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Targeted transgenesis: the Hypoxanthine phosphoribosyl transferase (HPRT) gene locus

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August, 2003

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Masters of Science.

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Acknowledgements

"It takes a village to raise a child" – African proverb

On January 2002, I was introduced to the world of genetics. It was a whole new world, new ideas, new people who all had essential roles in my education. I would like to take this opportunity to express my utmost gratefulness to all who helped me in the culmination of this thesis.

First and foremost, Dr. Alan Peterson who accepted me as a student, a beginner. You lighted up my interest, guided me through this project, and sharpened my skills. You taught me how to think right and to be confident but critical of myself. Working with you taught me how to be persistent and never give up. To you, I extend my deepest thanks for your teachings and support.

I would also like to express my most gratitude to Dr. Hana Friedman. You encouraged me to excel. You were by my side in every step of the way. You put up with my never ending questions and did all what you could to help me. Thank you.

I, also, would like to specially thank Dr. Eric Denarier for his extreme assistance in both my academic and laboratory work.

To my colleagues and friends, Nancy Dionne, Samar Dib, Dr. Pierre Dumas, Farnaz Forghani, Christine Dy and the rest of the laboratory members. Thank you all for being my friends and having a genuine interest in seeing me succeed and

achieve my goals. Thank you for taking me in and making me feel part of the family.

A great thank you also goes to Priscila Valera, Heidi Furtenbaecher and France Bourdeau for their technical expertise.

Last but certainly not least to my beloved husband, Sherif who supported me in every step I took. You gave me inspiration and motivation to succeed. Thank You.

Inas El-Gouhary

August, 2003

ABSTRACT

To overcome the limitations that accompanied traditional transgenesis using pronuclear injections, multiple methods have been employed to target a single copy of a transgenic sequence to a chosen location in the genome. One of which is the introduction of a construct as a single copy, in a known orientation, upstream of the hprt (hypoxanthine phosphoribosyl transferase) gene. Such hprt targeting is becoming a widely used tool for site-specific transgenesis. The present study is designed to characterize the influence this locus could impose on the expression capability of the docked sequence.

Consistent ectopic expression of reporter constructs bearing different Myelin Basic Protein (mbp) regulatory elements and docked at the hprt locus was noted in cardiomyocytes and major CNS blood vessels; two sites in which mbp is not thought to be expressed.. This ectopic expression originated from endogenous hprt enhancer activity as similar mbp reporter constructs, randomly inserted, did not reproduce the same expression phenotypes. Moreover, analysis of a reporter construct bearing a minimal heat shock protein (HSP) promoter; an enhancer trap construct; docked at hprt extensively expressed in these two sites emphasizing that an hprt enhancer effect is uniquely responsible for the observed ectopic expression. The outcome of this study contributes to the general understanding of the hprt targeting strategy and helps in the accurate interpretation of results obtained from utilizing this transgenesis approach.

In addition, this work also unraveled aspects of the complex nature of mbp gene regulation. The results obtained from this study reveal that reporter

constructs containing mbp enhancer elements docked in hprt locus expressed ectopically in the cartilagenous cells of the vertebral bodies during the mouse mid-fetal development period. However, this was not deregulation caused by elements of the hprt locus as analysis of mice bearing similar mbp reporter constructs randomly inserted revealed similar ectopic expression. In addition, the "enhancer trap" construct, which contains no mbp sequences, did not express in the vertebral body cartilage. Taken together the above data indicate the presence of common regulatory elements between glia and cartilage cells.

Finally, analysis of the "enhancer trap" construct exposed additional ectopic expression in the muscles of the back and tongue uncovering further hprt enhancer activity. Surprisingly, when mbp enhancer sequences were added to the "enhancer trap" construct, marked reduction in muscle expression was noted. Hence, the possibility arises that such mbp regulatory sequences may contain cell-type specific repressors of expression which operate in the overall control of endogenous mbp locus. A study, such as this one, in which the in vivo expression phenotypes of multiple constructs can be directly compared, was required to reveal this type of inhibitory activity.

By identifying the major components of the mbp regulatory system and developing strategies to modulate their effects, potential, therapeutic interventions affecting myelin production in demyelinating diseases like Multiple Sclerosis (MS) might be achievable.

RESUME

Afin de palier aux limites inhérentes à la technique de transgénèse traditionnelle utilisant l'injection pronucléaire, plusieurs méthodes ont été développées pour cibler l'insertion d'une seule copie d'un transgène dans un locus prédéterminé. Dans l'une de ces techniques le transgène est inséré en amont du locus *hprt* (hypoxanthine phosphoribosyl transférase). Il est ainsi possible de contrôler le nombre de copie —une— et l'orientation du transgène au site d'insertion. Cette technique de transgénèse ciblée est maintenant devenue courante. Le présent travail cherche à caractériser l'effet possible du site d'insertion sur l'expression des séquences qui y sont placées.

Plusieurs contructions utilisant diverses séquences régulatrices du gène *mbp* (*myelin basic protein*) insérées au locus *hprt* ont montré une expression reproductible du gène rapporteur associé dans les souris transgéniques étudiées. Cette expression est ectopique puisqu'elle s'est retrouvée dans les cardiomyocytes ainsi que dans les vaisseaux sanguins principaux du système nerveux central (SNC), deux sites où l'on ne s'attend pas —*a priori*— à une expression de *mbp*. De plus, on peut affirmer que cette expression ectopique est due au site d'insertion *hprt* puisque des constructions similaires utilisant les mêmes régions régulatrices de *mbp*, mais insérées selon la transgénèse traditionnelle (injection pronucléaire aléatoire) n'expriment pas le gène rapporteur dans les cardiomyocytes ou les vaisseaux sanguins du SNC. De plus, les séquences régulatrices couplées au promoteur minimal de HSP (*heat shock protein*), un "*enhancer trap*" démontré, vont aussi permettre l'expression du gène rapporteur à ces sites ectopiques. Cela signifie donc que le site d'insertion *hprt* est seul

responsable de l'expression ectopique du transgène qui y est inséré. Les résultats de cette étude permettent donc une meilleure compréhension de la stratégie d'insertion de transgènes au site *hprt* qui est essentielle à la bonne interprétation des données découlant de cette approche.

De plus, ce travail a aussi permis de dévoiler la nature complexe de la régulation du gène *mbp*. Ainsi, nos résultats montrent que les constructions contenant les séquences régulatrices de *mbp*, lorsqu'insérées au locus *hprt*, vont induire l'expression du gène rapporteur dans le cartilage des corps vertébraux du foetus de souris à mi-développement. Cette expression apparemment ectopique ne semble cependant pas due à une dérégulation causée par le locus *hprt* puisque des constructions similaires insérées par transgénèse aléatoire classique produisent aussi cette expression dans les corps vertébraux. De plus, les constructions utilisant la "*enhancer trap*" HSP en absence de tout élément propre à *mbp* n'expriment pas dans le cartilage. Ces données suggèrent donc la présence d'éléments régulateurs communs entre les cellules gliales exprimant *mbp* et les cellules du cartilage.

Finalement, les constructions utilisant la "*enhancer trap*" ont aussi permis d'observer l'expression ectopique dans les muscles du dos et la langue des foetus, dévoilant d'autres influences *cis* causées par le locus *hprt*. De façon surprenante, l'addition de séquences régulatrices de *mbp* à la "enhancer trap" HSP a eu pour effet de réduire l'expression du gène rapporteur observée dans le muscle. Cela soulève la possibilité que les régions régulatrice de mbp contiennent des répresseurs spécifiques à certains types cellulaires qui participeraient de concert à

la régulation endogène de *mbp*. Seule une étude comme la nôtre, permettant l'étude comparative simultanée de plusieurs constructions *in vivo* peut permettre d'observer cette activité inhibitrice.

Ainsi, notre travail permet d'identifier les mécanismes complexes de régulation d'un gène comme *mbp* et de développer des stratégies pour élucider ceux-ci. Dans le cas de *mbp*, ces résultats pourraient aider au développement d'interventions thérapeutiques modulant la synthèse de myéline avec de possibles applications dans une maladies comme la sclérose en plaques.

Chapter I

Literature Review

TRANSGENESIS

What is a transgene?

Foreign DNA can be randomly or specifically integrated into the mammalian genome. Linear DNA fragments introduced into cells are rapidly ligated end to end by intracellular enzymes to become integrated into a chromosome at random or specific sites according to the method employed for DNA introduction. If the modified chromosome is present in the gametes (egg or sperm), the animal will pass these foreign genes on to its progeny. Animals that have the foreign DNA are termed transgenic, and the new locus is referred to as the transgene (Palmiter et al., 1986 & Boyd et al., 1993,).

Development of the transgenic science.

Retroviral gene transfer

At the mid-1970s, Dr. R. Jaenisch and Dr. R. Mulligan demonstrated genomic integration and germline transmission of proviral DNA after infection of pre and post-implanatation mouse embryos with retroviruses (Garcia Hernadez et al., 1997). However, this method was not widely used due to the high degree of mosaicism (many cells do not contain the new genetic material due to delayed integration) and consequently low rate of germline transmission. In addition, the size of the transgene was limited using this approach.

Pronuclear microinjection

Most commonly, the method for transgenesis in mice involves the nuclear microinjection of a purified experimental DNA into the fertilized mouse ovum. The first transgenic mouse using this method was successfully created at the start

of the 1980s (Palmiter., 1982) and currently most transgenic mice are produced using the following technique. The injected ovum is transferred to a pseudopregnant female. Progeny are analyzed for germline transmission of the transgene by southern blots and PCR of the progeny's tail DNA. Transgenic mice are mated with wild-type mice to establish a transgenic line (Gossenet et al., 1993).

This technique is termed random transgenesis because the transgene integrates anywhere in the mouse genome by non-homologous recombination (Jasin et al., 1996). Therefore, there is no control over the site of the transgene integration. Consequently, there are position effects i.e. ectopic activation of the transgene if it lands beside a strong enhancer or repression if it integrates into transcriptionally inactive chromatin (Gridley et al., 1991). There is, also, no control on the copy number of the integrated transgene (Misra, 2002). The transgenes will frequently get integrated as tandem arrays ranging from a few copies to several hundred copies. Moreover, deletions and rearrangements occur around the site of integration. Without the means to control these intrinsic variables, multiple independent transgenic lines must be generated and analysed to reach significant conclusions about the effect of the transgene. The cost of generating and maintaining multiple lines makes the detailed analysis of promoter structure and function invivo very difficult.

Embryonic stem cell-mediated gene transfer

By the end of the 1980s, a second major advance in mouse molecular genetics occurred. This was facilitated by the ability to grow pluripotent embryonic stem

(ES) cells, derived from the primitive ectoderm of a mouse blastocyst, and maintain their pluripotency in culture. ES cells have the unique ability to colonize all the tissues of a host embryo including its germ line (Babinet et al., 2001). Targeting vectors containing the DNA of interest can be introduced into ES cells (Mansour et al., 1990). The DNA would contain the cloned gene of interest flanked by homologous arms to facilitate specific integration of the transgene through homologous recombination into the ES cell genome (Ledermann et al., 2000). Since homologous recombination was a rare event, positive and negative selectable markers were included in the DNA to be able to select for the correctly targeted ES cells. The latter could be introduced into a blastocyst where they can contribute to the development of a chimeric mouse; including the germline (Koller et al., 1992 Moreadith et al., 1997).

Targeting to the ROSA26 locus

Once ES cell manipulation was possible, Friedrich and Soriano in 1991, infected ES cells with a promoterless retroviral gene trap Gen ROSAβgeo vector in order to identify and mutate developmental genes in mice. βgeo encodes a bifunctional lacZ/neomycin phosphotransferase gene. ES cells harboring a proviral copy were injected into blastocysts and transgenic mice were produced. Mice ubiquitously expressing the lacZ reporter contained within the retrovirus were thus generated. One such strain, ROSA26, has been studied extensively because of its general usefulness (Zambrowicz et al., 1997).

The ROSA26 locus was cloned later on (Zambrowicz et al., 1997). The characterization of this locus has led to two methods for generating transgenic

mice that express a transgene in a generalized fashion. First, it is possible to use the genomic locus to target transgenes (Soriano P., 1999). This has been used to produce a variety of transgenic mice harboring different genes of interest (Soriano P., 1999, Mao et al., 1999, Srinivas et al., Awatramani et al., 2001, Farley et al., 2000). Second, the promoter of the ROSA26 locus has been characterized in ES cells (Zambrowicz et al., 1997) and has been used for broad expression of randomly inserted transgenes in mice (Kisseberth et al., 1999). However, in this case expression was broad but not uniform. Therefore it seems that targeting to a single locus is more likely to produce uniform expression than random transgenesis with a ubiquitous promoter. Hence, in cases where uniform expression is desired, targeting to the ROSA locus might be the better way to go.

Targeting to the ROSA26 locus is achieved by introducing the desired gene into the first intron of the locus. A construct using the same splice acceptor as used in the original gene trap allele followed by the gene of interest and polyadenylation site is inserted at the ROSA26 locus. Positive and negative selectable cassettes are included in the targeting vector to allow for homologous recombination. Targeted clones can be verified using a flanking probe and later genotyped using oligonucleotides derived from the locus flanking the insertion.

The drawback to this method is that there are valid concerns that the presence of a selectable marker expression cassette can influence the expression of the neighboring genes (Fiering et al.,1995). Such interference can create a phenotype that does not reflect the role of the targeted gene and lead to misinterpretation.

Transgenesis using the Cre Recombinase system (Nagy A., 2000)

Cre recombinase of the P1 bacteriophage catalyzes recombination between two of its consensus 34 base pair DNA recognition sites (loxP sites) in any cellular environment and on any kind of DNA. The 34bp consensus sequence consists of a core spacer, 8bp, and two 13bp palindromic flanking sequences. By using the cre recombinase system, excision or inversion of loxP-flanked DNA segments or creation of intermolecular recombination between different DNA molecules can be achieved. The result of the Cre recombinase-mediated recombination depends on the location and orientation of the loxP sites. They can be located in cis or in trans. The utilization of trans Cre-mediated recombination allows site-specific insertion of a transgene into the genome. In this strategy, recombination occurs between a circular DNA (eg. a plasmid) and a linear DNA (a chromosome). If simple loxP sites are utilized, then the intermolecular recombination leaves two loxP sites in the chromosome, which will be placed in close vicinity and provide a cis substrate for intramolecular recombination (i.e. excision of the transgene). The latter occurs at much higher efficiency than the former, so the equilibrium favors the excised state over the "pop-in" state. A continous, strict and accurate selection system is required to correctly identify the ES cells with the "pop-in" state and derive clones from them. This is achieved by placing a promoterless positive selectable marker, eg. neomycin gene, preceded by a loxP site in the geneome. Cre-mediated insertion of the plasmid will integrate a promoter before the selectable marker and drive its expression. As long as the promoter is not excised and remains in the genome, the cell will survive the G418 selection.

Another alternative to attain site-specific integration of a DNA fragment into the genome using the loxP system is by taking advantage of the characteristic tolerance of cre recombinase to mutant loxP site recognition. If the mutation is a single point mutation at the site's margin, then the cre-recombinase enzyme could still recognize the mutant loxP sites and mediate recombination. The loxP site, as mentioned before, is composed of an asymmetric 8 bp spacer flanked by 13 bp inverted repeats. Nucleotide changes are introduced into the left 13 bp element (LE mutant lox site) or the right 13 bp element (RE mutant lox site) Recombination between the LE mutant lox site and the RE mutant lox site produces the wild-type lox P site and a LE+RE mutant site that is poorly recognized by Cre, resulting in stable integration (Albert et al., 1995). This means that after the insertion is achieved, the segment cannot be removed easily. Therefore, strict selection is not required like the previously described method of simple loxP sites. A variety of studies made use of such a strategy to target transgenes into specific site in the genome (Araki et al., 1995 & Araki et al., 1997).

A third way to use the Cre-recombinase system for site-specific insertion of transgenes is based on the utilization of a substitute recognition site for loxP which is lox511. This site differs from loxP site in the spacer sequence which is the 8bp between the palindromic sequences. Cre recombines two lox511 as efficiently as normal loxP sites but at a low efficiency between loxP and lox511 sites. If a loxP and a lox511 were used to flank the desired insertion site in the genome and another pair around the transgene in the vector, then transient cre expression in the ES cells catalyzes recombination between the similar lox sites

(i.e. between the pair of lox-P and lox511 sites but not between the two). This results in the insertion of the transgene in the genome (Soukharev et al., 1997). Recombination between the loxP and lox511 could still occur but with a low efficiency and therefore the "pop-in" state is favored and the transgene stays in position even if cre-recombinase is still around.

The disadvantage of this method is the possibility that the plasmid sequences may be present for many generations and integrate into the mouse genome (de Wit et al., 1998). In addition it does not have a 100% efficiency as there is always a slight possibility of transgene excision if the cre recombinase is still around. Finally, the recombination event has to take place as soon as possible to avoid mosaicism..

Targeting the region upstream of the hprt gene (Figure 1).

A report by Bronson et al. in 1996 described the introduction of transgenes by gene targeting into the hypoxanthine phosphoribosyl transferase (hprt) locus of ES cells. It is transcribed from a promoter lacking CAAT and TATAA boxes. It is a ubiquitously expressed, housekeeping gene, ubiquitously expressed albeit at different levels, in all tissues and cultured cells with mRNA accumulation to highest abundance in the brain (Magin et al., 1992).

Hprt is an enzyme that is involved in the salvage pathway of nucleotide synthesis. Mutations of this gene in humans lead to a deficiency of hprt enzyme activity and resulted in the development of the Lesch-Nyhan syndrome. This syndrome is characterized by severe neurological disorder including mental retardation, spasticity, and a compulsive form of self-mutilation. However, mice

with mutations in HPRT are phenotypically normal and fully fertile (Jiralerspong et al., 1996). Hprt is located on the X-chromosome. Therefore, it is hemizygous in XY ES cells. Bronson et al. used a naturally occurring hprt null male cell line. The size of the deletion was about 30Kb and included the promoter and exons 1 and 2 of hprt. The transgenes consisted of a mouse bcl-2 cDNA driven by the chicken or human B-actin promoter. The targeting vector consisted of the transgene and two flanking homologous sequences including the promoter and exons 1 and 2 of hprt. Gene targeting of ES cells restored the hprt locus simultaneously placing a single copy of the construct upstream of the hprt gene.

It took only about 17 days only to obtain correctly targeted ES cell clones that were ready for injection into blastocysts (Evans et al., 2000). Moreover, the hprt targeted transgenic mice could be generated in a short period of time because of a consistent contribution of the BK4 ES cells (with the hprt deletion) to the germ line (Evans et al., 2000). The results presented in Bronson's report are convincing that targeted integration of the transgene, as opposed to random transgenesis, yielded consistent expression levels of the transgene in both the cell clones and in mouse tissues.

