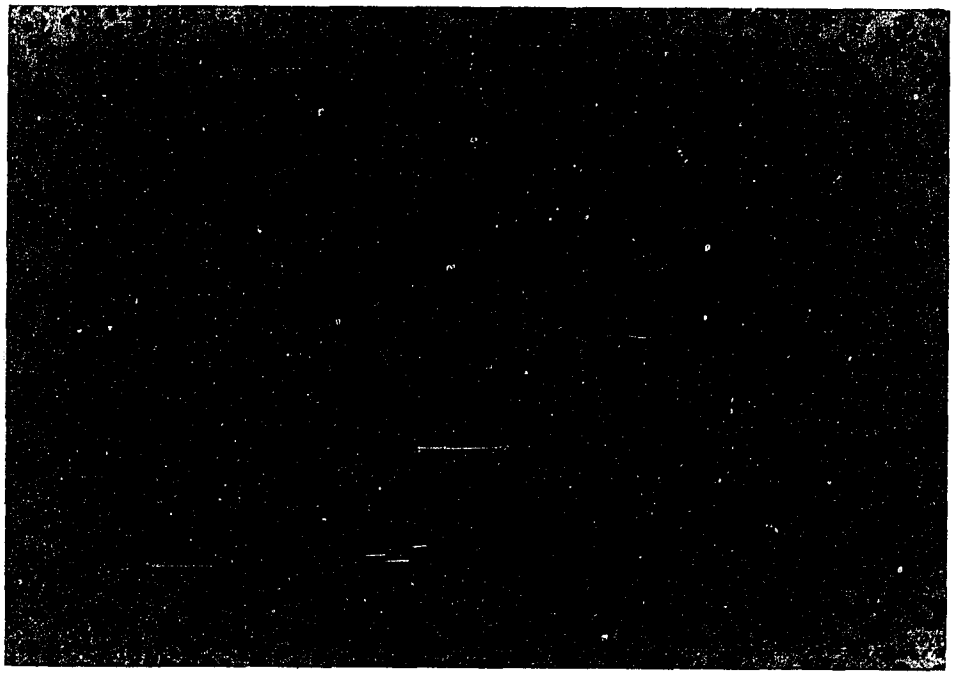


Figure 17
Histopathology

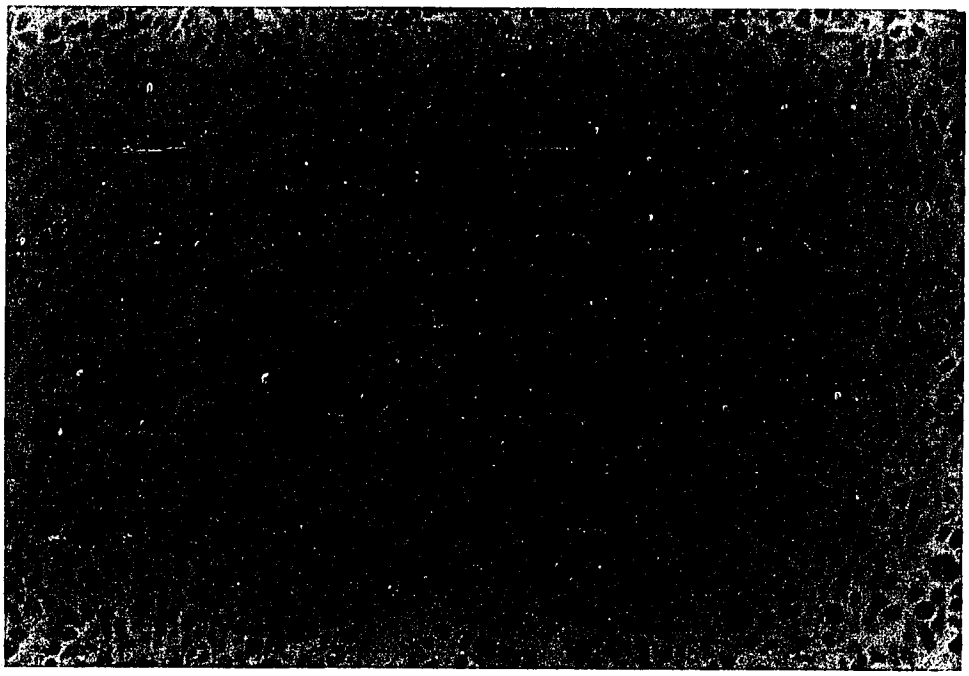


a. AbnL liver



b. AbnL spleen

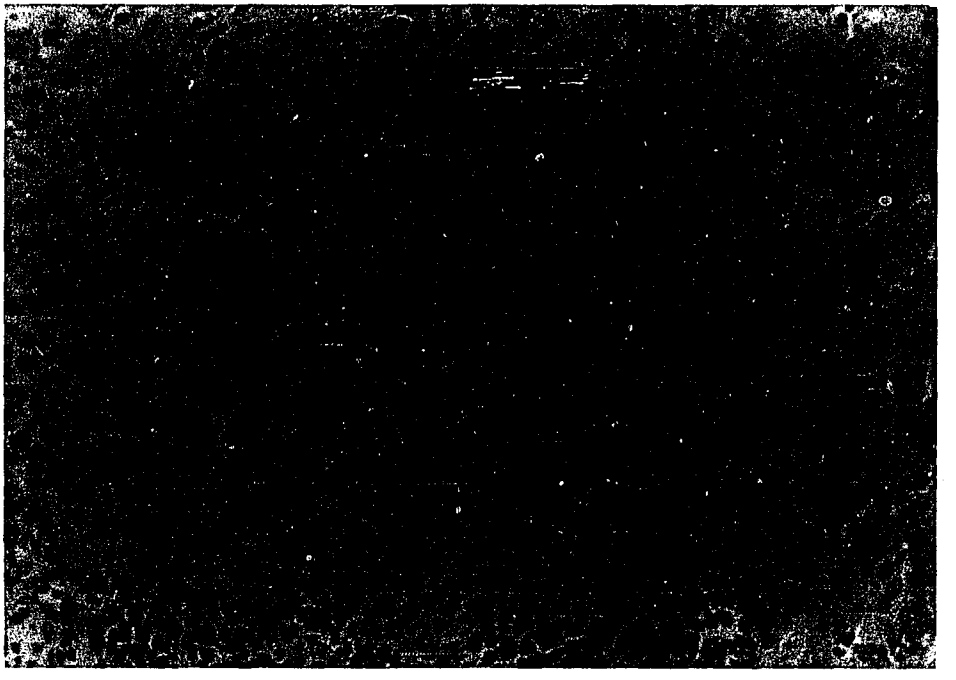
Figure 17
Histopathology



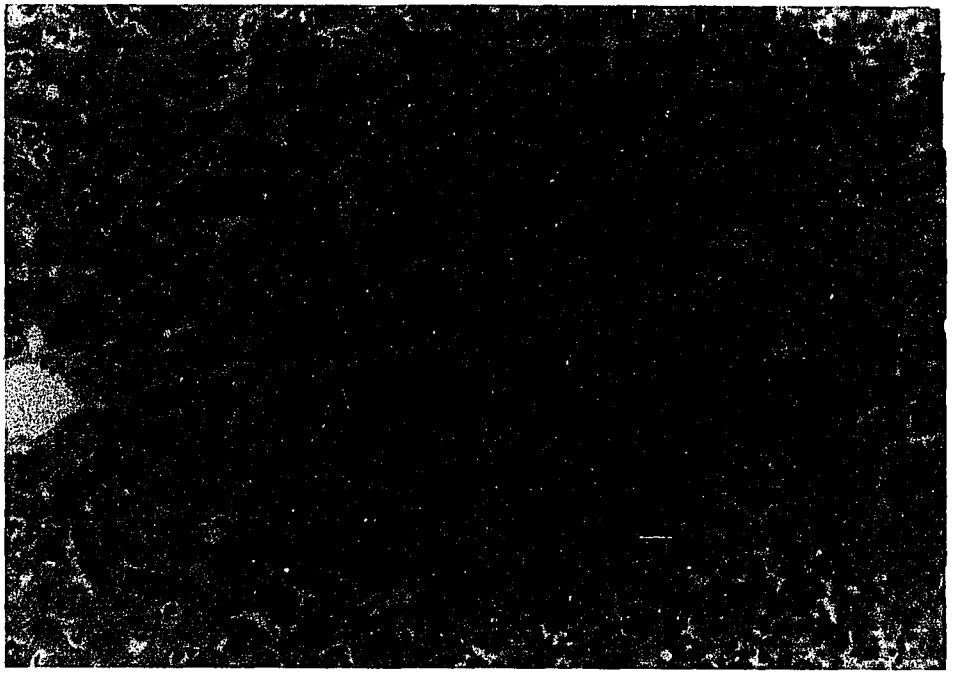
c. AbnL thymus

d. AbnL lymph node

Figure 17
Histopathology



e. F2.43 liver



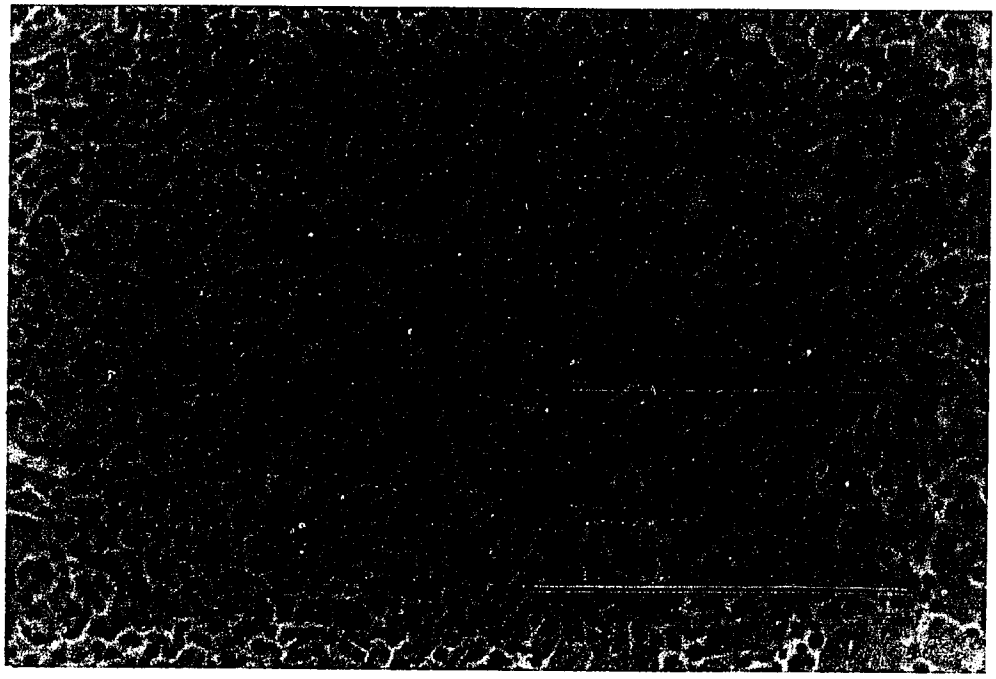
f. F2.43 liver

Figure 17
Histopathology

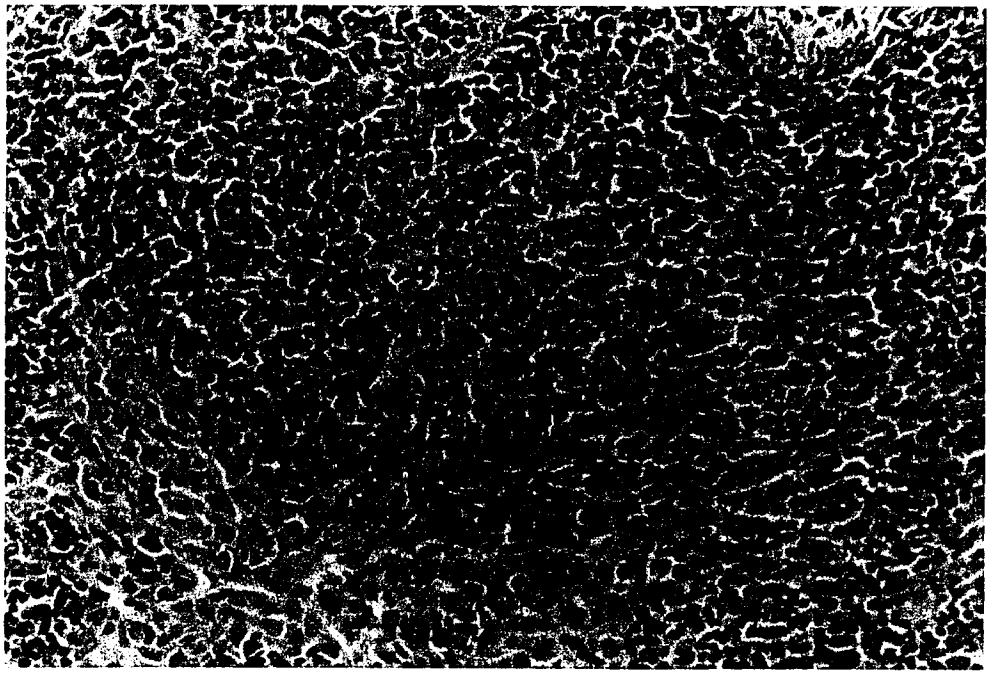
g. F3.22 lung

h. F3.22 liver

Figure 17
Histopathology

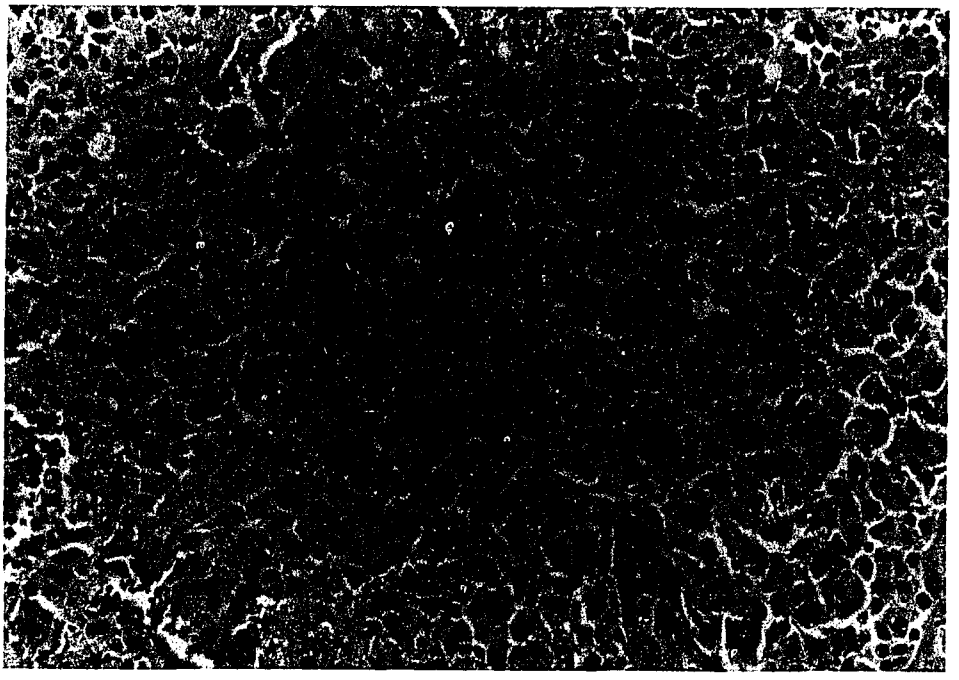


i. F3.31 liver

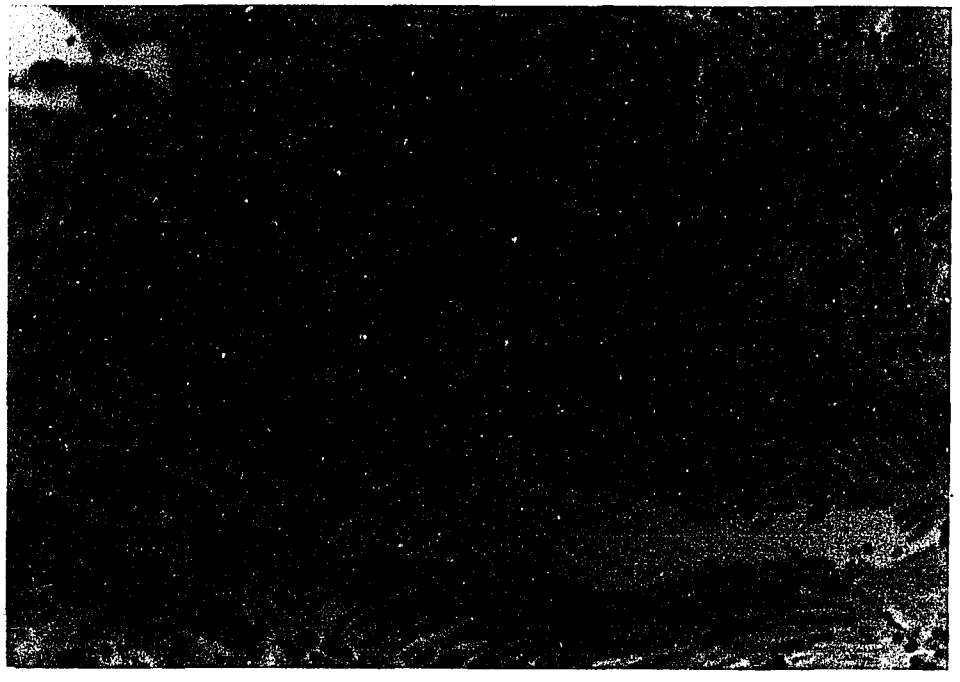


j. F3.31 spleen

Figure 17
Histopathology

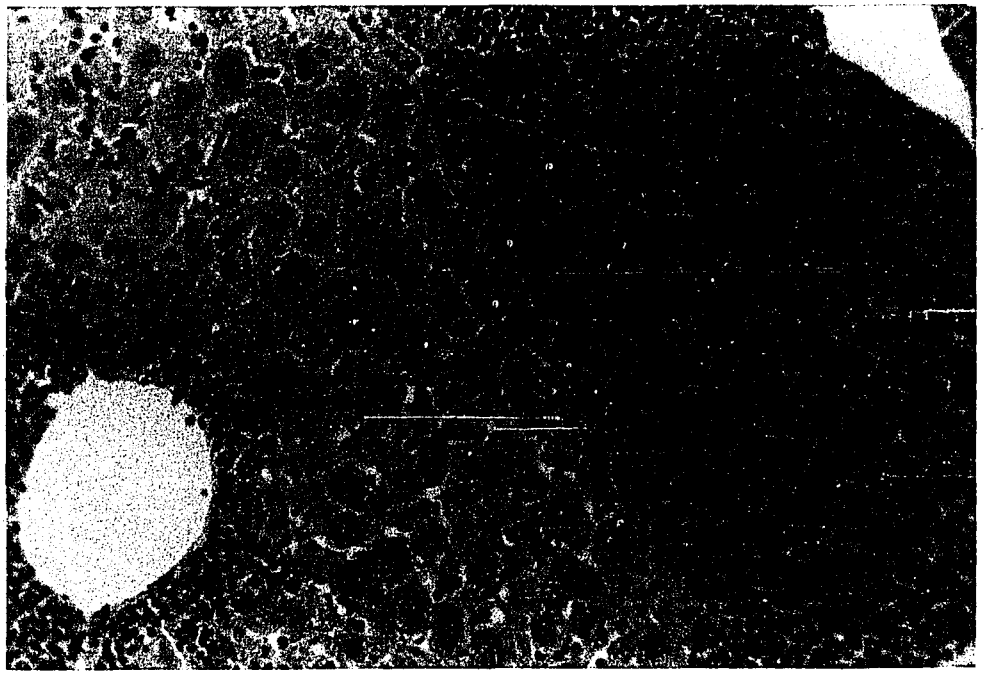


k. F2.12-3 liver

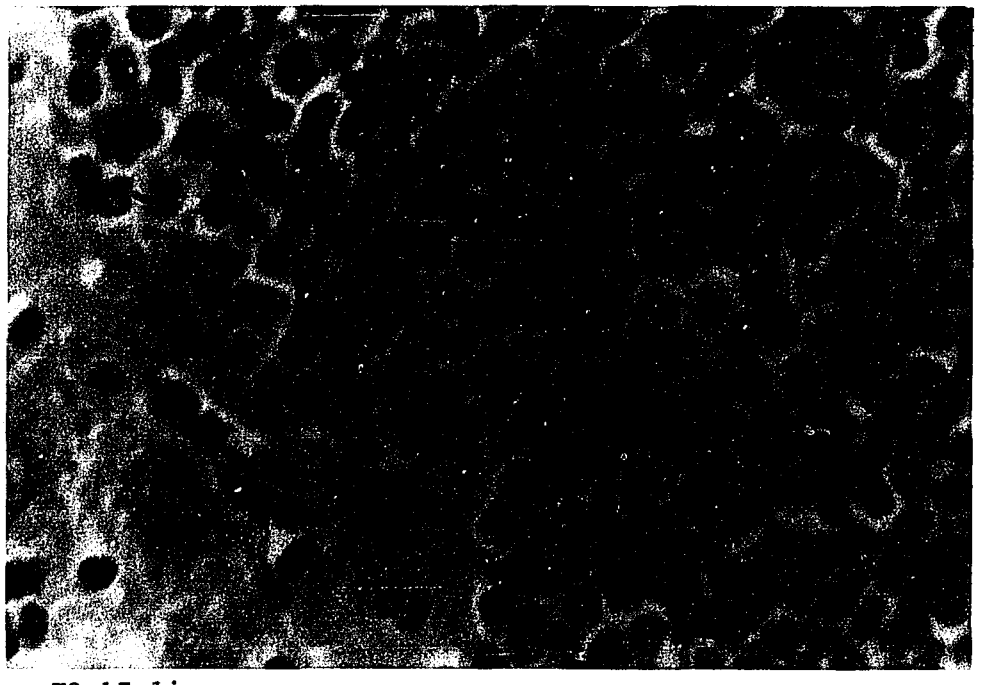


l. F2.12-3 kidney

Figure 17
Histopathology

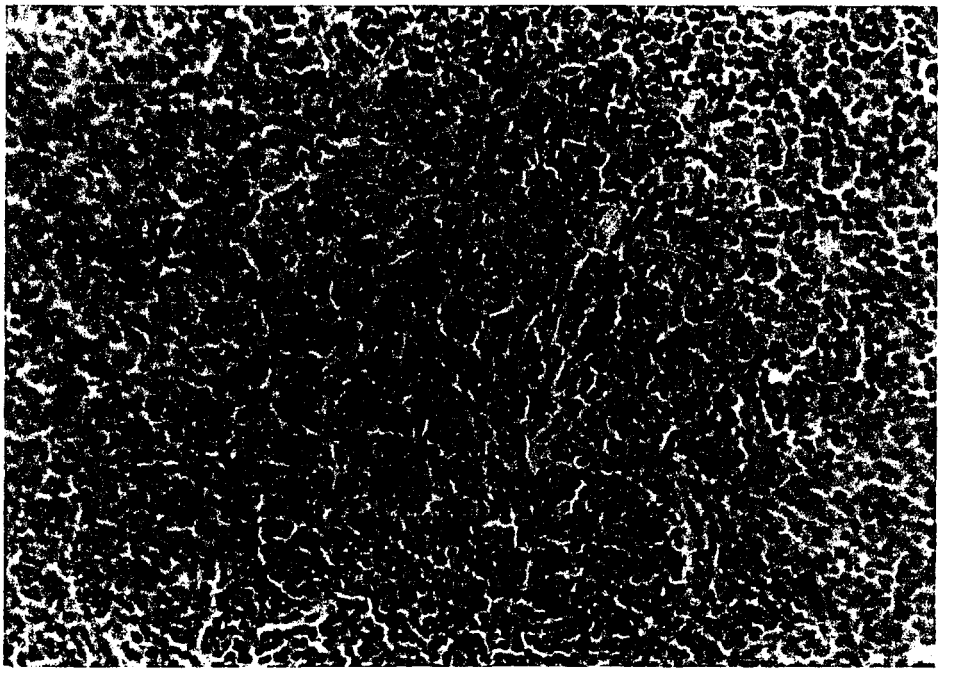


m. F3.17 liver

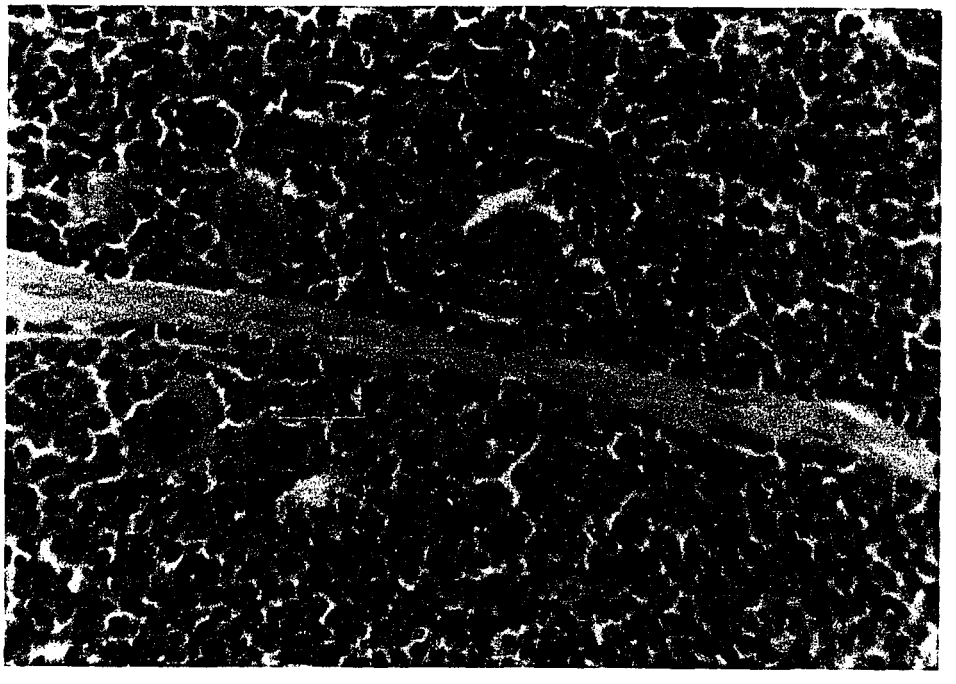


n. F3.17 liver

Figure 17
Histopathology

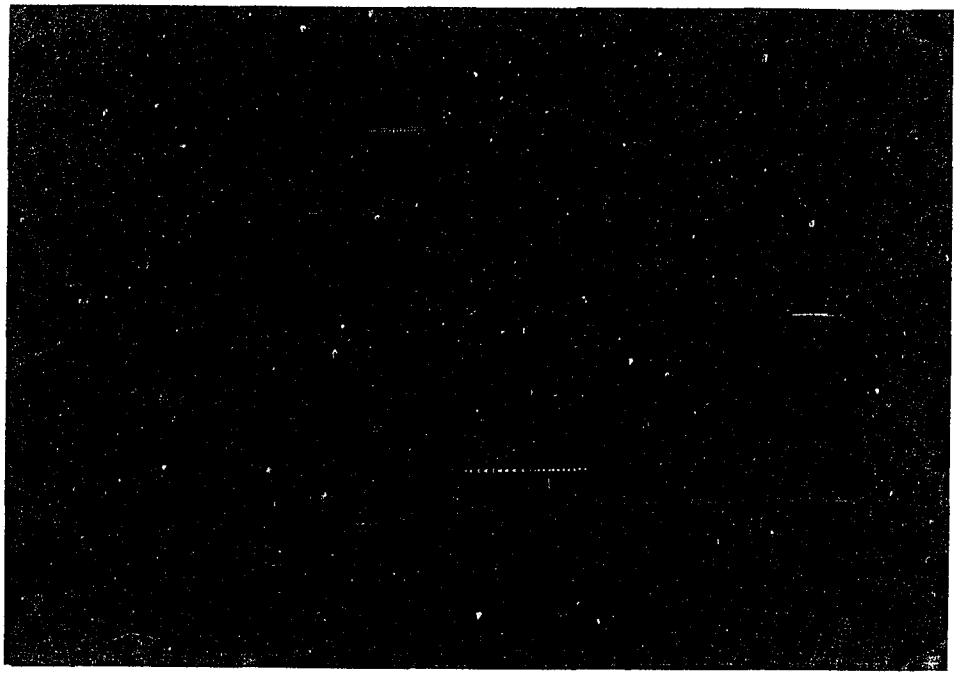


o. F3.17 spleen

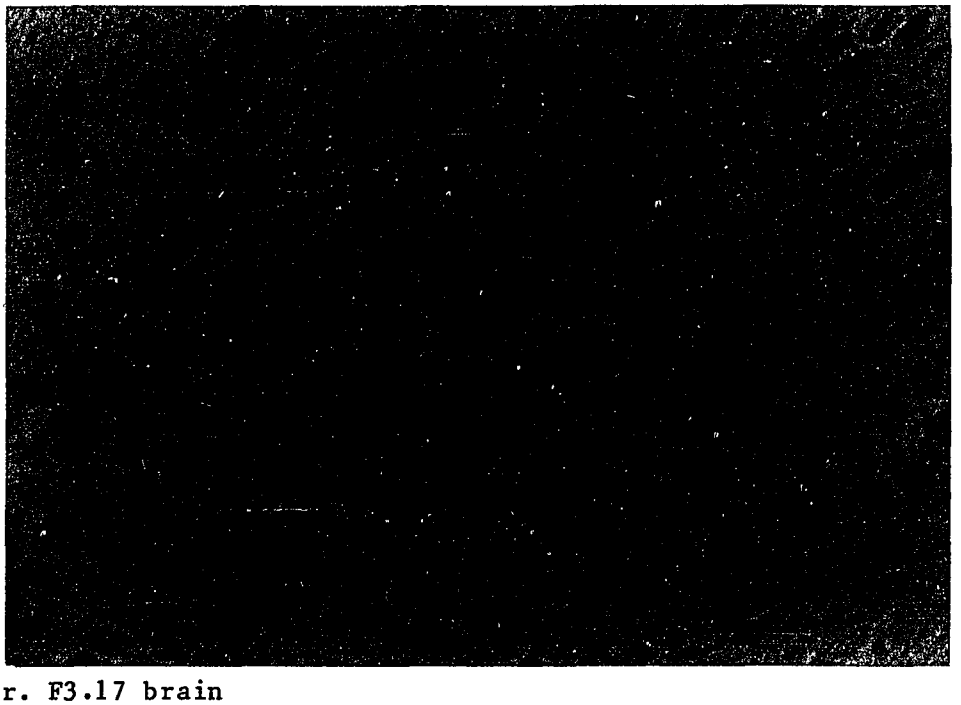


p. F3.17 spleen

Figure 17
Histopathology

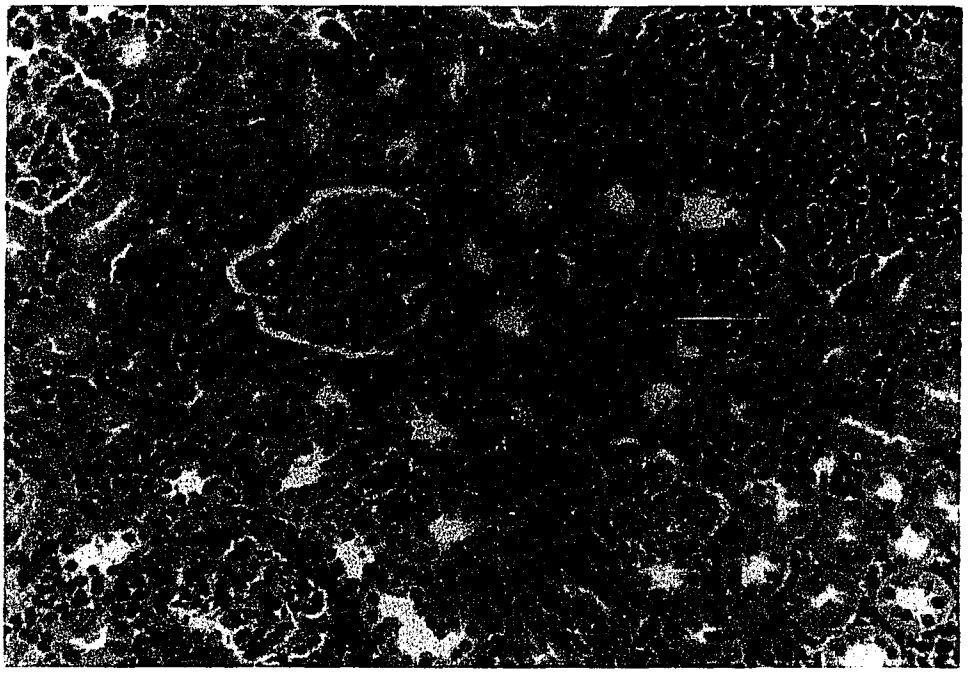


q. F3.17 brain

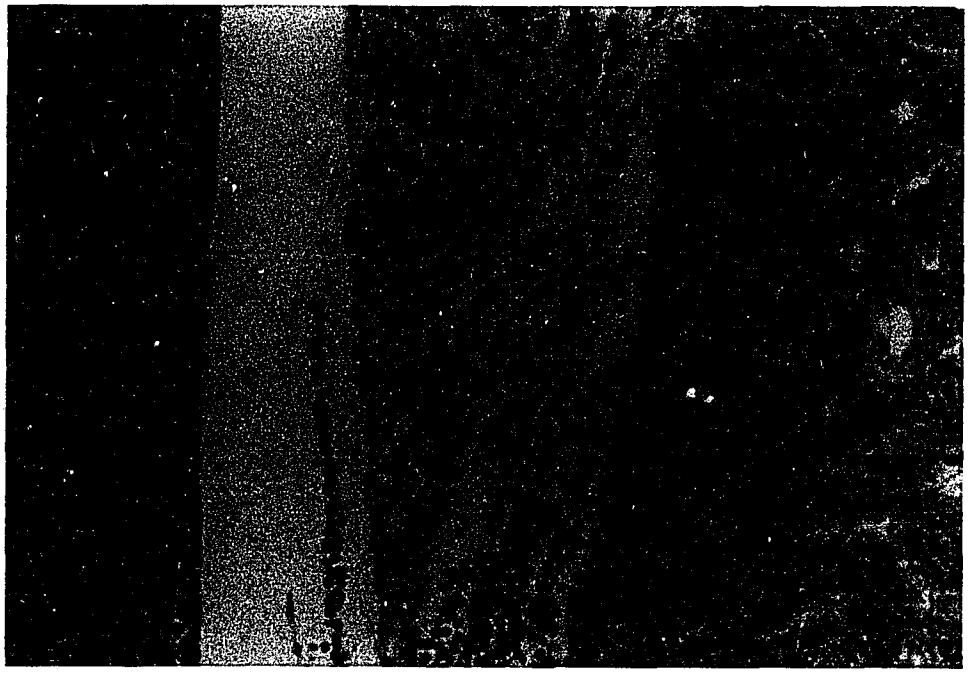


r. F3.17 brain

