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Expression of a Foreign Gene in a Line of Transgenic Mice Is Modulated by a Chromosomal Position Effect

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Unusual aberrant expression of a foreign gene in a particular transgenic mouse line is often attributed to chromosomal position effect, although proof of this is lacking. An alternative explanation is that expression has been modified by the arrangement of multiple copies of the foreign gene at the insertion site or by mutation or gene rearrangement. We have distinguished between these explanations in the case of one particular transgenic line by recovering the aberrantly expressed foreign DNA and reintroducing it into the mouse genome to produce secondary transgenic mice. The expression pattern of the gene in the secondary transgenic mice was normal, showing that this case of aberrant expression is due to a chromosomal position effect.

To date, most transgenic mice have been produced by embryo pronuclear microinjection. As far as is known, the foreign DNA is integrated into the chromosomes at random. Integration usually occurs at only one chromosomal site in a given embryo, but the number of copies of the input DNA that become integrated varies from one to hundreds (see, for example, Lacy et al. [22]). When integration occurs at more than one site, the different sites can be segregated from each other by mating the founder (G_0) animal to nontransgenic mice. A set of related mice all of which carry the same configuration of foreign DNA at the same integration site is a transgenic line.

Several studies have demonstrated that in transgenic mice derived from different microinjected embryos, the same foreign gene is expressed to different degrees. Although the number of copies of the foreign gene varies greatly from one integration site to another, there is no general relationship between copy number and level of expression (9, 17, 23). When the foreign gene is expressed in more than one tissue, variation in expression may confer on the mice different patterns of tissue-specific expression.

Because there is considerable variation in expression between individual mice, comparisons are most convincing when transgenic lines are established. By measuring expression levels in several individuals of a line, environmental variation and variation due to genetic background is canceled out. We previously reported results obtained in this way (1). The DNA fragment that we introduced into the mouse genome consists of a 2.2-kilobase-pair (kb) major urinary protein (MUP) gene promoter and 5′-flanking region linked to the herpesvirus 1 (HSV) thymidine kinase (TK) gene coding region. At its 3′ end, beyond the HSV TK gene polyadenylation site, the DNA fragment carries a copy of the bacterial SupF gene. In four of five transgenic lines, HSV TK activity and HSV TK mRNA are present in the liver, the tissue in which the endogenous MUP gene promoter is active. However, four lines have high levels of HSV TK activity in the preputial gland, and all five have high levels in the testis. Three of the lines are of particular value because each has a small number of foreign DNA sequences at the insertion site (1, 2, and 3 copies respectively). One of these lines (line 64) has negligible HSV TK activity in the preputial gland. The expression pattern of line 64 mice has been the same for four generations of outcrossing. Here we report experiments exploring the cause of the aberrant preputial gland expression in line 64.

The foreign DNA inserted into the chromosome of a transgenic line is the result of a unique set of molecular events that occur in the pronucleus after microinjection. A small set of foreign DNA molecules (effectively two in the case of line 64) is joined together and becomes integrated into a site on a single chromosome. In principle, unusual expression of the foreign DNA may be due to either of two sorts of effects. One class of possible effects relates to the foreign DNA itself; these include point mutation, damage due to breakage and rejoining, and effects due to the proximity and juxtaposition of more than one copy of the foreign DNA fragment. A second class of possible effects relates to the chromosomal environment into which the DNA has been inserted; regions of the same chromosome, which may be close to or far from the insertion site, may modulate the expression of the gene, perhaps in the way that enhancers modulate promoter activity or possibly in other ways. This second class has been called chromosomal position effect.