However, such a design of targeting to the upstream region of hprt could be accompanied by promoter occlusion effect, as it is well documented that neighboring promoters can influence each other (Jasin et al., 1996). In promoter occlusion, also known as transcriptional interference, RNA polymerases from an upstream gene fail to terminate and interfere with the ability of promoters downstream to function. This interference results from the tendency of run-away polymerases to displace transcription factors and other DNA-binding proteins that

may lie in their path and thus inactivating the gene (Karreman et al., 1996). In our laboratory, constructs were put in with the promoters of the transgene and hprt either going in opposite directions (i.e. on opposite DNA strands), to counteract promoter occlusion effect, or in the same direction. The results were the same in both.

Selection of HPRT targeted ES cells (Figure 2).

Correctly targeted ES cells are selected on Hypoxanthine, Aminopterin and Thymidine (HAT) medium. Aminopterin is an analogue of dihydrofolate, which blocks the de novo synthesis pathway of nucleotides. Mammalian cells, however, survive culture in the presence of aminopterin because they can utilize two salvage pathways, one of which utilizes hypoxanthine and is catalyzed by hprt. Hence, correctly targeted ES cells that had their hprt locus restored are able to survive in HAT medium.

This proved to be a very efficient means of selection because ES cells are a rapidly dividing population, very sensitive to aminopterin and will soon die on HAT medium if they do not have hprt. No selectable markers are employed in this technique because hprt itself is both the negative and positive selectable marker. Therefore, the side effect of selectable markers' integration in the genome, as mentioned above, is eliminated. Hprt acts as a negative selectable marker when choosing the naturally occurring BK4 ES cells with deletion in the HPRT gene. These cells are selected on 6-thioguanine (6-TG) medium. Hprt enzyme utilizes 6-TG to produce a toxic metabolite that kills the cells. Therefore, BK4 ES cells that have no HPRT can survive on 6-TG medium.

Genomic environment of the transgene.

As the above strategy to insert experimental constructs places them in close proximity to hprt, such transgenes might be influenced by the same regulation that controls the hprt gene. During investigations to characterize hprt transcription control elements required in embryonic stem (ES) cells, Magin TM et al., 1992 discovered a requirement for intron-1 sequences for expression in ES cells. The essential intron-1 element, which is 420 bp long, is located 230 bp downstream from the transcription start point and is shown to increase transcription from the hprt promoter in a position- and orientation-dependent manner. Likewise, it could also affect transgene expression that is docked upstream of the hprt promoter using Bronson's technique.

In addition the naturally occurirng 30kb deletion in these hprt ES cells places the transgene in close proximity to the upstream gene PHF6 (plant homeodomain zinc-finger 6 gene) which, like hprt, is a constitutively expressed gene. Consequently, influence emanting from these two genes might deregulate the expression program controlled by elements associated with the experimental construct.

Examples of genes targeted to the hprt upstream locus

Soon after the publication of Bronson's report, a number of studies started adopting the hprt targeting strategy; of which were studies on endothelial-specific genes. Little is known about the molecular bases of vascular diversity (Guillot et al., 2000). Various functions of the endothelium are differentially regulated in

time and space, giving rise to endothelial cell heterogeneity and vascular diversity (Minami et al., 2002). One approach to this problem is to study the mechanisms that underlie endothelial cell subtype-specific transcriptional regulation. Three studies (Guillot et at., 2000, Evans et al., 2000 & Minami et al., 2002) targeted a single copy of endothelial specific promoters to the hprt site in order to test their endothelial targeting capability. An understanding of the mechanisms that underlie differential gene expression would provide important insight into the molecular basis of vascular diversity.

1- Targeting of human eNOS promoter to the hprt locus leads to tissue-restricted transgene expression (Guillot et at., 2000).

The endothelial nitric acid synthases (eNOS) are a family of enzymes that are responsible for generating nitric oxide. eNOS is expressed predominantly in endothelium where it has been implicated in the control of vasomotor tone as well as vascular remodeling and angiogenesis. This study targeted a single copy of a transgene that contains 1,600 bp of the human eNos promoter coupled to LacZ reporter gene to the hprt locus of mice by homologous recombination. Analysis of mice demonstrated that the expression pattern was limited to a subset of endothelial cells, cardiomyocytes, and vascular smooth muscle cells. The x-gal reaction product was detected in larger vessels of the heart, spleen, skeletal muscle, brain, kidney and lungs. Finally, B-galactosidase activity within the endothelium was significantly downregulated in adult vs neonatal mice. In contrast, expression of lac Z in cardiac myocytes was relatively preserved with aging.

2- Targeting the hprt locus in mice reveals differential regulation of Tie2 gene expression in the endothelium. (Evans et al., 2000)

The endothelial cell-specific tyrosine kinase receptor, Tie2, is involved in the remodeling of blood vessels, the interactions of endothelial cells with extracellular matrix and perivascular cells, and the sprouting and growth of vessels from preexisting vasculature (angiogenesis). To study the in vivo expression of the murine Tie2 gene, this study targeted the hprt locus to generate two single-copy transgenic mice. These were T1, containing the 2100-bp Tie2 promoter upstream from the B-galactosidase (LacZ) gene, and T5, which also included an enhancing element originating from the first intron of the Tie2 gene. By comparison, moderate reporter gene activity was observed in the brain and kidney of T1 adults, whereas extensive lacZ gene expression was seen in the vasculature of most organs of the of T5 mice.

3- Differential regulation of the von Willebrand factor and Flt-1 promoters in the endothelium of hypoxanthine phosphoribosyl transferase-targeted mice. (Minami et al., 2002)

This study took advantage of the hprt single copy targeting approach to target the promoters of two different genes, namely the von Willebrand (VWF) and the Flt-1 to HPRT gene locus. VWF promoter was found to contain information for expression in a subset of endothelial cells in the heart, skeletal muscle, and brain. On the other hand, the Flt-1 promoter directs expression in all vascular beds except for the liver.

Unfortunately, none of these studies employed a control transgene to detect the influence of the hprt locus on gene expression. As described in detail below, the

influence of hprt elements on transgene expression is particularly pronounced for vascular tissues and the heart.

Hprt and X-chromosome inactivation

The presence of hprt on the X chromosome makes it subject to random X-chromosome inactivation. In female mammals, dosage compensation of X-linked genes between males and females occurs by genetic inactivation of one of the two X-chromosomes (Lyon et al., 1961). The choice of X-chromosome to be inactivated is a random process in somatic cells, i.e., either the paternally or maternally inherited X-chromosome is inactivated in a given cell. Once established, the inactivity is clonally maintained. X-chromosome inactivation occurs early in development. Both the paternally and maternally inherited X-chromosomes are active in preimplantation embryos after fertilization. The inactivation occurs around the time of implantation (Monk et al., 1979 & Takagi et al., 1982).

The bcl-2 transgene reported in the Bronson's article (Bronson at al., 1996) expressed equally in transgenic homozygous females as hemizygous transgenic males, indicating that it was indeed subject to X-inactivation. That lyonization process occurring in females was also observed in the study in which the Tie2 promoter was targeted to the hprt locus. In the latter, mice with one targeted allele in the absence of a wild-type allele (male hemizygotes) showed a stronger overall staining than in mice with both a targeted hprt allele and a wild-type allele (female heterozygotes) (Evans et al., 2000).

Targeting transgenes to other loci

In order to avoid the complication of X-chromosome inactivation, transgenes were targeted to autosomal loci in a similar approach as hprt. Application of this approach in ES cells to both the pim1 and oct4 loci (te Riele et al., 1990 & Mountford et al., 1994) was done. However, a selectable marker should be introduced with the transgenes for selecting the correctly targeted ES cells as these loci have no intrinsic selectability, like hprt. Selection for antibiotic resistance carried by the transgene does not all the time ensure that the insertion was correct. Most will be randomly inserted. Hence, a lot of positive clones must then be screened in order to find the correctly targeted ES cells The marker could be eliminated from the genome, for example, by transient expression of a cre expression system but this makes the procedure more complicated than the direct HPRT selectable strategy. It is, also, subject to the drawbacks of the cre recombinase system explained above. Therefore, the intrinsic selectability of the cells expressing hprt gave this system an enormous advantage.

The influence of chromosomal location on the expression of transgenes

The study by Hatada et al., 1999 tested the hypothesis that the choice of the site of transgene insertion would cause a predictable influence on the tissue-specific expression of an inserted transgene. The study examined the influence of two chromosomal locations, hprt and apoAI-CIII., on the expression of two transgenes in mice. The apoAI-CIII. is a gene which codes for appolipoprotein. The results demonstrate that the chromosomal location of a transgene markedly and with a considerable degree of predictability affects the level of transgene expression. The same transgene when docked at hprt expressed at lower levels in

the liver and small intestine as compared to its expression at apoAI-CIII locus, which is highly expressed in the liver and small intestine. This study shows how expression levels in various tissues is affected by interaction between the promoter elements of the introduced transgene and the enhancers that reside near the location of the transgene. Therefore, it is of utmost importance to characterize the nearby enhancers of the site of chromosomal integration to make targeted transgenesis a versatile approach for achieving the in vivo function of the transgene.

The Hatada study, however, did not use the same targeting strategy as the one mentioned in Bronson's report. Instead, the targeting vector had the region of homology 4kb of DNA 5' to the hprt locus and 1.6kb of DNA extending 3' from HindIII (restriction) site in intron 2 to an EcoRI (restriction) site in intron 3 of the hprt gene. This vector was transfected into the partially deleted hprt gene of the BK4 ES cells. This resulted into the integration of the transgene immediately upstream of the hprt 3rd exon; further disrupting the hprt gene. Since the targeting vector contained a neo cassette, the correctly targeted ES cells were selected on G418 instead of HAT medium.

Insight into the myelin basic protein (mbp) gene.

Our laboratory uses hprt targeting to study the regulatory elements of the Myelin Basic Protein gene (mbp). One aim of this project was to gain new insight into the functions of some of these regulatory sequences. For this reason, it is important to have a clear understanding of the regulatory influences that might emanate from the hprt docking site to influence expression of mbp reporter constructs.

A means of rapidly conducting action potentials over long distances is essential for normal nervous system activity. In vertebrates, this function is achieved by large caliber axons that are invested with myelin (Foran et al., 1992). Myelin is composed of lipids and myelin proteins. Myelin proteins are among the most abundant in the nervous system and they are considered to be expressed only in myelin-forming cells namely the oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS) (Landry et al., 1998). The membranes of these cells ensheath the axons. One of the major classes of myelin proteins is the Myelin Basic Protein (mbp). Mbp is one of the most important proteins of the myelin sheath (Deber et al., 1991 & Smith et al., 1992). Its significance is demonstrated in the shiverer mouse mutants, which have only a small amount of structurally unstable myelin because the gene for mbp is mostly deleted (Roach et al., 1985 & Mateu et al., 1996). CNS myelin is largely absent in shiverer mutants and, when present, appears as abnormal whorls of cytoplasm-filled membranes that are uncompacted at the major dense line (Privat et al., 1979 & Kirschner et al., 1980). This trait is recessive and inherited in a Mendelian manner, indicating that mbp is coded for by a single gene (Ridsdale et

al., 1997). Moreover, the mbp gene maps to mouse chromosome 18, as does the shiverer mutation (Sidman et al., 1985). When the cloned mbp gene was reintroduced into the shiverer genome, it successfully corrected the dysmyelinating phenotype (Readhead et al., 1987). In mammals, the gene consists of seven exons, and differential splicing of the primary mbp mRNA accounts for the different isoforms of mbp (Campagnoni et al., 1988 & de ferra et al., 1985).

The mbp gene encodes a family of at least six intimately related membrane proteins, which collectively make up 30-40% of the central nervous system (CNS) myelin protein and 5-15% of peripheral nervous system (PNS) myelin protein (Lees et al., 1984). These related proteins are all small (14.0-21.5 KD), remarkably basic, and required for normal myelination (Lemke et al., 1988). They have been localized immunocytochemically to the major dense lines of myelin (Omlin et al., 1982) and are therefore limited to the cell's interior. The larger forms of mbp containing exon II may play a more significant role in the early stages of myelin wrapping, since they are relatively abundant at that time and relatively scarce later on (Whitaker et al., 1981). The myelin ensheathments of the major axons in the CNS and PNS are remarkably similar in architecture regardless of the fact that CNS myelin is elaborated by oligodendrocytes, a cell of neural tube origin, while PNS myelin is synthesized by Schwann cells which are from the neural crest origin (Gow et al., 1992). Although some myelin proteins are unique to each type of cell, several proteins like MBP and proteolipid protein (plp), and myelin-associated glycoprotein (MAG) are shared between both cell types (Lemke et al., 1988, Whitaker et al., 1981, Puckett et al., 1987). Consequently, it is logical to believe that the genes encoding these proteins are

coordinately regulated. In other words, these genes may be controlled by a set of cis-acting elements and trans-acting proteins that stimulate transcription in both cell types (Gow et al., 1992).

In a study done by Wiktorowicz and Roach, 1991, nuclear transcription run-on assays have demonstrated that the developmental expression of mbp is regulated primarily at the transcriptional level. The latter study showed that in the shiverer brains, the transcription rates for the intact 5' end of the mbp gene follow closely those seen in wild-type animals up to the age at which maximal myelination normally occurs. Thus the shiverer deletion does not remove information required for efficient, developmentally regulated transcription, and the low level of myelin basic protein gene transcripts in this mutant must be a result of their reduced stability (Wiktorowicz et al., 1991). Taken together, this implies that the transcriptional regulatory elements within the 5' flanking sequence of the mbp gene primarily modulate MBP expression. Forghani et al., 2001 demonstrated that the majority of the mbp expression program can be accounted for by the first 12kb of flanking sequence. Specifically, the first 9kb of 5' flanking sequence, when included in reporter constructs, tracks the mbp gene by promoting expression of reporter gene specifically to both the oligodendrocytes and Schwann cell populations. No interference with the function of mbp regulatory elements emanating from sequences outside the mbp 5' flank extending to –12kb was observed in Forghani's study. Consequently, this implies that all mbp regulatory elements are in close association, significantly less dispersed than what encountered in seemingly related myelin genes (eg. PMP 22) (W. Orfali, J. Snipes and A. Peterson-unpublished data). Forghani's study also located and defined an

autonomous Schwann cell enhancer (SCE1), a 584bp sequence using randomly integrated reporter constructs. The autonomous activity of SCE1 began to define a modular structure for the mechanisms that regulates mbp expression which was further revealed by inter-species sequence comparisons of the mbp gene.

Human-mouse sequence comparisons revealed highly conserved non-coding sequences in the mbp 5' flanking sequence referred to as modules. Four modules showing conservation in 70% identities or greater in 100 bp were identified; namely M1, M2, M3 and M4. Each module was found to drive a unique regulatory program when included in reporter constructs docked at the hprt site. (Hooman Farhadi et al., 2003). For example, M1 targeted expression to oligodendrocytes during primary myelination but transgene expression was not detectable beyond P30. The addition of the M2 containing sequence extending to -794 bp, resulted in higher levels of reporter gene expression at P18 and maintenance of expression further into adulthood. The regulatory elements within M3, when combined with a minimal 300bp hsp promoter, were found to be sufficient to target expression to oligodendrocytes of both young and old mice. In contrast, M4 sequences, which overlap with part of SCE1, conferred expression of reporter constructs to Schwann cells (Hooman Farhadi, submitted). Taken together, the above data demonstrate that a major component of the mbp regulatory phenotype is conferred by quantitative regulatory functions; as was shown previously for the endo-16 locus of sea urchin (Yuh et al., 1998). Functional characterization of the endo-16 gene revealed different sub regions or cis-regulatory modules each of which displays a specific regulatory function when linked with the basal promoter i.e. each module independently stimulates the

basal transcription complex. These modules function positively in that, in isolation, each is capable of promoting expression. The modules also interact with one another with one module increasing the output of the other (Yuh et al., 1996).

KROX-20 IN MYELIN & BONE FORMATION

The identification of numerous transcription factors that exert a pivotal role in Schwann cells has shed light on the molecular mechanisms governing myelination (Ghislain et al., 2002). Amongst them is the Krox20/Egr2 (Erlebacher et al., 1995). The Krox-20 gene was originally identified as a serum response immediate-early gene which encodes a protein with three C2H2-type zinc fingers (Chavrier et al., 1988 & Gilardi et al., 1991). The Krox-20 protein was, then, shown to bind a specific DNA sequence and to constitute a transcription factor (Chavrier et al., 1990 & Nardelli et al., 1991). During embryogenesis, Krox-20 is first expressed in two prospective hindbrain segments, the rhombomeres 3 and 5 (Wilkinson et al., 1989).

Topilko et al., 1994 created a null allele in the mouse Krox-20 gene by in-frame insertion of the Escherichia coli lacZ gene, and showed that hindbrain segmentation is affected in Krox-20-/- embryos. They, also, demonstrated that Krox-20 is activated in Schwann cells before the onset of myelination and that its disruption blocks Schwann cells at an early stage in their differentiation, the premyelinating state, thus preventing myelination in the peripheral nervous system. In Krox-20-/- mice, Schwann cells wrap their cytoplasmic processes only one and a half turns around the axon, and although they express the early myelin marker, myelin-associated glycoprotein, late myelin gene products are absent, including those for protein zero and myelin basic protein. Taken together, Krox-20 is likely to control a set of genes required for completion of myelination in the peripheral nervous system.

The same group in 1996 (Levi et al., 1996), using the same mutant Krox-20 mice, showed that Krox-20 is also activated in a subpopulation of growth plate hypertrophic chondrocytes and in differentiating osteoblasts and that its disruption severely affects endochondral ossification. During skeletogenesis, mesenchymal cells aggregate and give rise to condensations which subsequently differentiate leading to generation of mature bone or cartilage (Erlebacher et al., 1995). It is possible to distinguish between two major modes of bone development: intramembranous and endochondral. The first one involves the direct differentiation of precursor cells into osteocytes (bone forming osteoblasts) and it is typical of the flat bones of the skull. The second one is characterized by the gradual conversion of an initial cartilage model into bone. This occurs by the concomitant deposition of bone matrix by the perichondrial osteoblasts (periosteal bone) and within the cartilage scaffold by osteo-progenitor cells (endosteal bone) (Levi et al., 1996). Krox-20 seems to control the second type of ossification.

Krox-20-/- mice develop skeletal abnormalities including a reduced length and thickness of newly formed bones, a drastic reduction of calcified trabeculae and severe porosity. The periosteal component to bone formation and calcification does not appear to be affected in the homozygous mutant suggesting that the major role for Krox-20 is to be found in the control of the hypertrophic chondrocyte-osteoblast interactions leading to endosteal bone formation (Levi et al., 1996).

The identification of the Krox-20 target genes in osteogenic cell types should shed more light on the molecular mechanisms of bone formation. It will be

interesting to find out whether some of these target genes are conserved between the different systems in which Krox-20 plays a regulatory role e.g. PNS myelination and bone formation. As described in detail below, enhancers from the myelin protein gene (mbp) led to early expression in bone cartilege. Thus, it is likely that certain molecular regulatory mechanisms are shared between the two systems.

