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Genetics

Multiplex gene regulation: A two-tiered approach to transgene regulation in transgenic mice

(herpes simplex virus/immediate early transactivation)

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ABSTRACT **Transgenic mice have been used to study gene function and regulation by introducing inducible or tissue-specific transgenes. This approach is generally limited to studying gene function in adult mice since ectopic expression of many interesting genes is disease causing or may be lethal to the developing embryo. To extend the utility of the transgenic mouse system to the early stages of embryogenesis, we have developed a two-tiered method of gene regulation to control transgene expression. Our multiplex gene regulatory system (MGR) allows the establishment of transgenic lines that harbor inducible potentially lethal transgenes. These inducible transgenes are activated only when mated to a second transgenic animal. Induction in the MGR system provides a high degree of temporal and spatial control over transgene expression and should be suitable for engineering "gain of function mutations" for many developmental genes.**

The multiplex gene regulatory (MGR) system consists of a transactivating gene product and its target transresponding promoter sequence. Our system is based on the observed transactivation of the immediate-early (IE) genes of herpes simplex virus (HSV-1) by the transactivator virion polypeptide VP16, (Vmw65, TIF) during HSV-1 infection. All of the IE genes of HSV-1 contain a cis-acting sequence (TAATGARAT) that is necessary and sufficient for transactivation by the VP16 gene product (1–5). The VP16 protein is not a DNA binding protein but mediates transactivation of the IE promoters by forming a protein–protein complex with a cellular DNA binding factor (6–9). The cellular factor involved in this complex appears to be the ubiquitously expressed octamer binding protein (9, 10). The MGR system utilizes these components [the IE promoter of infected cell polypeptide (ICP4) and the VP16 transactivator] to produce the inducible two-tiered regulatory network illustrated in Fig. 1. One transgenic mouse line, the transresponder, contains the gene of interest regulated by the ICP4 promoter element. A second transgenic line, the transactivator, contains the VP16 gene regulated by an inducible or tissue-specific promoter. When the transresponder and transactivator lines are mated, the offspring that inherit both transgenes (boxed region of Fig. 1) should exhibit specific induction of the IE-regulated transgene. The pattern of IE expression in these offspring is dependent on the pattern of VP16 expression. The time and pattern of IE-regulated gene induction are thus limited only by the availability and specificity of the promoter element used to control VP16 expression.

Any transactivator promoter pair can potentially be used to form a two-tiered regulatory network. Khillan *et al.* (13) have demonstrated transactivation of the long terminal repeat (LTR) from the human immunodeficiency virus (HIV) by the *TAT* gene product in the eyes of transgenic mice. Similarly, Nerenberg has shown transactivation of the human T-cell lymphotropic virus type 1 (HTLV-1) LTR by the *tax* gene product (14, 15). In both of these studies, the LTR-regulated target gene exhibited high basal levels of activity, and expression of the transactivator (*TAT* and *tax*) produced deleterious oncogenic phenotypes (14–16). Unlike the oncogenic HIV and HTLV-1 viruses, HSV-1 is a lytic virus with a very broad host range. While small portions of the HSV-1 genome can transform tissue culture cells (17, 18), these sequences are not associated with the VP16 transactivator. Moreover, transformation of tissue culture cells by HSV-1 is independent of viral gene expression. The broad HSV-1 host range and the apparent nontransforming character of VP16 suggested to us that a combination of the IE promoter element and the VP16 transactivator might produce a useful two-tiered regulatory network capable of controlling transgene expression in most murine tissues.