To distinguish between the two classes of effects, one a property of the foreign DNA itself and the other a property of the chromosome in the neighborhood of the foreign DNA, we recovered the foreign genes from line 64 DNA by molecular cloning and reintroduced them by embryo pronuclear microinjection into the genome of mice. We refer to first cycle transgenic animals such as line 64 mice as primary transgenic mice and to transgenic mice produced in the second cycle as secondary transgenic mice. If the arrangement of the DNA at the insertion site of the primary transgenic animal is the cause of its unusual expression, we would expect to observe the same expression pattern, i.e., a lack of expression in the preputial glands, in the secondary transgenic mice. If, on the other hand, the unusual expression is due to a chromosomal position effect, then the expression pattern of the secondary transgenic mice should be the same, in general, as that of primary transgenic mice, i.e., with expression occurring in the preputial glands in most cases. The results show that the lack of expression in

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the preputial glands of line 64 mice is due to a chromosomal position effect.

MATERIALS AND METHODS

Transgenic mice and TK assays. Transgenic mice were produced by pronuclear microinjection as described previously (1). The same report contains a description of the TK assay procedure. Transgenic mice were routinely identified by the polymerase chain reaction, using tail DNA as the template and primers specific for the SupF gene.

Recovery of the foreign gene insertion from line 64 DNA. High-molecular-weight DNA was prepared from a crude preparation of liver nuclei by standard sodium dodecyl sulfate-proteinase K extraction and deproteinization. A 200-μg sample of DNA was partially digested with *Mbo*I and fractionated on a 10 to 40% sucrose density gradient. Fractions containing 15- to 25-kb fragments were identified by agarose gel electrophoresis, pooled, and used for library construction. DNA from the bacteriophage lambda vector EMBL3A (11) was digested with *Bam*HI, the DNA was recovered and annealed, and the vector arms were isolated by gradient centrifugation. Each ligation was performed with 1 μg of vector arms and 0.2 μg of fractionated line 64 DNA. In vitro packaging with Gigapack Plus extract (Stratagene) gave a yield of 3.8×10^6 plaques per μg of mouse DNA when assayed on a $SupF^+$ *Escherichia coli* host (ED8654 [3]). When plated on a Sup^0 host (MC1061 [5]), the number of viable plaques obtained was lower by a factor of approximately 2.5×10^5. Of 12 plaques, 8 reacted with an HSV TK gene probe.

DNA blotting procedures. To map restriction enzyme sites to genomic DNA, 10-μg samples were digested appropriately and run on 0.8 or 0.5% agarose gels. The DNA was depurinated and transferred to Hybond-N (Amersham Corp.) and fixed by UV cross-linking. HSV TK-specific bands were detected by using the *Pst*I fragment of the HSV TK gene, labeled with ^{32}P to a specific activity of 10^8 to 10^9, and the hybridization method of Church and Gilbert (6).

Recombinant clones were mapped by using probes specific for the HSV TK gene, the MUP BS6 gene promoter fragment, and the SupF gene. Transfers were by the alkaline blotting method of Reed and Mann (24), and hybridization was in 1× SSC (0.15 M NaCl, 0.015 M sodium citrate) containing 0.3% low-fat dried milk and 50 μg of denatured salmon sperm DNA per ml.

RESULTS

Structure of the foreign DNA and patterns of tissue-specific expression in lines 58, 64, and 78. The structures of the chromosomal insertions of foreign DNA in the primary transgenic lines 58, 64, and 78 are summarized in Fig. 1. The structure of the line 64 insertion was determined by mapping bacteriophage lambda clones (see below). Those of the line 58 and line 78 insertions were deduced from Southern blots of genomic DNA (data not shown). Line 64 contains two copies of the foreign DNA in direct tandem arrangement. The two copies are separated by a short (about 500 base pairs) intervening DNA sequence of unknown origin. The two copies and the flanking regions of chromosomal DNA are contained within a 17.5-kb *Hin*dIII fragment. Line 58 contains three copies of the foreign DNA in direct tandem arrangement, with short terminal deletions at the junction points between fragments. All three copies are contained within a 20-kb *Hin*dIII fragment. Line 78 contains about 1.3