Figure 17
Histopathology

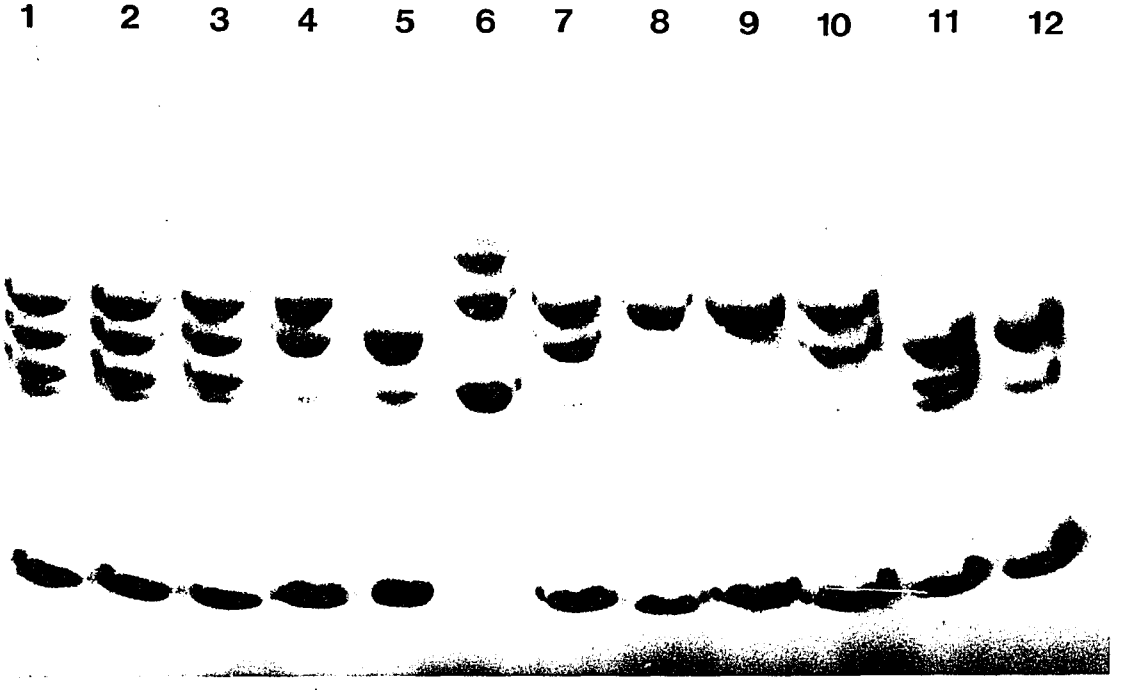


s. F3.17 kidney



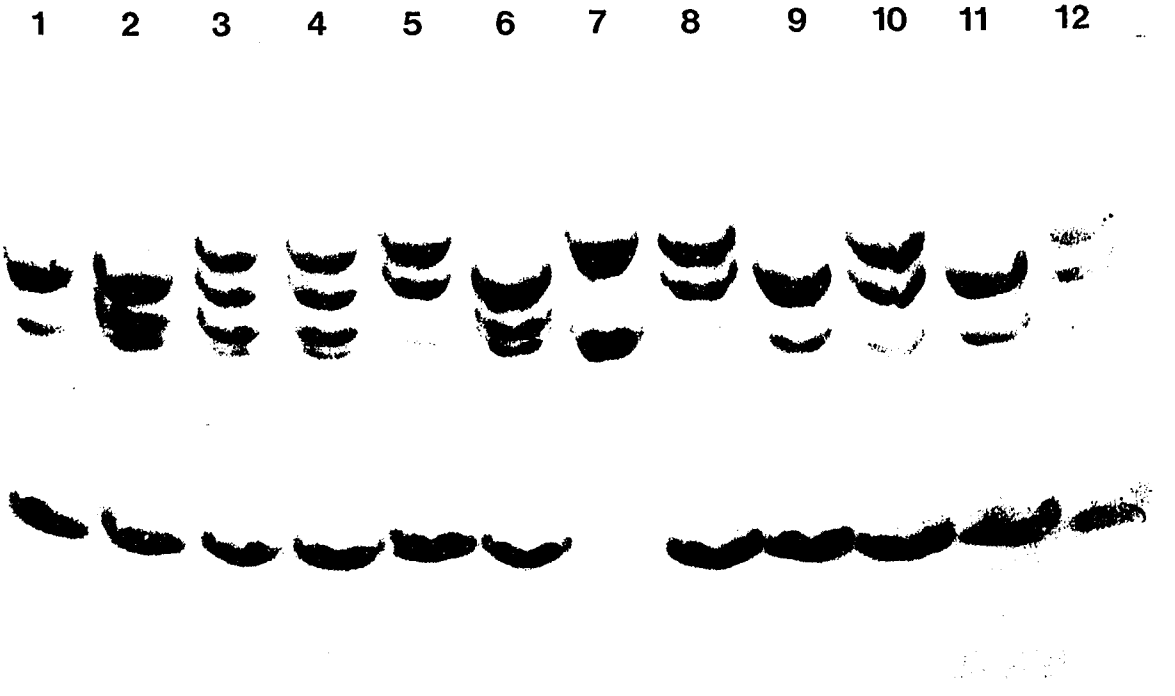
t. F3.17 kidney

Figure 18a
Triton gel analysis of globin protein



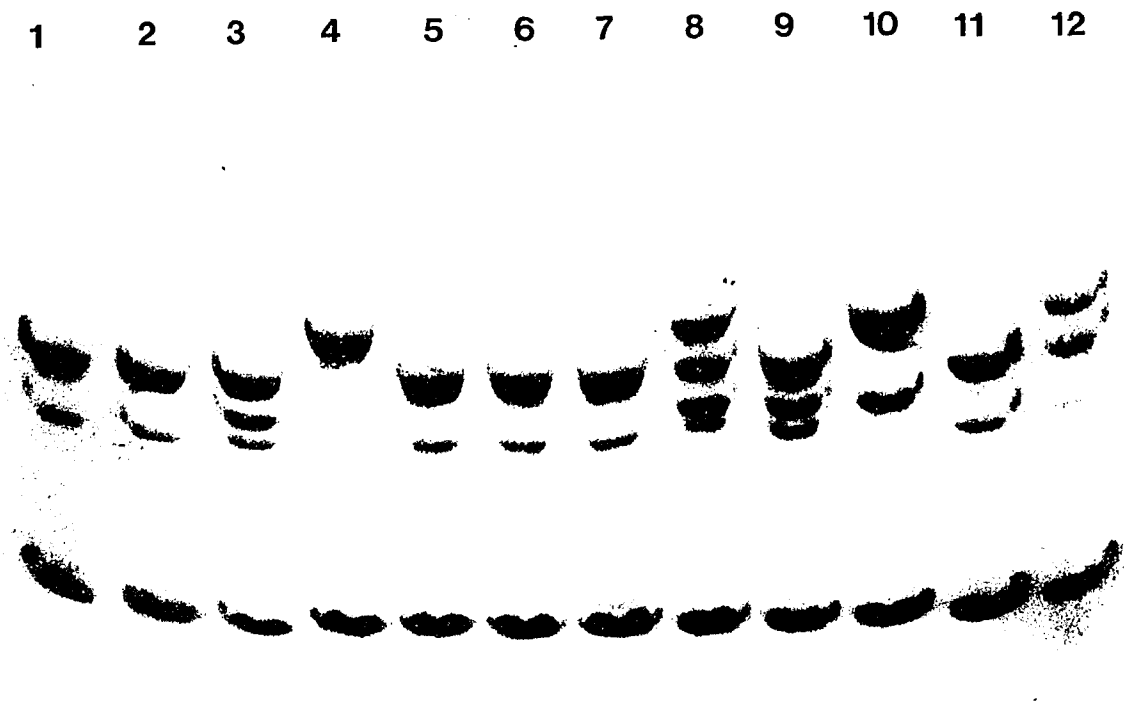
Lane	Sample
1	80.4
2	80.9
3	β80 founder
4	80.3
5	80.7
6	Human cord + adult blood
7	80.B
8	C57 ($\beta^s\beta^s$)
9	CD-1
10	B6D2 ($\beta^d\beta^s$)
11	80.12
12	80.10

Figure 18b
Triton gel analysis of globin protein



Lane	Sample
1	80.18
2	80.20
3	80.23
4	80.25
5	80.32
6	80.34
7	Human adult blood
8	B6D2 ($\beta^{d}\beta^{s}$)
9	F2.3
10	F2.9
11	F2.10
12	F2.12

Figure 18c
Triton gel analysis of globin protein

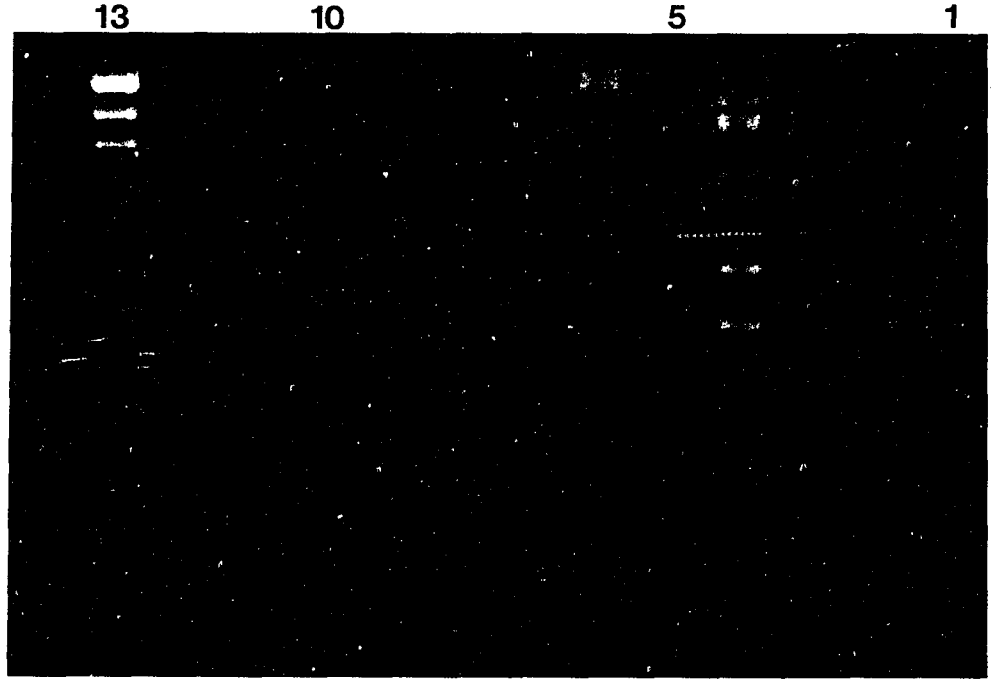


Lane	Sample
1	80.38
2	F2.4
3	F2.5
4	F2.14
5	F2.15
6	F2.16
7	F2.17
8	F2.18
9	F2.20
10	F2.21
11	Z (+/+)
12	B6D2 ($\beta^d\beta^s$)

Table 6
Cosmid clone analysis:
Tabulation of Southern blots

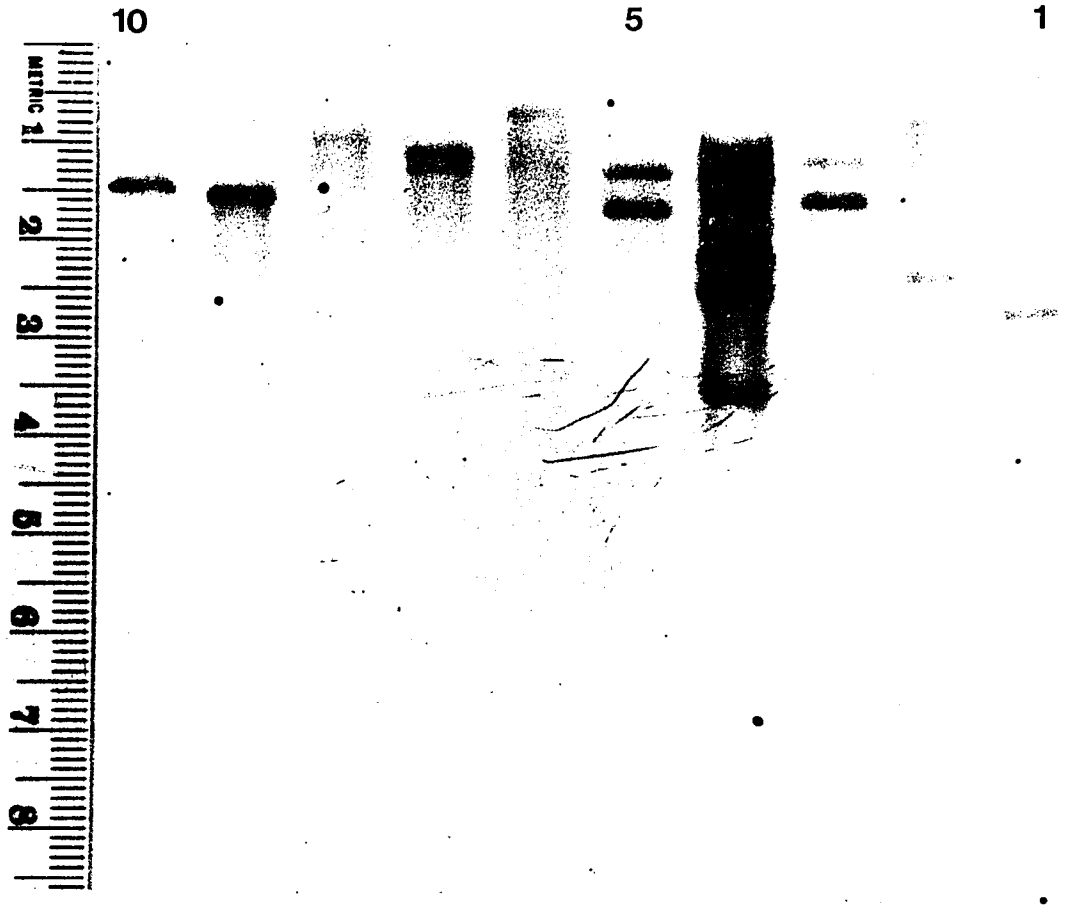
Figure #:	Restriction enzyme	letter:	EtBr/probe
19	EcoRI	a	Ethidium bromide stain
20	BamHI and HindIII	b	lambda probe
21	SalI and KpnI	c	λEB probe
22	BalI	d	pBR322 probe
23	EcoRV	e	genomic mouse DNA probe
24	BglII	f	ßHindIII fragment probe
		g	3.1kb EcoRI fragment probe
		h	λHεG1 probe (Ch4A arms)