METHODS

Plasmids and DNA Preparation. The plasmid pPOH14 contains the 360-base-pair (bp) *Sma* I/*Bam*HI promoter fragment of ICP4 (IE175) linked to the coding sequences for chloramphenicol acetyltransferase (CAT) along with the simian virus 40 splice and polyadenylylation signals (19). This insert was excised from the plasmid and the 2.1-kilobase (kb) IE–CAT transgene was gel-purified prior to microinjection. The coding and polyadenylylation sequences for the HSV-1 transactivator VP16 were derived from pCA15 (19). The murine neurofilament promoter (NF-L) was kindly provided by Nicholas Cowan (New York University School of Medicine). The 1.7-kb *Bam*HI/*Asu* II fragment of pCA15, which contains 65 bp of 5′ untranslated leader sequences, the entire VP16 coding region, and the endogenous polyadenylylation signals, was subcloned to form pTIF. A 1.5-kb *Hin*dIII/*Sma* I fragment from the 5′ regulatory region of NF-L was subcloned 5′ of the VP16 coding sequences to form pNFT. This 1.5-kb NF-L promoter fragment contains the TAATA box and ≈90 bp of 5′ untranslated leader sequence. The 3.2-kb neurofilament-regulated VP16 transgene (NFT) was excised from the plasmid and gel-purified prior to microinjection.

Microinjection and Southern Blot Analysis. DNA for microinjection was resuspended at 2 μg/ml in 10 mM Tris·HCl, pH 7.5/0.25 mM EDTA and injected into the male pronuclei of (CD-1 × B6D2)F_1 fertilized eggs. Injected eggs were transferred to the oviduct of pseudopregnant CD-1 females (20). Transgenic offspring were identified by Southern blot hybridization (21) using genomic DNA (10 μg) extracted from tail samples. For the IE–CAT lines, the DNA was digested with *Bam*HI or *Pvu* II, both of which cut only once within the

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Abbreviations: MGR, multiplex gene regulatory; IE, immediate early; HSV-1, herpes simplex virus 1; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; NFT, neurofilament-regulated VP16 transgene.

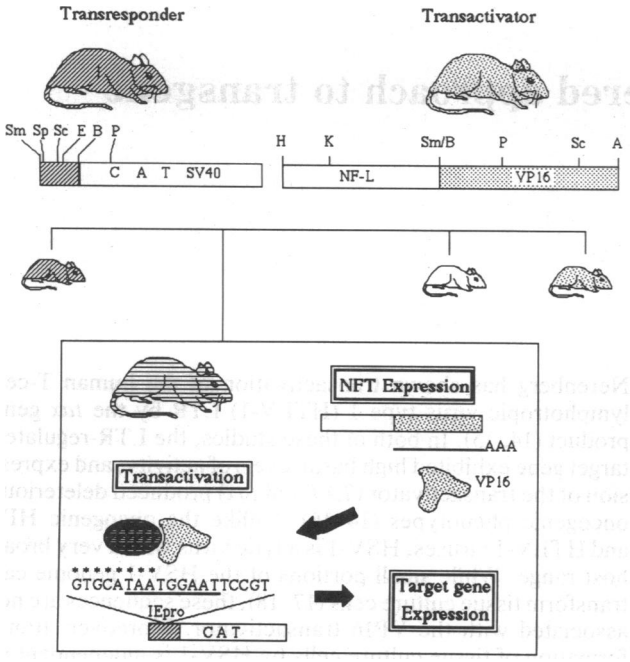

FIG. 1. A diagrammatic representation of the two-tiered multiplex regulatory system. Two transgenic mouse lines are represented. One transgenic line, the transresponder (left), contains a target gene (CAT) regulated by the HSV-1 IE promoter element (hatched area). In the transresponder line, there is no expression of the target gene. The second transgenic line, the transactivator (right), contains the HSV-1 transactivator VP16. In this example, VP16 is regulated by a neurospecific promoter element from the murine NF-L gene (11, 12). The two lines are crossed. The offspring inherit the transresponder (left), transactivator (far right), neither (small white animal), or both transgenes (boxed region). In the double transgenic offspring, VP16 is expressed in a neurospecific pattern. In the cells expressing VP16, VP16 forms a protein–protein complex with the ubiquitous octamer binding protein (oval). This protein complex specifically activates the IE promoter, resulting in neurospecific expression of the target gene. One of the TAATGARAT domains (underlined) in the 360-bp ICP4 promoter is shown. Immediately 5′ of the TAATGARAT sequence (*) is a potential octamer binding site. B, *Bam*HI; E, *Eco*RI; K, *Kpn* I; P, *Pvu* II; Sp, *Sph* I; Sc, *Sac* II; Sm, *Sma* I.