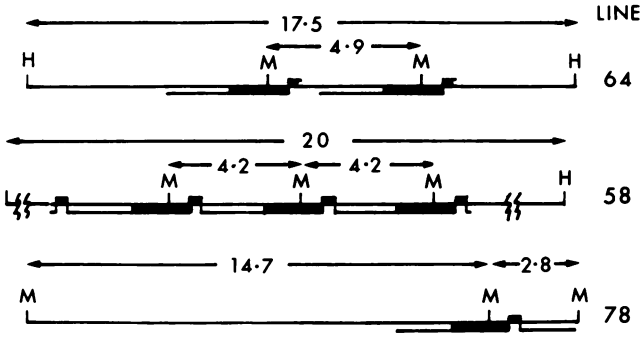

FIG. 1. Structures of the foreign gene chromosomal insertions in primary transgenic lines. The map of the line 64 insertion is based on cloned sequences (Fig. 2); the others are based on DNA blotting data. The lengths shown are in kilobases. Symbols: continuous line, mouse chromosomal DNA; open box, MUP BS6 gene promoter region; filled box below line, HSV TK structural gene; filled box above line, SupF sequence; open-ended box, uncertainty about the precise junction point; vertical wavy line, shortening of the map. Restriction enzyme sites: H, *Hin*dIII; M, *Sma*I.

copies of the foreign DNA, again in direct tandem arrangement and with a short deletion at the junction between the nearly complete 5′ copy and the truncated 3′ copy. Two *Sma*I fragments with a combined length of 17.5 kb contain the line 78 insert and extensive flanking regions. From these results it is clear that each line carries a small number of foreign genes in a unique arrangement.

The pattern of expression of HSV TK in these three lines and two others, each of which contains 10 or more copies of the gene, has been described in some detail (1). The expression observed in the liver in each case (Table 1) was expected in that the resident MUP gene promoters are active in liver. Expression was also observed in two tissues in which the resident MUP gene promoters are not active, namely, the testes and preputial glands. Expression in the preputial gland appears to be a function of the MUP gene promoter, since it has been observed in other transgenic mice carrying different MUP gene constructs (19). Of the five lines originally described, four express the hybrid gene in the preputial glands and all five express it in the testis (1). In fact, HSV TK activity in preputial glands of line 64 mice was above the background assay level observed with nontransgenic preputial glands (Table 1). However, the activity was respectively 20 and 60 times less than the activities present in line 58 and line 78 preputial glands. The liver activity level in line 64 mice was also lower than activities in lines 58 and 78. The levels of testis activity were much the same in the three lines.

Recovery of the line 64 foreign DNA by molecular cloning. Several previous studies have used the bacterial SupF gene to facilitate recovery of foreign DNA from transfected (33) and retrovirus-infected (26, 32) cells, and the principle of the method is well known (30). We have routinely included a 3′-terminal SupF gene in foreign DNA introduced into the mouse germ line and have presented evidence that in at least one case it does not influence expression of the gene with which it is associated (1).

Clones carrying the SupF gene and positive to a HSV TK gene probe were isolated from a genomic library of line 64 DNA constructed in the lambda vector EMBL3A (see Materials and Methods). The restriction sites of the cloned fragments were congruent with each other and with a restriction site map of the relevant region of line 64 genomic DNA, showing that the DNA had not become rearranged during

TABLE 1. Expression of the BS6 TK-SupF hybrid gene in tissues of primary and secondary transgenic male mice