Mechanisms of Transcriptional Repression

In addition to enhancers that target mbp expression to myelinating cells, repressor elements that act to restrict expression might also be found in mbp regulatory sequence. Hence it is important to know the potential mechanisms of how repressors operate to limit gene expression. For most genes the initiation of transcription is the principle point at which their expression is regulated (Gaston et al., 2003). Transcription factors, some of which bind to specific DNA sequences, generally either activate or repress promoter activity and in so doing control transcription initiation. Some transcriptional repressor proteins counteract the activity of positively acting transcription factors (activators) by either competing with them for DNA binding or sequestration of such activators. Others interact with the basal (core) transcriptional machinery and hence impede subsequent binding of RNA polymerase. Some interfere with the methylation state of the DNA (Japsen et al., 2002) Moreover; the repression of transcription is often intimately associated with chromatin re-organization. Chromatin structure has a pivotal role in the regulation of gene expression. Transcriptional repression of a gene requires the recruitment of chromatin remodeling complexes. These complexes modulate the higher order structure of chromatin, hinder accessibility of transcriptional activators, and prevent the establishment of a transcriptional preinitiation complex. Histone deacetylases are examples of such remodeling complexes (Burke et al., 2000).

The regulation of gene expression by transcriptional control is required for many cellular events and for the proper development of an organism. Nervous

system development requires the specification of numerous neural stem cells. Consequently, these stem cells divide in a spatially and temporally controlled way to create the diverse cell types found in the different layers of the nervous system. Lineage specification is brought about by transcriptional regulators, which often act as transcriptional repressors (Edenfeld et al., 2002).

In the last 5 years, many co-repressors have been identified in eukaryotes that function in a wide range of species, from yeast to Drosophila and humans. Co-repressors are co-regulators that are recruited by DNA-bound transcriptional silencers and play essential roles in many pathways including differentiation, proliferation, programmed cell death, and cell cycle. Accordingly, it has been shown that aberrant interactions of co-repressors with transcriptional silencers provide the molecular basis of a variety of human diseases (Burke et al., 2000). Taken together, the overall picture suggests that transcriptional silencers and co-repressors act in analogy to transcriptional activators and co-activators, but with the opposite effect leading to gene silencing.

CHAPTER II

Hprt TARGETED TRANSGENESIS

INTRODUCTION

Variable gene expression amongst transgenic lines occurs due to copy number and to random associations of incoming DNA with chromosomal elements at the site of integration. Preselection of genomic sites for the introduction of transgenes by gene targeting improves the reproducibility of transgene expression and provides an efficient means of single copy transgene introduction by homologous recombination.

The method described by Bronson et al. (1996) uses homologous recombination in ES cells to generate mice having a single copy of a transgene integrated 5' to the X-linked hprt locus by a directly selectable homologous recombination event.

The hprt gene is transcribed from a promoter lacking CAAT and TATAA boxes. It is expressed ubiquitously in all tissues and cultured cells, therefore, its locus is presumably relaxed and transcriptionally favorable. Hence, transgenes that are inserted into the locus are likely to be free of some of the constraints of higher order gene regulation.

But is it enough to conclude that the hprt locus is a neutral place and does not affect transgene expression? Is the hprt transgenesis strategy appropriate for analysis of all genes? Two caveats in Bronson's report pointed to the possibility of influences from this locus. For example, B-actin is a constitutively expressed

gene as is apparent from their analysis on the endogenous B-actin locus. However, when the B-actin transgene was inserted in hprt, low or undetectable transgene expression was noted in liver and kidney. The transgene expression was monitored by Northern analysis, rather than by expression within individual cells and because of potential RNA degradation, it is difficult to draw firm conclusions regarding accurate transgene expression. In order to test the neutrality or influence of the hprt locus on tissue specific expression of a transgene, the transgene should have a tissue-specific expression pattern rather than a ubiquitously expressed gene like B-actin.

In our laboratory, analysis of mice bearing hprt docked reporter constructs bearing regulatory elements of the mbp gene (expressed exclusively in myelin forming cells of CNS and PNS) revealed to a greater extent that such transgenes reproduce the expression phenotype seen with random insertions. For example, as mentioned before, (Farhadi et al.,) showed that reporter constructs bearing M4 sequences have the intrinsic capability to express in Schwann cells. These studies were carried out using random transgenesis. M4 reporter constructs docked at HPRT also revealed expression in Schwann cells. Similar agreement was observed between the phenotype of randomly inserted and hprt-docked constructs. It was, thus, concluded that the hprt site is permissive and does not deregulate mbp targeting and that hprt is an ideal and neutral place to dock transgenes. However, analysis of mice bearing reporter constructs docked at hprt revealed consistent B–galactosidase activity in the major blood vessels of the CNS; a site in which mbp is not thought to be expressed. In addition, this ectopic expression was not noted in random transgenic lines. The identification of an hprt influence on blood vessels' gene expression limits the use of the hprt targeting strategy.

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was not noted in random transgenic lines. The identification of an hprt influence on blood vessels' gene expression limits the use of the hprt targeting strategy.

In this project, I attempted to characterize the origin and significance of the "deregulated" expression observed above. Achieving this understanding could be crucial for interpreting future results obtained from hprt docked constructs; whether from mbp or any other gene. This study helped, too, in providing novel insight into the regulatory elements that might control mbp expression.

Three specific questions were pursued:

First, I determined where and when mbp reporter gene expression occurs in hprt transgenic mice with particular attention to times and cell types that are not obviously related to myelination and mbp expression.

Second, I asked if regulatory activity associated with the hprt docking site equally influences the expression of all inserted constructs. If yes, I would conclude that the hprt enhancer effect is simple but if different reporters are differentially expressed, I would conclude that unique partnerships develop between hprt regulatory sequences and enhancers contained within the docked constructs.

Finally, if mbp associated regulatory elements are recognized by transcription factors expressed in both glia and ectopic sites, comparisons between variously

deleted constructs might reveal the location of such elements and along with inter-tissue comparisons, might illuminate relevant transcription factors.

MATERIAL AND METHODS

Mouse analysis: was performed at mid-fetal development, mostly at E13.5 because at this age all organs have formed. Moreover, the size of such embryos allowed for the preparation of full sections of the body, whereas later stages require dissection of the entire body increasing the chance of missing expressing cells. Further analysis of embryos at E9.5, E10.5 and E15.5 was done to determine the developmental expression pattern of the transgenes.

Embryo derivation: Embryos were obtained from mating between transgenic males carrying hprt-docked reporter constructs and wild-type females. This was done to avoid the depletion of transgenic females that are required to maintain the colony. The females used in this mating program were derived from C57BL6 inbred strain. Pregnant females were sacrificed, by cervical dislocation, mostly at E13.5 and to a lesser extent E9.5, E10.5 and E15.5.

Tissue preparation: Embryos were dissected out and placed in Webster's fixative for 1 hour. Webster's fixative is made up of 0.5% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffer 0.1M. Embryos were then left in 30% sucrose in 0.1M phosphate buffer overnight. The following day, embryos were frozen in isopentane and cooled by liquid nitrogen and stored at –80. Meanwhile the placentas of embryos were dissected and frozen at –20 for DNA extraction.

DNA extraction: Since only the female embryos will carry the transgene on their X-chromosome (hprt is located on X-chromosome), PCR for sry, a y-linked

gene, was done on placental DNA to distinguish females and males. The placenta of each embryo was placed in lysis buffer (0.1M Tris (pH 8.5), 0.005M EDTA (pH 7.0), 0.2% SDS, 0.2M NaCl) and proteinase k. 100μg proteinase k / ml lysis buffer. This "activated solution" was freshly prepared before each use. The tissues were left overnight in the activated lysis buffer solution. The following day, DNA extraction was performed by isopropanol precipitation followed by ethanol precipitation, and DNA used for PCR. The testis determining gene on the Y-chromosome, Sry, is only present in normal male embryos, thus the PCR reaction amplification of a fragment of Sry gene is diagnostic of a male embryo. The PCR primers and amplification conditions are from Gubbay et al., 1992. Amplification consists of denaturation at 94C for 3 minutes, followed by 30 cycles at 96°C for 40 seconds, 65°C for 30 seconds, and 72°C for 45 seconds. The sequences of primers used were as follows: Sry 5': AGAGCATGGGAGGGCCAT and Sry 3': CCACTCCTCTGTGACACT. For visualization, 8μl of sample from each reaction is electrophoresed on a 2% agarose/TBE gel containing 0.1μg/ml ethidium bromide. Male embryos were analyzed as controls.

LacZ staining: Sagittal sections, 10μm thick, of frozen embryos were prepared using a cryostat. Slides were left in formol sucrose (7.5gm sucrose, 10ml formaldehyde in 100ml phosphate buffer 0,1M) for 15 minutes and stained overnight for B-galactosidase activity. LacZ is a bacterial gene used here as a reporter for histological localization of transgene expression. The lacZ gene product B-galactosidase catalyzes the hydrolysis of the substrate X-gal to produce a blue color that is easily visualized with a microscope. The stain was made up of 102 mg potassium ferricyanide, 130 mg potassium ferrocyanide, 200ul 1M

$MgCl_2$, 2mls X-gal (40mg/ml), 0.01% sodium deoxycholate and 0.02% CA630 in 0.1M phosphate buffer pH 7.2.

Light microscopy: On the following day, the sections were washed twice by phosphate buffer, slides mounted and examined for blue staining cells under a light microscope using different magnifying lenses. Due to random X-chromosome inactivation in females, only about half the cells should have an active transgene bearing X-chromosome and hence one expects a mosaic pattern of expression in any expressing population of cells. Photographs of sections were taken using a digital camera mounted on a photomicroscope (Zeiss).

Electrophoretic Mobility Shift Assay (EMSA) : was performed on 4 sequences around M3 that were suspected to have Krox-20 binding sites. The actual bp locations relative to the ATG of MBP is as follows: oligo1:-3019_-3038, oligo2: -3192_-3212, oligo3: -4242_-4262, oligo4: -4458_-4476. Using MacVector (computer software), 4 putative binding sites were characterized which were further analyzed for their Krox-20 binding by EMSA. Oligonucleotides were designed and sent to Biocorp for synthesis. Double stranded oligonucleotides (annealed sense and antisense) were labelled by radioactive (gamma 32P) ATP and T4 polynucleotide kinase. After labeling, labeled oligonucleotides were purified using the QIAquick Nucleotide Removal Kit (Quiagen). Oligos were eluted from the column with 100 µl 10 mM Tris, pH8.0. The probes were then ready for use. They were incubated with a solution containing bacterial extract of Krox-20 protein. The binding reaction occurs under specific salt/pH conditions in a binding buffer. Poly-dIdC was added to prevent nonspecific binding of proteins to the oligonucleotide probe and by this to reduce

background. For a competition test, a "cold" oligonucleotide probe was added in excess to the binding reaction mixture before adding the labeled probe. To test for binding specificity, the protein extract was incubated with a non-specific oligonucleotide. The protein extract was also incubated with labelled probe of the consensus Krox-20 sequence as control. After 30 minutes of incubation, the reaction products were analyzed on a nondenaturing 5% polyacrylamide gel (acrylamide12.5ml, 1X TBE 37.5ml, ddH2O 24.4ml, ammonium persulfate 525ul, TEMED 60ul). The gel was then transferred to Whatman 3MM, dried for an hour and exposed to autoradiography film.

RESULTS

Expression pattern of endogenous mbp locus at E13.5.

In order to determine the pattern of expression of the endogenous mbp locus, I took advantage of previous work in which mbp exon 2 was replaced with the sensitive LacZ reporter gene. Only those transcripts originating from the mbp promoter were demonstrated to encode enzymatically active B-galactosidase thus providing a unique opportunity to track mbp promoter driven expression (Bachnou et al., unpublished data). Analysis of the embryos carrying the knock-in allele at E13.5 revealed LacZ expression in the progenitors of myelin forming cells; a small number of oligodendrocyte precursor cells; in the CNS. No other expressing cells were reported at E13.5. Therefore, the presence of any other population of cells expressing the hprt targeted constructs containing mbp elements would be considered ectopic expression.

Ectopic expression of MBP reporter constructs at E13.5

I started by analyzing the expression of the constructs that contained all four conserved non-coding mbp modules (M1, M2, M3 and M4), docked in hprt. If such modules are the site of the major regulatory sequence, I would expect these constructs to behave like the endogenous locus. If so, they should express only in a few oligodendrocyte precursor cells, like the knock-in allele. Surprisingly that was not the case. Rather, two similar constructs (-9.08kb and -9.5kb 5' MBP LacZ) containing the 4 conserved mbp modules expressed ectopically in the heart (cardiomyocytes) (figures 4A&4B) and blood vessels of the brain (figures 4C&4D).

Are the hprt enhancers responsible for that "deregulated" expression in the heart and CNS blood vessels? If yes, then is it a pure hprt enhancer effect or is it a partnership between hprt enhancers and the enhancers within the docked constructs?

Analysis of 2 independently generated random insertion lines (lines 17 and 32) carrying similar reporter constructs as the ones examined above (-9kb 5' MBP LacZ), that contained all the 4 conserved mbp modules, revealed no such ectopic expression (figure 5A&5B). However, in one of the random insertion lines, expression was observed in the endothelium of blood vessels. Subsequently, I demonstrated that all the hprt docked constructs revealed a similar pattern of ectopic expression in cardiomyocytes and blood vessels. Of more relevance, I analyzed embryos carrying a construct in which the minimal promoter (300bp) of the heat shock protein68, HSP, was linked to LacZ. This construct does not contain any mbp associated sequence. On its own it is incapable of driving expression and consequently, it has been used in many studies as a neutral enhancer trap. In my analysis, it was expressed in heart and blood vessels (figure 6).

The above results suggested that hprt associated regulatory activity is uniquely responsible for the observed ectopic expression in the cardiomyocytes and blood vessels. (Table 1).

The developmental stage at which this ectopic expression occurs.

I analyzed embryos carrying reporter constructs docked at hprt at E9.5. No expression in cardiomyocytes was observed (figure 7A).

I, next, analyzed embryos carrying reporter constructs at E10.5. In some embryos there was expression in the cardiomyocytes (figure 7C&7D) while there was none in others (figure 7B). This could be attributable to the developmental variability that could be seen in different embryos at the same gestational age.

These results indicate that expression in the cardiomyocytes starts around E10.5

Analysis of mice at P0, P9, 1 month, 2 months, and 11 months showed consistent expression in the cardiomyocytes (figure 8) and brain blood vessels regardless of the specific reporter constructs docked at hprt.

This indicates that once expression is turned on in cardiomyocytes, it is continuous.

Ectopic expression of reporter constructs containing mbp enhancers in cartilage cells of vertebral bodies at E13.5.

Reporter constructs containing the four conserved mbp modules (-9.08kb&-9.5kb 5' MBP LacZ) were also found to express in cartilage cells of the vertebral bodies whether the construct was hprt docked (figure 9A&9B) or randomly inserted (figure 9C&9D). This apparently ectopic expression was not influenced by the hprt site as the HSP LacZ construct did not similarly express in these cells (figure 10).

To map elements in the mbp promoter that could be responsible for expression in cartilage cells of the vertebral bodies, I compared the cartilage expression phenotypes of multiple constructs. I analyzed a minimal mbp promoter reporter construct containing part of module 1 (M1) (i.e. -116bp 5' MBP LacZ). This construct does not drive expression in the oligodendrocytes in CNS of adult mice. Although this reporter construct expressed in the heart and blood vessels of the brain, it did not show expression in the vertebral body cartilage (figures 11A&11B). Similarly, analysis of a reporter construct which could target expression to oligodendrocytes in the adult mouse and which contains the full length M1, and another with both M1 and M2 (-330bp and -750bp 5'MBP LacZ respectively) did not show expression in the vertebral body cartilage (figures 11C&11D.

The results so far suggest that expression in cartilage cells is not conferred by elements in the proximal mbp promoter.

I then analyzed mice bearing a reporter construct that had 1.1 kb of M4 sequences (linked to minimal hsp promoter) and which is capable of targeting to Schwann cells in the adult mouse. Expression in the vertebral body cartilage cells was noted (figure 12A and inset). A smaller sub-sequence of M4 (0.2kb) similarly expressed in the vertebral body cartilage suggesting that the 0.2 kb sequence contains the relevant elements (figure 12B).

Analysis of reporter constructs carrying M3 sequence linked to minimal hsp promoter that is shown to have targeting ability to oligodendrocytes in adult mice also showed cartilage expression at E13.5 (figure 13A).

However when sequences in the proximal mbp promoter (i.e., -256bp) were added to M3, it repressed its expression capability in cartilage cells of vertebral bodies (figure 13B). On the other hand, when sequence around M3 (i.e. -6_-3 kb) was added to the latter construct, it alleviated this repression and cartilage cell expression reappeared (figure 14A). In an attempt to discover the elements in the sequences around M3 that were able to counteract the repression imposed by the proximal mbp promoter on M3, I used MacVector (computer software) as well as the online TFSEARCH program supported by TRANSFAC databases. Four putative Krox-20 binding sites, which are similar in sequence to the consensus for Krox-20, were found in the sequences around M3 (-3019_-3038, -3192_-3212, -4242_-4262 & -4458_-4476). Band shift experiments revealed that at least one of the 4 sites (-4458_-4476) binds Krox-20 protein (figure 14B).

The above data suggest that the regulation of myelination and cartilage gene expression may share common regulatory elements. Krox-20 is a possible candidate (Tables 2 & 4).

Ectopic expression in muscles of tongue and back.

Analysis of HSP LacZ construct docked at hprt also expressed in muscles of the tongue and back (figures 15A, 15C & 16A, 16C). When M4 and M3 were linked to HSP and docked in hprt, they did not similarly express (figure 15B, 15D& 16B, 16D respectively). This might suggest the presence of repressor elements in the M3 and M4 sequences especially because when only part of M4 (0.2 kb) was linked to HSP and docked in hprt, there was some expression in the

muscles of the tongue (figure 15B inset), indicating that a repressor element might have been eliminated in the shorter M4 sequence. This possibility is also indicated by the fact that the level of LacZ expression was higher in the heart, blood vessels, tongue and back muscles in mice bearing the HSP LacZ construct than in mice bearing constructs that also contained M3 and M4.

Taken together, the above data suggest the possibility of the presence of repressors within the mbp enhancer elements used (Table 3).