Figure 19a
EcoRI-digested cosIII clones:
Ethidium bromide stain



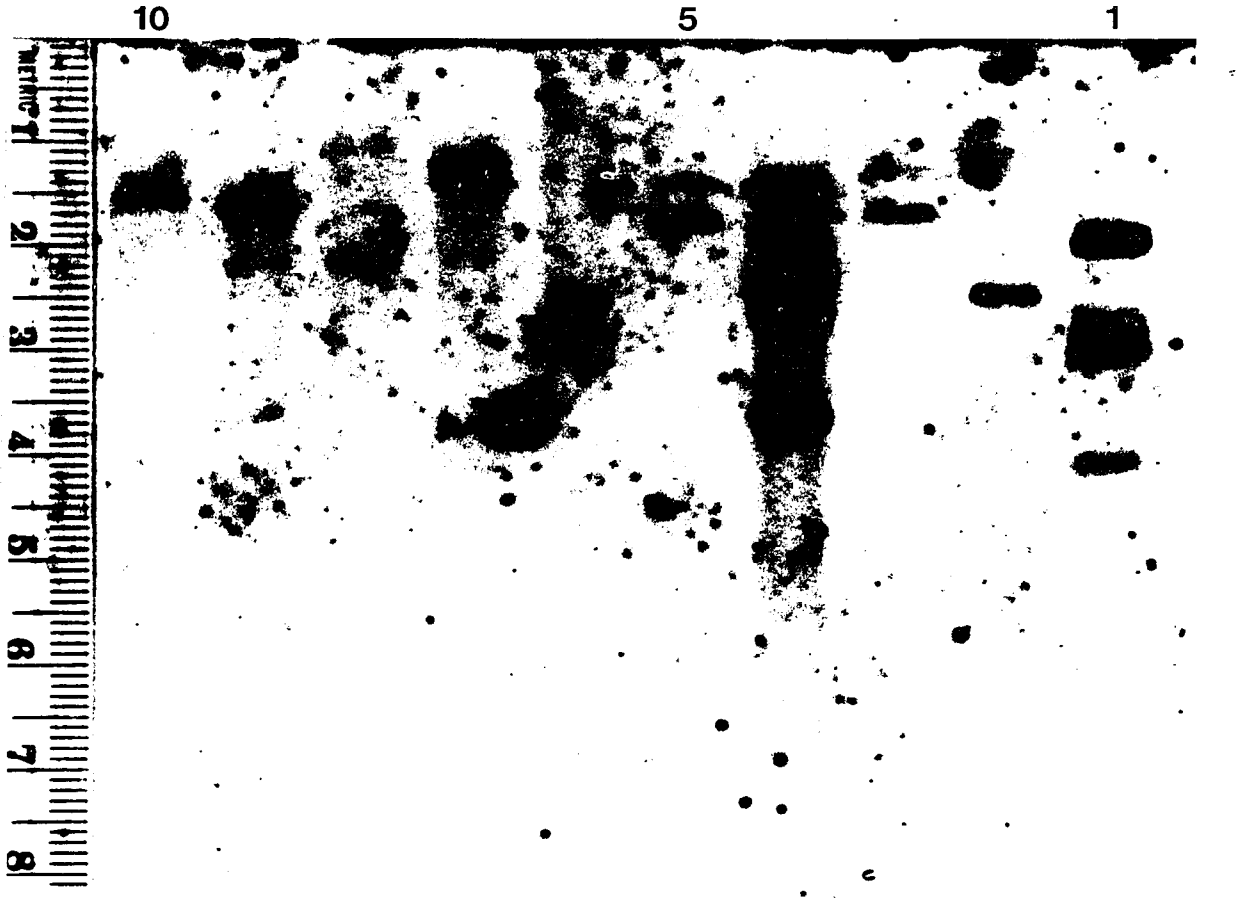
Lane	Clone
1	cosHG28tk
2	4-4α
3	7-6α
4	10-5α
5	12-1α
6	13-3α
7	14-2α
8	14-7α
9	15-4α
10	5-1
13	λ/HindIII control

Figure 19b
EcoRI-digested cosIII clones:
lambda probe



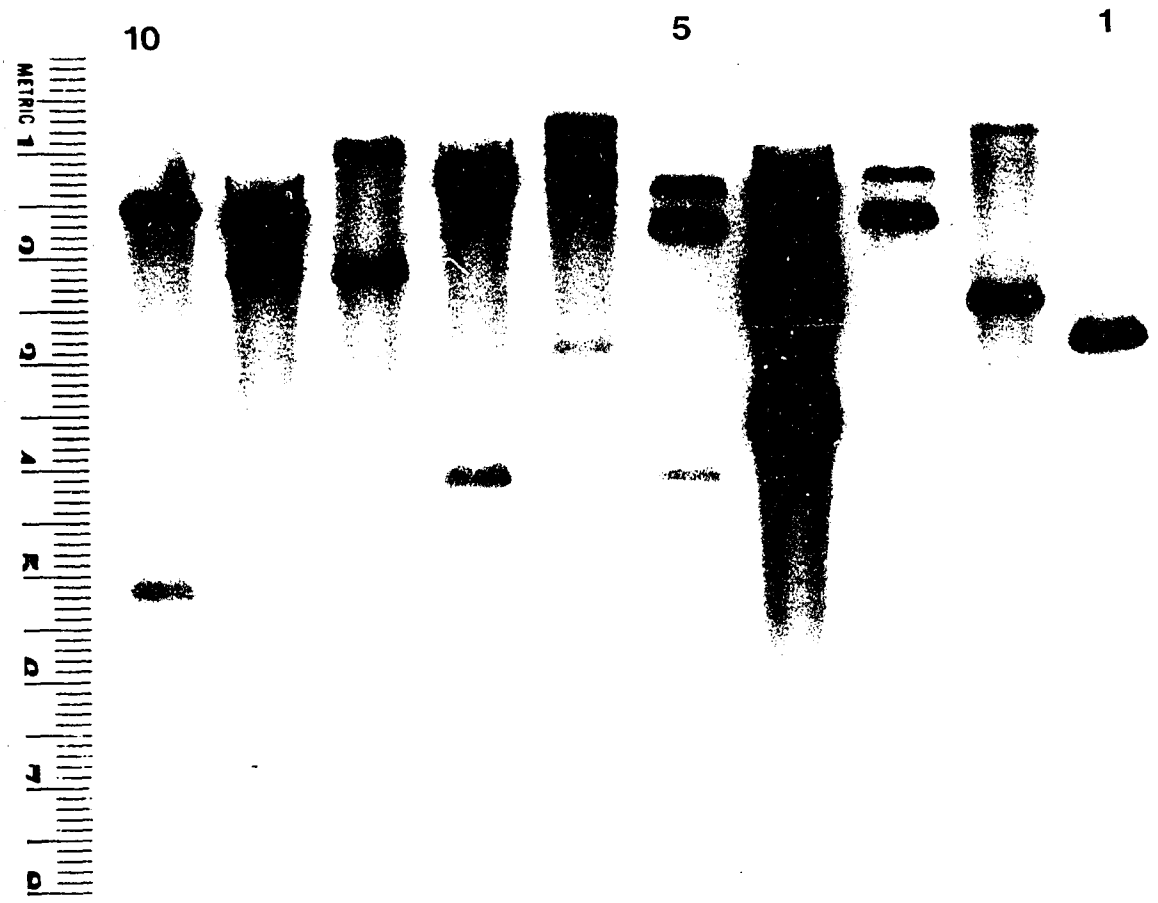
Lane	Clone
1	cosHG28tk
2	4-4α
3	7-6α
4	10-5α
5	12-1α
6	13-3α
7	14-2α
8	14-7α
9	15-4α
10	5-1

Figure 19c
EcoRI-digested cosIII clones:
λEB probe



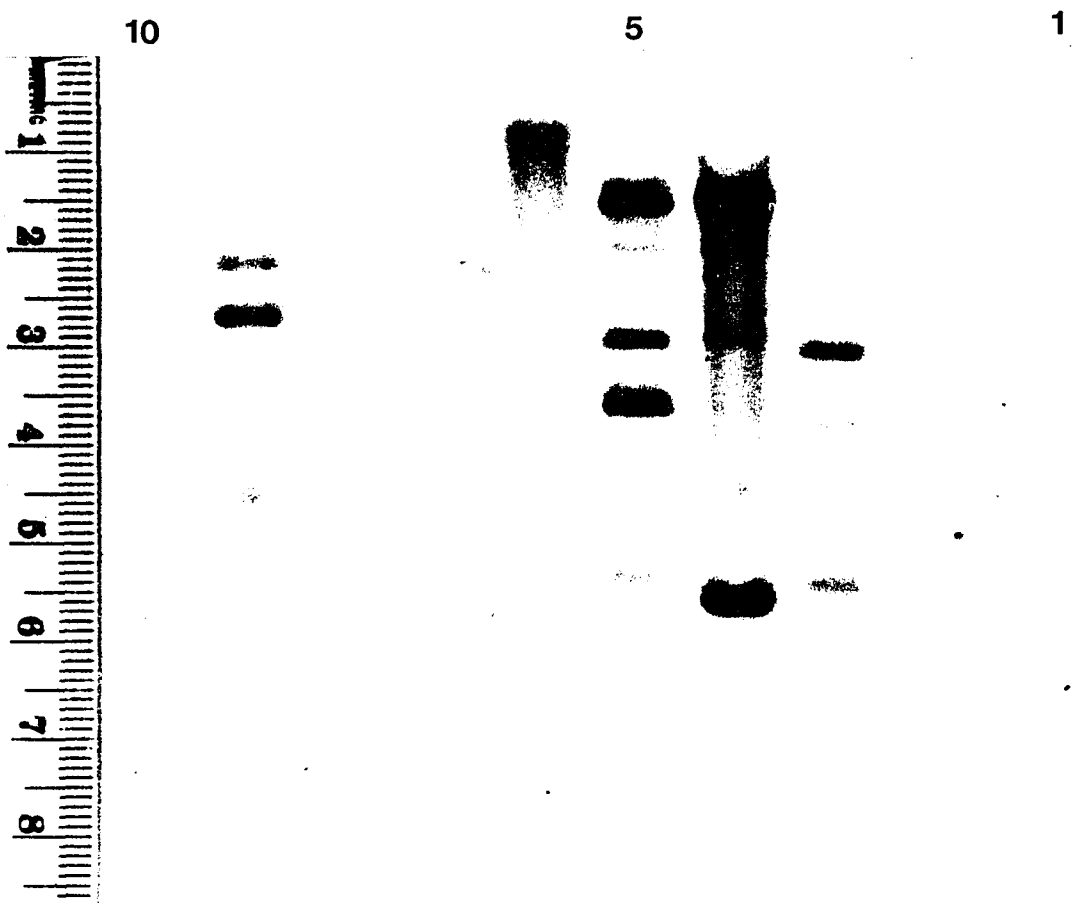
Lane	Clone
1	cosHG28tk
2	4-4α
3	7-6α
4	10-5α
5	12-1α
6	13-3α
7	14-2α
8	14-7α
9	15-4α
10	5-1

Figure 19d
EcoRI-digested cosIII clones:
pBR322 probe



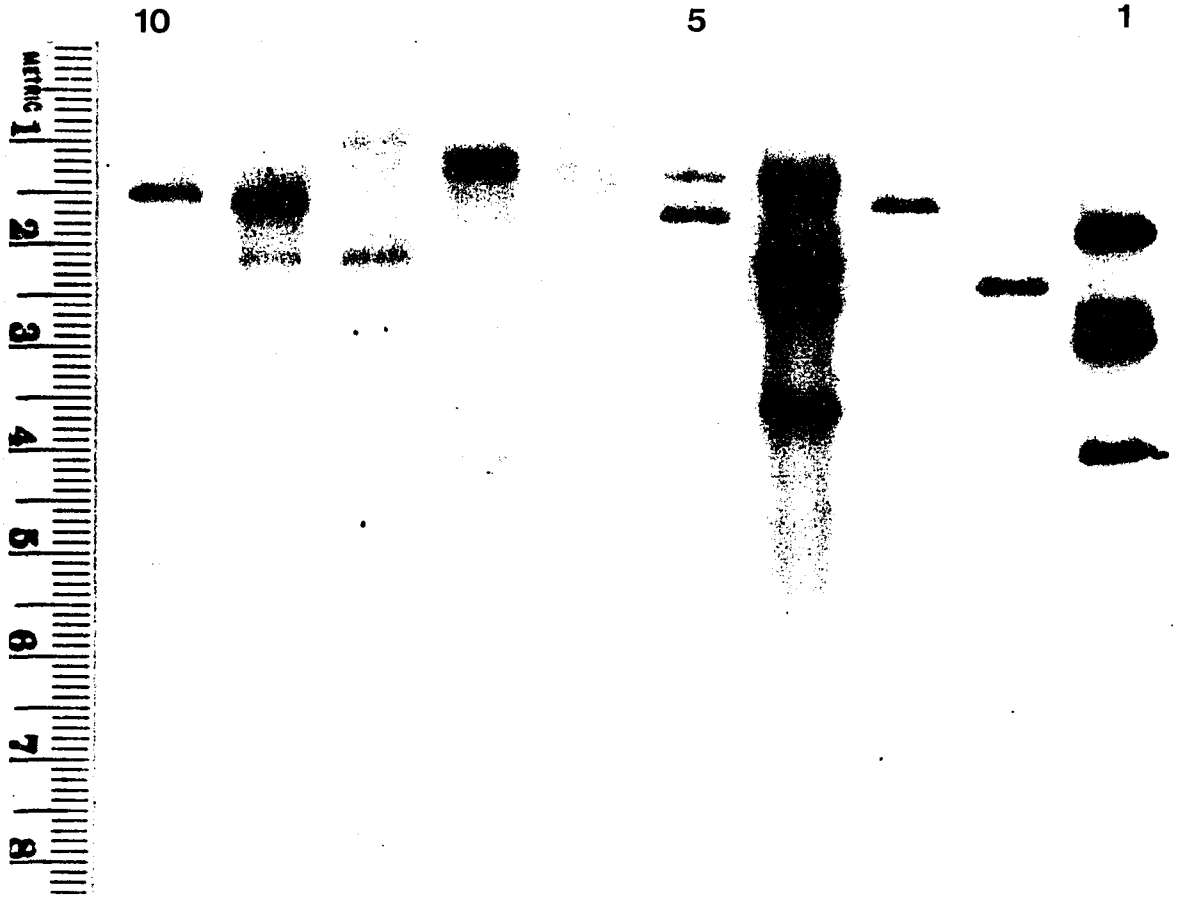
Lane	Clone
1	cosHG28tk
2	4-4α
3	7-6α
4	10-5α
5	12-1α
6	13-3α
7	14-2α
8	14-7α
9	15-4α
10	5-1

Figure 19e
EcoRI-digested cosIII clones:
wild-type genomic mouse DNA probe



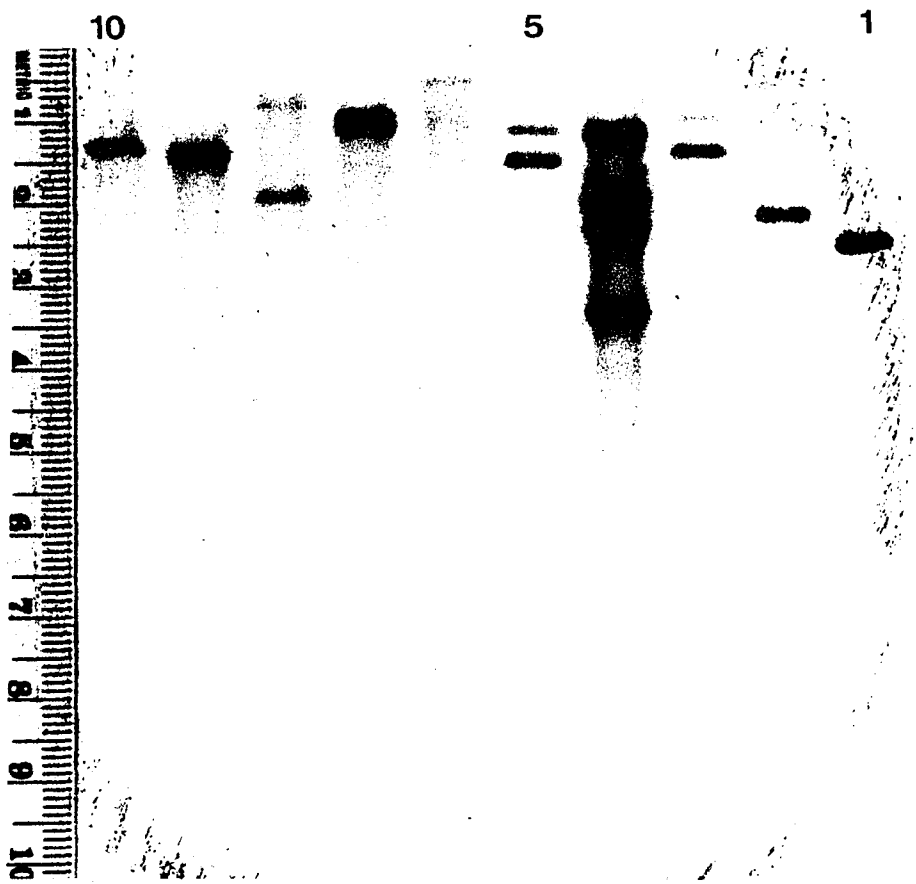
Lane	Clone
1	cosHG28tk
2	4-4α
3	7-6α
4	10-5α
5	12-1α
6	13-3α
7	14-2α
8	14-7α
9	15-4α
10	5-1

Figure 19f
EcoRI-digested cosIII clones:
βHindIII probe



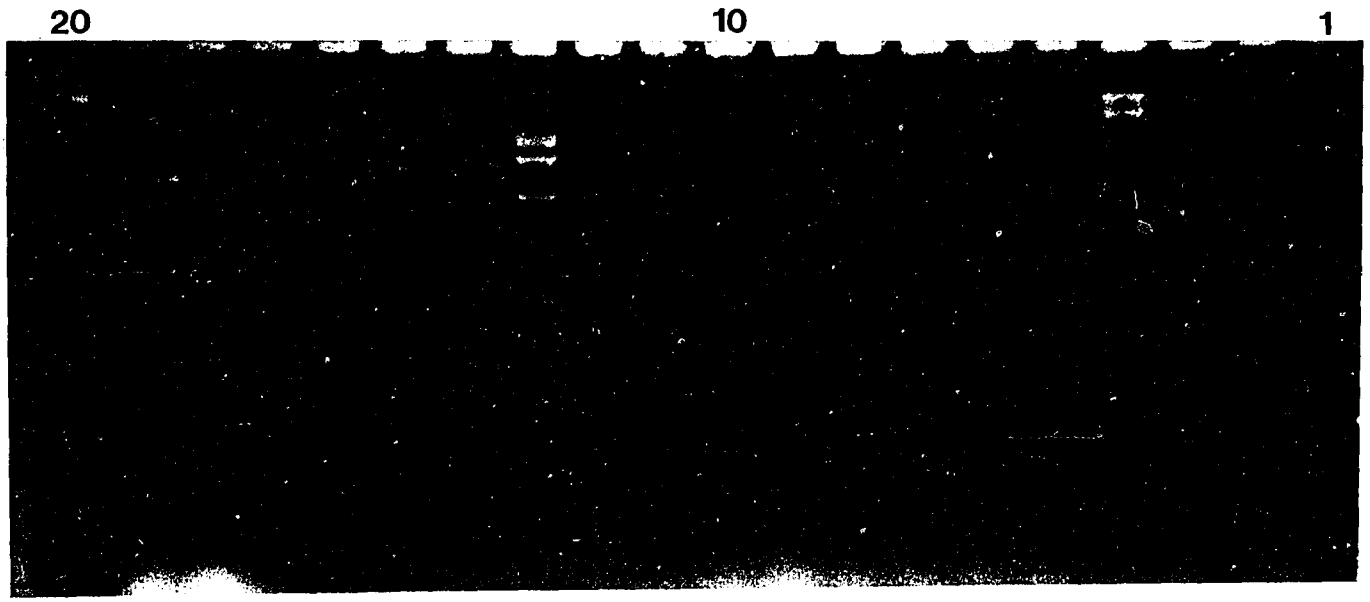
Lane	Clone
1	cosHG28tk
2	4-4α
3	7-6α
4	10-5α
5	12-1α
6	13-3α
7	14-2α
8	14-7α
9	15-4α
10	5-1

Figure 19g
EcoRI-digested cosIII clones:
3.1kb EcoRI fragment probe



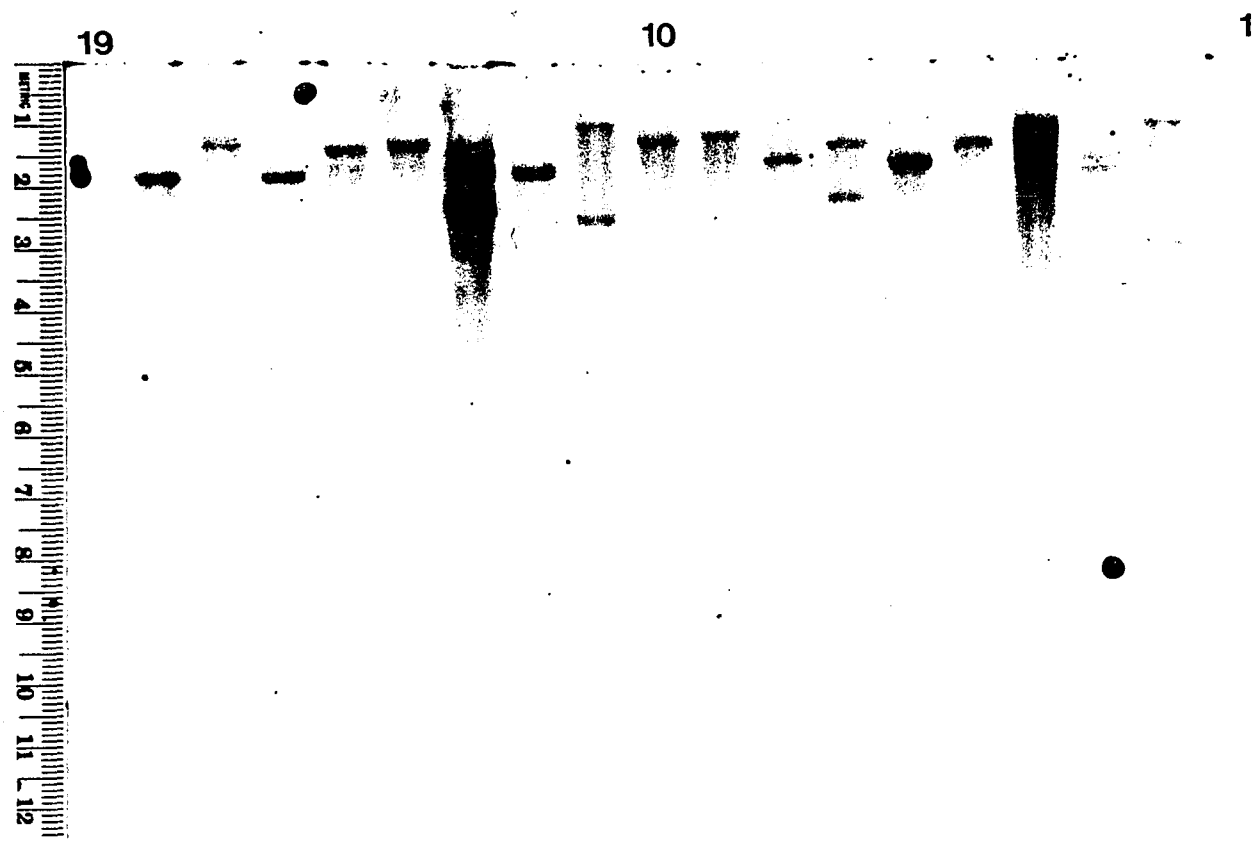
Lane	Clone
1	cosHG28tk
2	4-4α
3	7-6α
4	10-5α
5	12-1α
6	13-3α
7	14-2α
8	14-7α
9	15-4α
10	5-1