Group	TK activity[a] (pmol/min per mg of protein)		
	Testis	Preputial gland	Liver
Control	0.25 ± 0.08 (6)	0.23 ± 0.10 (7)	0.02 ± 0.004 (7)
Primary transgenic line			
40[b]	140 ± 6.7 (3)	12.0 ± 0.39 (2)	0.05 ± 0.04 (4)
46[b]	139 ± 17.6 (3)	83.1 ± 19.9 (2)	1.9 ± 0.32 (8)
58[b]	31.1 ± 10.5 (3)	9.9 ± 2.6 (3)	5.8 ± 1.15 (3)
78[b]	62.4 ± 8.4 (3)	32.1 ± 5.3 (3)	12.7 ± 1.85 (3)
64	40.8 ± 2.9 (7)	0.65 ± 0.17 (7)	1.3 ± 0.23 (7)
Secondary transgenic mouse nos. (cloned line 64 insert reintroduced)			
A6[c] (64.1.2)	36.7	17.6	0.01
A17[c] (64.1.2)	24.9	47.9	3.14
A45 (64.1.2)	330	196	19.6
A60 (64.1.2)	38.8	80.3	2.26
B27 (64.2.1)	50.6	7.8	0.42
B50[c] (64.2.1)	5.6	1.4	1.20

[a] Mean ± standard error (number of observations).
[b] From Al-Shawi et al. (1).
[c] Fertile nontransmitting male; other males were sterile.

reisolation (Fig. 2). One clone (not shown) had the structure expected to result from a reciprocal recombination event between nonidentical copies of the foreign DNA during bacteriophage multiplication; that is, it contained a single copy of the foreign DNA sequence flanked by the same regions that flanked the tandem gene pair in the other clones.

Expression pattern in secondary transgenic mice of the foreign DNA insertion recovered from line 64. Clones 1.2 and 2.1 were chosen for introduction into the mouse genome. Two clones were used rather than one because a single clone might have suffered a mutation during recloning and growth in the *E. coli* host. If the two clones behave in the same way in the secondary transgenic mice, this may be assumed not to be the case. Both clones contained the entire (9 kb) tandem insertion and similar amounts of flanking chromosomal DNA (Fig. 2). In each case, the entire cloned fragment, consisting of the tandem insert and the flanking DNA, could be separated from the vector arms by digestion with *Sal*I, which cleaves the small polylinker sequences of EMBL3A but does not cleave either cloned fragment. The fragments were freed from vector arms by agarose gel electrophoresis and introduced into mouse embryos by direct pronuclear microinjection.

An analysis of HSV TK activity in tissues of six G_0 males is summarized in Table 1. Three of these mice were fertile but did not father transgenic offspring, and the others were sterile. Both types of reproductive behavior are characteristic of primary G_0 transgenic males containing the BS6 TK-SupF hybrid gene; of five primary G_0 males, two were fertile but nontransmitting and three were sterile (1). Fertile nontransmitting G_0 males are mosaics, and males of the five established lines are sterile (1). HSV TK activity was present in the preputial glands of all six males (Table 1). The activity levels in different G_0 males were highly variable, but the highest value (male A45) was greater than the highest value observed among the primary transgenic lines (Table 1 and reference 1). Furthermore, the distribution of values did not overlap that of seven line 64 males. The significance of this is the greater since the secondary transgenic male with the lowest preputial gland expression is a mosaic. Thus, the negligible enzyme activity in the preputial glands of line 64 mice was not preserved when the tandem gene pair was recovered and introduced into secondary transgenic animals. It may therefore be concluded that the very low preputial gland activity is due to the chromosomal environment around the insertion site in line 64, in other words, to chromosomal position effect. Since the cloned DNA contained the regions immediately flanking the line 64 foreign DNA, the position effect presumably originates from more distant regions of the chromosome.