DISCUSSION

Hprt ENHANCER ACTIVITY

The results of this study demonstrated endogenous hprt enhancer activity in the smooth muscles of the heart, muscles of the tongue and back as well as the large CNS blood vessels. The availability of the "enhancer trap" construct facilitated this observation. The latter reporter construct consisted of the minimal promoter of the heat shock protein, HSP, driving the LacZ reporter gene. This promoter is used by many studies as a heterologous promoter which by itself is incapable of driving gene expression. Theoretically, if the hprt locus is a neutral site, then expression from the HSP LacZ construct is an unexpected event. To rule out the possibility that the endogenous mammalian B-galactosidase enzyme is causing the hydrolysis of the substrate X-gal present in the stain to produce the blue color observed, a control experiment was performed. Tissues from male, nontransgenic mice did not similarly stain blue. This suggests that the "enhancer trap" construct is "trapping" enhancer element in the nearby locus. The nearest regulatory elements to the docked constructs using the targeting transgenesis described by Bronson et al., are those of hprt gene. A study done by Magin et al., (1992), reported that intron-1 sequence of the hprt gene, which is 230bp downstream from the transcription start point, was found to increase transcription from the hprt promoter in ES cells. However, in the hprt targeting strategy described by Bronson et al., intron-1 sequence was not included in the targeting vector used for transfection of BK4 ES cells and, therefore, its contribution to the hprt enhancer effect noticed in my study is nil. The upstream gene (PHF6) is 10kb away from the docked construct. The latter could still exert an influence on the docked construct; although this possibility is slightly remote.

Analysis of mice carrying a mbp knock-in allele, where exon 2 of mbp was replaced by the reporter gene LacZ, did not show this deregulated expression in the heart and large CNS blood vessels. Moreover, when similar reporter constructs carrying the mbp promoter elements were randomly inserted, they did not reproduce that ectopic expression; all indicating that the mbp regulatory elements are not responsible for the observed ectopic expression.

The morphology of the cells that stained blue in the heart resembled cardiomyocytes. Nonetheless, it was difficult to differentiate whether it was the endothelial or the smooth muscles cells of the blood vessels that were expressing the transgene. Immunohistochemical analysis is required to identify these cells.

SIGNIFICANCE

A study such as this one may be important in the interpretation of future results obtained from the genes docked at the hprt locus. The identification of an influence of the hprt gene in some tissues renders results obtained from genes expressed in these tissues inconclusive and misinterpreted. For example, the study on the Tie2 promoter compared the difference of expression patterns of two promoter elements; one contains an enhancer originating from the first intron of the Tie2 gene (T5) and the other does not (T1). Results show moderate reporter gene activity in the brain and kidney of T1mice. In contrast, expression was absent in the heart, the lung, the spleen and the liver. The results reported in my study show extensive reporter gene activity with the control "enhancer trap" construct in the heart and lung. This indicates that not only does T1 not contain enhancer elements but points to the possibility of harboring repressor elements for endothelial gene expression. On

possibility of harboring repressor elements for endothelial gene expression. On the other hand, extensive LacZ gene expression was seen in the vasculature of most organs of the T5 mice. The identification of endogenous hprt activity in the blood vessels raises the question of whether the potentiated endothelial gene expression obtained with the T5 is a unique effect of the enhancing endothelial element that it contained within T5 or the multiplexed effects are the sum of the partnership between the hprt enhancer elements and the enhancer of the Tie2 gene.

One of the advantages of the hprt targeting strategy is that it limited the vast numbers of germlines that had to be generated for a single construct when the transgene was randomly inserted. The randomness of the site of integration, the variability in copy numbers of the transgenes in addition to the different expression patterns of the transgene according to the site of integration were all variables in the random transgenesis that were compensated for by the generation of multiple independent lines in order to reach significant conclusions about the function of the transgene in vivo. By targeting a single copy of the transgene to the hprt locus, fewer lines are required. At the extreme, the study on the Tie2 promoter generated only one mouse line for each construct. Moreover, that study did not include a control construct e.g. a non-endothelial cell specific transgene to illustrate that the observed results are the pure consequences of docking the Tie2 promoter elements in hprt. In my report, the results obtained from both the "enhancer trap" construct and the mbp promoter elements are sufficient to conclude that hprt enhancer activity influences the expression of the of the transgenes in the blood vessels.

The study on the eNOS promoter, another endothelial cell-specific gene, also docked in hprt, reported transgene expression in both the smooth muscle and endothelial cells of the blood vessels. Additionally, the hprt-targeted eNos LacZ transgene was also expressed in cardiac myocytes. Despite the close correlation between hprt-targeted eNos lacZ activity and endogenous eNos, there was discordance of expression in the vascular bed of the liver where there was no detectable transgene expression yet the endogenous gene was expressed. Interestingly too, the pattern of eNos LacZ expression was found to be predominant in the right ventricle as opposed to the more uniform distribution of the endogenous gene in the heart. The latter two discrepancies between the eNos endogenous and transgene expression all point out to an external regulatory activity being imposed on the transgenes and that being from the closest gene; hprt.

Similarly, in the study by Minami et al., (2002), which showed the differential regulation of the von Willebrand factor (VWF) and Flt-1 promoters in the endothelium of hprt-targeted mice, again there were inconsistencies between endogenous and the transgene expression patterns. First, in the VWF-LacZ-hprt mice, there was limited distribution of the B-galactosidase activity in general as opposed to the more widespread expression of the endogenous VWF gene. This study also detected B-galactosidase activity in the cardiomyocytes of Flt-1 transgenic mice; a site in which the endogenous Flt-1 is not expressed. This suggests that, just like mbp, the Flt-1 transgene is ectopically expressed in the heart emphasizing the HPRT enhancer activity.

Taken together, these results indicate that the hprt targeting strategy does not seem to be an appropriate tool for studying endothelial or muscle specific genes. Figures 17&18 show the similarities in ectopic expression obtained from all the studies that targeted any transgene to the hprt site. Irrespective of the transgene docked at hprt, similar ectopic expression was observed in the cardiomyocytes and blood vessels. Indeed, the list of hprt-targeted endothelial cell-specific transgenes is growing at a fast rate without the knowledge that hprt enhancer activity is likely to lead to the faulty interpretation of results.

MYELIN AND CARTILAGE

The study by Levi et al., (1996) showed that the knock-out of Krox 20; a zinc finger transcription factor needed for the control of the chondrocyte-osteoblast interactions, led to defective bone formation. The same group, in 1994, also showed that in mice homozygous for null Krox 20 alleles, Schwann cells ensheath axons but fail to elaborate myelin demonstrating that Krox-20 plays an essential role in Schwann cell maturation and myelination. Forghani et al., (2001), using MacVector software, encountered a perfect match to a Krox-20 binding site near the 3' end of SCE1 which is capable of targeting expression to Schwann cells in adult mice. However, when a 5 bp mutation was introduced to this sequence that abolished Krox-20 binding, high level B-galactosidase expression was still observed in Schwann cells throughout the PNS. With the results of the band shifting assays in my report, it could be that the Krox-20 binding sequence that is

present in the sequence between -6 and -3kb (i.e. -4458_-4476), and that was also included in the reporter construct used in Forghani's study, could be sufficient to cause the high-level B-galactosidase expression observed in Schwann cells.

M3, however, does not contain putative Krox20 binding sites; yet, it confers expression to cartilage cells suggesting the presence of another shared transcription factor between cartilage and myelin-forming cells which still remains to be identified. Another explanation is that effective binding site for Krox20 protein in vivo might be quite different than the consensus.

The fact that Krox-20 is expressed simultaneously by two populations of cells, which have different developmental histories, might reflect similar responses to common inducing signals. The actions of fibroblast growth factors (FGFs) are mediated via a family of four closely related FGF receptor genes (FGFRs 1-4). Peters et al., (1993) used in situ hybridization analysis to show that FGFR3 has a unique pattern of expression during organogenesis. At 1 day postpartum and in the adult brain, FGFR3 was expressed diffusely and localized in cells with morphologic characteristics of glia, a pattern distinctly different from the discrete neuronal expression of FGFR1. Outside the nervous system, the highest level of FGFR3 expression was found in the cartilage rudiments of developing bone. During endochondral ossification, FGFR3 was expressed exclusively in resting cartilage, a pattern distinct from FGFR1 and FGFR2. It would be interesting to see the effect of a FGFR3 knock-out.

Taken together, the above data suggest that common regulatory elements as well as similar responses to common inducing agents are shared between glia and cartilage cells. By identifying common pools of transcription factors and signaling pathways shared between these two cell types, the regulation of myelination could be further elucidated.

Transcriptional repression of the mbp gene

The investigation by Forghani et al., (2001) reported activity of a developmentally regulated cell-specific repressor located in the more proximal mbp promoter. Of the three random insertion SCE1-hsp68 lines examined, all consistently revealed transient B- galactosidase expression in a small subpopulation of cells in the neural tube, brain and retina. However, constructs bearing SCE1 with the 6kb proximal promoter were not similarly expressed. This indicated putative negative regulatory elements serving to repress mbp expression in these cell populations might be located within the first 6kb of mbp 5' flanking sequence. Likewise, my results showed that all reporter constructs that contained any parts of the proximal 6kb mbp promoter (i.e. -116bp, -256bp, -256bp &-750bp) did not express in cartilage cells. Moreover, when such sequences (e.g. -256bp) were added to M3, they repressed its targeting ability to cartilage cell of vertebral bodies.

The results of my investigation also indicate the presence of repressors of ectopic expression in M3 and M4 sequences. This is demonstrated by the observed repression of LacZ gene expression in tongue and back muscles when M3 and M4 sequences were added to the "enhancer trap" construct.

APPLICATION IN GENE THERAPY

Recent scientific developments make it possible for medicine to target diseases at the molecular level, and thus offer the prospects of effective new therapies. The genes of somatic cells can be manipulated by drugs to modify a disease in an individual. If genes in germ cells are modified, a genetic disease may be prevented in progeny as well.

Regulation of the amount of protein produced is achieved through a number of mechanisms, among which is the production of repressor and activator proteins, which prevent the appearance of too much or too little protein, respectively. The knowledge of how genes are regulated is essential in designing an effective, targeted approach to treating disease. Studies by Haggiag from the Revel group laboratory (1997-2002) identified the paired homeodomain gene, Pax-3, as a major target for interleukins 6 (IL-6), a drug used in Multiple Sclerosis therapy. Addition of IL6 to cultured embryonic dorsal root ganglia causes complete disappearance of Pax-3 correlating with the induction of myelin genes like MBP and P0. The switch of myelin genes by IL-6 appears to be correlated with the increase in the ratio of transcription factors of myelin genes, like Sox10, over repressors like Pax-3. By further identifying repressor elements, the hope is to elucidate how myelin gene expression is controlled and could be triggered when needed, as in demyelinating diseases like MS.

Figure 1A: shows mouse MBP gene. M1, M2, M3 & M4 are four highly conserved non-coding sequences in the mbp 5' flanking sequence revealed by human-mouse sequence comparisons. Their positions in nucleotide numbers upstream of the transcription start site as well as their sizes are shown.

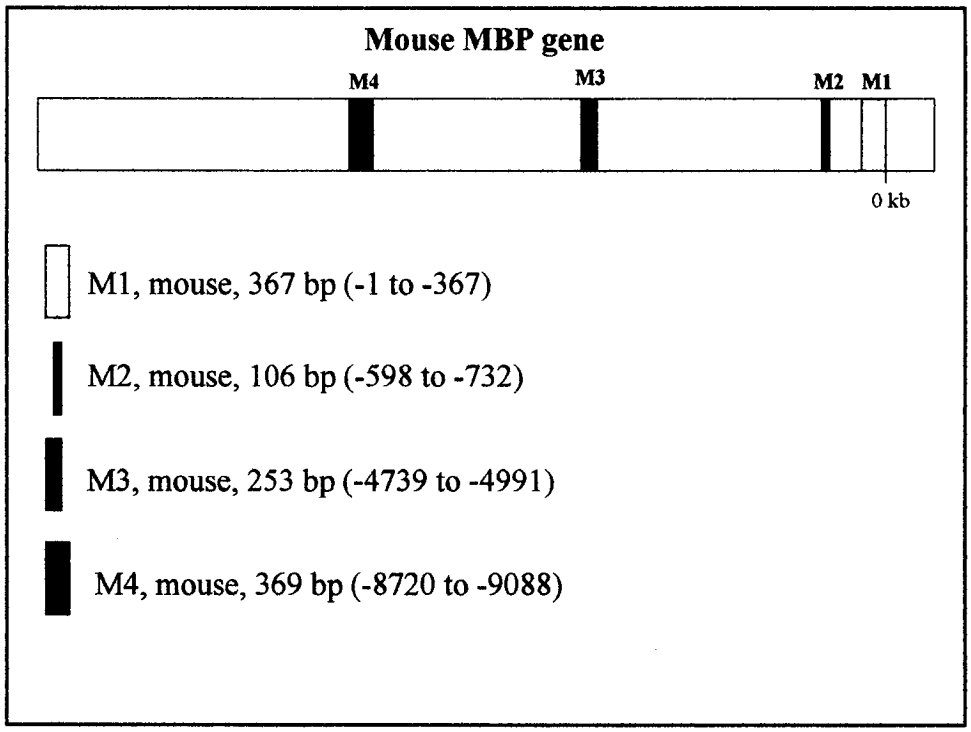
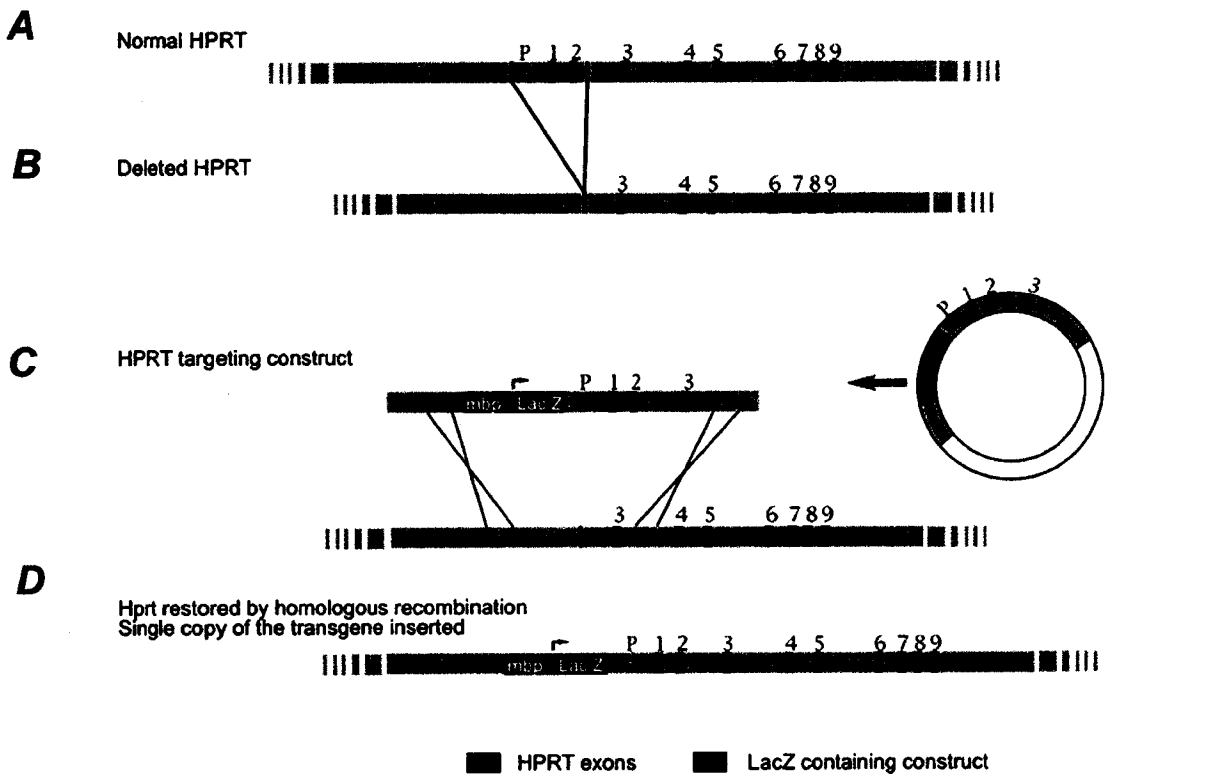

Mouse MBP gene
M4
M3
M2
M1
0 kb
M1, mouse, 367 bp (-1 to -367)
M2, mouse, 106 bp (-598 to -732)
M3, mouse, 253 bp (-4739 to -4991)
M4, mouse, 369 bp (-8720 to -9088)

Figure 1B: shows the hprt targeting strategy. A: wild-type hprt gene. B: the partially deleted hprt gene of BK4 ES cells. C: linearized vector used for transfection of ES cells. D: genotype of hprt locus of correctly targeted ES cells. The transgene is placed as a single copy upstream of the hprt gene by homologous recombination.

HPRT Targeting Strategy

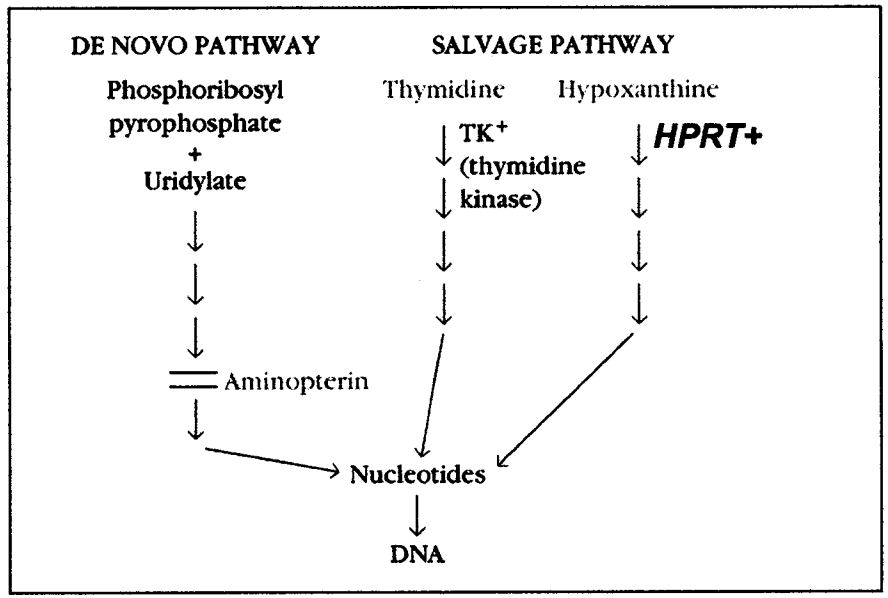


(After Bronson et al, PNAS 1996, 93:9067-9072)

Figure 2: shows how correctly targeted ES cells survive on HAT (hypoxanthine, aminopterin, thymidine medium (in blue). In the presence of aminopterin and absence of hprt enzyme, ES cells cannot utilize the salvage pathway for nucleotide synthesis which is catalyzed by hprt and die on HAT medium.

Figure 3: Genomic environment of the transgene which lies immediately upstream of the hprt gene and approximately 10 kb downstream of the PHF6 gene.