transgene. DNA of the NFT mice was digested with *Pvu* II, which cuts at a single site in the VP16 coding sequences. The digested DNA was fractionated on 0.8% agarose and transferred to nitrocellulose paper. The IE–CAT filters were probed with a 280-bp *Bam*HI/*Eco*RI fragment from the CAT coding region. The NFT blots were hybridized with a 370-bp *Pst* I fragment of the NF-L promoter. Both probes were ^{32}P-labeled, single-stranded DNA probes derived from primer extension with M13.

HSV-1 Infection. Transgenic mice (6–8 weeks old) were anesthetized with Avertin and infected with HSV-1 (KOS) by ocular scarification. The cornea of each eye was scratched with the point of a 21-gauge needle and 10–40 μl of virus (10^6 plaque-forming units/ml) was applied to each eye. The infected animals were housed in sterile filtered cages for 3–7 days, at which time the eyes were processed for CAT assays.

CAT Assay. Tissue samples were collected and suspended in 50–200 μl of 0.25 M Tris·HCl, pH 7.8/1 mM EDTA. The tissues were homogenized in Eppendorf tubes using a small plastic pestle and then frozen at −70°C. Two cycles of 5-min freezing (−70°C) and 5-min thawing (37°C) were used to lyse the cells. After the last thaw, the samples were heated to 60°C for 5 min (22), centrifuged, and the supernatants were collected. Supernatants were stored at −20°C until used. CAT assays were as described by Gorman (23).

RESULTS

To test the MGR system, we produced eight lines of transgenic animals that contained a 360-bp IE promoter fragment from ICP4 linked to the bacterial reporter gene CAT (IE–CAT). Several laboratories (1, 19, 24, 25) have shown that this IE–CAT construct is strongly activated in tissue culture cells either by cotransfection with VP16 or by HSV-1 infection. Southern blot hybridization analysis indicated that two of the IE–CAT mouse lines contained rearranged transgenes and were not studied (data not shown). The remaining lines contain from 2 to 20 head to tail concatameric repeats of the IE–CAT transgene. Four of these lines were analyzed for their basal level of CAT activity and for their ability to express CAT when activated by HSV-1 infection. A variety of tissues from transgenic newborn mice were assayed for CAT activity for each of the four lines. No significant CAT activity was observed in any of the tested tissues, even when a large excess of protein was used for the assays. The results for two of these lines (IE–CAT8 and IE–CAT35) are shown in Fig. 2*A*.