Figure 20a
BamHI- and HindIII-digested cosIII clones:
Ethidium bromide stain



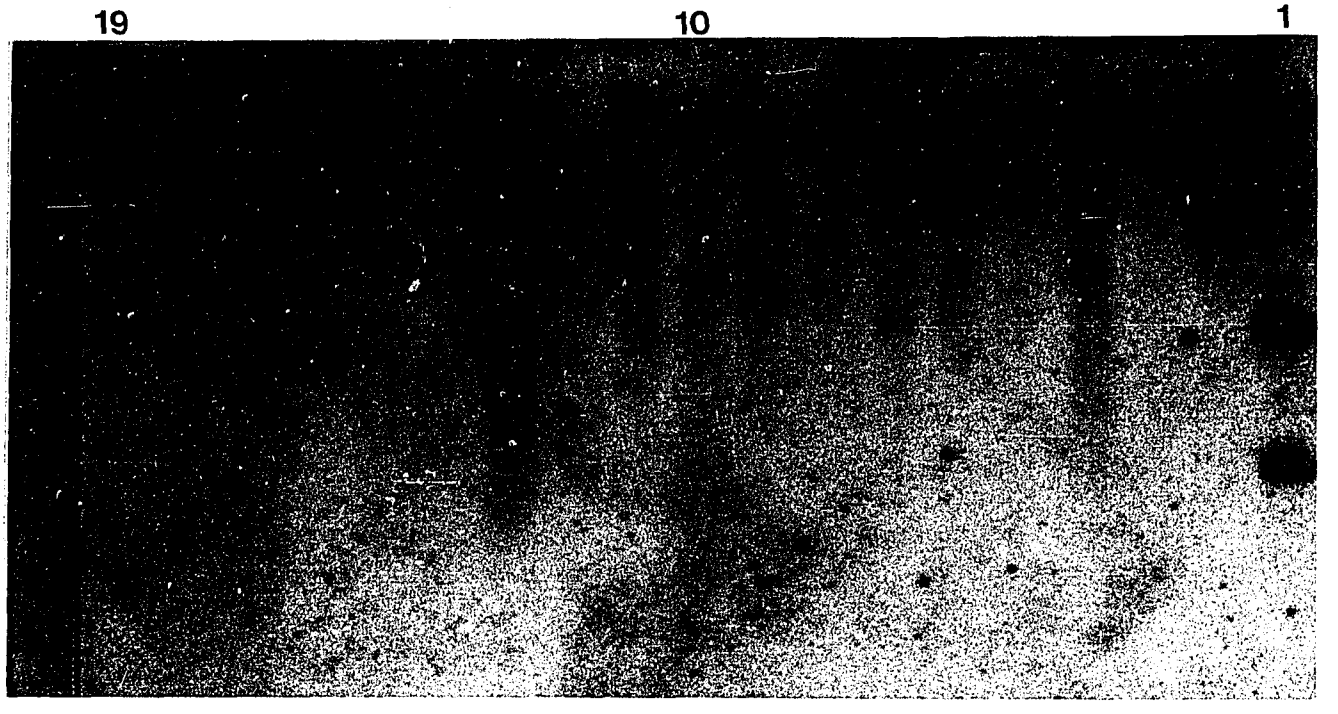
Restriction Enzyme		clone
BamHI	HindIII	
Lane		
1		cosHG28tk
2	11	4-4α
3	12	7-6α
4	13	10-5α
5	14	12-1α
6	15	13-3α
7	16	14-2α
8	17	14-7α
9	18	15-4α
10	19	5-1
	20	λ/HindIII control

Figure 20b
BamHI- and HindIII-digested cosIII clones:
lambda probe



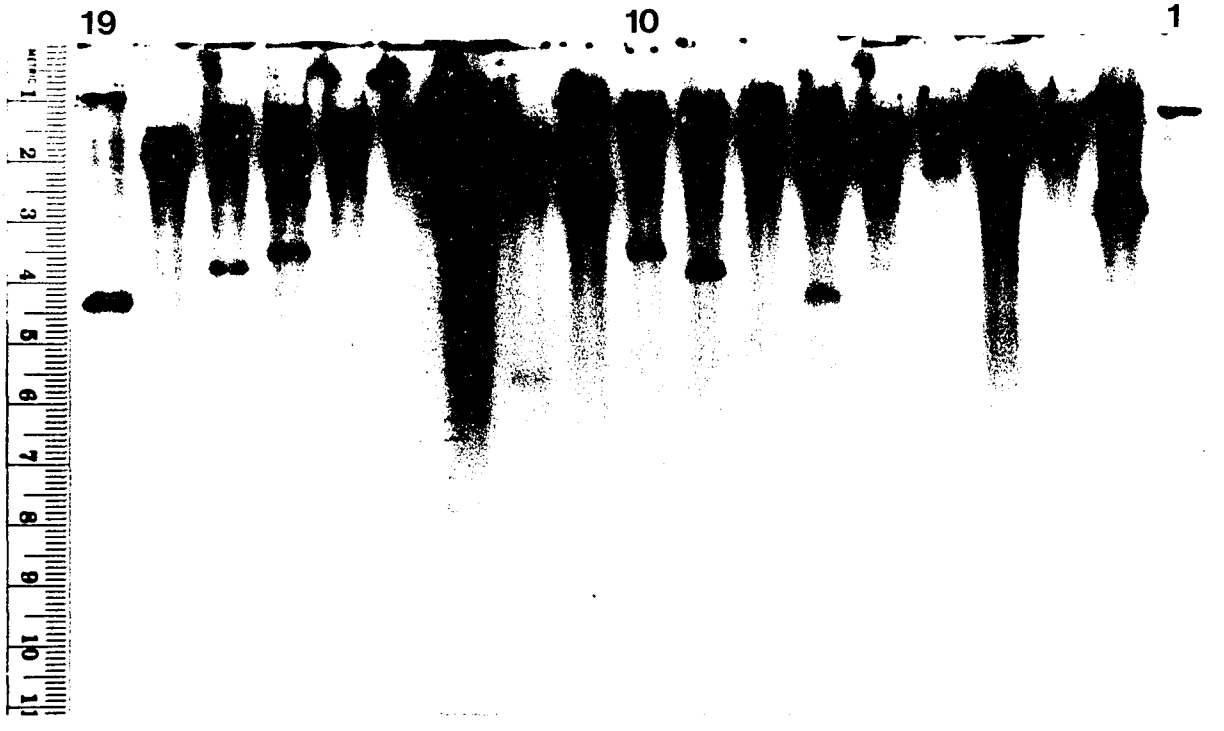
Restriction Enzyme		clone
BamHI	HindIII	
Lane		
1		cosHG28tk
2	11	4-4α
3	12	7-6α
4	13	10-5α
5	14	12-1α
6	15	13-3α
7	16	14-2α
8	17	14-7α
9	18	15-4α
10	19	5-1

Figure 20c
BamHI- and HindIII-digested cosIII clones:
λEB probe



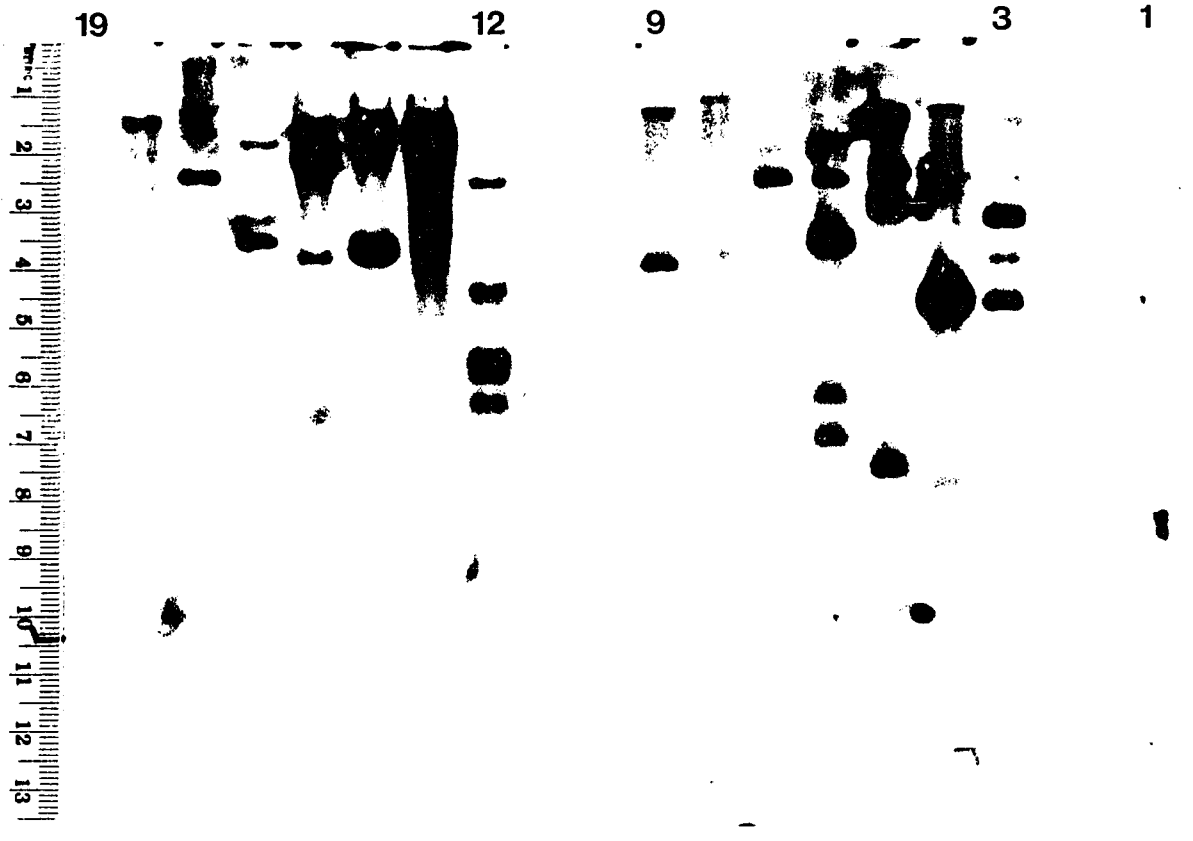
Restriction Enzyme		clone
BamHI	HindIII	
Lane		
1		cosHG28tk
2	11	4-4α
3	12	7-6α
4	13	10-5α
5	14	12-1α
6	15	13-3α
7	16	14-2α
8	17	14-7α
9	18	15-4α
10	19	5-1

Figure 20d
BamHI- and HindIII-digested cosIII clones:
pBR322 probe



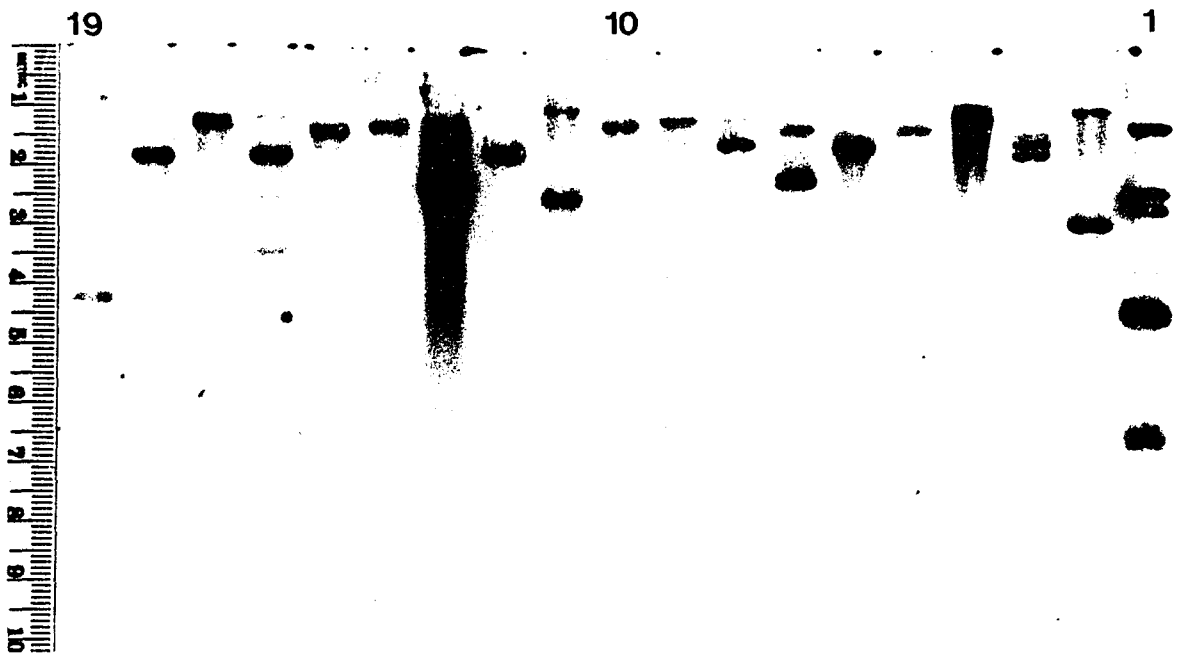
Restriction Enzyme		clone
BamHI	HindIII	
Lane		
1		cosHG28tk
2	11	4-4α
3	12	7-6α
4	13	10-5α
5	14	12-1α
6	15	13-3α
7	16	14-2α
8	17	14-7α
9	18	15-4α
10	19	5-1

Figure 20e
BamHI- and HindIII-digested cosIII clones:
wild-type genomic mouse DNA probe



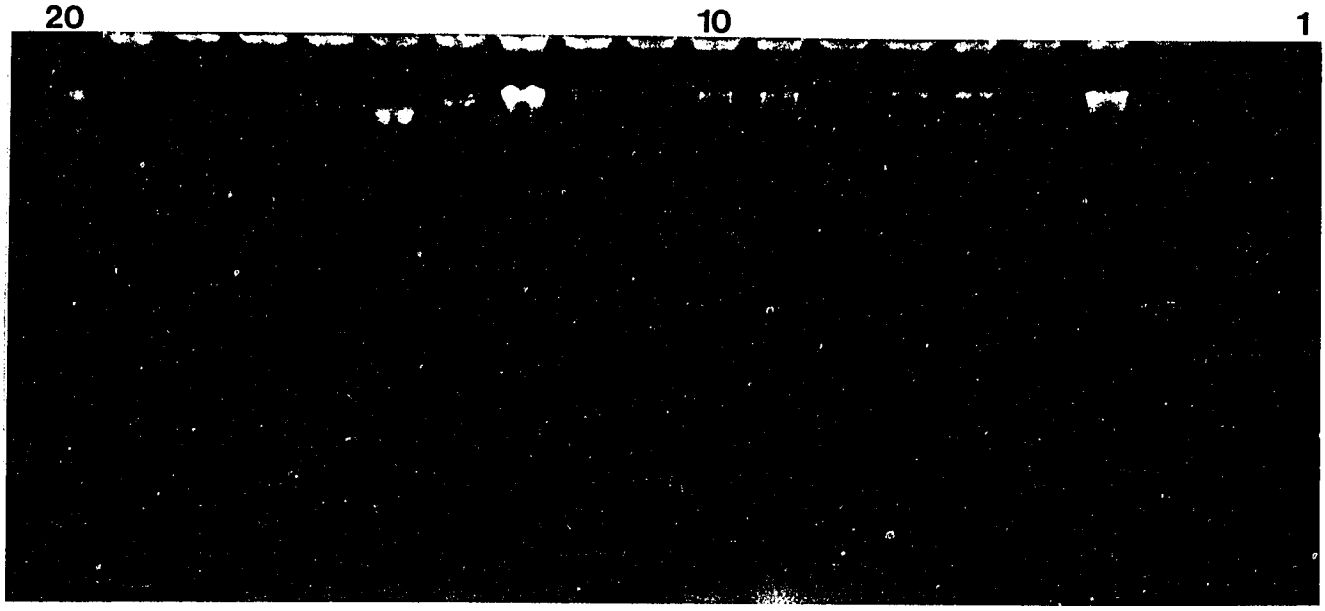
Restriction Enzyme		clone
BamHI	HindIII	
Lane		
1		cosHG28tk
2	11	4-4α
3	12	7-6α
4	13	10-5α
5	14	12-1α
6	15	13-3α
7	16	14-2α
8	17	14-7α
9	18	15-4α
10	19	5-1

Figure 20f
BamHI- and HindIII-digested cosIII clones:
βHindIII probe



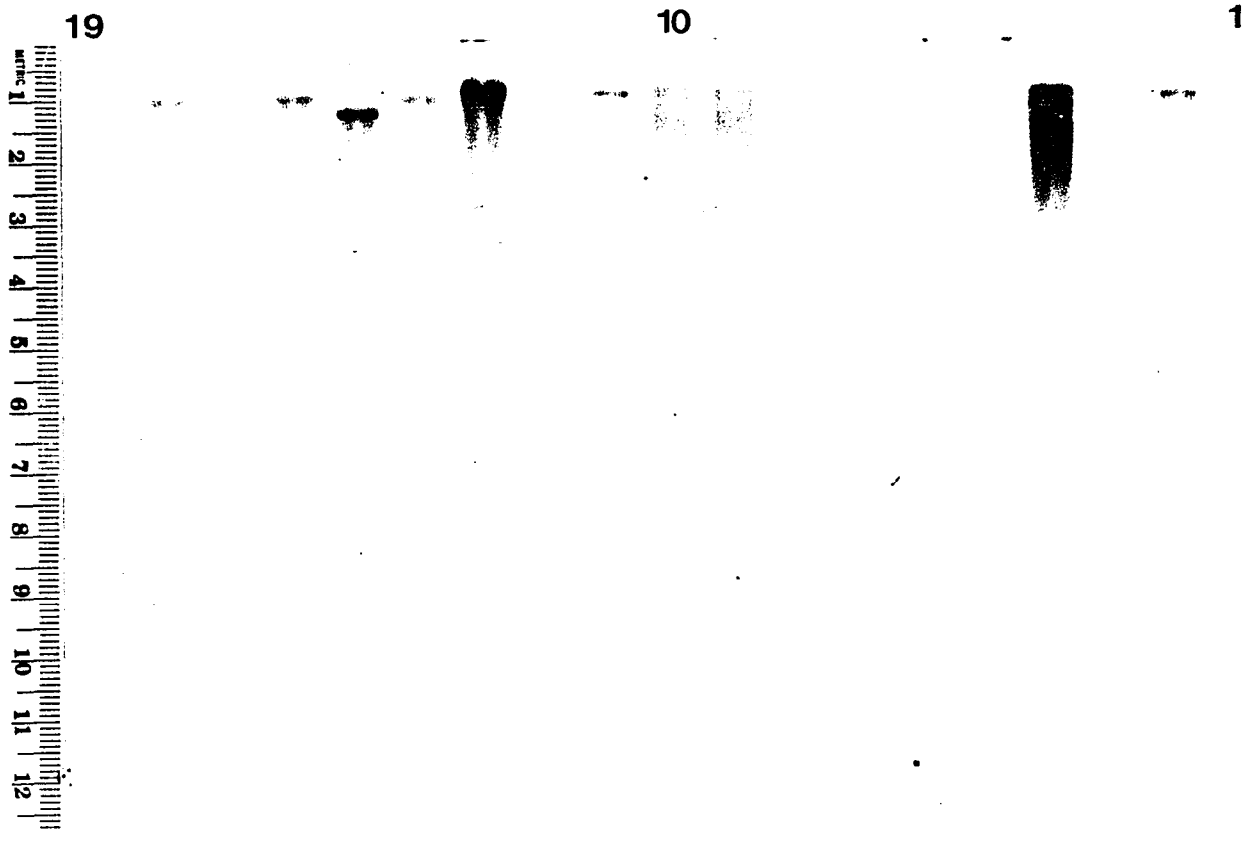
Restriction Enzyme		clone
BamHI	HindIII	
Lane		
1		cosHG28tk
2	11	4-4α
3	12	7-6α
4	13	10-5α
5	14	12-1α
6	15	13-3α
7	16	14-2α
8	17	14-7α
9	18	15-4α
10	19	5-1

Figure 21a
SalI- and KpnI-digested cosIII clones
Ethidium bromide stain



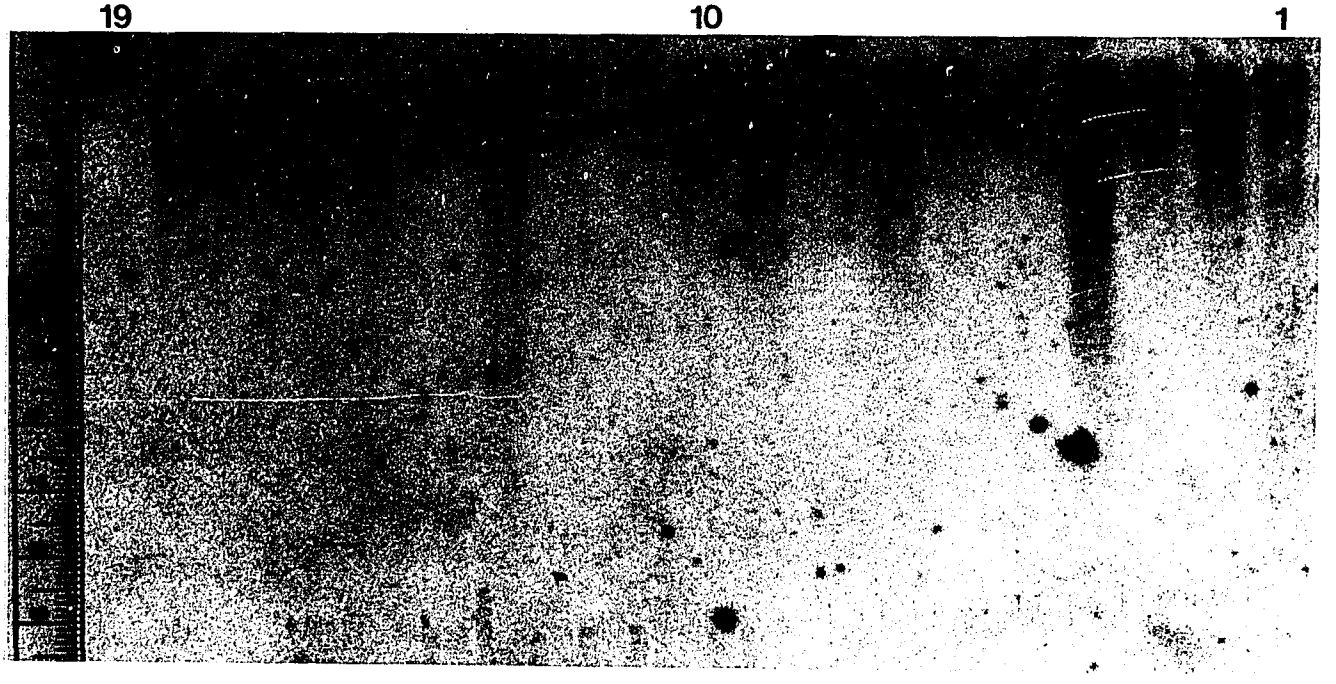
Restriction Enzyme		clone
SalI	KpnI	
Lane		
1		cosHG28tk
2	11	4-4α
3	12	7-6α
4	13	10-5α
5	14	12-1α
6	15	13-3α
7	16	14-2α
8	17	14-7α
9	18	15-4α
10	19	5-1
	20	λ/HindIII control