Means of Selection of HPRT-targeted ES cells



Genomic Environment of the Transgene

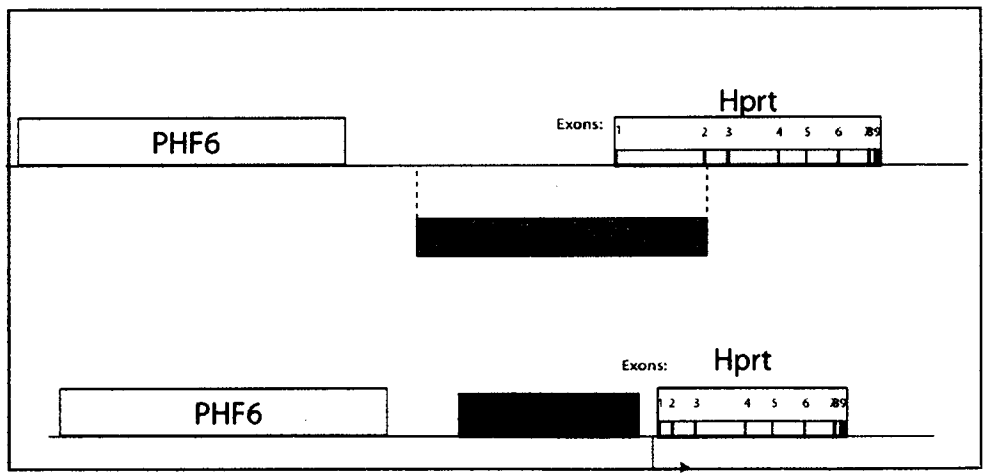


Figure 4: Sagittal sections of E13.5 transgenic mouse embryos bearing hprt-docked MBP reporter constructs shown. 9.08kb for A&C and 9.5kb for B&D. A&B: X5 magnification of the heart, arrow: LacZ positive cells in the heart. C&D: X10 magnification of the brain, arrow: cerebral blood vessels. Inset in C: X40 magnification showing blue cells in the blood vessel's wall.

HPRT-docked constructs: Ectopic LacZ Expression in Heart & Blood Vessels of Brain

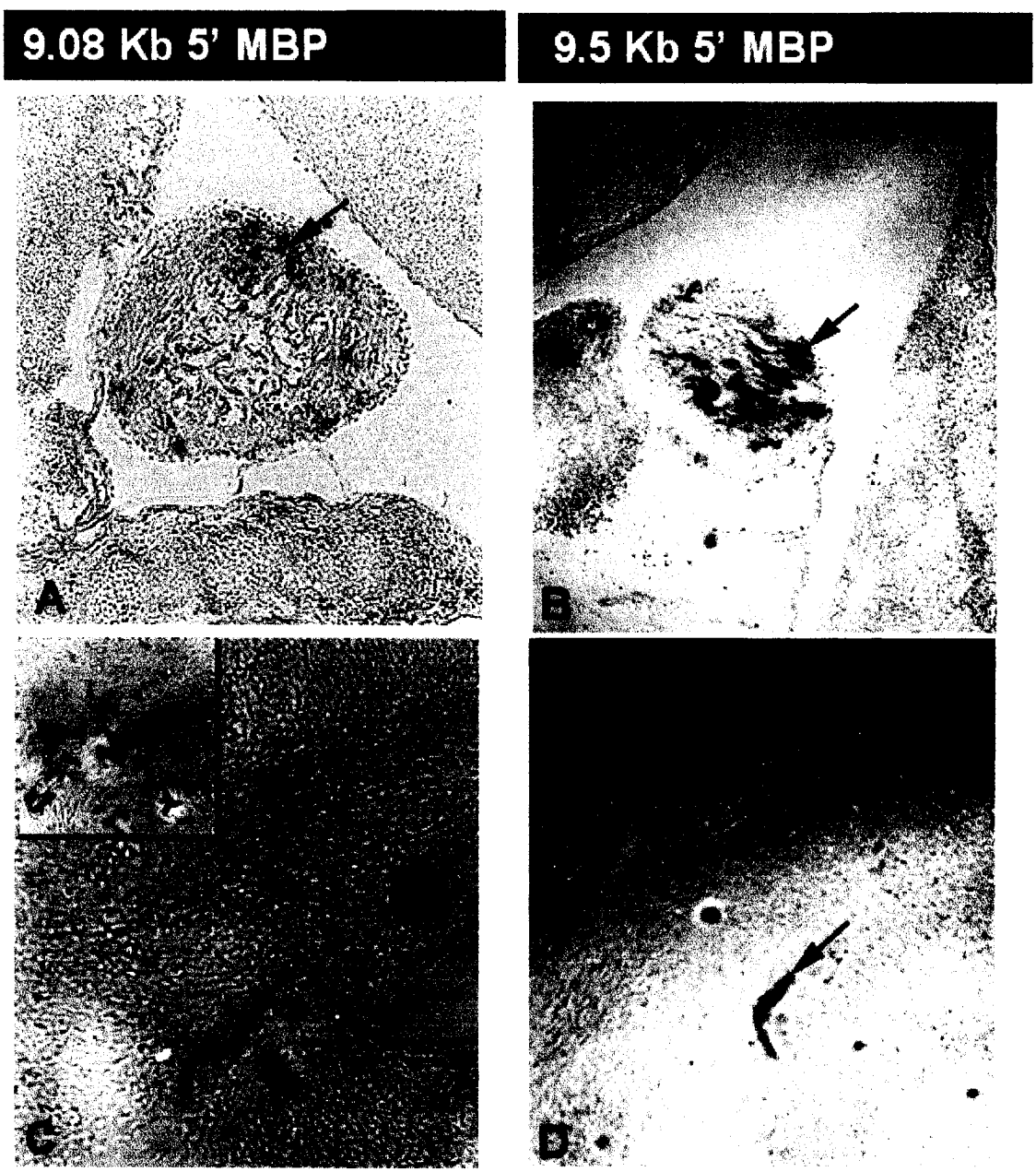


Figure 5: Sagittal sections of E13.5 transgenic mouse embryos bearing randomly inserted mbp reporter construct shown. A & B are from lines 17 and 32 respectively; X5 magnification of the heart. No lacZ expression as compared to figure 4.

Random Lines: No Ectopic LacZ Expression in Heart

9 Kb 5' MBP

Line-17 **Line-32**

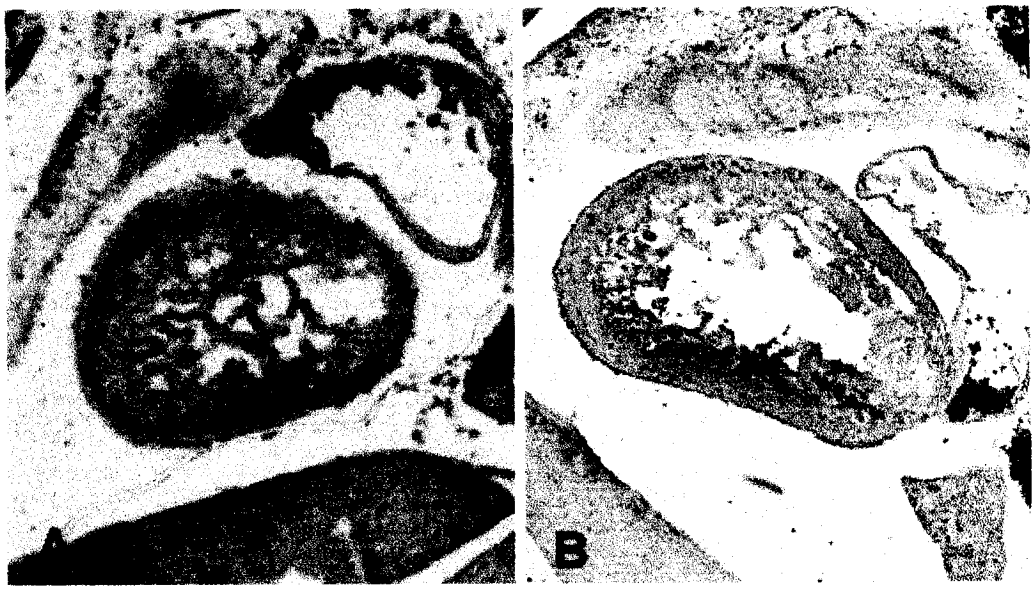


Figure 6: Sagittal section of E13.5 transgenic mouse embryo bearing the hprt- docked mbp reporter construct shown. A: X1.25 magnification, arrowhead: cerebral blood vessels. B: heart X5 magnification. Note the pattern of LacZ positive cells that resemble cardiomyocytes. C: X10 magnification of brain; arrowhead: cerebral blood vessel staining blue. D: X5 magnification of transverse section of umbilical cord showing the 3 umbilical vessels. Note the blue cells in the vessels' walls.

HSP-HPRT: Ectopic Expression in Heart & Blood Vessels

[redacted]

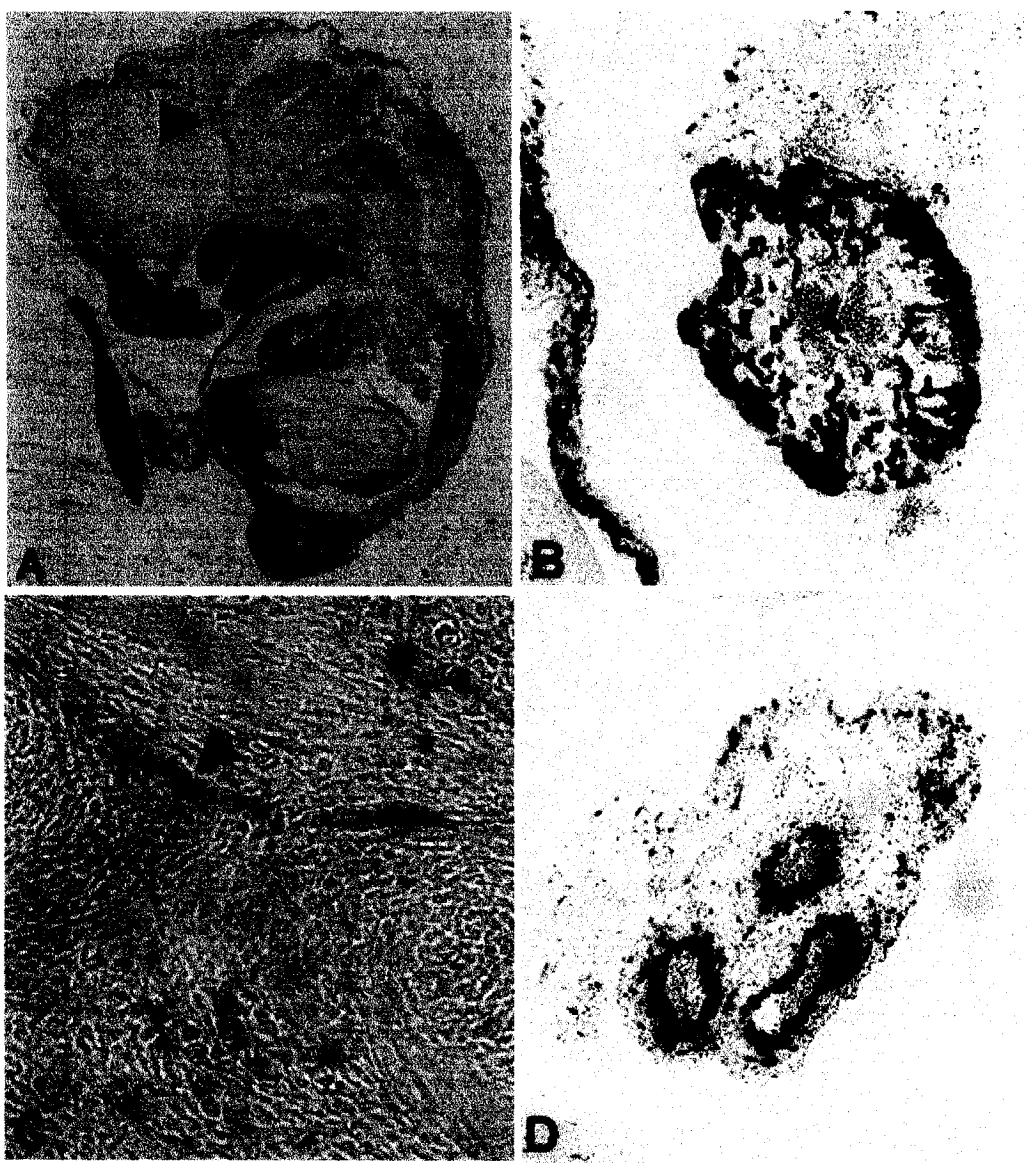


Figure 7: Sagittal section of E9.5 & E10.5 transgenic mouse embryos bearing different hprt-docked mbp reporter constructs. A&B: X5 magnification of the heart showing no blue cells. C&D: X5 magnification of the heart showing blue, LacZ positive cells.

Developmental stages of Ectopic Expression in the Heart (1)

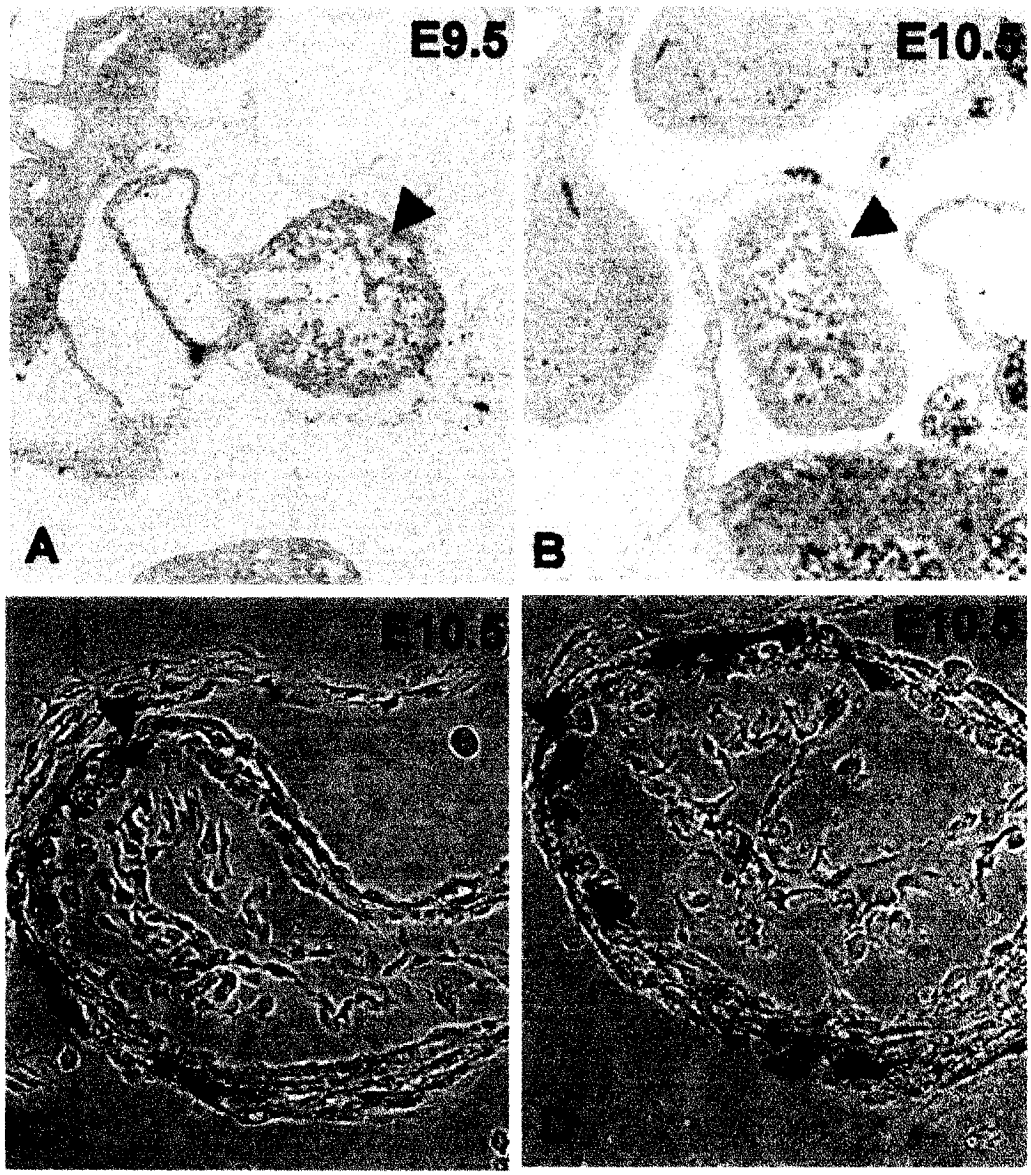


Figure 8: shows whole mount photomicrographs of hprt-targeted mbp-LacZ mice. Incubation of whole organs with 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside (X-Gal) substrate reveals LacZ staining in the heart as shown at P0, P9, P30, 2m, 6w & 11m.

Developmental stages of Ectopic Expression in the Heart (2)

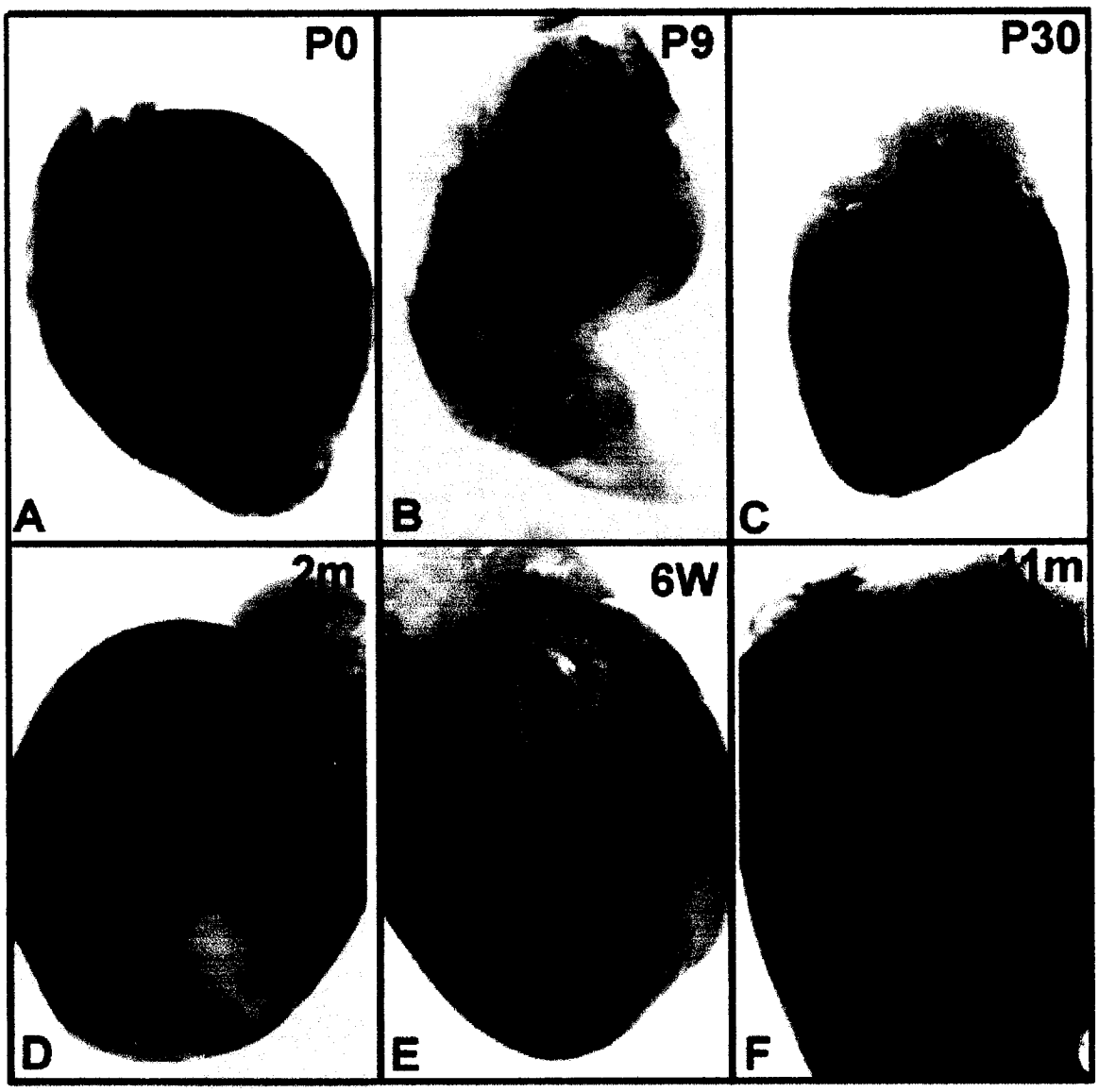


Figure 9: Sagittal sections of E13.5 transgenic mouse embryos bearing mbp reporter constructs shown on top of each photograph. Arrows: LacZ positive cartilage cells in vertebral bodies seen in both hprt-docked (A&B) and randomly inserted (C&D) reporter constructs.