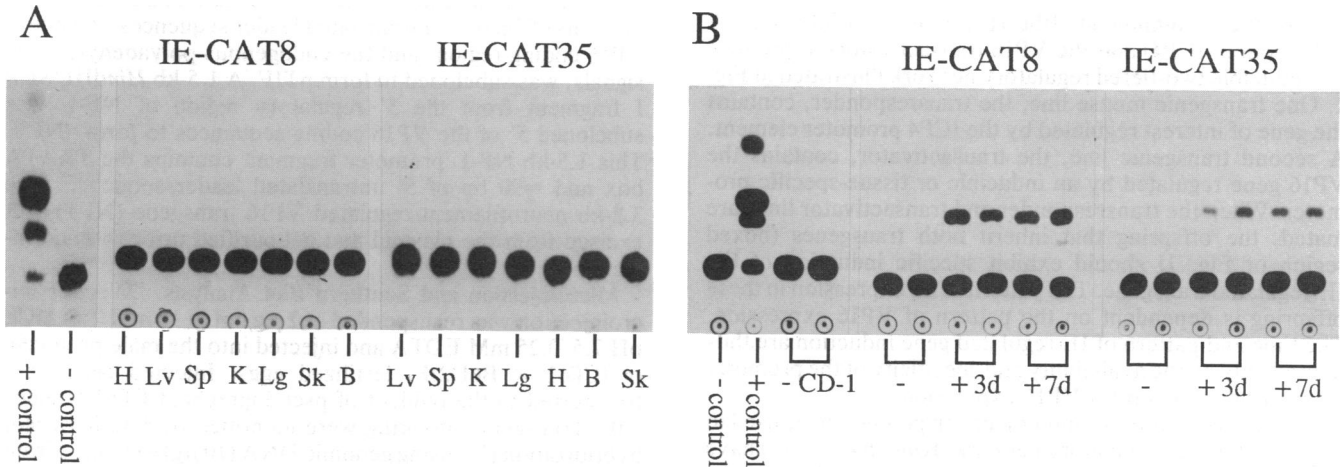

FIG. 2. An analysis of two IE–CAT transgenic mouse lines. (*A*) Basal level of CAT expression in the IE–CAT8 and -35 lines. A series of tissues from newborn transgenic offspring of each line was tested for the presence of CAT. The positive control is a protein extract from mouse L cells transfected with the IE–CAT plasmid. Relative to the positive control a 50- to 100-fold excess of protein was used in the CAT assays for the transgenic tissue samples. No significant levels of basal CAT activity could be detected. (*B*) CAT induction by HSV-1 infection. Adult transgenic animals of the IE–CAT8 and -35 lines were infected with HSV-1 (KOS) by ocular scarification. Samples of both the left and right eyes were collected at either 3 or 7 days postinfection. Both lines exhibit CAT induction at 3 days postinfection. No CAT activity was detected in the uninfected transgenic animals (−) or in the normal CD-1 mice. The positive control is the same as in *A*. B, brain; H, heart; K, kidney; L, liver; Lg, lung; Sk, skin; Sp, spleen.

To determine whether the IE–CAT transgene was inducible, adult transgenic animals from each line were infected by ocular scarification with HSV-1 (KOS). All of the tested lines exhibited specific CAT induction in the infected animals. Strong induction of CAT was observed in lines IE–CAT8 and -35 (Fig. 2*B*), while the other two lines exhibited easily detected, although lower, levels of CAT activity (data not shown). The differences in CAT activity between the four lines may be due to integration site-specific position effects or, alternatively, due to differences in the degree of infection and subsequent viral regulation of the IE promoters. These experiments demonstrate that the IE promoter element, in transgenic mice, is a tight promoter with very little activity in the absence of VP16. Moreover, the lack of activity in the uninduced state appears to be maintained at a high frequency since four of four independent transgenic lines all exhibited no CAT activity in a variety of tissues, yet they maintained their inducible character.

We have also derived three founder mice that contain a murine NFT transgene (11, 12). Only one of these animals (NFT4) expresses the VP16 gene product as assayed by its ability to induce CAT when mated to the IE–CAT8 line (Figs. 3 and 4). When crossed to a female homozygous for the IE–CAT8 transgene (Fig. 3*B*), the NFT4 founder segregated two unique VP16-specific restriction patterns (Fig. 3*A*), suggesting that the NFT4 male has two unlinked sites of integration. One integration site yields two restriction fragments of 2.8 and 3.2 kb (Fig. 3*B*, lanes 1, 6, and 8) and appears to be inactive since offspring with this integration site exhibit no CAT activity in any tissues (Fig. 3*C*, lanes 1, 6, and 8; Fig. 4). The other integration site is active and produces a single 3.2-kb restriction fragment (Fig. 3*A*, lanes 2, 4, 7, and 9). The offspring that inherited this active integration site have easily detected levels of CAT activity in the brain and spinal cord