The activity in the liver and testis of the secondary transgenic G_0 males was also variable (Table 1). In this respect, the secondary transgenic lines resembled the five primary transgenic lines. Interestingly, in both primary and

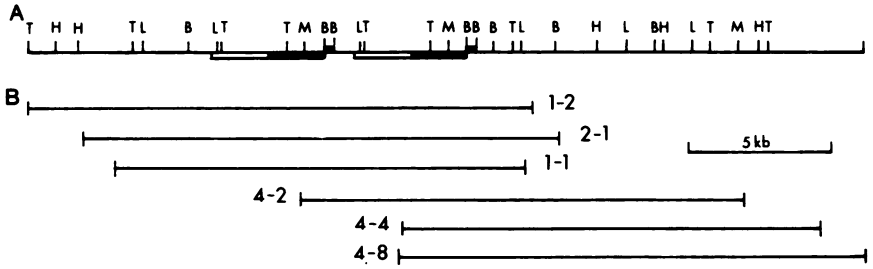

FIG. 2. (A) Cloned segments of line 64 DNA. Symbols: continuous line, mouse chromosomal DNA; open box, MUP BS6 gene promoter region; filled box below line, HSV TK structural gene; filled box above line, SupF sequence. Restriction enzyme sites: B, *Bam*HI; H, *Hin*dIII; T, *Sst*I; M, *Sma*I; L, *Bgl*II. The precise junction points of the two foreign DNA fragments with the chromosome and with the extra fragment of DNA between them have not been precisely mapped. (B) Length of sequence present in six lambda EMBL3A recombinants.

TABLE 2. Expression of the BS6 TK-SupF hybrid gene in tissues of second-generation secondary transgenic male mouse lines

G_1 no.	TK activity (pmol/min per mg of protein)		
	Testis	Preputial gland	Liver
A49.1	121	47.2	0.82
B65.2	387	251	27.1
B56.5	0.74	40.4	0.00
B56.11	0.82	56.3	0.02
B56.9	3.74	28.3	0.06

secondary transgenic mice, the levels of expression in the three tissues varied independently of each other. TK activity levels were determined in five other tissues, lachrymal gland, submaxillary gland, brain, skeletal muscle, and kidneys. As in the case of the primary transgenic mice (1), the TK activities in these tissues were very low and indistinguishable from those observed with control nontransgenic mice (data not shown).

Structures of the foreign DNA in the secondary transgenic mice. As an alternative, the loss and subsequent recovery of peputial gland expression might be explained by DNA rearrangement. If the particular arrangement of the genes in line 64 caused the loss of expression, for example by interactions between the two genes, then expression might be recovered in the secondary transgenic mice if the genes became rearranged in such a way as to reverse the original effect.

It is inherently unlikely that an appropriate rearrangement would have occurred in all of the nine secondary transgenic mice examined (Tables 1 and 2). However, it is necessary to show that this DNA fragment does not undergo a consistent rearrangement and also to demonstrate that the secondary transgenic mice carry the intended DNA fragment. The organization of the DNA in several secondary transgenic mice was therefore examined by Southern blot analysis, using as a probe a fragment of the HSV TK gene. Digestion with *Sst*I released from line 64 DNA four fragments that hybridized with the probe. Two of these were 2.2 kb in size, and the others were 2.6 and 2.8 kb. Digestion with *Bgl*II released probe-positive fragments of 4.9 and 5.5 kb (Fig. 3A). The presence of *Sst*I or *Bgl*II fragments of other sizes would indicate that rearrangements had occurred, the most likely of which is a junction between the foreign DNA and the chromosomal DNA of the secondary transgenic animal. The DNA of four G_0 secondary transgenic mice was examined after separate digestion with *Sst*I and *Bgl*II. In each case the predominant hybridizing bands were the expected bands (Fig. 3A), showing that most of the foreign DNA fragments were not rearranged in a way that disturbed these patterns. The *Bgl*II digests of DNA samples A17 and B27 each showed an additional faint hybridizing band (Fig. 3B). These bands probably represent junction fragments between chromosomal DNA and the foreign DNA insert.

The lambda 2.1 recombinant contains a single *Hin*dIII site. If the DNA in the secondary transgenic lines takes the usual form of a direct tandem array, digestion with *Hin*dIII will produce mainly unit-size HSV TK-positive fragments. In addition, each insertional array will have a flanking junctional fragment of a different length at each end. In most cases, such fragments may be expected to hybridize with the probe. The same is true of lambda 1.2, which has two *Hin*dIII sites close together, except that the predominant fragment will be shorter than unit length by an amount equal to the 0.8-kb length of DNA between the two *Hin*dIII sites. The DNA of three secondary transgenic mice was examined after *Hin*dIII digestion. In each case, the expected band was present (Fig. 3B and data not shown).