Figure 21b
SalI- and KpnI-digested cosIII clones:
lambda probe



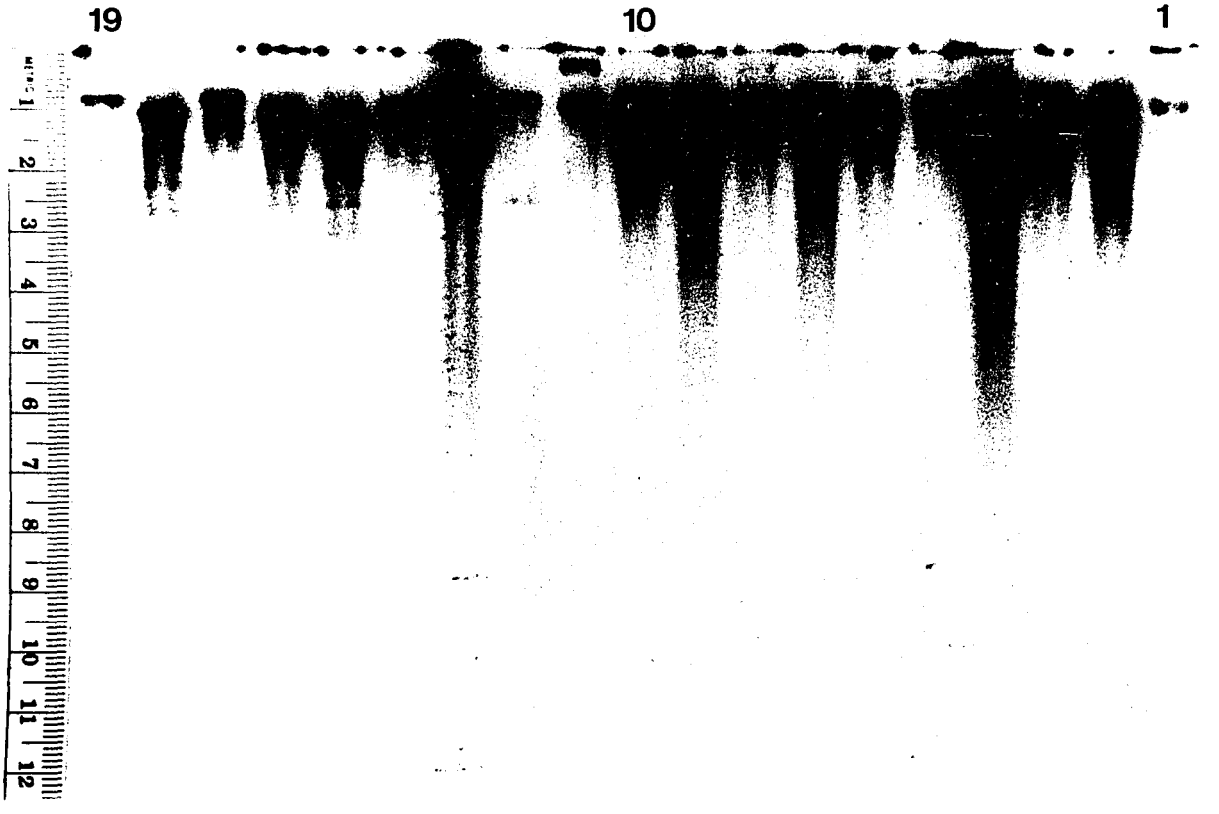
Restriction Enzyme		clone
SalI	KpnI	
Lane		
1		cosHG28tk
2	11	4-4α
3	12	7-6α
4	13	10-5α
5	14	12-1α
6	15	13-3α
7	16	14-2α
8	17	14-7α
9	18	15-4α
10	19	5-1

Figure 21c
SalI- and KpnI-digested cosIII clones:
λEB probe



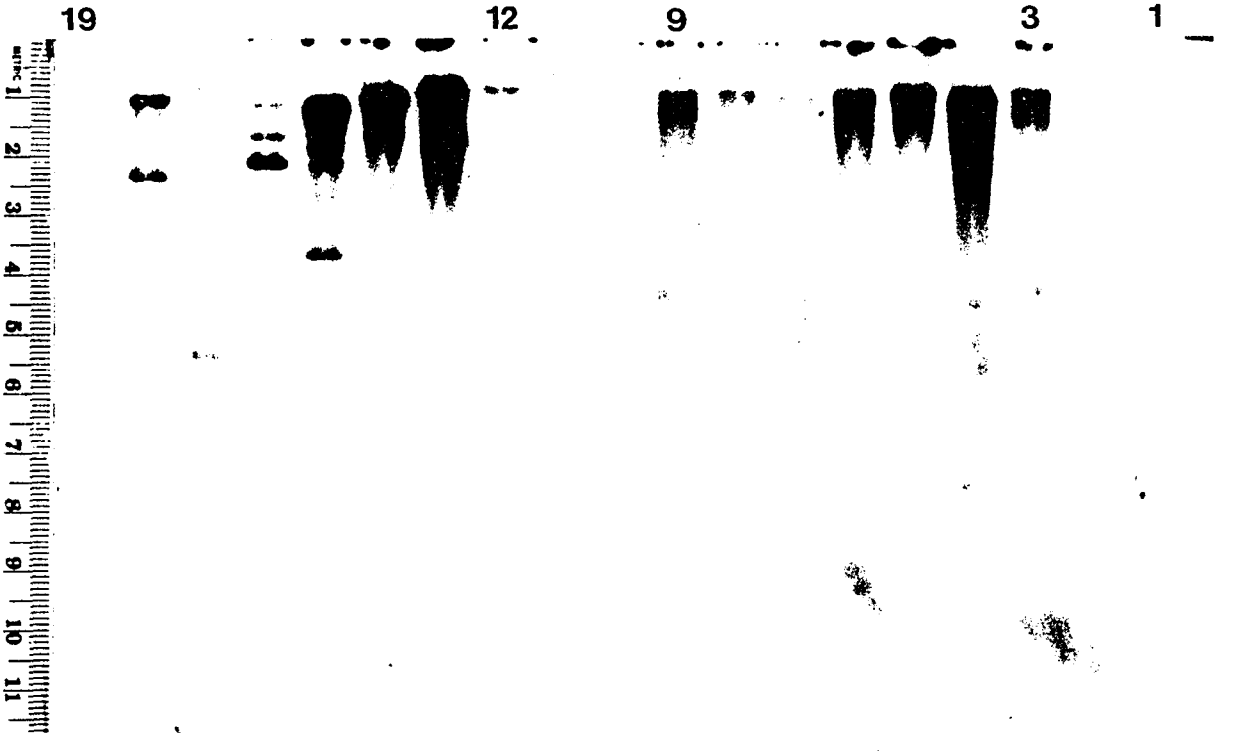
Restriction Enzyme		clone
SalI	KpnI	
Lane		
1		cosHG28tk
2	11	4-4α
3	12	7-6α
4	13	10-5α
5	14	12-1α
6	15	13-3α
7	16	14-2α
8	17	14-7α
9	18	15-4α
10	19	5-1

Figure 21d
SalI- and KpnI-digested cosIII clones:
pBR322 probe



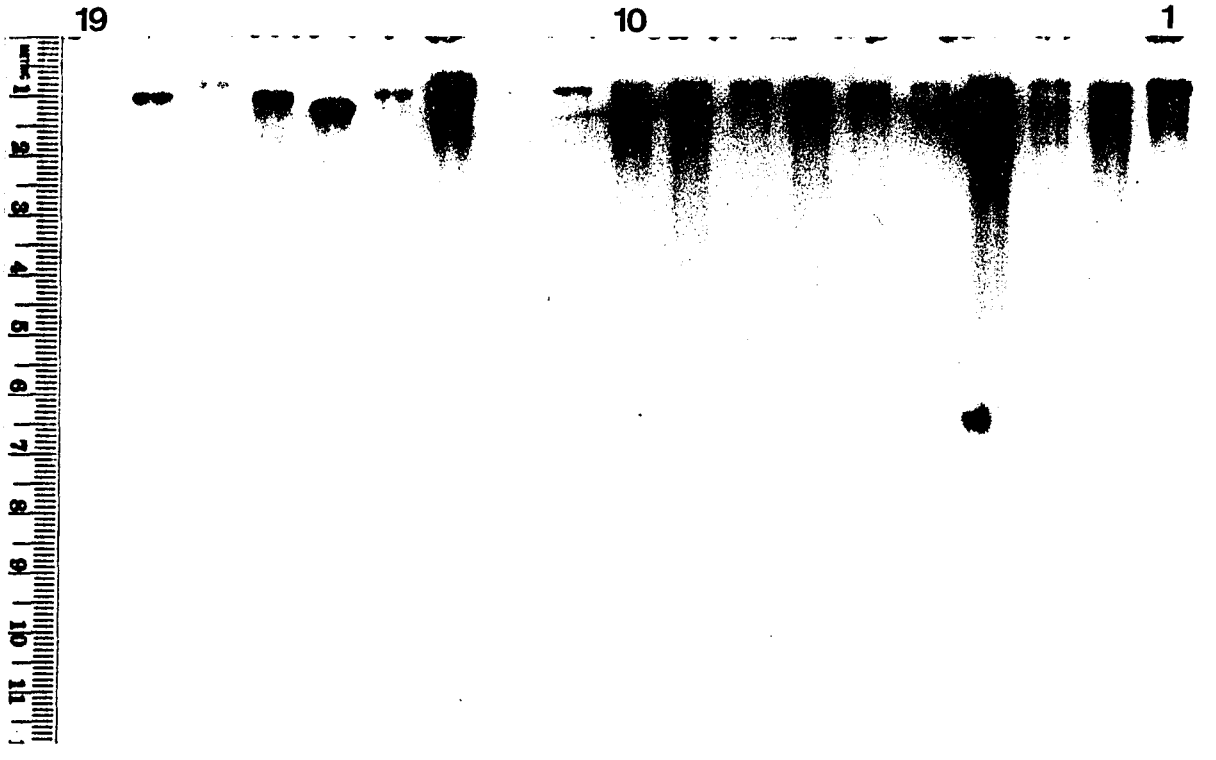
Restriction Enzyme		clone
SalI	KpnI	
Lane		
1		cosHG28tk
2	11	4-4α
3	12	7-6α
4	13	10-5α
5	14	12-1α
6	15	13-3α
7	16	14-2α
8	17	14-7α
9	18	15-4α
10	19	5-1

Figure 21e
SalI- and KpnI-digested cosIII clones:
wild-type genomic mouse DNA probe



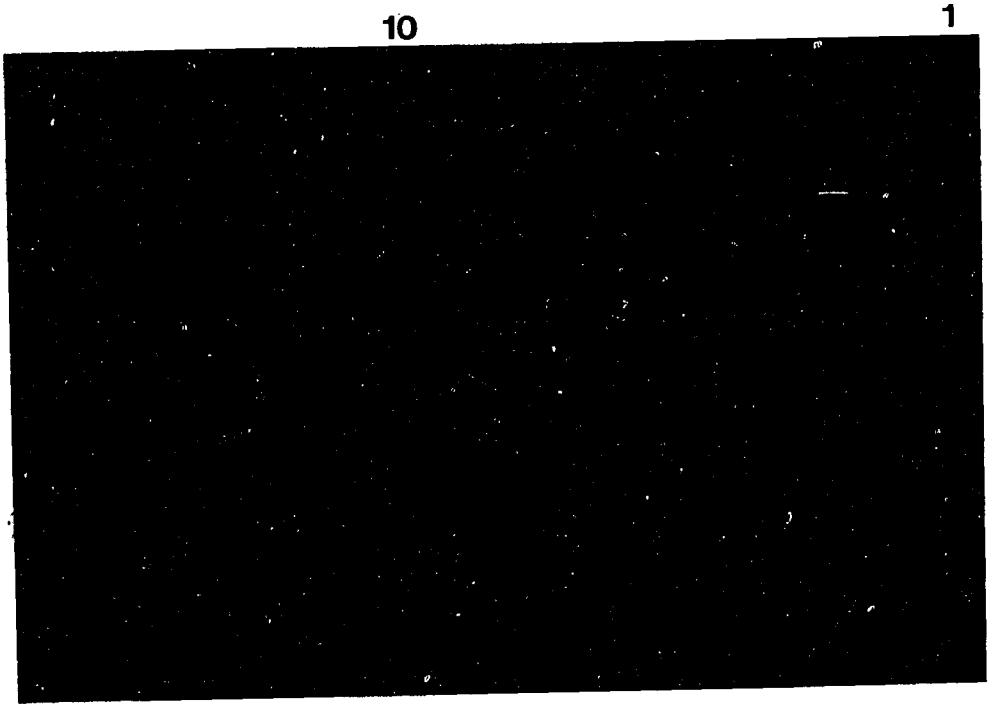
Restriction Enzyme		clone
SalI	KpnI	
Lane		
1		cosHG28tk
2	11	4-4α
3	12	7-6α
4	13	10-5α
5	14	12-1α
6	15	13-3α
7	16	14-2α
8	17	14-7α
9	18	15-4α
10	19	5-1

Figure 21f
SalI- and KpnI-digested cosIII clones:
βHindIII probe



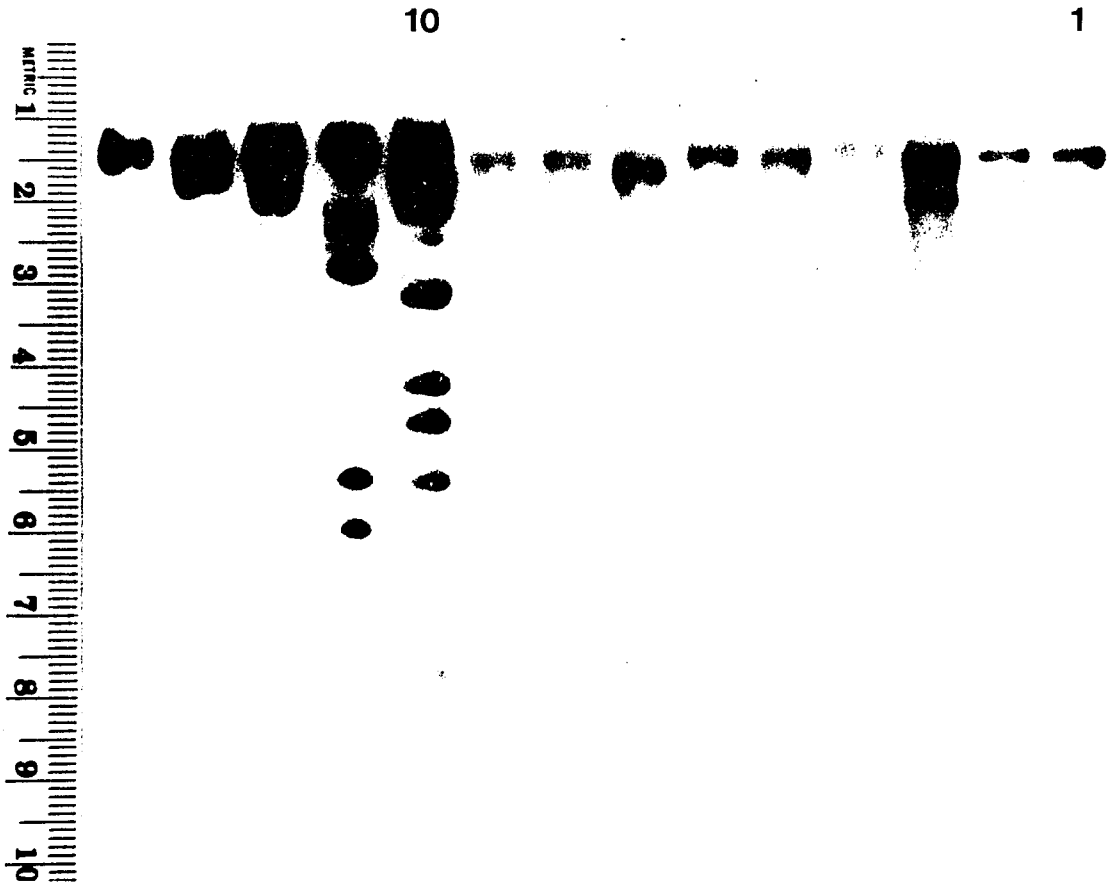
Restriction Enzyme		clone
SalI	KpnI	
Lane		
1		cosHG28tk
2	11	4-4α
3	12	7-6α
4	13	10-5α
5	14	12-1α
6	15	13-3α
7	16	14-2α
8	17	14-7α
9	18	15-4α
10	19	5-1

Figure 22a
BalI-digested cosIII clones:
Ethidium bromide stain



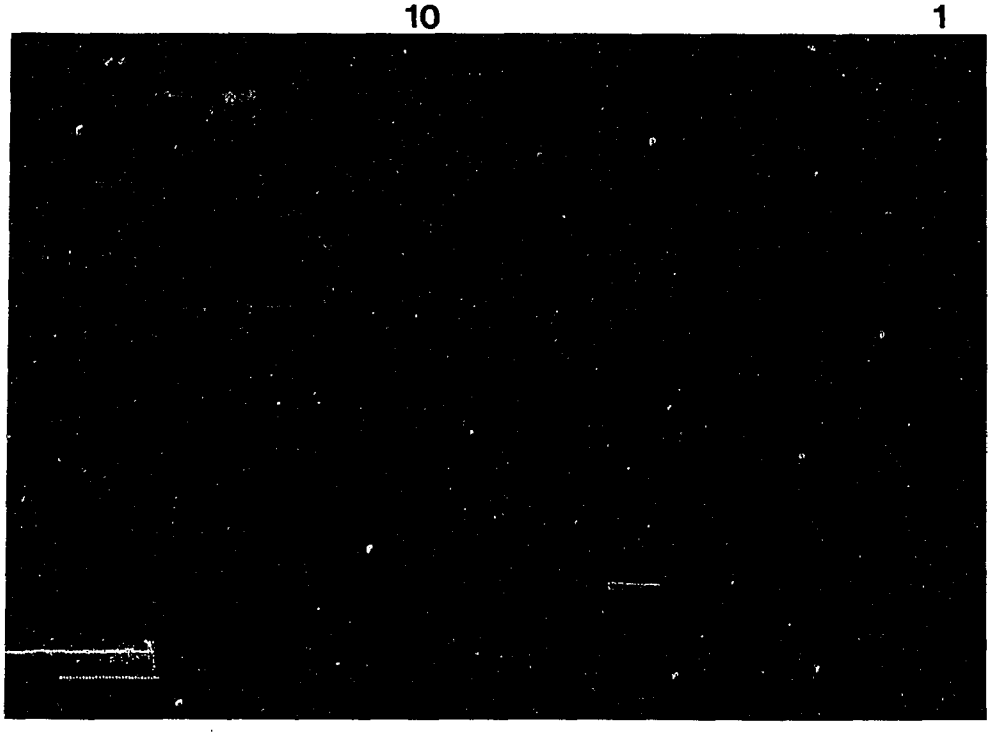
Lane	Clone
1	4-4α
2	7-6α
3	10-5α
4	12-1α
5	13-3α
6	14-2α
7	14-7α
8	15-4α
9	5-1
10	λHeG1/BalI
11	λHeG1/EcoRI
12	λ/EcoRI
13	λ/HindIII
14	λ/EB

Figure 22h
BalI-digested cosIII clones:
λHeG1 probe



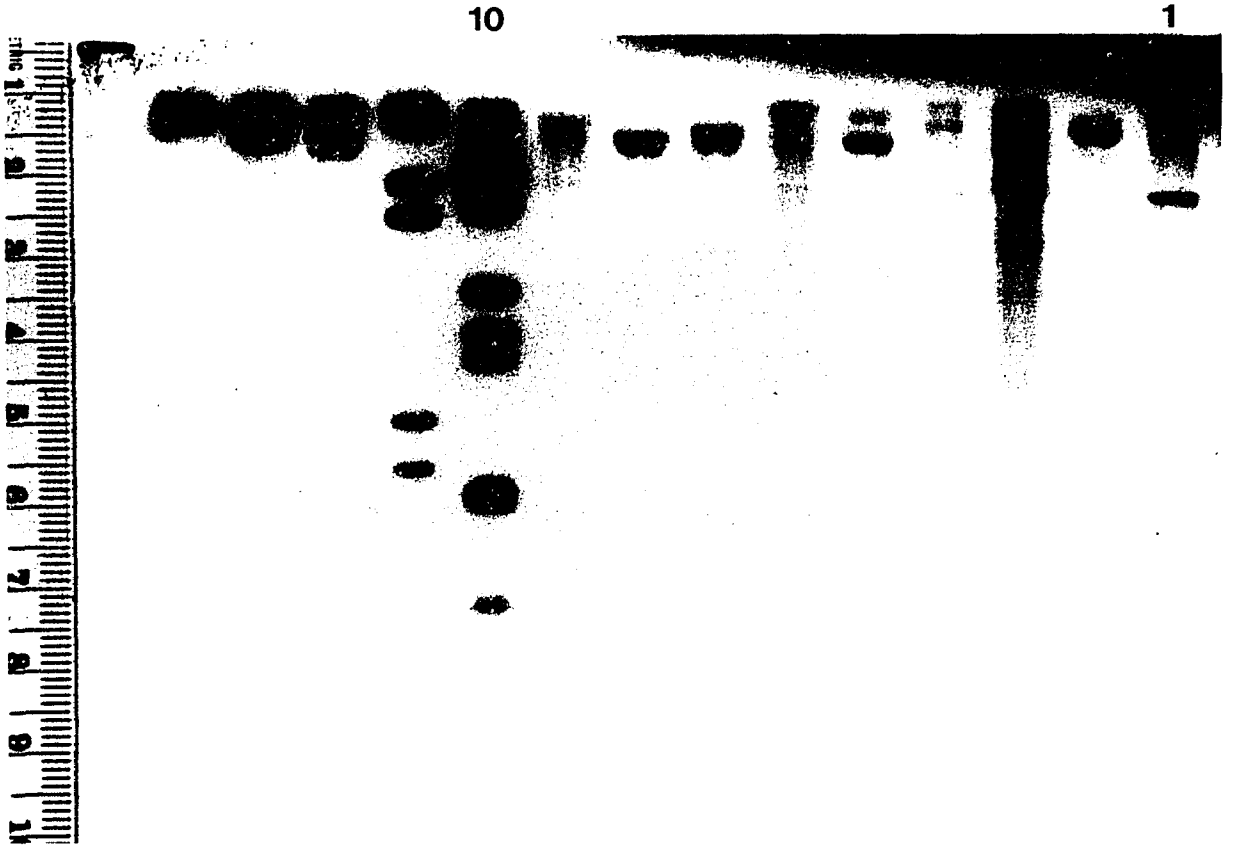
Lane	Clone
1	4-4α
2	7-6α
3	10-5α
4	12-1α
5	13-3α
6	14-2α
7	14-7α
8	15-4α
9	5-1
10	λHeG1/BalI
11	λHeG1/EcoRI
12	λ/EcoRI
13	λ/HindIII
14	λ/EB

Figure 23a
EcoRV-digested cosIII clones:
Ethidium bromide stain



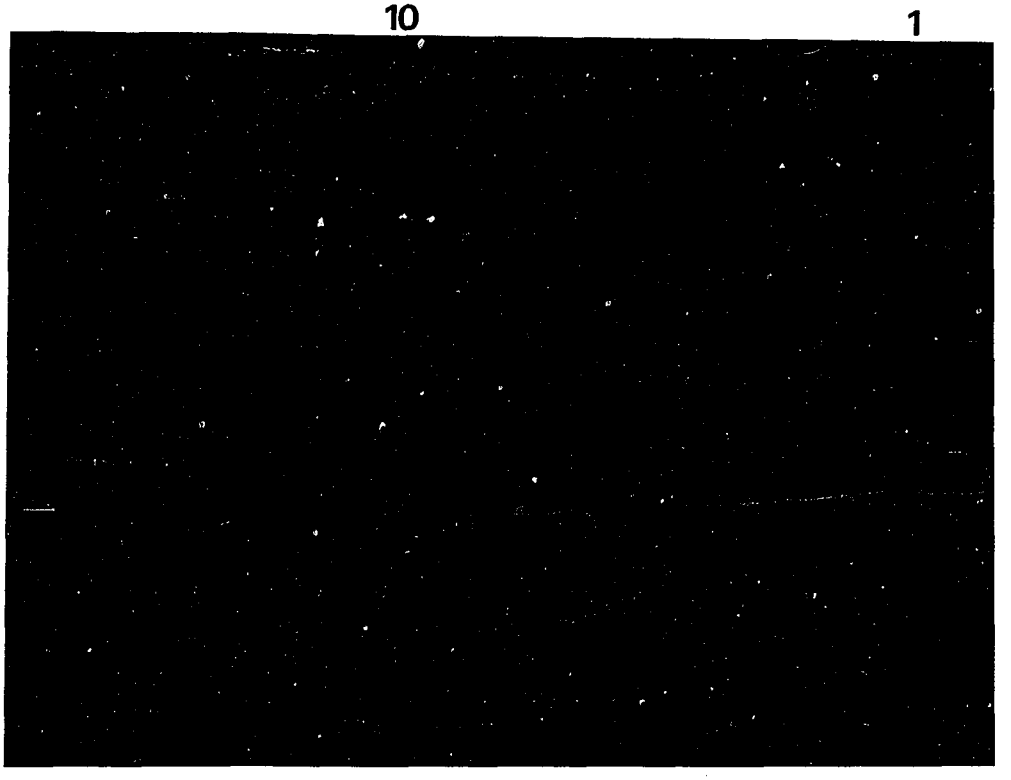
Lane	Clone
1	4-4α
2	7-6α
3	10-5α
4	12-1α
5	13-3α
6	14-2α
7	14-7α
8	15-4α
9	5-1
10	λHeG1/EcoRV
11	λHeG1/EcoRI
12	λ/EcoRI
13	λ/HindIII
14	λ/EB