LacZ Expression of HPRT-docked & Randomly Inserted Transgenes in Cartilage Cells of Vertebral Bodies

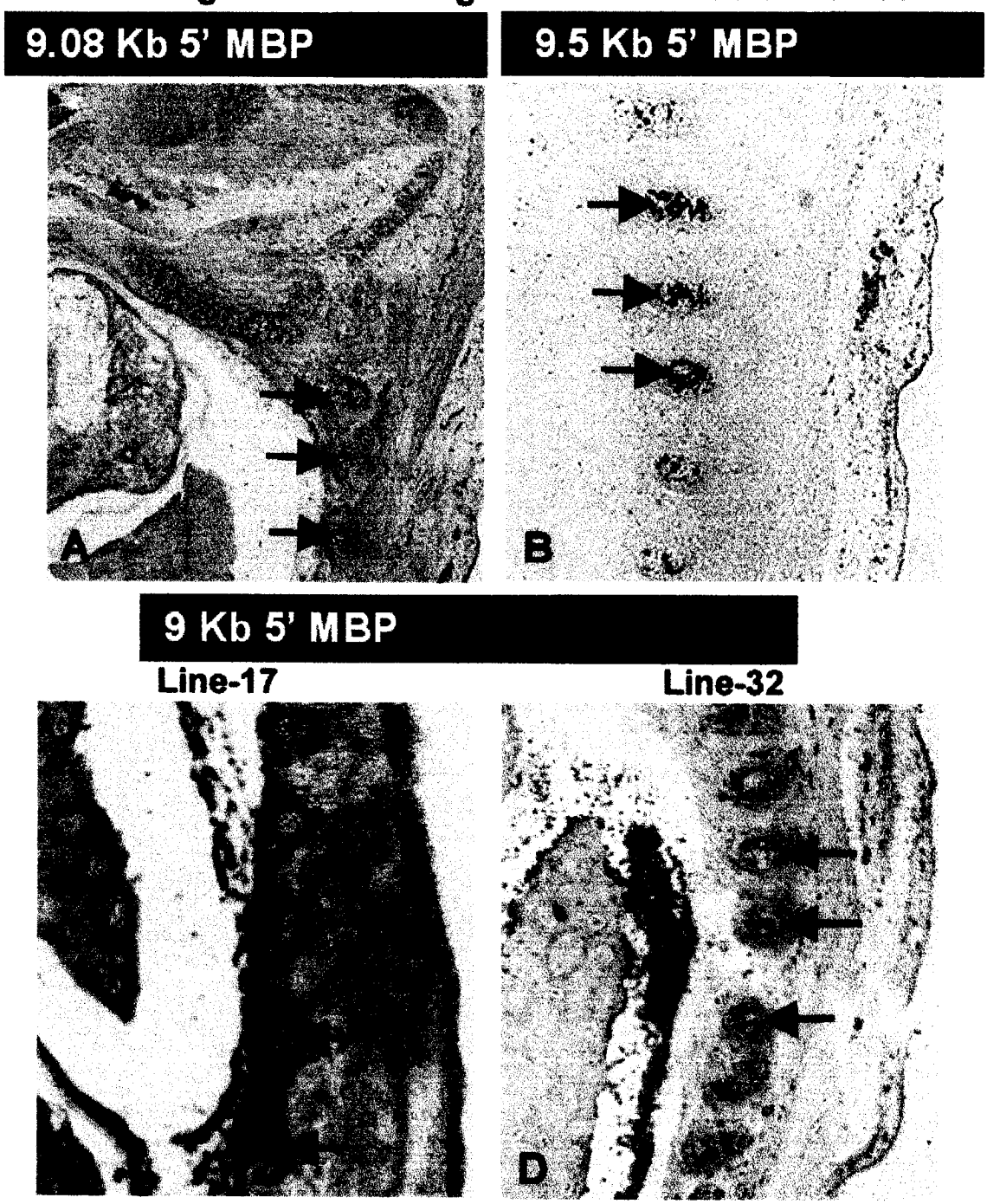


Figure 10: Sagittal sections of E13.5 transgenic mouse embryo bearing the reporter construct shown. A: X5 magnification of upper vertebral column; arrow: no lacZ expression in cartilage cells of vertebral bodies. B: X5 magnification of lower part of vertebral column. No lacZ positive cartilage cells noted as also shown by the inset: eosin stain, X10 magnification of vertebral bodies. BV (blood vessel).

HSP-HPRT: No lacZ Expression in Cartilage Cells of Vertebral Bodies

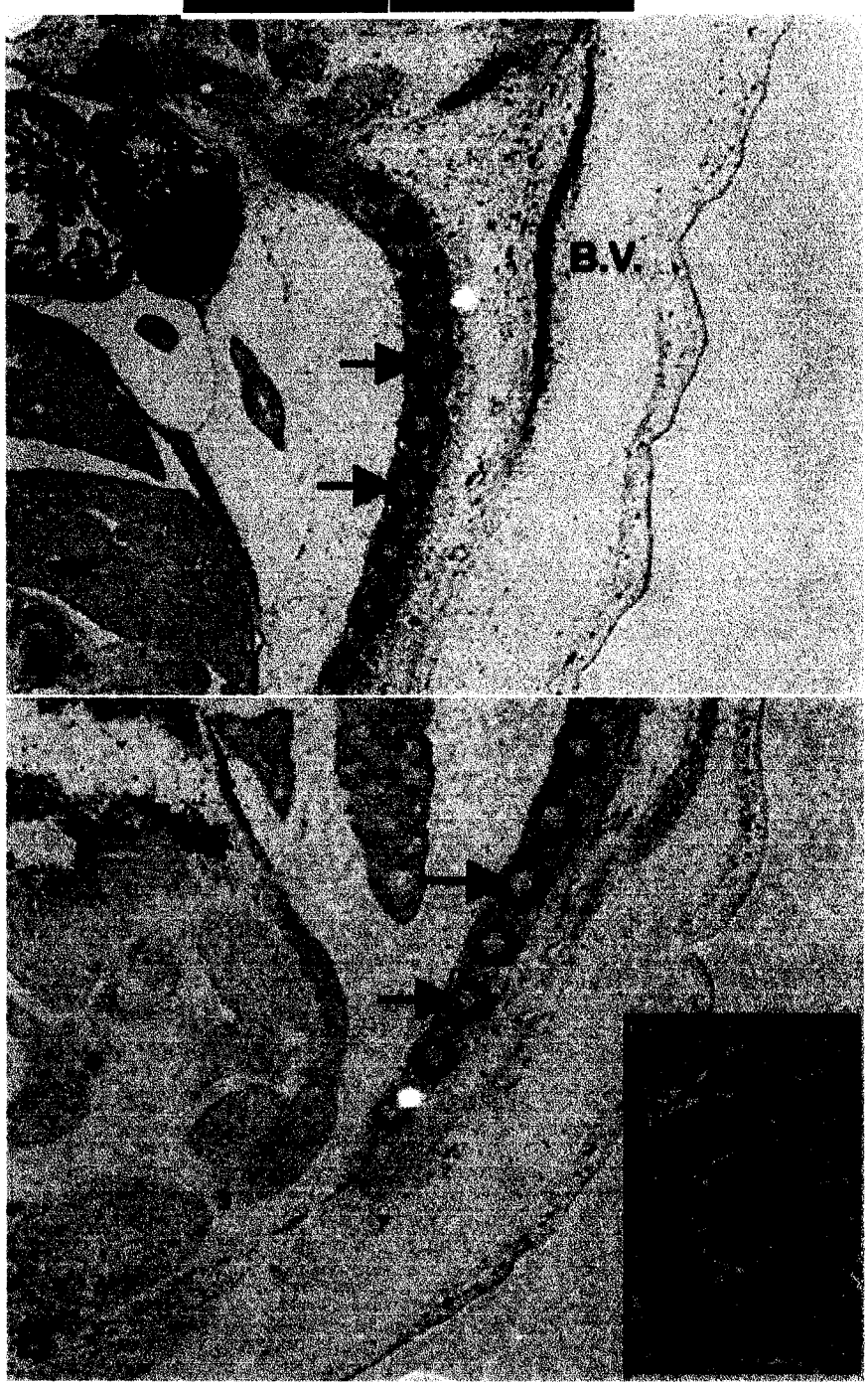


Figure 11: Sagittal sections of E13.5 transgenic mouse embryos bearing the hprt-docked reporter constructs shown on top of each photograph. SCE1 is an autonomous Schwann Cell Enhancer located and defined by Forghani's study using randomly integrated reporter constructs. It is a 584bp sequence. A: X1.25 magnification of the entire body. B: X5 magnification of the vertebral column of the embryo shown in A, arrow: cartilage cells of vertebral bodies; no LacZ staining. C: Sagittal section, inset on bottom left: X5 magnification of the vertebral column of the embryo showing no blue cells. D: Sagittal section showing no LacZ positive cartilage cells in the vertebral bodies, inset: blue cells in the heart; a pattern which resembles cardiomyocytes.

Cartilage Expression Not Conferred By Elements in Proximal MBP Promoter

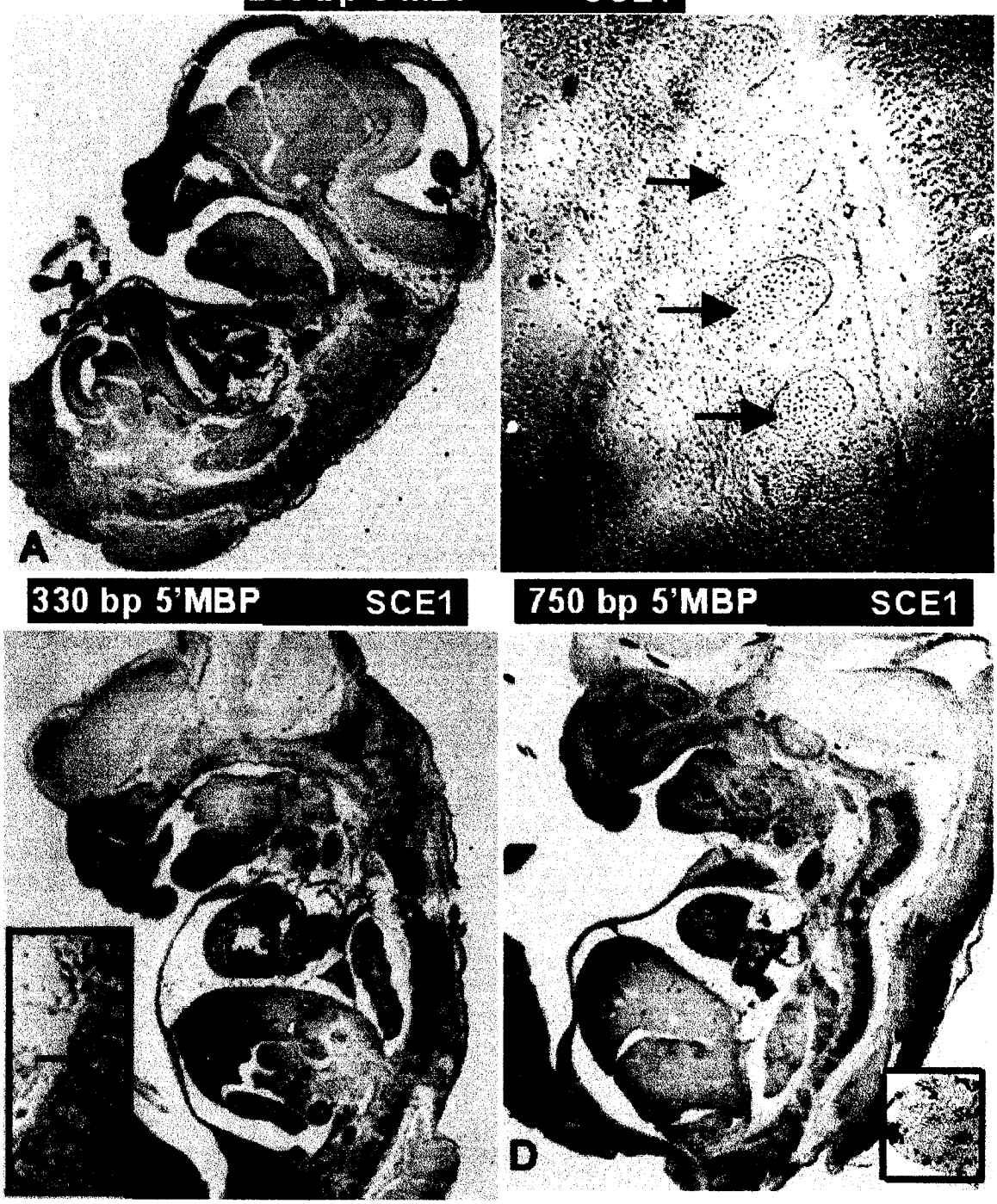


Figure 12: A: Sagittal sections of E13.5 transgenic mouse embryos bearing the hprt-docked reporter constructs shown on top of A (M4-HSP-LacZ); inset: X5 magnification of vertebral column; arrowheads point to LacZ positive cartilage cells in the vertebral bodies. B: X5 magnification of vertebral column of a different embryo bearing the reporter construct shown on top of B (M4(0.2kb)-HSP-LacZ). Note the blue cartilage cells in the vertebral bodies.

M4-HSP Targets LacZ Expression to Cartilage Cells of Vertebral Bodies

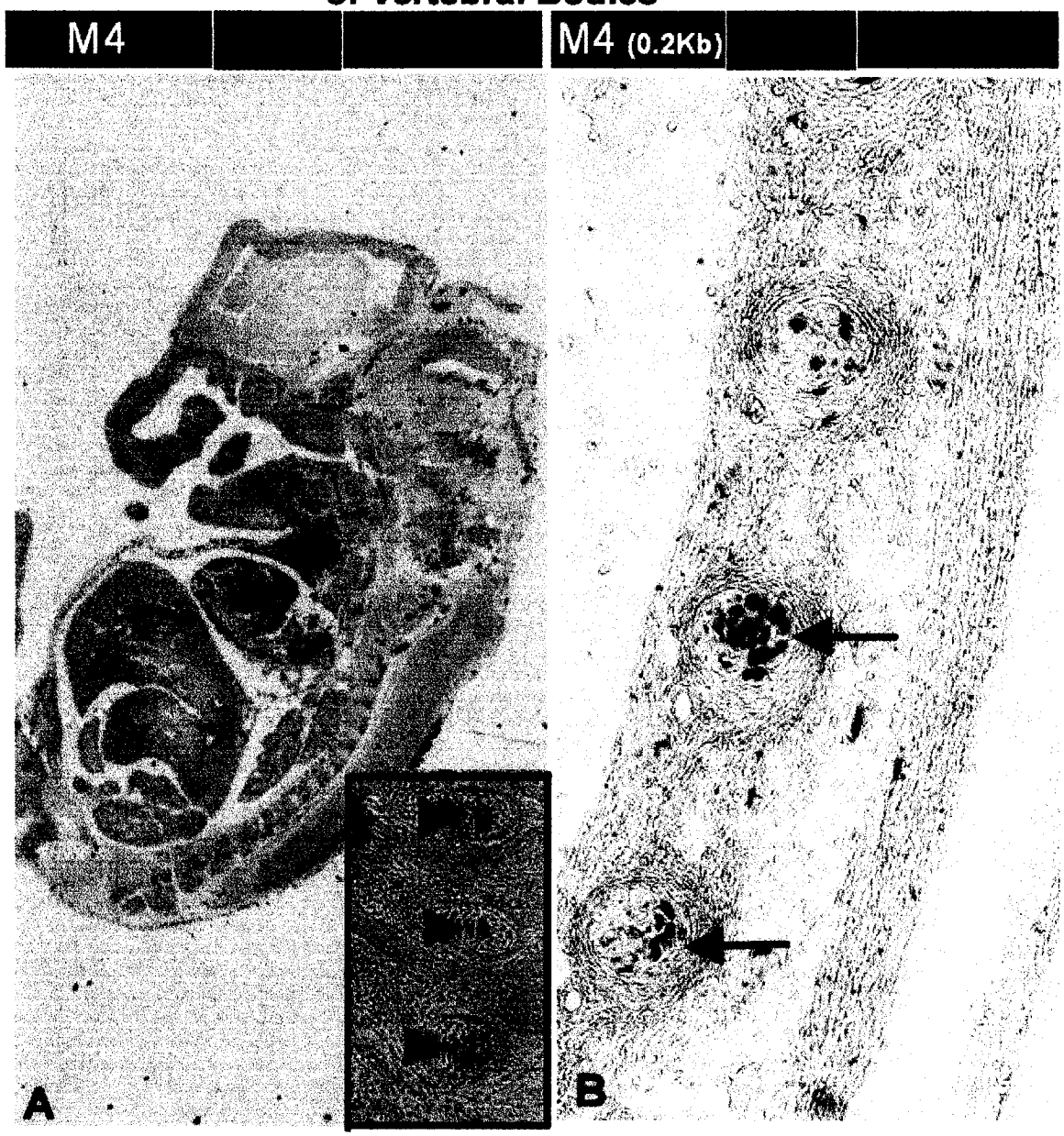


Figure 13: X5 magnification of vertebral column of transgenic embryos at E13.5 bearing the reporter constructs shown on top of A (M3-HSP-LacZ) and B (M3-P-LacZ-SCE1). P is the minimal promoter of MBP (256bp). A: arrowheads point to LacZ positive cartilage cells in the vertebral bodies. B: arrowheads point to cartilage cells which are not staining blue like A.