Male descendants of secondary transgenic females. The five primary transgenic lines that carry the BS6 TK-SupF hybrid gene are all male sterile, and the founder in each case was a transgenic female (1). All of the male transgenic mice in each of the primary lines inherit their foreign genes from the mother, and it is therefore possible that the foreign gene insert in line 64 is genetically imprinted (15, 25, 29, 37) during female germ line maturation. If so, the property of being imprinted must be peculiar to the insertion of foreign DNA in line 64, since the foreign gene is expressed in the preputial glands of the other primary transgenic lines. Imprinting, if it occurs, might be due either to a chromosomal position effect or to the arrangement of the foreign DNA at the insertion site. In either case, the secondary transgenic G_0 males would

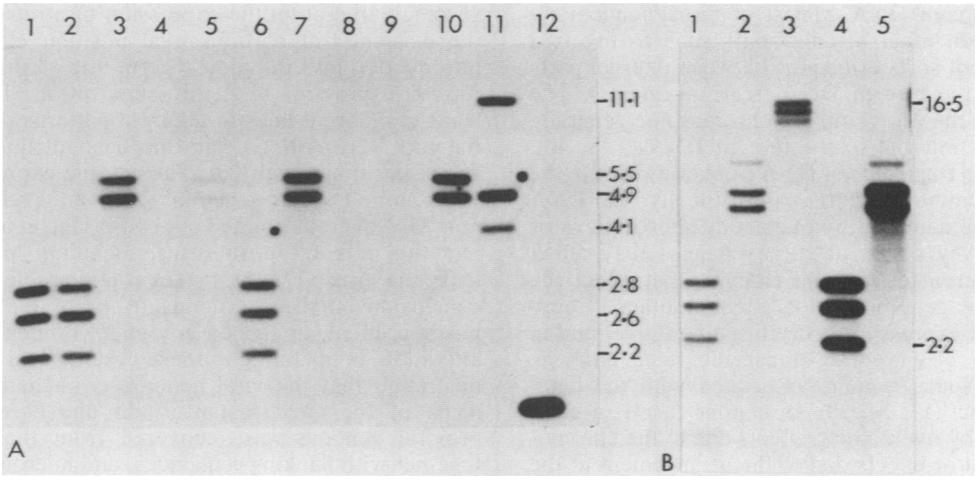

FIG. 3. Southern blot analysis of G_0 secondary transgenic mice. (A) *Sst*I and *Bgl*II digests. Lanes: 1, lambda 2.1 × *Sst*I (five-copy marker); 2, A6 × *Sst*I; 3, A6 × *Bgl*II; 4, A17 × *Sst*I; 5, A17 × *Bgl*II; 6, B27 × *Sst*I; 7, B27 × *Bgl*II; 8, B50 × *Sst*I; 9, B50 × *Bgl*II; 10, lambda 2.1 × *Bgl*II (five-copy marker); 11, lambda 2.1 × *Sma*I (five-copy marker); 12, kilobase size ladder (Bethesda Research Laboratories). (B) Lanes: 1, A17 × *Sst*I; 2, A17 × *Bgl*II; 3, A17 × *Hin*dIII; 4, B27 × *Sst*I; 5, B27 × *Bgl*II.

not show the imprinted phenotype (i.e., the same phenotype as line 64) because the foreign genes that they carry have not passed through the female (or any) germ line since they were recovered by molecular cloning. Thus, up to this point we have not excluded the possibility that negligible expression in the preputial gland is due to a gene arrangement or to some other alteration that invites imprinting.