Figure 23h
EcoRV-digested cosIII clones:
λHeG1 probe



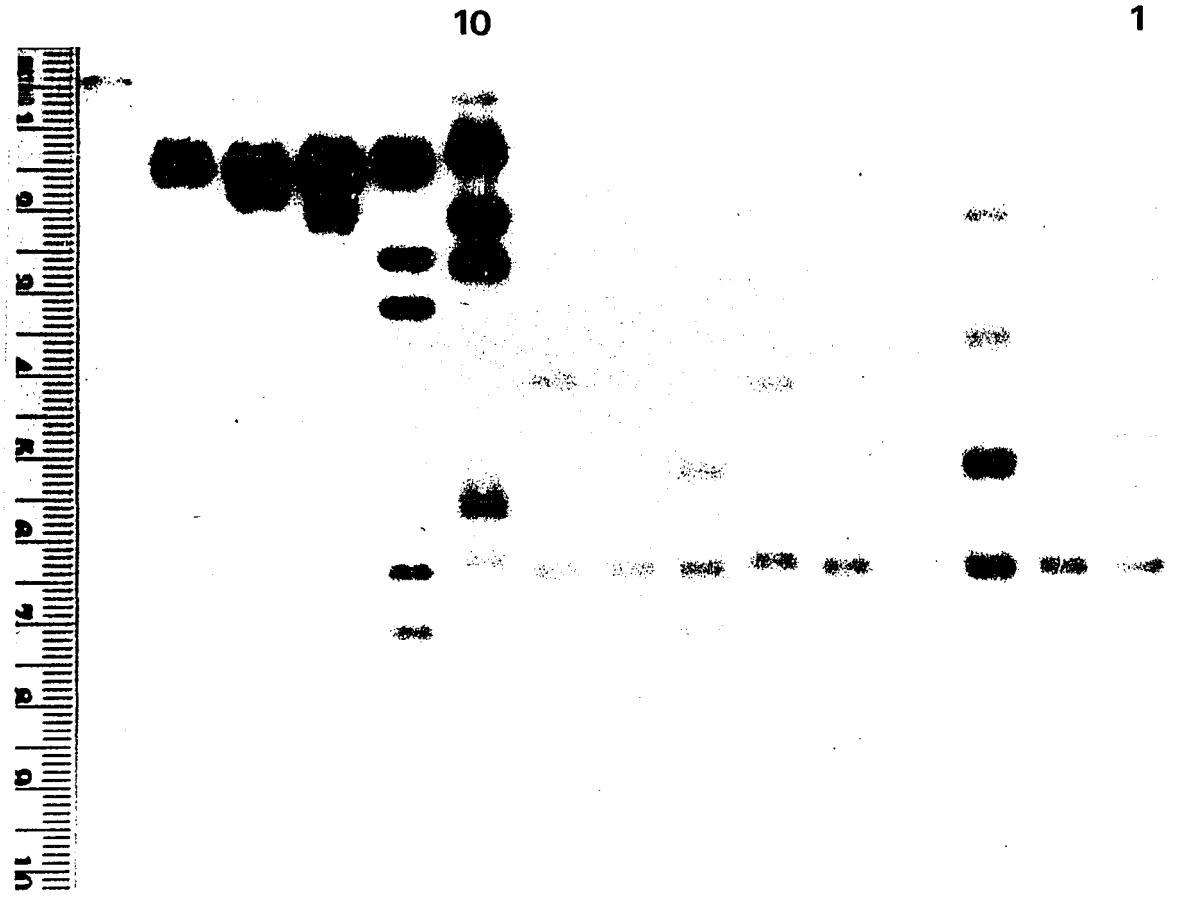
Lane	Clone
1	4-4α
2	7-6α
3	10-5α
4	12-1α
5	13-3α
6	14-2α
7	14-7α
8	15-4α
9	5-1
10	λHeG1/EcoRV
11	λHeG1/EcoRI
12	λ/EcoRI
13	λ/HindIII
14	λ/EB

Figure 24a
Bg1II-digested cosIII clones:
Ethidium bromide stain



Lane	Clone
1	4-4α
2	7-6α
3	10-5α
4	12-1α
5	13-3α
6	14-2α
7	14-7α
8	15-4α
9	5-1
10	λHeG1/Bg1II
11	λHeG1/EcoRI
12	λ/EcoRI
13	λ/HindIII
14	λ/EB

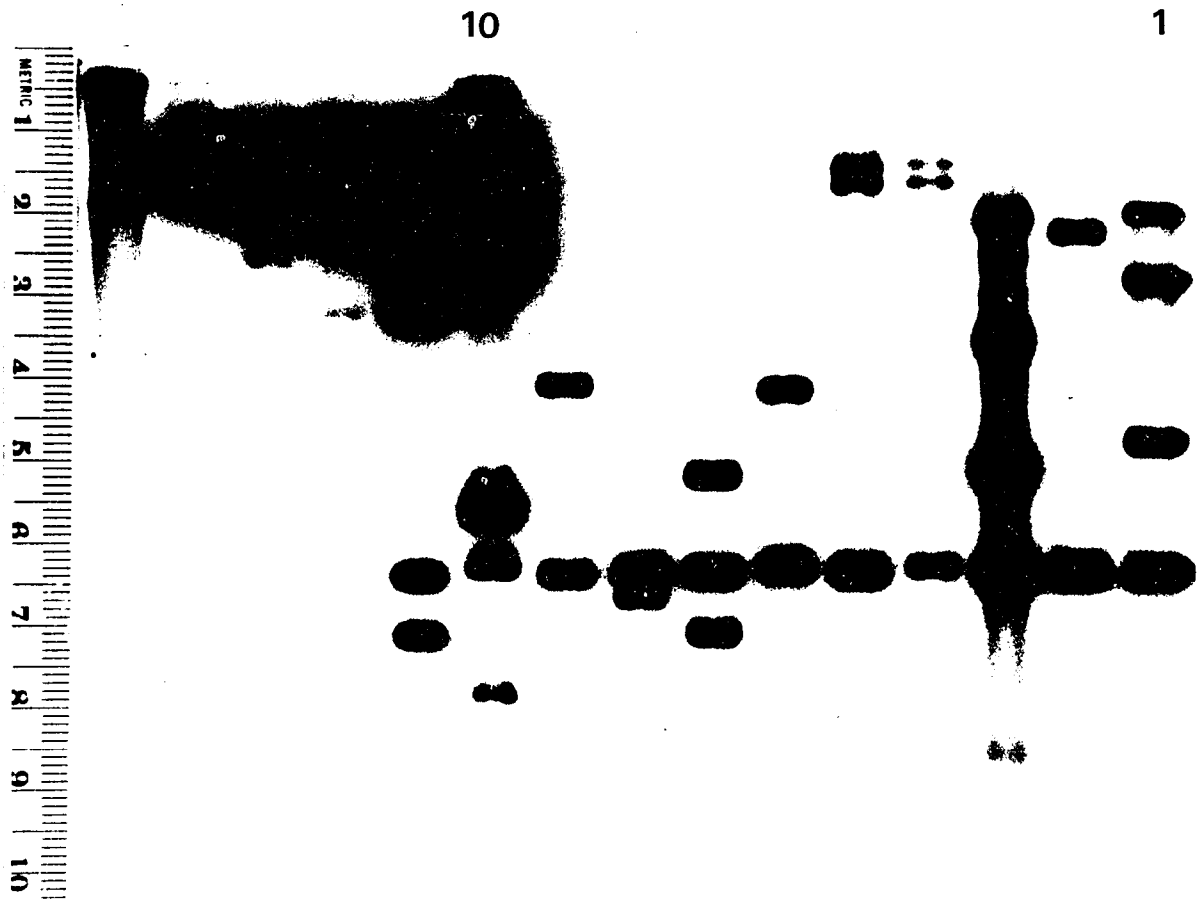
Figure 24h
Bg1II-digested cosIII clones:
λHeG1 probe



Lane	Clone
1	4-4α
2	7-6α
3	10-5α
4	12-1α
5	13-3α
6	14-2α
7	14-7α
8	15-4α
9	5-1
10	λHeG1/Bg1II
11	λHeG1/EcoRI
12	λ/EcoRI
13	λ/HindIII
14	λ/EB

Positive controls 10 and 11 were not heated, which allowed the cohesive ends spanning the cos site to anneal.

Figure 24b
Bg1II-digested cosIII clones:
λHeG1 probe
Long exposure



Lane	Clone
1	4-4α
2	7-6α
3	10-5α
4	12-1α
5	13-3α
6	14-2α
7	14-7α
8	15-4α
9	5-1
10	λHeG1/Bg1II
11	λHeG1/EcoRI
12	λ/EcoRI
13	λ/HindIII
14	λ/EB

Table 7
Overlapping cosmid clones: comigrating fragments

enzyme	clones	fragment	probe	figure
EcoRI	7-6α, 12-1α	14kb; 9kb	λ	19b
			λEB	19c
			pBR322	19d
			3.1kb	19g
	12-1α, 14-2α	2kb	pBR322	19d
	14-7α, 15-4α	5.5kb	pBR322	19d
	7-6α, 12-1α; 10-5α?	4kb; 1.5kb	mouse	19e
	10-5α, 12-1α	9kb	mouse	19e
BamHI	?		λ	20b
	?		λEB	20c
	?		pBR322	20d
	7-6α, 12-1α	2.1kb	mouse	20e
	7-6α, 15-4α	2.3kb	mouse	20e
	13-3α, 14-2α	4.8kb	mouse	20e
HindIII	?		λ	20b
	?		λEB	20c
	?		pBR322	20d
	7-6α, 14-7α	4.7kb	mouse	20e
	12-1α, 13-3α	3kb	mouse	20e
SalI	?			21b-e
KpnI	?			21b-e
BalI	?		λHεG1	22h
EcoRV	?		λHεG1	23h
BglII	1.8kb	all clones	λHεG1	24h
	4-4α, 10-5α	4.9kb	λHεG1	24h
	10-5α, 14-7α	2.1kb	λHεG1	24h
	12-1α, 13-3α	10kb, 11kb	λHεG1	24h
	14-2α, 5-1?	3kb	λHεG1	24h

Figure 25
Somatic cell hybrid analysis: mapping of β80 locus
A12 probe

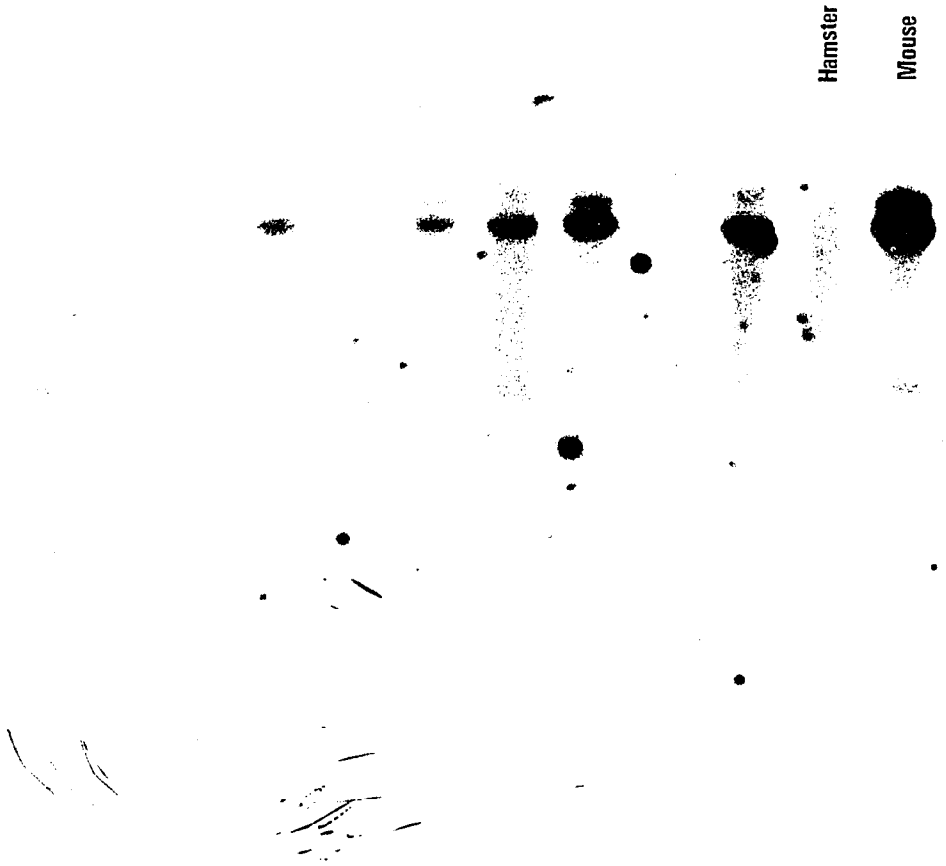
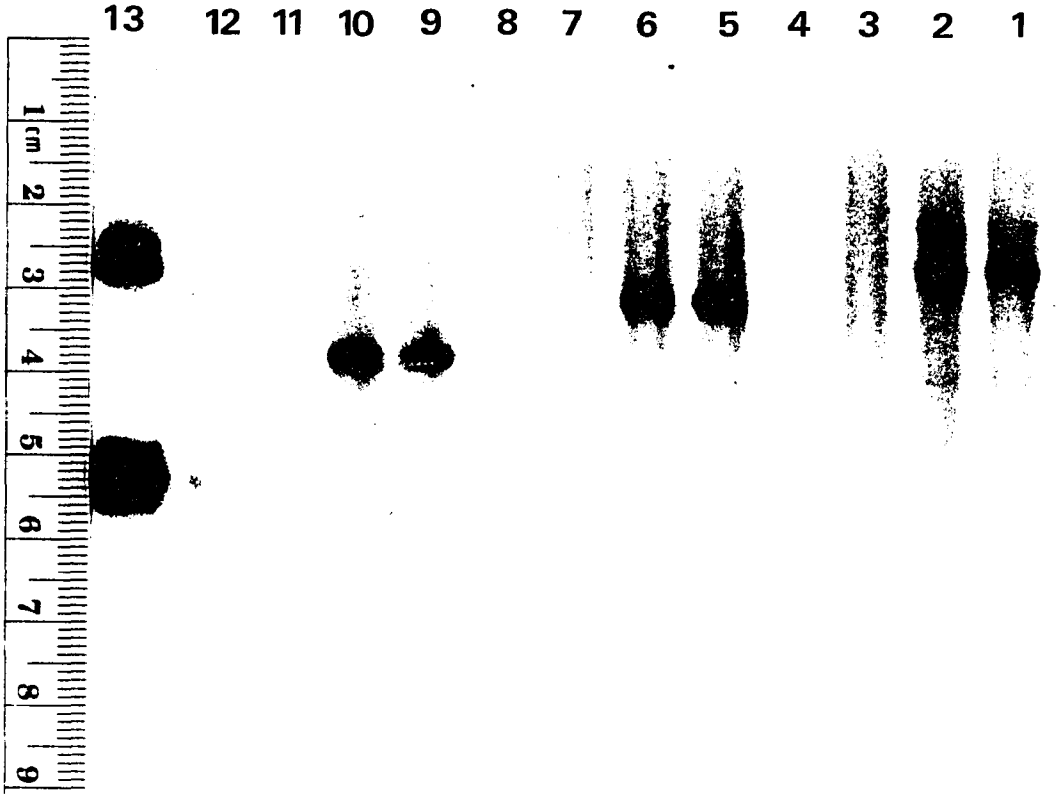
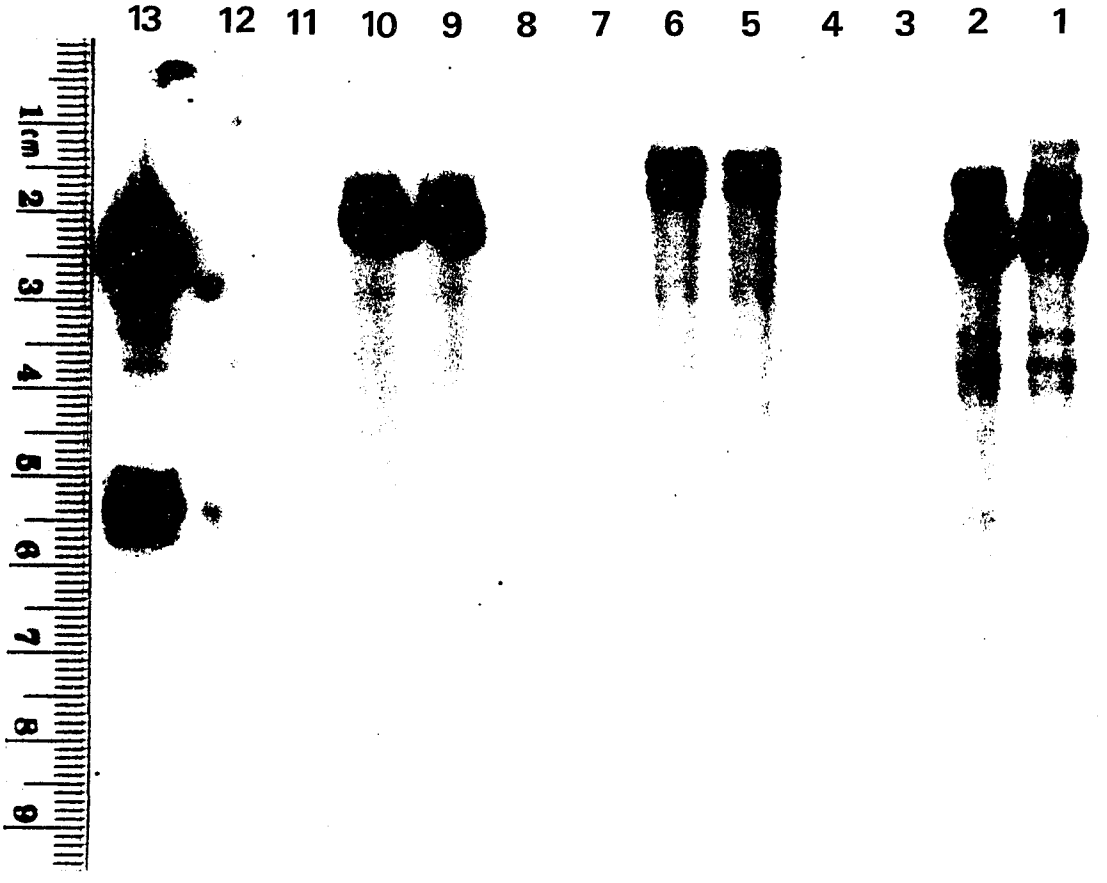


Figure 26a
RFLP analysis:
A9 probe



Lane	DNA sample	Restriction enzyme
1	mouse -/-	EcoRI
2	β80 +/-	EcoRI
3	hamster	EcoRI
4	human	EcoRI
5	mouse -/-	BamHI
6	β80 +/-	BamHI
7	hamster	BamHI
8	human	BamHI
9	mouse -/-	PstI
10	β80 +/-	PstI
11	hamster	PstI
12	human	PstI
13	A2, A9, A12	EcoRI

Figure 26b
RFLP analysis:
A12 probe



Lane	DNA sample	Restriction enzyme
1	mouse -/-	EcoRI
2	β80 +/-	EcoRI
3	hamster	EcoRI
4	human	EcoRI
5	mouse -/-	BamHI
6	β80 +/-	BamHI
7	hamster	BamHI
8	human	BamHI
9	mouse -/-	PstI
10	β80 +/-	PstI
11	hamster	PstI
12	human	PstI
13	A2, A9, A12	EcoRI

DISCUSSION I.

The ß80 line displays spontaneous development of cancer with associated congenital anomalies. The high propensity for malignancy in a line of transgenic mice carrying human globin genes is a new finding, and contrasts with the numerous other lines of transgenic mice carrying globin genes. In all reported transgenic and retrovirally-transfected mice which develop cancer, functional oncogenes have been transferred. However, the observations seen in the ß80 line cannot be adequately explained by this mechanism. Causes of the ß80 mutant phenotype may include cryptic effects of foreign globin gene expression, insertional oncogene activation, or a biochemical defect induced by insertional mutagenesis.

Data from other ß-globin transgenic mice support the view that foreign gene expression is not the cause of mutation in transgenic mice. A high level of foreign globin expression does not result in malignancy in any of several lines examined (Townes et al., 1985; Magram et al., 1985; Chada et al., 1985, 1986; Kollias et al., 1986). The aspect of dominant cancer inheritance in this line is particularly difficult to reconcile with foreign gene expression, since λHßG1 contains no oncogenes.

Several lines of evidence indicate low or absent expression of λHßG1 in ß80 mice. The inhibitory effect of vector sequences on expression of microinjected globin genes (Lacy et al., 1983; Chada et al., 1985; Townes et al., 1985) predicts inactivity of globin genes in the ß80 line. Demonstration that transgenic mice with Charon 4A vector sequences do not express foreign globin in erythroid lineages (Lacy et al., 1983; Humphries et al., 1985) indicates that the Charon 4A vector may participate in vector inhibition of foreign globin expression in the ß80 line.

The demonstration that human globin mRNA is translatable in mice, and that the resulting globin chains persist in mouse red blood cells (Townes et al., 1985) indicates that the lack of human globin protein in mouse blood cells of the ß80 line can be equated with a lack of mRNA expression. Although it is possible that human globin genes are expressed in ectopic tissues, it is unlikely that levels of expression would be appreciable, since inappropriate expression occurs at low levels and does not approach levels seen in erythroid cells (Lacy et al., 1983; Chada et al., 1985). The

demonstration that human globin protein was not found in a phenotypically abnormal mouse shown to have a homozygous genotype provides evidence that foreign globin gene expression does not occur in the ß80 line and is not a factor in the phenotypes seen. The above considerations indicate that cryptic effects of human globin gene expression are not responsible for the phenotypes seen in this line.

Genotypic alterations resulting in malignancy fall into three major classes: promoter insertion (Hayward et al., 1981; Neel et al., 1981; Payne et al., 1982), endogenous oncogene activation (Knudson, 1986), and chromosome rearrangement (Klein, 1983; Cory, 1986). The first of these possiblities, that λHßG1 provided a promoter which activated an endogenous oncogene, is unlikely, since this construct contains no strong promoters. A putative globin enhancer has been identified at the 3' end of the adult ß-globin gene (Karlsson and Nienhuis, 1985), leaving the possibility that the globin construct provided an enhancer which activates an oncogene in hematopoietic cell lineages.

Most hereditary cancers (Knudson, 1986) are dominantly inherited, as for example neurofibromatosis and polyposis coli. These are thought to involve a gene which dominantly predisposes to malignancy, without the necessity of further mutation. Another class of hereditary cancer is thought to involve recessive cancer genes, including retinoblastoma and Wilm's tumor. The affected locus has been termed an antioncogene, since the presence of one functional allele confers an antioncogenic effect. A second mutation event causing loss of heterozygosity is required to elicit the transformed phenotype (Cavenee et al., 1983; Koufos et al., 1985). When inherited, transmission follows an autosomal dominant pattern, since only one event is required for tumor formation.

Endogenous oncogene activation might involve mutation by the ß80 insert of a classical oncogene to an activated state, or inactivation of an antioncogene; in either case chromosomes 1, 3, 8, or 9 would be linked to the affected oncogene. (If there is a translocation, the A12 locus does not necessarily identify the chromosome on which the oncogene resides.) The likely effect of insertion into a classical oncogene would be inactivation of structural or promoter regions, although insertion into a repressor locus could occur. A cryptic position effect would also be possible (Shapiro, 1982).

The alternative, antioncogene inactivation, would be consistent with the observation of cancer in ß80 heterozygotes, and would require the occurrence of a second genetic event; either loss of heterozygosity at the same locus, or a mutation which interacts with this locus. The association of antioncogenes with congenital anomalies in the human lends credence to this mechanism. This would be an exciting possibility, since the affected antioncogene could be cloned due to genetic linkage with the isolated ß80 locus.

Two examples of chromosome translocations consistently associated with malignancy are c-myc translocations (Leder et al., 1983; Varmus, 1984; Cory, 1986), which cause acute lymphocytic leukemia and Burkitt lymphoma, and the Philadelphia chromosome (Nowell and Hungerford, 1960), a reciprocal translocation between chromosomes 9q and 22q (Rowley, 1973), seen in over 90% of patients with chronic myelogenous leukemia. Chromosome rearrangement has been reported in transgenic mice, but has not been associated with malignancy (Overbeek et al., 1986). The cytogenetic study in progress will address the question of whether a consistent translocation is found in the ß80 line.