M3-HSP Targets LacZ Expression to Cartilage Cells of Vertebral Bodies While M3-MBP Does Not

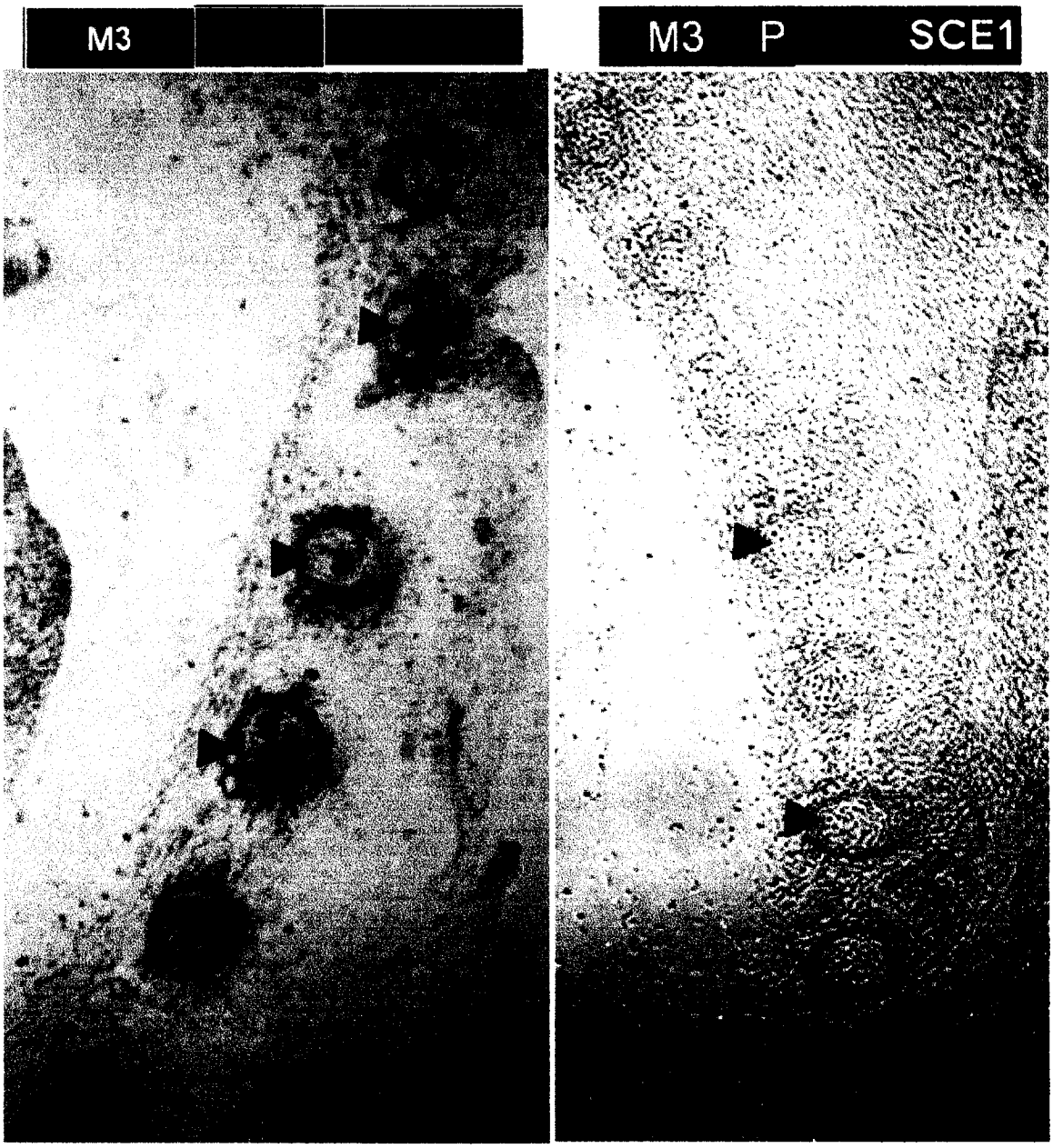


Figure 14: A: X5 magnification of sagittal section of a transgenic mouse embryo at E13.5 showing the middle part of the body. The transgene is shown on top; arrowheads: LacZ positive cartilage cells in the vertebral bodies. B: Electromobility shift assay, Kc: consensus sequence for Krox-20 binding, oligos 1-4: sequences of mbp in respect to the ATG site as follows: oligo 1:- 3019_-3038, oligo2: -3192_-3212, oligo3: -4242_-4262, oligo 4:-4458_-4476. Lanes 1-3: Binding of radioactive consensus sequence of Krox-20 in lane 1 was partially competed for by the cold probe (lane2) but not by a non-specific sequence (lane 3) as noted from the decrease in band intensity in Lane2. Radioactive Oligos 1 & 3 did not bind to Krox-20 protein as shown in lanes 4&10 respectively. Lane 13: radioactive oligo 4 bound to Krox-20 protein, partially competed for by the cold probe (lane14) but not by the non-specific oligo (lane 15). The results from oligo2, however, are not conclusive, as the non-specific oligo was able to partially compete with the radioactive oligo 2 (lane 9).

Krox-20:Possible Common Regulatory Element Between Cartilage & Glia

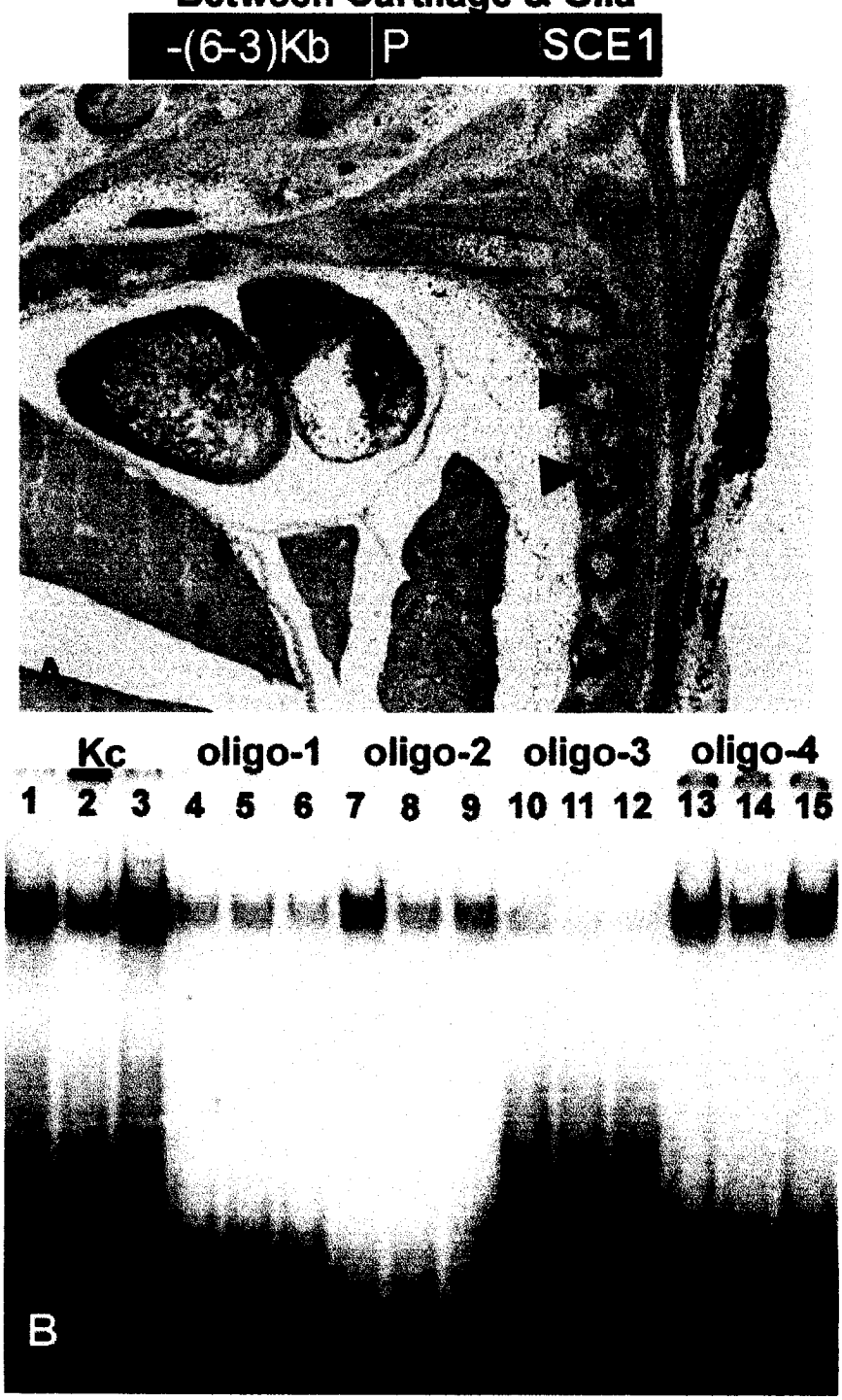


Figure 15: Sagittal sections of transgenic mouse embryos at E13.5.

Transgenes for A&C and B&D are shown on top of A and B respectively. A&B: X5 magnification of tongue; inset in B: X5 magnifiaction of tongue of embryo bearing a reporter construct which contains only 200bp of the full 1kb sequence of M4. Note the presence of LacZ positive cells in the tongue in the inset as compared to their absence in B. C: X40 magnification of muscles of the back showing LacZ positive myocytes. D: X5 magnification, arrowheads: muscles of the back with very few LacZ positive cells. Note in D the LacZ positive cartilage cells of vertebral bodies infront of the muscles of the back.

M4 Suppress Ectopic Expression in Muscles of Tonque & Back

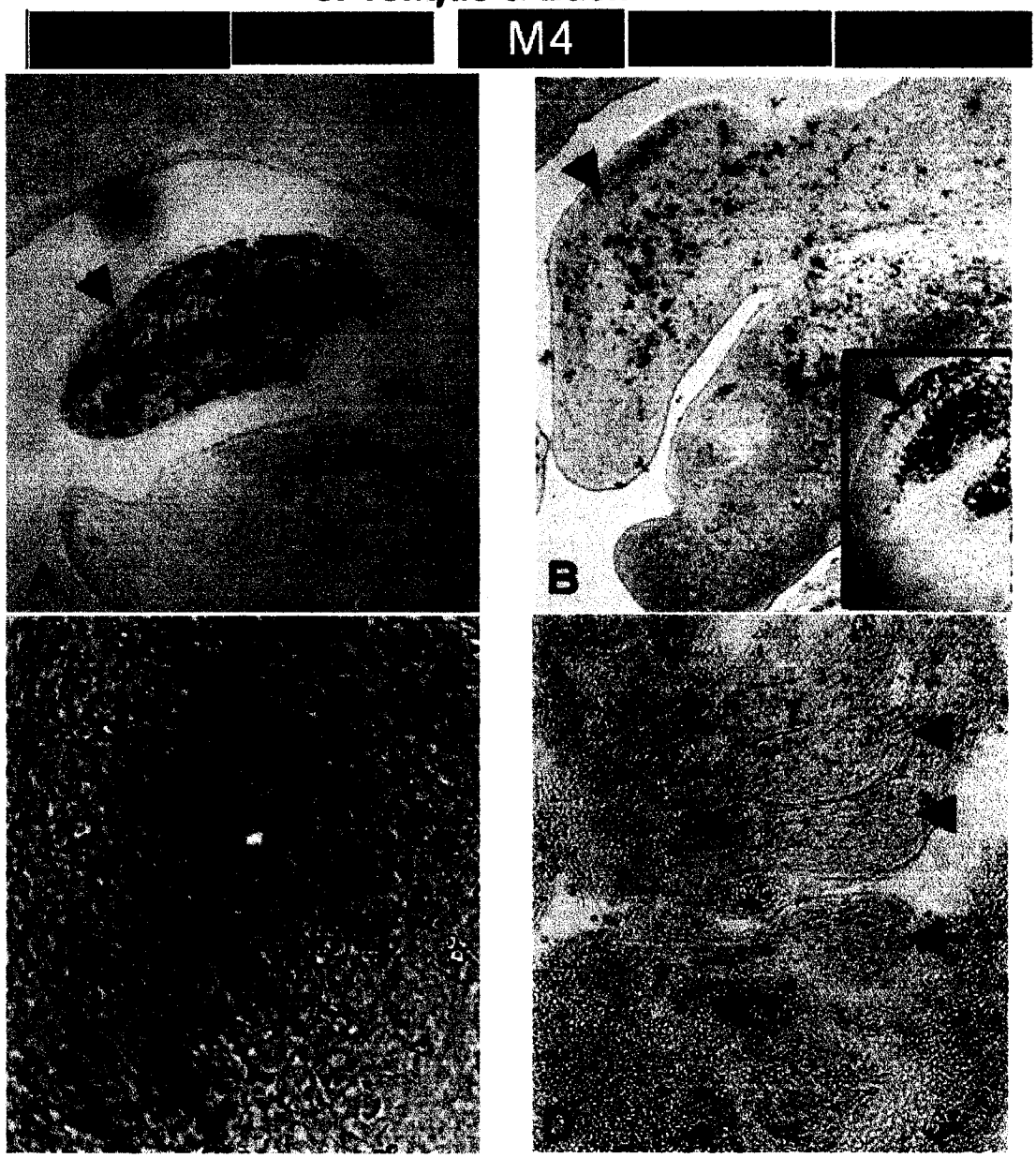


Figure 16: Sagittal sections of transgenic mouse embryos at E13.5.

Transgenes for A&C and B&D are shown on top of A and B respectively. A&B: X5 magnification of tongue (arrows); A, C: eosin stain. Refer to Fig.6A for the section of the entire body and note the intensely stained tongue. C&D: X5 magnification of back muscles (arrowheads). Note the intense LacZ expression in tongue and back muscles of HSP-HPRT construct (A,C) as compared to the near absence of any LacZ tracing in M3-HSP-HPRT construct (B, D)

M3 Suppress Ectopic Expression in Muscles of Back & Tongue

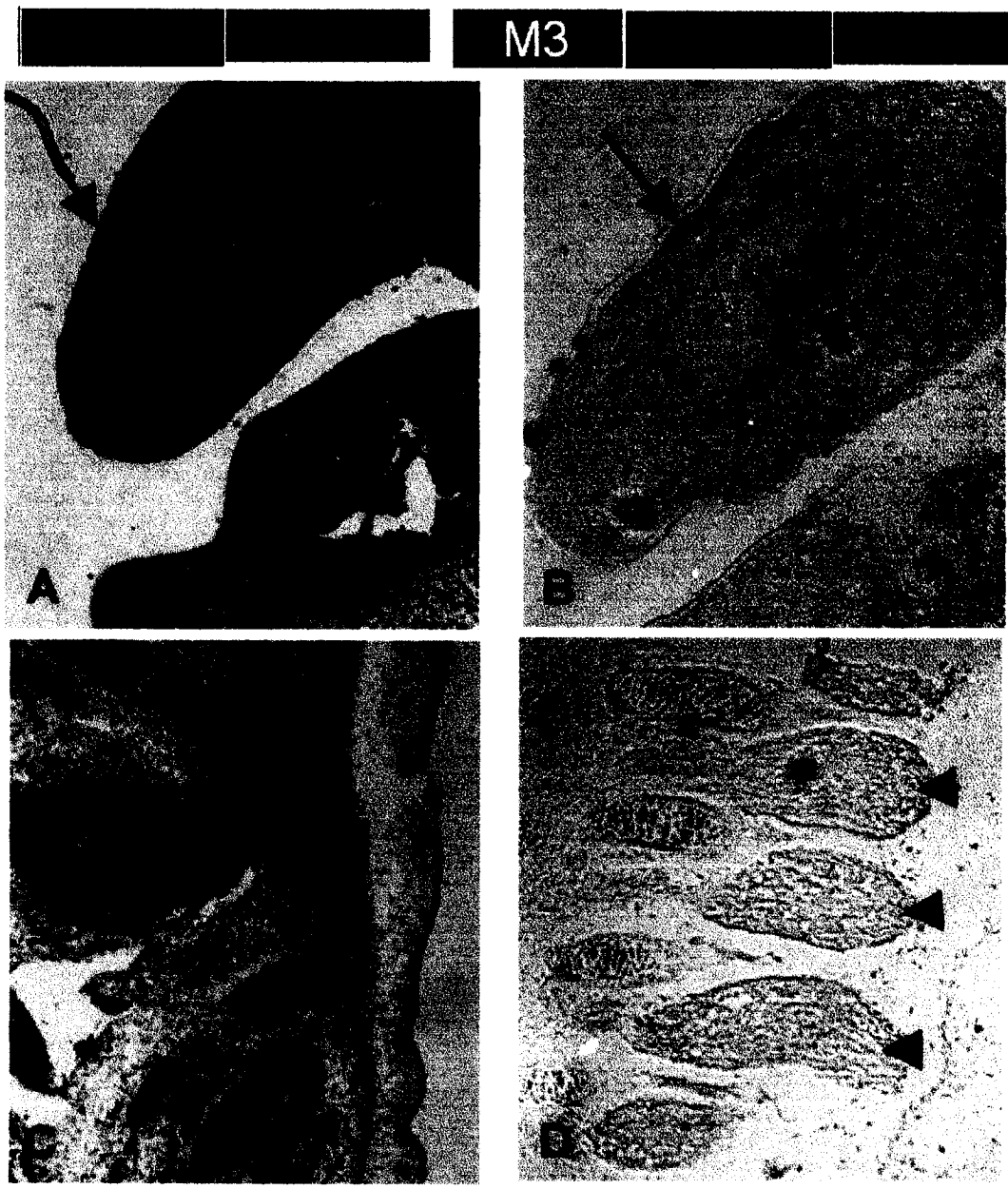


Figure 17: A-H: whole mount photomicrographs of hprt-targeted endothelial nitric oxide synthase (eNOS) LacZ heart (A), brain (B), Flt-1 LacZ heart (C), brain (D), von-Willebrand factor (VWF) LacZ heart (E), brain (F), myelin basic protein (mbp) lacZ heart (G), brain (H). Incubation of whole organs with 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside (X-Gal) substrate reveals LacZ staining as shown. X-gal staining is present in the superficial arteries of brain as shown in B, D, F & H. Whether the transgene is an endothelial specific or muscle specific promoter (e.g. eNOS, Flt-1, VWF) or not (e.g. mbp), expression is still noted in blood vessels and cardiomyocytes.