This possibility was tested by analyzing the progeny of three secondary G_0 transgenic females. Three transgenic male descendants of B56 and also transgenic male descendants of A49 and B65 were analyzed (Table 2). As was the case with two of the G_0 males, expression in the preputial glands of all of these males was high. This experiment excludes the possibility that the low preputial gland expression in line 64 is related to a genomic imprinting effect dependent on the structure of the foreign gene or to the immediate chromosomal environment. It remains possible that the chromosomal position effect that we have identified acts through imprinting, but this does not affect the central conclusion that it is a position effect.

DISCUSSION

When a foreign DNA fragment is introduced by embryo pronuclear microinjection, the chromosomal integration sites usually contain many copies of the fragment. These are generally arranged in a direct tandem array (other arrangements are found infrequently), and the arrays probably arise by homologous recombination between different identical copies of the foreign DNA fragment. The process of integration is essentially stochastic and cannot be controlled. Consequently, any set of transgenic mice made by introducing the same foreign DNA fragment is inevitably made up of individuals that carry different numbers of fragments at the chromosomal integration site (or at each site if there are more than one). It is generally found that the level of expression of the foreign gene is independent of its copy number. Underlying this, each individual chromosomal integration complex is associated with a different level of expression. As we (1) and others (9, 39) have shown by comparing transgenic lines, the expression level is a heritable property of the integration complex.

Integration complexes differ in a number of ways. First, there is the copy number referred to above. Then, although most arrays of foreign DNA consist of direct tandem repeats, some contain other arrangements such as inverted repeats and different sorts of fusions between internal parts of two copies of the foreign DNA (rearrangements). The junctions between the array and the chromosome generally involve sequences internal to the foreign DNA (7, 8, 40). Arrays may contain fragments of DNA from elsewhere in the genome or from unknown sources (40). Finally, the DNA may in principle be damaged by nucleotide substitutions or small deletions. Any or all of these changes may affect expression in different cases, and changes that affect the expression of one gene may not affect another. Copy-number-dependent expression in erythroid fetal liver and in T cells has been observed in transgenic mice carrying particular chromosomal regions associated with the beta-globin and CD2 loci (13, 14, 28). In neither case is it clear whether these act by overcoming effects due to the chromosomal environment or effects due to the arrangement of the gene array, where the copy number is greater than one.

The question we have addressed here is whether the level of expression is in some way dictated by chromosomal location, as opposed to the effects listed above. We selected a transgenic line with unusual expression and with a low copy number so that the foreign DNA could be isolated easily. The expression pattern of the foreign gene in this line is unusual in that the level of expression is extremely low in just one of the three tissues in which it is expressed in other lines. Thus, the gene is not totally inactive as it would be if it had suffered a major lesion or if it were uniformly methylated. When the foreign gene pair was reisolated from line 64 and reintroduced into secondary transgenic mice, expression in the preputial gland was restored. The same general approach was previously used to study the cause of the inactivation of an HSV TK gene that had become expressionally inert while inserted into the chromosomes of an L-cell line. In that case too, the activity of the gene was restored when it was recovered and reintroduced into the genome of a cell (4). At least two of our secondary transgenics showed some rearrangement of the foreign DNA. Any one of these rearrangements could in principle have reversed the effect of a previous rearrangement. It is highly unlikely, however, that this could have happened in every case, while on the other hand it is very likely indeed that the foreign genes were integrated into entirely new chromosomal locations in the secondary transgenic lines. Thus, the conclusion that negligible preputial gland expression in line 64 is due to a chromosomal position effect is a strong one. Since the immediate chromosomal flanking regions were reisolated and reintroduced along with the insert, the position effect must be due to influences originating from more distant parts of the chromosome.

P-element-mediated transformation of the *Drosophila* genome leads in most cases to the insertion of solitary foreign DNA molecules into the chromosome. This system therefore lends itself to the detection of chromosomal position effects without the complication of tandem arrays and major rearrangements of the foreign DNA. Moderate negative effects attributed to chromosomal position have been reported (12, 36). In these cases, mutational changes and small rearrangements of the foreign DNA were not excluded as possible causes of the reduced expression.