The pathology of these mice provides a diagnosis of lymphoma and stem cell leukemia. These kinds of malignancy alert to possible oncogenes involved. Some oncogenes have been chromosomally mapped in the mouse. The c-abl gene (Goff et al., 1982) and the c-src gene (Blatt et al., 1984) are on chromosome 2, c-mos maps to chromosome 4 (Swan et al., 1982), c-fes and c-Has-ras-1 map to chromosome 7 (Kozak et al., 1983a), c-myc (Crews et al., 1982) and c-sis (Kozak, 1983b) map to chromosome 15. None of these are implicated by the ß80 mapping data. However, if a translocation is involved, one end would be unidentified by the probe, so no genes are ruled out. Oncogenes which might be involved because of relation to hematopoietic differentiation are c-fms, c-abl, c-fes, c-fps, c-myc, and c-myb (Adamson, 1987), with mutation of c-myb fitting the pathology seen (Sheiness and Gardinier, 1984; Duprey and Boettiger, 1985; Emilia et al., 1986).

Genetics of the ß80 Line

Although the genetics of the ß80 line is not fully understood, it is clear that no abnormal animals are seen which do not contain the foreign insert. Therefore, the ß80 locus is 100% concordant with the ß80 mutation, and the ensuing considerations concern issues of dominance and penetrance, heterozygosity and homozygosity.

A striking aspect of the ß80 line is the pleiotropic nature of the mutations seen (table 4). Single gene mutations often manifest themselves in syndromes in humans and mice. For example, Danforth's short-tail (Sd) affects the axial skeleton, the gut, and the urogenital system (Green, 1981). A gene with pleiotropic manifestations can exert a dominant effect for certain aspects of phenotype, and a recessive effect for others. This is illustrated by the lethal A^y mutation (Green, 1981; Copeland et al., 1983), in which a yellow coat color and an increased propensity for the development of certain cancers are seen in heterozygotes, but embryonic lethality is manifested in homozygotes. The genetics of the ß80 line suggests that many homozygotes die in utero. The observation of rare homozygous mice with congenital abnormalities is consistent with decreased penetrance of homozygous lethality, which unmasks the skeletal deformity by permitting animals to survive to birth. That these mutations are homozygous is supported by Southern blotting and statistical analysis. In addition, among 328 animals derived from heterozygous vs. wild-type matings, none were similar to the surviving homozygotes. The AbnL mouse showed a dissimilar phenotype, as one limb was normal, one limb was more deformed than previous mice, growth retardation was less severe, survival well beyond days or weeks occured, and the AbnL mouse developed cancer at six months of age. (It is worth pointing out that other limb deformities have been observed unilaterally, and generally have increased penetrance on the left side; Green, 1981.)

Malignancy in this line is seen in mice heterozygous for the ß80 insert. The narrow age of onset also indicates that the mutant gene dosage is equivalent in these mice. The average age of onset of leukemia was 14 months, excluding the AbnL mouse, and the earliest onset seen was 13 months. The AbnL mouse does not follow the same phenotypic pattern. In addition, no congenital malformations were seen in heterozygotes which developed malignancy, or in other heterozygotes, further contrasting the AbnL mouse from the usual phenotypic pattern.

Similarity of the ß80 Mutant Phenotype to Fanconi's Anemia

Several rare genetic syndromes which have chromosome instability and predisposition to malignancy are repair defective, implicating chromosome mutation in the etiology of cancer (German, 1978). These include Bloom's syndrome (BS), ataxia-telangiectasia (AT), Xeroderma Pigmentosum (XP), Werner's syndrome (WS), and Fanconi's anemia (FA) (German, 1969, 1973a, 1973b, 1980, 1983; Carter, 1981; Schroeder, 1982; Ray and German, 1983). The further association between congenital defects and cancer in these diverse syndromes (Fraumeni and Miller, 1967; Miller, 1968; Welshimer and Swift, 1982) indicates that DNA instability is the primary defect causing the various manifestations of these diseases.

An intriguing similarity exists between the abnormalities seen in the ß80 line and those seen in Fanconi's anemia (Fanconi, 1927, 1967), a familial hypoplastic anemia which is associated with skeletal abnormalities and a high incidence of cancer. Inheritance is believed to occur via an autosomal recessive mode (Reinholt, 1952; Schroeder, 1976). Since Fanconi's original description, the following have been recognized as cardinal features of this syndrome: congenital abnormalities, pancytopenia, increased chromosomal aberrations, and a predisposition toward the development of leukemias and other malignancies. Congenital anomalies include radial aplasia, thumb deformity, abnormal wrist bones, small stature from birth, hyper- and hypopigmentation, hypogonadism, renal malformations, microcephaly, decreased intelligence, strabismus, micropthalmia, ear deformities and deafness (Visfeldt and Mortensen, 1970; Wintrobe, 1981; Welshimer and Swift, 1982; McKusick, 1983). It has been estimated (Alter and Potter, 1983) that leukemia is a terminal event in 5-10% of FA patients. Fanconi's anemia is twice as common in males as in females; in addition, there is a lower age of onset of pancytopenia in males than in females (Alter and Potter, 1983). There is a highly variable clinical picture with respect to associated anomalies and age of onset of pancytopenia, which is a hallmark of FA (Altay et al., 1975). Thus, although Fanconi's anemia is highly pleiotropic in its presentation, much clinical variation is presumed to be due to the presence of diverse interacting genetic factors. The conclusion of Schroeder et al. (1976) of an autosomal recessive mode of inheritance would indicate incomplete penetrance, the potential existence of more

than one complementation group, and/or variation with genetic background. Some of these aspects have been explored experimentally.

The similarity of ß80 mice to the Fanconi's anemia syndrome includes the association of skeletal defects of the forelimb and forearm, pancytopenia, hypogonadism, sex distortion, growth retardation, possible neurologic disease, and lymphoma and leukemia. Precise correspondence of phenotype would be unexpected, since pleiotropy can be due to second pathways after an initial biochemical defect, illustrated by the ability to modify tail length and urogenital anomalies independently in Sd heterozygotes (Gruneberg, 1963); furthermore these second pathways presumably vary among species. Identification of analogous mutations would depend on demonstration of a similar underlying biochemical defect, not on identical genetic patterns of mutation.

Cytogenetic analysis of untreated, cultured cells from Fanconi anemia patients revealed an innate chromosomal instability in these cells (Schroeder, 1964; Bloom et al., 1965, 1966; German et al., 1965). Chromatid-type aberrations, including constrictions, gaps, and breaks were frequently found in FA (Schroeder, 1964; Schmid, 1967), but no consistent translocations were seen. In addition to spontaneous chromosome anomalies, FA chromosomes have an increased susceptibility to viral transformation, radiolytic damage, and clastogenic (chromosome breaking) agents (Todaro et al., 1966; Swift and Hirschhorn, 1966; Todaro and Aaronson, 1968; Aaronson and Todaro, 1968; Dosik et al., 1970; Young, 1971; Higurashi and Conen, 1971, 1973; Potter and Potter, 1975; Remsen and Cerutti, 1976; Lubiniecki et al., 1977).

Despite the undetermined nature of the biochemical defect(s) in FA, hypersensitivity to clastogenic agents has been used as a reliable assessment of FA, and has been successfully applied in prenatal diagnosis (Auerbach et al., 1986). Various clastogenic agents such as diepoxybutane (DEB) and mitomycin C (MMC) have been used to assess the FA phenotype (Auerbach and Wolman, 1976, 1978; Cohen et al., 1982, 1983) by counting induced chromosomal anomalies and subjecting these data to statistical analysis. These studies are reliable only for homozygotes; the broad range of response in the control and parental (obligate heterozygote) groups makes heterozygote assessment by this method impractical. These definitive tests provide an opportunity to determine if ß80

transgenic mice carry a mutation which corrsponds to FA or a related syndrome. Mice can be bred, and chromosomes from transgenic fetal fibroblasts can be studied for their response to clastogenic agents. If increased susceptibility is observed we will have strong evidence that globin gene insertion has induced a mutation similar to that of FA. In this instance, cloning of DNA around the ß80 insertion should for the first time permit a molecular analysis of genes affected in chromosome instability syndromes.

In summary, the ß80 line possesses a new genetic mutation which is unlike that previously seen in other transgenic mice. The mechanism whereby the ß80 syndrome is manifested is unclear, but may relate to one of the mechanisms discussed above. It is also possible that a combination of these mechanisms are involved. For example, a translocation might inactivate a Fanconi-like locus in the mouse; then, cytogenetic analysis of ß80 cells would seemingly contradict the lack of a consistent translocation seen in FA cells, but the biochemical defect would be equivalent. Similarly, antioncogene inactivation might occur by insertional inactivation or inactivation via translocation. Another consideration is that the phenotypic manifestations of an underlying defect such as chromosomal instability can vary between the mouse and the human, which could account for differences between FA and the ß80 line. For this reason, it is possible that another chromosome instability syndrome could represent the analogous biochemical defect seen in ß80 transgenic mice. These questions will be addressed by studies currently in progress.

BACKGROUND II.

The structural characteristics of certain highly repetitive sequences and pseudogenes in eukaryotic genomes provide evidence for the reverse flow of genetic information from RNA to DNA. Reverse transcription was first demonstrated for retroviruses (Baltimore, 1970; Temin and Mizutani, 1970) and later shown in hepatitis B virus and cauliflower mosaic virus (Summers and Mason, 1982; Pfeiffer and Hohn, 1983; Varmus, 1983). Eukaryotic transposable elements such as Ty elements in yeast and copia-like elements in Drosophila also utilize reverse transcription in their transposition life-cycle and appear to encode functional reverse transcriptase (Clare and Farabaugh, 1985; Boeke et al., 1985; Garfinkel et al., 1985; Mellor et al., 1985; Saigo et al., 1984).

Two types of DNA sequences in vertebrates bear evidence of having undergone processing via RNA intermediates, although they do not themselves encode reverse transcriptase. These include pseudogenes and interspersed repetitive DNA sequences. For pseudogenes (Vanin, 1985), the combined observations of 1) precise loss of intervening sequences, 2) start site in some cases coincident with the site of transcriptional initiation, 3) presence of a poly(A) addition signal and adenine tracts, with homology to the normal gene ending abruptly at the poly(A) addition signal, 4) transposition to a new chromosomal site, and 5) insertion site duplication, bear evidence that many processed pseudogenes have resulted from reverse transcription of an mRNA intermediate (Weiner et al., 1986). For example, the mouse α-3 globin pseudogene has precisely lost both its intervening sequences in accordance with the G-T/A-G splicing rule of RNA (Nishioka, et al., 1980) and is located on a different chromosome than the mouse alpha-globin cluster (Leder et al., 1981). Multiple intronless human dihydrofolate reductase (DHFR) pseudogenes are no longer syntenic with the functional DHFR gene on chromosome 5, and some of these bear adenine-rich tracts at the 3' end (Shimada et al., 1984). A processed immunoglobulin gene (Hollis et al., 1982) bears spliced J-C regions, a poly(A) tail, and is flanked by a 9-base pair direct repeat indicative of insertion site duplication, and has been dispersed to a new chromosome. Three pseudogenes, human ß-tubulin (Wilde et al., 1982), human metallothionein (Karin and Richards, 1982), and rat α-tubulin

(Lemischka and Sharp, 1982), in addition to loss of introns, presence of poly(A) tails and short flanking direct repeats, also abruptly lose homology to the functional gene 5' to the start of transcriptional initiation. Van Arsdell et al. (1981) have suggested that small nuclear RNA pseudogenes have also been created via reverse transcription of an RNA intermediate. Although the assertion of transposition via an RNA intermediate is inferential since it is based solely on sequence data, Shimotohno and Temin (1982) demonstrated that a mouse α-globin which was cloned into a retrovirus lost its intervening sequences during viral replication. Moreover, most pseudogenes which remain linked to their functional counterparts also retain their introns, but most processed pseudogenes have lost their introns and are no longer syntenic with their homologous, active genes. The structural characteristics of processed pseudogenes appear to be the footprints left by a reverse transcription activity operational during the meiotic life cycle, and the prevalence of these elements indicates that reverse transcription plays a role in shaping the eukaryotic genome during the course of evolution (Syvanen, 1984; Baltimore, 1985).

The existence of repeated sequences in eukaryotic genomes was first indicated by the rapid renaturation rate of DNA (Britten and Kohne, 1968). These sequences were subsequently shown to consist primarily, in human DNA, of a single family of sequences called the Alu family due to a conserved site for the restriction enzyme AluI (Houck et al., 1979; Rinehart et al., 1981). The Alu family exists as a set of 300bp sequences with approximately $3\text{-}5 \times 10^5$ dispersed members (Hwu et al., 1986) arranged in a short-period interspersion pattern (Schmid and Deininger, 1975; Deininger and Schmid, 1976). The elements are homogeneously divergent (Rinehart et al., 1981) from one consensus sequence (Deininger et al., 1981; Schmid and Jelinek, 1982) to which they bear an average 80% homology (Jagadeeswaran et al., 1981; Sharp, 1983).

The DNA structural characteristics of Alu sequences and their transcriptional activity have led several investigators to suggest that they comprise a class of transposable elements (Van Arsdell et al., 1981; Jagadeeswaran et al., 1981; Sharp, 1983; Baltimore, 1985). Structurally, the primate Alu sequence is composed of a head-to-tail dimer formed by two 130bp homologous monomers, each with an A-rich region at the 3' end; the first sequence has a 31bp deletion (Deininger et al., 1981; Pan

et al., 1981; Weiner et al., 1986). Several of the sequenced elements have been shown to be flanked by 7-20bp direct repeats corresponding to insertion site duplication; these are considered to be the hallmark of insertion at a staggered nick, the mechanism used by the many various classes of transposons (Bell et al., 1980; Calos and Miller, 1980; Schmid and Jelinek, 1982; Sharp, 1983; Shapiro, 1983); in contrast, insertion of transfected SV40 DNA does not result in target site duplication (Stringer, 1982).

Duncan et al. (1979) demonstrated the presence of elements along the human ß-globin cluster which serve as templates for in vitro RNA polymerase III (RNA pol III) transcription; these were shown to be Alu elements upon sequence analysis (Jelinek et al., 1980). These and other Alu elements contain an internal split RNA pol III promoter (Duncan et al., 1981; Fuhrman et al., 1981; Ciliberto et al., 1983; Perez-Stable et al., 1984) which becomes incorporated into the resulting RNA; thus, transposed elements can in theory serve as templates for subsequent dispersion events (in contrast to RNA pol II-promoted transcripts). A key observation from these studies is that the RNA pol III transcripts initiate at the first base pair of the repeat, immediately after the 5' flanking direct repeat, strongly implying the existence of an RNA intermediate in the formation of the Alu template. The transcript continues through the entire element, into the 3' poly(A)-rich region, through unique DNA sequences, terminating in a U-rich region.

These observations have culminated in a model for Alu transposition via self-primed reverse transcription (Van Arsdell et al., 1981; Jagadeeswaran et al., 1981). According to this model, an Alu transcript is initiated at an internal RNA pol III promoter, and is extended through and beyond the Alu element, as described above. The U-rich region at the 3' end of the RNA transcript then forms a hairpin turn and binds to the internal oligo(A) sequence, providing a primer for reverse transcriptase. This is then copied into a cDNA, integrated into genomic DNA at a staggered nick, followed by gap filling, thereby creating flanking direct repeats.

Despite the overwhelming circumstantial evidence that Alu elements have undergone transposon-mediated dispersion, Alu dispersion has never in fact been observed in the laboratory. By microinjecting transcribable human Alu sequences into mouse embryos, we have provided a template

for such a transposition event to occur. We present restriction enzyme evidence for the first in vivo observation of this event, and predict the DNA structural characteristics which may be discovered from sequencing data.

METHODS II.

100 mice were screened for the presence of microinjected DNA sequences. DNA was extracted according to the method of Blin and Stafford (1976), Southern blotted (Southern, 1975) and hybridized (Wahl et al., 1979). Probes for Southern analysis included λHßG1 (Fritsch et al., 1980), ßpst (4.4kb genomic fragment with ß-globin and two downstrean Alu sequences; a gift of F. Ruddle et al.), ß31A (a λHßG1 EcoRI subclone with the 3.1kb and 5.5kb fragments), pBR322, pBLUR8 (Jelinek et al., 1980, Deininger et al., 1981), and lambda phage.

Test digestions were performed to prove the λHßG1 phage lacked a recognition site for XhoI so that this enzyme could be used to determine the number of independent integration events in the ß19 line (figure 7). λHßG1 and lambda were each digested with 1) no enzyme, 2) XhoI, or 3) SmaI to display the size of restriction fragments released, establish enzyme activity, and show efficacy of the other reaction components. The λHßG1/XhoI reaction comigrated with undigested λHßG1 and lambda samples, and did not release any new fragments. To rule out resistance to digestion by XhoI in the λHßG1 samples due to contamination, λHßG1 and lambda were digested in the same reaction; no new fragments were seen in XhoI or SmaI digestions. Therefore λHßG1 contains no XhoI sites.

RESULTS II.

The ß19 Founder Mouse Coat Color Phenotype

The first anomaly noted in the founder mouse was a premature greying of its black coat (figure 27). The greying pattern consisted of a patchy distribution of white hairs. This patterned greying is in contrast to the diffuse, salt-and-pepper distribution, both of which have been described in relation to age-related greying (Morse et al., 1985). A similar correlation between age of onset of greying and greying pattern was seen, in that the early onset within a few weeks of age was associated with a patchy distribution of white hairs. This patchy loss of pigmentation is similar to that described by Jaenisch (1980) for a mouse with multiple somatically acquired copies of MoMuLV proviral DNA. This observation provided the first indication that the founder mouse might have numerous sites of foreign DNA insertion. The greying phenotype was not seen in offspring, indicating that the mutation did not exist in the germ line, and providing evidence that the founder mouse was a mosaic.

Southern Analysis of the ß19 Founder Mouse

Figure 28 demonstrates the original pattern of "blur" hybridization seen in the ß19 mouse when spleens of putative transgenic animals were digested with EcoRI and screened with whole nick-translated phage for the presence of microinjected sequences. Comparison to the ethidium bromide stain shows that equal amounts of spleen DNA were loaded in all 14 tracts. Therefore, unequal loading of DNA could not account for the large increase in hybridization seen for the ß19 spleen sample; this experiment was repeated several times for verification. This atypical blur pattern is in striking contrast to the pattern of discrete bands seen with all transgenic mice reported to date (see figure 15).

The blur pattern was reproducible with PstI, HindIII, and MspI, enzymes which recognize sites within the phage clone. These results suggested extensive rearrangement of microinjected sequences, and indicated possible dispersion of microinjected sequences.

The possibility of contamination with intact phage DNA was investigated by hybridization of undigested DNA from ß19. If contamination accounted for the pattern seen, one would expect this same pattern to appear in this experiment, with low molecular weight hybridizable fragments migrating away from the undigested genomic DNA. Undigested spleen DNA was accordingly Southern blotted and probed with λHßG1 phage. No hybridization was seen, demonstrating that the genomic DNA was not degraded, suggesting that the hybridizable material in the sample was integrated.

Having eliminated the preceding possibilities for anomalous hybridization, we attempted to determine the number of integration sites by restriction with XhoI, an enzyme which recognizes no sites within the phage clone (figure 7). Each band seen on a Southern blot of DNA digested with a non-cutting enzyme must derive from restriction of mouse DNA flanking the phage DNA. With this method, each resulting band corresponds to a unique site of integration. This experiment was performed to determine if the blur pattern represented one integration site of extensively rearranged donor material, or multiple sites. In the former case one band would be expected, while in the latter instance a blur would again appear.

Figure 29 shows that digestion of ß19 spleen DNA with XhoI again resulted in a blur pattern of hybridization, indicating numerous independent integration sites. Furthermore, since XhoI is methylation-sensitive, many genomic sites would be expected to be undigested, thus underestimating the number of integration sites. These results demonstrate extensive dispersion of microinjected sequences in the ß19 mouse. This experiment contrasts the integration mechanism in the ß19 mouse with that previously observed in transgenic mice, and indicates that a novel integration mechanism was operational during the insertion of foreign sequences in this mouse.

To more precisely define the content of the dispersed sequences, ß19 spleen DNA was probed with three subcomponents of the λHßG1 probe. First, HindIII and MspI digests of ß19 spleen DNA were reprobed with ß31A, a subclone containing the 3.1kb EcoRI fragment with two Alu sequences and the 5.5kb fragment consisting of 5' ß-globin sequences and the transcriptionally active Alu-like element upstream of the ß-globin gene (Carlson and Ross, 1983). This clone hybridized more

intensely than the whole λHßG1 probe, producing a darker smear, indicating that hybridizable material formed a larger fraction of this clone than λHßG1.

Next, ß19 spleen DNA was digested with EcoRI and probed with a plasmid containing a 4.4kb PstI fragment which spans the ß-globin gene and contains the two Alu sequences downstream of the ß-globin gene (figure 2). This probe showed a faint blur of hybridization to ß19 DNA (figure 30). Cross- hybridization to mouse globin was seen in mouse DNA samples, and to the 5.5kb and 2.25kb phage bands containing, respectively, ß- and δ-globin. In addition, hybridization was seen against the 3.1/3.2kb phage doublet containing pairs of Alu sequences, indicating that the probe hybridized to Alu sequences on this blot. Therefore, the blur may have been produced by homology of Alu sequences in the probe to dispersed DNA in the ß19 mouse.

The ß19 mouse was bred and then sacrificed to analyze its somatic tissues. DNA from brain, skeletal muscle, kidney, liver, testis, heart and lung was digested with EcoRI and Southern blotted using whole nick-translated phage as a probe. The blur pattern of hybridization was not observed in any tissue other than spleen using this probe. This indicated that the ß19 mouse was a mosaic, as first suggested by non-transmission of the coat-color phenotype, or that the conformation of hybridizable sequences in spleen differed substantially from that in other somatic tissues.

Southern Analysis of ß19 Offspring

Spleens of 22 offspring were similiarly blotted with whole nick-translated phage as a probe; these appeared negative. This indicated that a) most or all of hybridizing material was not integrated into the germline and/or b) only a small subcomponent of microinjected DNA was present. The analysis of the ß19 founder mouse, coupled with knowledge that Alu sequences in mouse and man are dispersed in the genome provided a rationale to probe for human Alu sequences in ß19 progeny. Figure 31 shows a Southern blot of spleen DNA digested with EcoRI and probed with pBLUR8 (Jelinek et al., 1980; Deininger et al., 1981), which contains a cloned human Alu sequence. Of six progeny blotted, three contained a single 7.5kb hybridizing fragment which was clearly absent from the negative control and the ß80 transgenic mouse. DNA from these mice did not, however,

hybridize with a lambda probe. Furthermore, the 7.5kb EcoRI fragment was transmitted to second generation progeny. These results show that some human Alu sequences were present in the germ line of the ß19 founder mouse and were transmitted to ß19 progeny. Detection of these fragments with the cloned Alu probe but not the entire phage indicates that Alu sequences were preferentially retained in these mice.