LacZ Expression of HPRT-docked Constructs in Heart & Blood Vessels of Brain

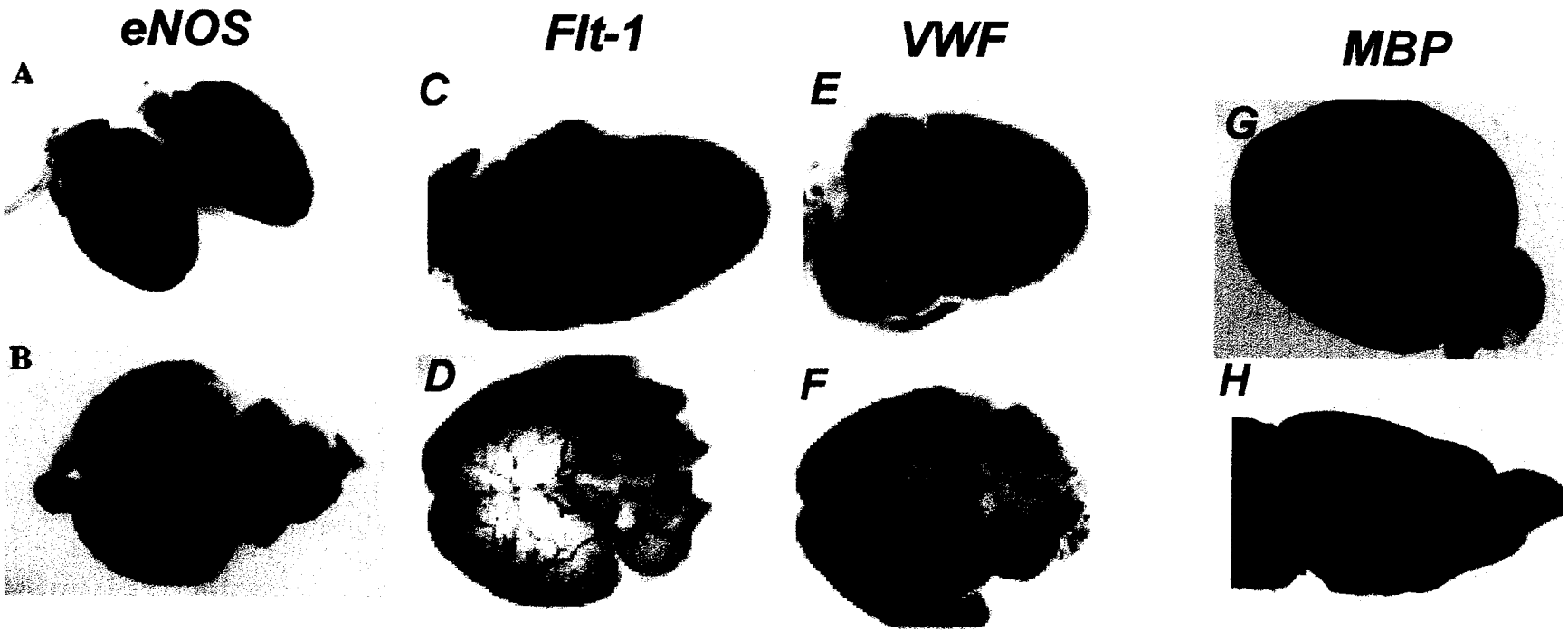
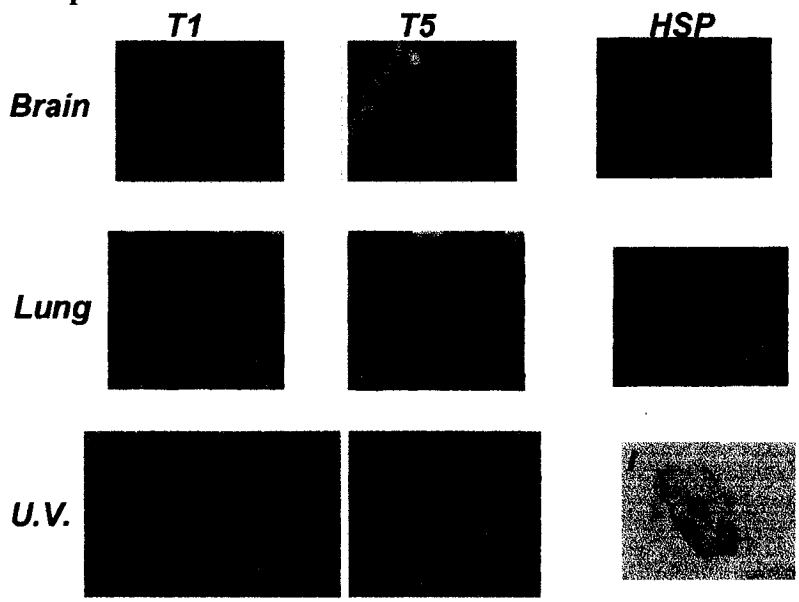


Figure18: Sections of T1 (Tie2 promoter-LacZ) for A,C&E, T5 (Tie2 promoter-LacZ-enhancer) for B,D&F and HSP (minimal promoter of heat shock protein-LacZ) for G,H&I. Brain (A, B&G), Lung (C, D&H) and umbilical vessels (G, H&I). Arrows in A, B and G indicate positively stained vessels. Magnifications are as follows: A, B: X100- C, D: X200- E, F: NA, G: X10, H: X40, I: X10. F: sections were incubated with thrombomodulin (endothelial cell-specific marker) to verify that X-Gal staining is confined to endothelial cells. Note the LacZ positive cells in specimen derived from both endothelial specific promoters (T1 and T5) and non-endothelial specific promoter (HSP).

LacZ expression of HPRT-docked constructs in Blood Vessels



Tables 1: Summary and conclusion1: hprt docking site influences the expression of docked constructs in the cardiomyocytes and blood vessels.

Summary & Conclusion 1

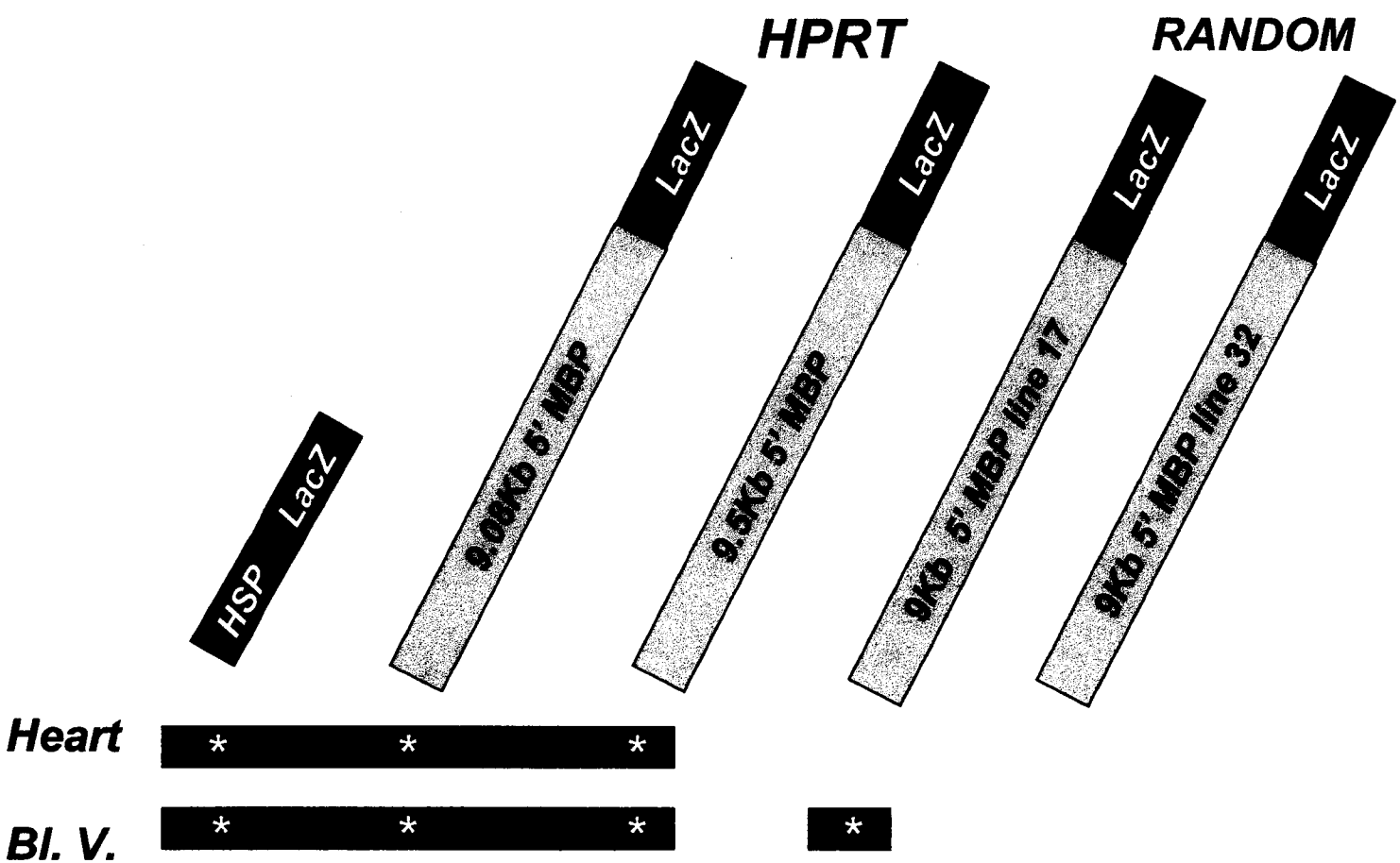


Table 2: Summary and conclusion 2: The regulation of myelination and cartilage gene expression may share common regulatory elements.

Summary & Conclusion 2

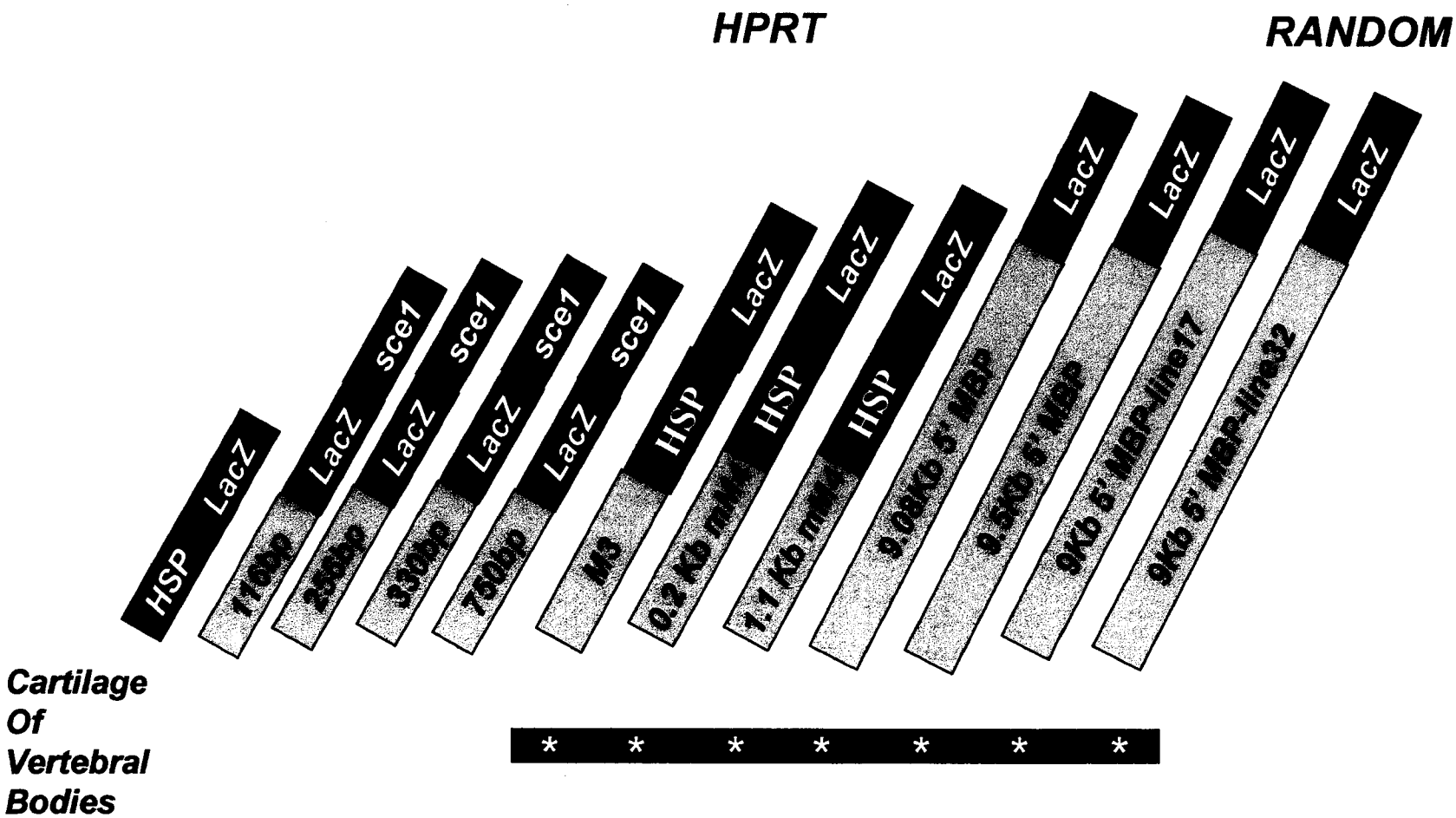
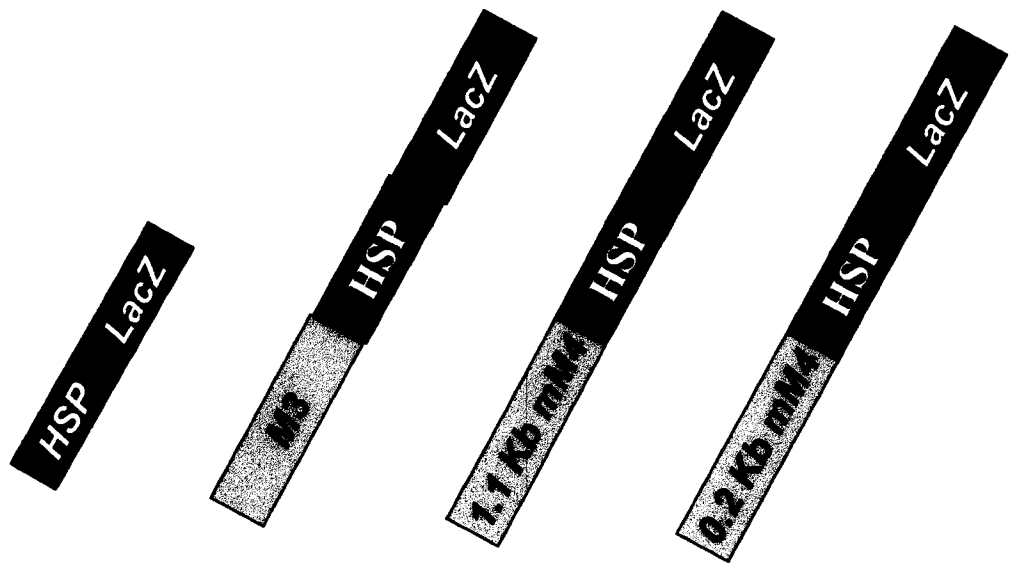


Table 3: Summary and conclusion 3: Possibility of repressors in M3 and M4 sequences.

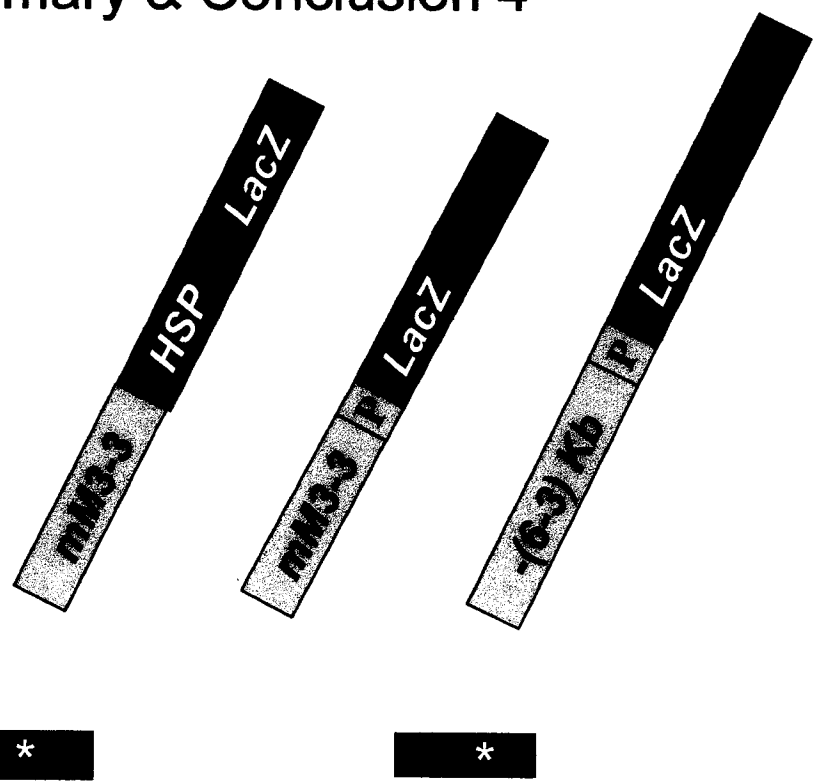
Summary & Conclusion 3



muscles of tongue	*			***Faint***
muscles of back	*			
level of B-gal activity	+++	+	+	+

Table 4: Summary and conclusion 4: Krox-20 binding site in the – (6-3) Kb alleviated the repression imposed on M3 from the minimal mbp promoter (P) in cartilage cells of vertebral bodies.

Summary & Conclusion 4



Cartilage of Vertebral Bodies

SUMMARY

1- The environment surrounding the hprt docking site influences the expression of inserted constructs. Appropriate targeting to oligodendrocytes and Schwann cells is conferred to reporter constructs by mbp regulatory sequences. Further, the majority of the diverse cell types in the developing and mature mouse do not "ectopically" express such reporter constructs. However, this investigation has revealed that the hprt-associated enhancer activity drives transgene expression in cardiomyocytes and blood vessels of developing and mature mice. Consequently the hprt transgenesis strategy does not appear appropriate for the analysis of cardiovascular or muscle regulatory mechanisms (as reported by ref. 2&3)

2- Features of myelin and cartilage gene regulation are shared. Unknown transcription factors (possibly including Krox-20) might be shared components of the regulatory mechanism expressed in these two cell types during development.

3- M3 and M4 are both known to contain enhancer elements sufficient to drive reporter gene expression in myelinating cells. My observations also point to the possibility that they may contain repressors of ectopic expression. A study such as the one I have carried out was required to reveal this type of regulatory element.

CONCLUSIONS

The results of this investigation contribute to our understanding of the regulatory environment associated with the hprt docking site. This insight will be useful for the interpretation of all past and future investigations that make use of this strategy. This understanding may also lead to modifications of the hprt docking site that minimize or eliminate such insertion side effects. In addition, new insights into the complex nature of mbp gene regulation have been obtained. Through this investigation, I have extended the characterization of regulatory functions from enhancers to include the activity of putative cell-type specific repressors that may serve to control the overall expression of the endogenous mbp locus.

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