The provirus form of a retrovirus is inserted into the chromosome by a special mechanism at any one of a very large number of potential sites (32) as a solitary sequence between direct repeats of the proviral long terminal repeat. Since it does not form integrated arrays, evidence of line-specific variation in the expression of a retrovirus suggests the existence of chromosomal position effects (34). The introduction into the mouse germ line of single copies of a retroviral provirus (e.g., Moloney murine leukemia virus) leads in different lines to different patterns of expression of the viral RNA (20, 21). This has been attributed to chromosomal position effect (20). The ectopic expression of retrovirus-borne cellular genes (35) may be a related phenomenon. However, as pointed out by Soriano et al. (35), the high mutation rate of retroviruses, including Moloney murine leukemia virus (27, 31), makes it impossible to draw a firm conclusion on these grounds. A proof that chromosomal position effect, or indeed any effect other than mutational alteration, is operating in these cases would require a demonstration that the viral genome is unchanged in at least some of the cited instances. In one case (Mov-3), the proviral genome was recovered from the infected line, together with flanking sequences, and microinjected into the cytoplasm of mouse embryos (18). A single mouse that had integrated the provirus into its genome was recovered and analyzed. Viral sequences were expressed at a high level in skeletal muscle, an ectopic tissue with respect to normal

Moloney murine leukemia virus expression. This experiment has crucial weaknesses, however, as a demonstration of chromosomal position effect, principally because only a single mouse (integration event) was studied. The route by which the microinjected provirus became integrated into the genome of the embryo is unclear, but the flanking chromosomal sequences are known to have been removed in some way. If integration involved transcription from the proviral genome, followed by reverse transcription and integration by the normal retroviral route, then it is possible that the properties of the virus were altered by point mutation, frameshift, or deletion (27). If it was integrated by illegitimate recombination between the incoming provirus (with flanking sequences) and chromosomal DNA, the loss of flanking sequences shows that DNA rearrangement has occurred, and this could have extended to the viral long terminal repeat. We conclude that while effects of chromosomal position may modulate the expression of retroviral proviruses, this explanation has the status of a hypothesis at this time.

In the few well-studied cases of foreign genes that are expressionally inactive in some but not all transgenic animal or cell lines, the foreign DNA was hypermethylated. The HSV TK gene which spawned expressionally inactive variants while resident in L cells was hypermethylated in most cases (4). Similarly, the murine line-specific inactive Mov proviruses studied by Jähner and Jaenisch (21) become hypermethylated during early development and are later selectively demethylated in a line- and tissue-specific manner. As pointed out above, expression of the line 64 foreign hybrid genes cannot be uniformly and irreversibly inactivated by methylation, since these genes are expressed in the liver and testis. However, it is possible that there is tissue-specific hypermethylation (or failure of demethylation) in the preputial glands of this strain. This possibility is now under investigation.

Causes of chromosomal position effect have been indicated or identified in some special cases. None of these findings exclude hypermethylation as a concomitant of inactivation. The powerful selection possible in cell transfection experiments has allowed the identification of chromosomal enhancers in the region of hyperactive foreign genes (2, 16). Similarly, cell lines transfected with retroviruses have been used to study chromosomal position effects. In one study, a transcriptionally active provirus was found to have been integrated at a chromosomal site which, in uninfected cells, was within a DNase-sensitive region of the chromatin (10). In another, an enhancer sequence that activated the proviral LTR was found close to the site of integration of a provirus that was unexpectedly active in an EC cell line (38). These examples relate to gene activation, as do the tissue-specific DNase-hypersensitive sites which activate the transcription of globin and other genes (13, 14, 28). The causes of tissue-specific inactivation, like that observed in line 64, must be of a different nature.

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