Figure 27
β19 Founder Mouse

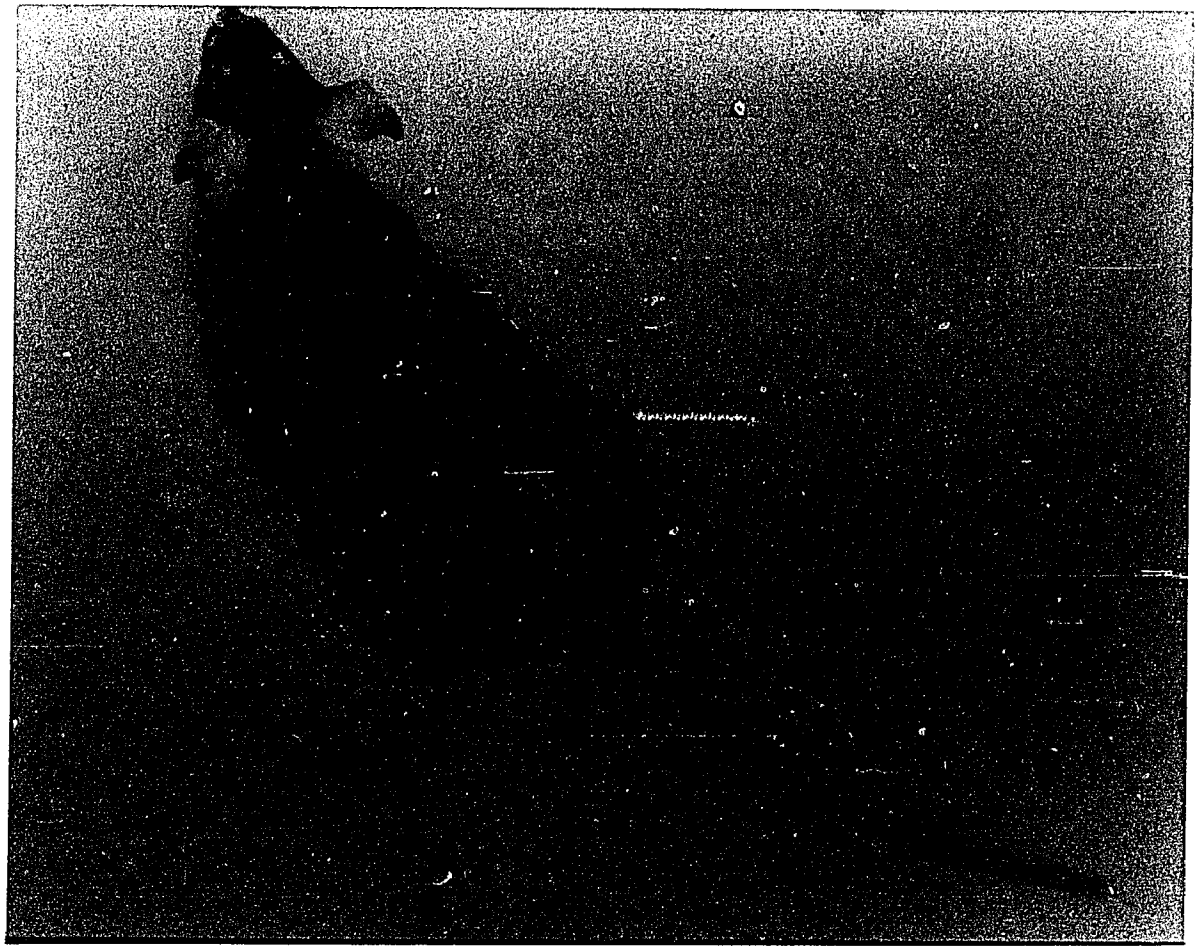
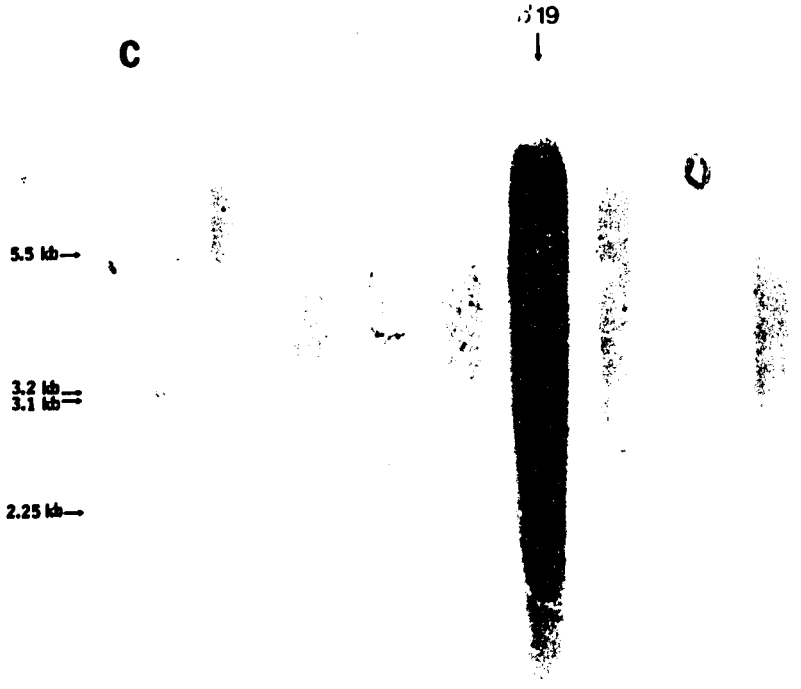


Figure 28
EcoRI-digested spleen DNA from microinjected mice
λHβG1 probe



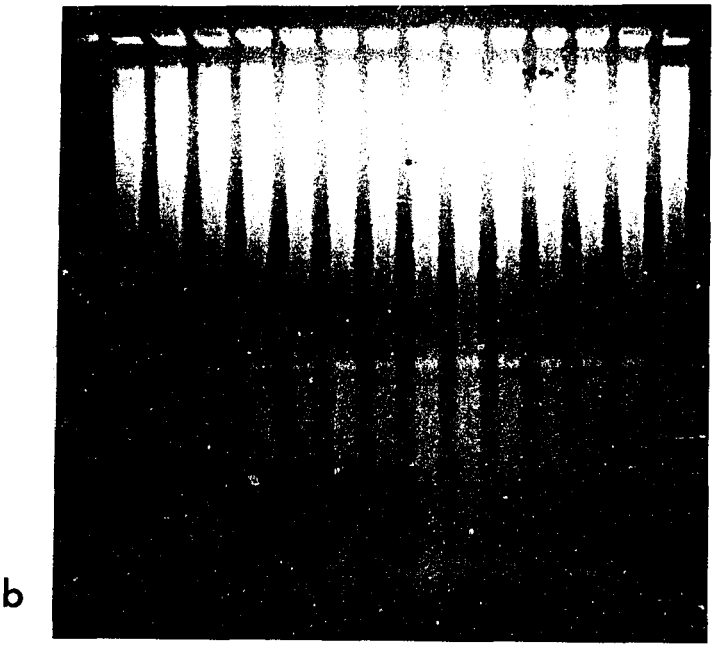


Figure 29
EcoRI-digested spleen DNA from microinjected mice
βPst probe

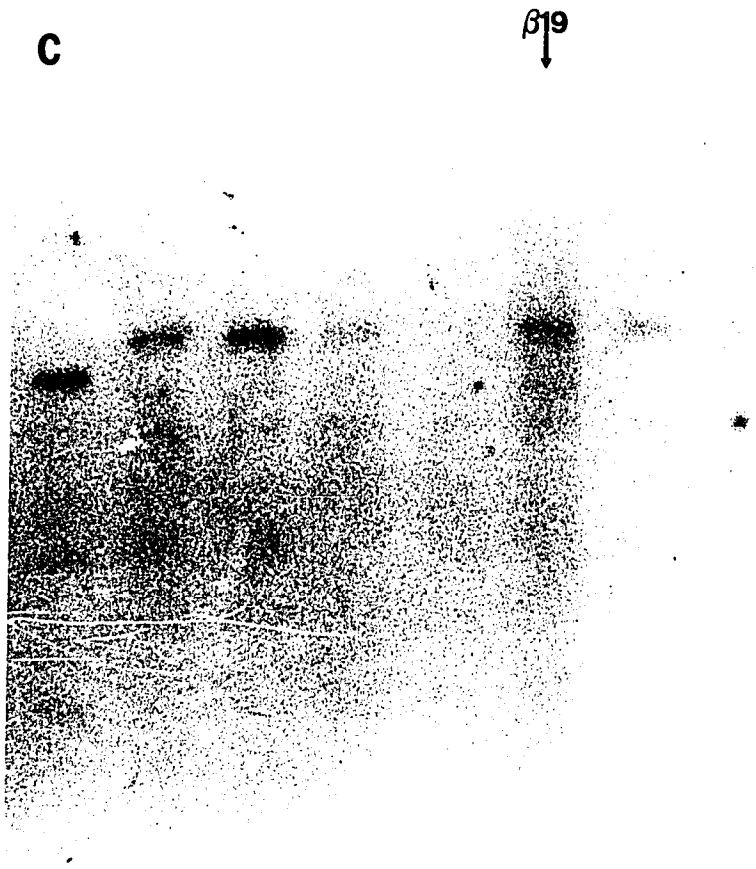
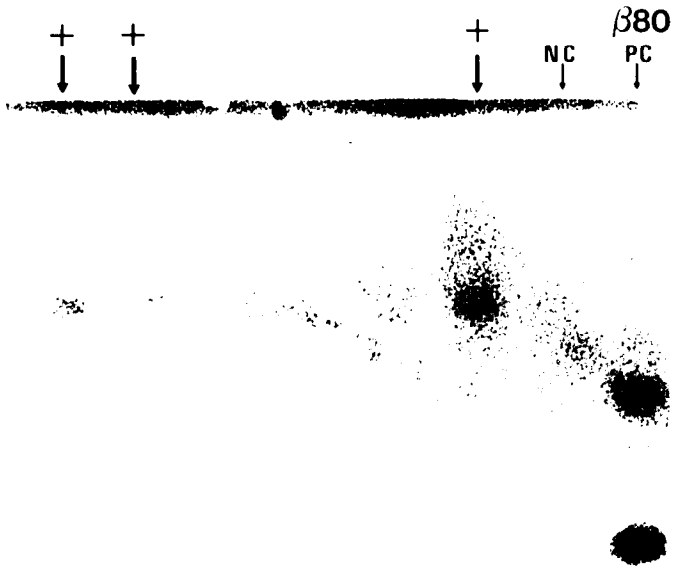


Figure 30
XhoI-digested spleen DNA from β19 and control mouse
λHβG1 probe

Figure 31
EcoRI-digested spleen DNA from β19 offspring
pBLUR8 probe



DISCUSSION I I.

Microinjection of a human genomic globin clone with Alu sequences resulted in extensive DNA dispersion in the spleen of a transgenic mouse. This integration pattern is in marked contrast to the one or two sites of insertion seen in all previously reported transgenic mice (Lacy et al., 1983; Storb et al., 1984; Overbeek et al., 1986). DNA dispersion followed a mosaic pattern in somatic and germline tissues of the founder mouse. Offspring were shown to possess a unique hybridizing fragment with homology to a human Alu sequence, indicating that DNA dispersion was mediated by Alu sequences.

It is possible that somatic tissues other than spleen of the founder ß19 mouse contained Alu sequences which went undetected. Since a 50kb probe was used to blot these tissues, and only 3% of this probe possessed Alu homology, the probe was relatively insensitive to Alu sequences.

Britten and Kohne (1968) predicted that saltatory replication of Alu sequences should be observable based on the homogeneous nature of dispersed repetitive elements. Microinjection of foreign DNA tests whether putative enzymatic activities can mediate DNA dispersion of microinjected DNA. According to the transposition model previously proposed, these activities are hypothesized to include: RNA polymerase III (RNA pol III) activity, reverse transcriptase activity, and enzymes for integrating extrachromosomal DNA which cause target site duplications. In order for new sequences to gain access to the germ line and have relevance to evolutionary change, these activities must be present in the early embryo or germ cell lineage.

RNA pol III-promoted transcription of mouse B2 repeated sequences (Kramerov et al., 1982) in the normal embryo was demonstrated by Murphy et al. (1983). That RNA pol III-promoted transcription of mouse B2 repeated sequences occurs after fertilization, during early embryogenesis, was shown by Vasseur et al. (1985), suggesting that the first condition is satisfied. This work takes previous in vitro transcription studies (Haynes and Jelinek, 1981; Duncan et al., 1981) a step further, by showing that repetitive sequence transcripts are produced during early mouse development. Thus, the endogenous transcriptional machinery of the fertilized egg is poised to act upon an exogenous

DNA template, as was provided in the microinjected clone. Potential transcripts derived from the exogenous template are described below.

Two potential sources of reverse transcriptase in the early embryo include intracisternal A-particles (IAP's) (Bernhard, 1958) and the L1 family of repetitive sequences (Voliva et al., 1983). IAP's are retrovirus-like intracellular vesicles (Kuff et al., 1981; Ono and Ohishi, 1983; Christy et al., 1985) containing RNA (Wong-Staal et al., 1975; Lueders et al., 1977; Paterson et al., 1978) and reverse transcriptase (Wilson and Kuff, 1972; Yang and Wivel, 1974), which bud into the rough endoplasmic reticulum. IAP's have been reported to be widespread in embryonic (Wivel and Smith, 1971; Calarco and Szollosi, 1973; Chase and Pikó, 1973; Biczysko et al., 1973) and transformed (Dalton et al., 1961; Wivel and Smith, 1971; Lueders and Kuff, 1975; Shen-Ong and Cole, 1982) mouse tissues. DNA sequences related to IAP RNA are highly reiterated in the mouse genome (Lueders and Kuff, 1977, 1980; Ono et al., 1980) and appear to be movable elements (Kuff et al., 1983) capable of causing insertional mutations (Hawley et al., 1982; Ymer et al., 1985).

Long interspersed repeated segments (LINEs) (Singer, 1982) also appear to be mobile repetitive elements (Voliva et al., 1984) which have become dispersed during mouse evolution (Brown and Dover, 1981). Evolutionary conservation of a long open reading frame suggested that a functionally significant protein is encoded by these elements (Martin et al., 1984). Recently, this open reading frame was shown to possess conserved homology to reverse transcriptase (Hattori et al., 1986), and to the I factor which controls transposition in I-R hybrid dysgenesis in D. melanogaster (Fawcett et al., 1986). Furthermore, there is evidence that LINEs are transcribed in pluripotential embryonic cells (Skowronski and Singer, 1985). Either of these two kinds of elements might be proposed to supply the reverse transcriptase activity in the early embryo to satisfy the second condition for the transposition model. As neither of these elements have yet been demonstrated to provide functional reverse transcriptase activity to the embryo, sequence data derived from a reverse-transcribed template would provide direct evidence of this activity.

The last condition of an insertion mechanism resulting in target site duplication is indicated by the duplicated DNA borders of dispersed families of DNA elements and pseudogenes. Although

enzymes with the ability to catalyze insertion and target site duplication reactions have not been discovered in the embryo, conservation of enzymatic mechanism in similar reactions implies that such a class of proteins does exist. The integration mechanism which operated in the ß19 founder mouse contrasts fundamentally with the normal insertion mechanism previously seen in transgenic mice. Sequence data will resolve whether this insertion mechanism produced target site duplications.

The existence of processed globin pseudogenes indicates that globin genes can serve as transcriptional templates in the early mouse embryo. However we interpret the limited blur hybridization to the ßpst probe, and the specificity of this probe for globin as demonstrated by cross-hybridization to mouse globin and delta globin, to indicate that globin sequences did not comprise a significant part of dispersed DNA in the ß19 founder mouse.

The pair of Alu sequences 5' to the delta globin gene (Coggins et al., 1980, 1981) includes a 5' sequence which is not transcribed in vitro, possibly due to a 2bp substitution in its RNA pol III promoter (Poncz et al., 1983), similar to that reported in an epsilon globin-associated Alu sequence (Di Segni et al., 1981), and a 3' sequence which is transcribed by RNA pol III in vitro (Duncan et al., 1979, 1981; Fritsch et al., 1981); the in vitro transcript terminates before reaching the delta globin gene. This Alu repeat region has been implicated in hemoglobin switching during ontogeny (Weatherall and Clegg, 1982; Spritz and Forget, 1983), and has been shown to correlate with transcriptional activation of adult globin genes in MEL-human hybrids (Zavodny et al., 1983).

The pair of Alu sequences 3' to the beta globin gene similarly contain an inactive 5' sequence, possibly due to a 24bp deletion in the RNA pol III promoter consensus sequence (Poncz et al., 1983), and a 5' sequence which is transcribed in vitro in an orientation away from beta globin (Fritsch et al., 1981) and which is coordinately expressed in vitro with adult globin (Zavodny et al., 1983).

Carlson and Ross (1983) have reported a sequence with Alu homology upstream of ß-globin, which is transcribed by RNA pol III in vitro from transfected globin templates in HeLa cells, and in vivo in bone marrow and reticulocytes. Transcripts extended into the globin coding region, were spliced at the 5' IVS-1 splice site, bound to oligo-d(T) columns, and were detected in vivo at approximately 20 to 50 copies per cell.

Sequencing data will indicate which Alu element(s) were involved in the dispersion event and will be used to determine the derivation of the Alu sequence in ß19 offspring. The unique sequence of each Alu element in the ß-globin cluster, and the unique sequences downstream of these elements will be compared to the sequence in ß19 offspring and will be used to determine which was involved in the ß19 dispersion event.

Evidence of poly(A) sequences which do not correspond to genomic DNA in the λHßG1 clone, and corresponding adenylation signals which do correlate with genomic sequences, would strongly indicate transposition via an RNA intermediate. In addition, coincidence of the 5' end of the Alu element, as defined by a target site duplication (if present) with the beginning of an Alu transcriptional unit derived from λHßG1, would demonstrate the insertion of a transcriptional unit. Precise excision of sequences within a microinjected clone at a site defining the start of a transcriptional unit would be most logically explained by reverse copying of an RNA intermediate. Since no introns are present, only these criteria can provide direct evidence of RNA processing.

In transgenic mice it is highly unusual to see a small component of DNA selectively integrated instead of the whole microinjected unit; this would still leave the mechanism of dispersion unaddressed. The demonstration of selective integration of a transcriptional unit of a microinjected clone would strongly suggest that dispersion is intimately linked to transcription (Boeke et al., 1986). Although other models may be proposed to explain the data in the ß19 line, the model best supported is DNA dispersion via an RNA intermediate.

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June 5, 1987

To: Ms. W. Rubinstein
From: Albert M. Jonas, D.V.M.
Subject: Pathology report

Thank you for sending the glass slides intact! This is a short report based on reading material sectioned after processing by paraffin embedding and then stained by the H&E method. The quality of the preparations varied from satisfactory to unsatisfactory. One case (F3-32) showed extensive autolysis, but the sections were readable.

Case AbnL: This is a 6 month old, male mouse (CD-1 x B6D2F1).
Liver - There is an extensive cellular infiltrate throughout the parenchyma with destruction of hepatocytes in some regions. These cells are large, have slightly pleomorphic nuclei, some of which are vesiculated or vacuolated. Some nuclei are slightly cleaved or indented.
Thymus - This tissue replaced with tumor cells. See description "liver".
Lymph node - This section replaced with tumor cells. See description "liver".
Spleen - There is massive infiltration of tumor cells replacing normal architecture. See description "liver".

Diagnosis: Lymphoma, malignant (B-cell or FCC large cell)

Case F2.43: This is a 15 month old, male mouse (CD-1 x B6D2F1).
Liver: This section is infiltrated with tumor cells which are large, have elongated nuclei, and are consistent with Type A cells (reticulum cell sarcoma).

Diagnosis: Lymphoma, malignant (reticulum cell sarcoma).

Case F3.32: This is a female mouse (CD-1 x B6D2F1), approximate age 1 year.
Liver: The sinusoids (spaces of Disse') are filled with tumor cell infiltrates. The cells are discrete with moderate amounts of cytoplasm. The nuclei tend to be spherical, but many nuclei are indented. In some regions there are myeloblasts and cells maturing towards the neutrophilic series. Whether these clusters of cells represent extramedullary myelopoiesis or differentiation of stem cells is uncertain. There is parenchymal

autolysis (artifact) and artifactual necrosis.

Salivary glands: This slide is MISLABLED "thymus". There is extensive artifactual necrosis (autolysis). However, there is no evidence of tumor infiltrates.

Kidney: There is extensive autolysis (artifact). There are no cellular infiltrates consistent with tumor.

Adrenal: There is autolysis, and no evidence of pathology.

Spleen: There is massive replacement of the normal architecture with tumor cells. The type and character of the cell population is similar to that seen in the liver. Since extramedullary myelopoiesis can occur in both spleen and liver, the presence of myelogenesis in itself is not diagnostic of tumor origin.

Pancreas: There is autolysis, but no evidence of pathology.

Diagnosis: Stem cell leukemia (lymphoma). Without bone marrow and more complete tissues, this diagnosis is only tentative.

Case No. F3.22: This is a 14 month old, female mouse (CD-1 x B6D2F1):

Lung: Pleural and parenchymal vessels (pulmonary arteries and pulmonary veins) are filled with tumor cells. This is an excellent case to study leukemia using tissue sections. Nuclei have both donut and pinched morphologies. The tumor cells are primarily restricted to the vascular system. These cells are large, have prominent nuclei, and abundant cytoplasm.

Liver: There is marked infiltration of tumor cells similar to those described under "lung". In addition there is marked extramedullary erythopoiesis (myelopoiesis). The latter is in response to the tumor.

Kidney: No visible pathology.

Adrenal: This tissue MISLABLED as lymph node. There is autolysis, but no evidence of pathology.

Adrenal: This section has a small medullary adenoma (pheochromcytoma), non-malignant. This is not an uncommon tumor.

Ovary: There are normal follicles indicating normal cycling.

Heart: No visible pathology. However, the section is inadequate for full review of this organ.

Salivary glands and lymph node: This section is MISLABLED "thymus". The parotid and submaxillary salivary glands have no visible pathology. The adjacent submaxillary salivary gland has extensive tumor replacing the central portion of the node. There are numerous mitotic figures. The cell type is similar to that described above.

" ? " : Pancreas - There is no visible pathology.

Diagnosis: Malignant lymphoma (immunoblastoma).

Case No. F2. 12-1: This is a female mouse (M x CD-1) ? . The tissue section is identified as "uterine vesicle". The section is that of uterus with regions of hyperplastic endometrium consistent with subacute endometritis. The dilated portion may be secondary to that process, but insufficient tissue is present for complete examination. This is not an uncommon finding.

Diagnosis: Endometritis, subacute, with endometrial hyperplasia.

Case No. F3.31: This is a female, 16 month old mouse (CD-1 x B6D2F1).

Liver - There is an extensive leukemic infiltration with large, clear, sometimes cleaved cells.

Spleen - There is extensive replacement of normal architecture with a large, histiocytic type cell. In addition, there is prominent extramedullary erythropoiesis. The tumor cells are similar to those seen in the liver.

Thymus - No visible pathology.

Blood film - Poor preparation, poor staining. Unable to read.

Diagnosis: Lymphoma, malignant, leukemia. This tumor could be classified as a reticulum cell sarcoma.

Case No. F2.12-3: This is a female, 14 month old mouse (CD-1 x B6D2F1).

Liver - There is massive infiltration with very pleomorphic cells. The nuclei have clumped chromatin, the cytoplasm is abundant, and mitotic figures are common.

Thymus - There is a thymic remnant embedded in fat. No visible pathology.,

Kidney - There are fibrin thrombi, and large tumor cells trapped within glomerular capillaries. There is mesangial cell increase and mesangial thickening.

Spleen - There is a solitary, large tumor nodule composed of pleomorphic cells which have prominent cytoplasm. Many have cleaved nuclei. These tumor cells are consistent with those found in other organs.

Diagnosis: Lymphoma, malignant, leukemia. This tumor could be classified as a stem cell leukemia.

Case No. F3.17: This is a male, 14 month old mouse (CD-1 x B6D2F1):

Liver - There are moderate to severe small cell tumor infiltrates in the spaces of Disse' (liver cords) and in aggregations around central veins.

Spleen - There is a nodular pattern of dense tumor cell infiltrates with splenomeglia.

Brain - There is focal hemmorhage and secondary demyelination, a few regions of leukemic cell infiltrates, and a striking presence of leukemic cells within capillaries and vessels.

Kidney - There is a massive infiltration of tumor cells in the cortex. The glomeruli are trapped within the tumor infiltration. There are tumor infiltrates into perirenal fat. A structure adjacent to the kidney is completely replaced with tumor cells.

Diagnosis: Lymphoma, malignant, leukemia. This tumor can be classified as a small cell lymphoblastic lymphoma.

Comments:

The tumors seen in this series of mice are all lymphomas. The tumors classified as stem cell leukemias reflect their undifferentiation. The presence or absence of specific organ involvement in this series is of interest. There is nothing unusual in observing tumors of this type in aging mice.

In order to properly characterize these transgenic mice, a

prospective study with statistically appropriate temporal sampling for pathology (clinical and tissue) is required. The health status of the mice must be documented and their environment standardized (caging, animal husbandry practices, nutrition, bedding, temp., humidity).

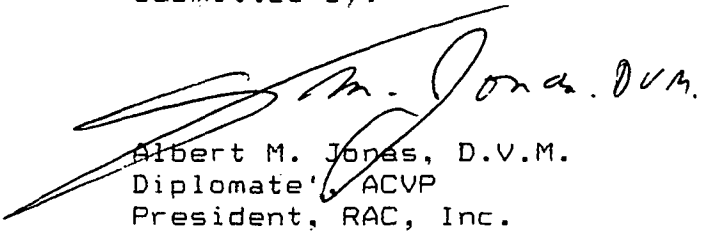
Necropsy protocols must be established, which detail the method of euthanasia, perfusion procedure (fixatives), organ and tissue collections (usually 20-30 per animal), the histologic methods (stains), and report methodology for computer entry and retrieval.

Clinical pathology is very helpful and can be accomplished without killing the animal. Temporal studies of this type are very interesting in some cases.

A more modest approach in understanding the disease processes in this study is to carefully evaluate each animal that dies. This requires daily evaluation and euthanasia of moribund mice to avoid post mortem change (autolysis).

If you require further consultation, please contact my office. Best wishes on your presentations!

Submitted by:



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President, RAC, Inc.

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