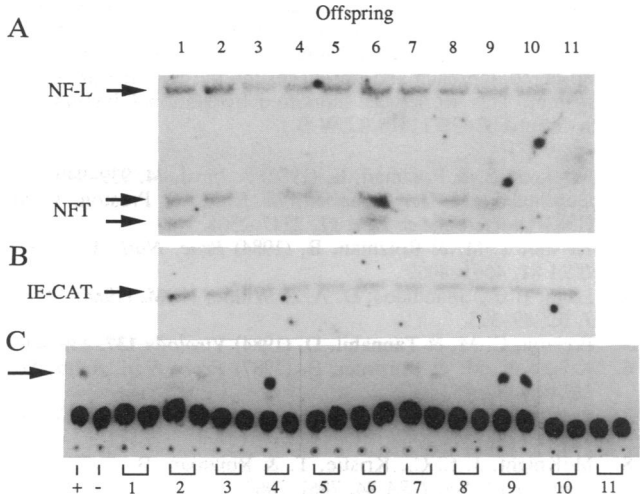

FIG. 3. Induction of CAT by mating with an NFT mouse. A female homozygous for the IE–CAT8 transgene was mated to the NFT4 founder male. (*A*) Southern blot hybridization analysis of the newborn offspring hybridized with a 370-bp *Pst* I fragment of the NF-L promoter. Two different restriction patterns are evident. Offspring 3, 5, 10, and 11 did not inherit the VP16 transgene. Offspring 1, 6, and 8 exhibit two transgene-specific restriction fragments (NFT arrows) of 2.8 and 3.2 kb. Offspring 2, 4, 7, and 9 exhibit a single 3.2-kb transgene restriction fragment. (*B*) The same filter probed with the 280-bp *Bam*HI/*Eco*RI fragment of CAT. All offspring inherited the IE–CAT8 transgene. (*C*) CAT analysis of brain (*Left*) and spinal cord (*Right*) tissues for each of the offspring. Only those offspring (lanes 2, 4, 7, and 9) with the single 3.2-kb restriction fragment exhibit CAT activity within the neuronal tissues. No significant CAT activity is present in the offspring that lack the VP16 transgene or contain the 2.8- and 3.2-kb restriction pattern. Equal volumes of protein extract were used for each CAT assay.

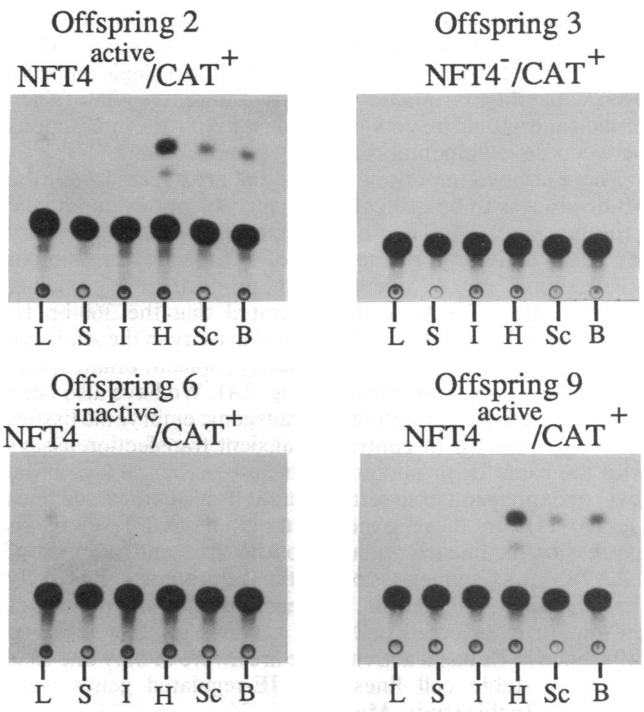

FIG. 4. Tissue specificity of the NFT4 founder. The offspring in Fig. 3 were further analyzed for the tissue specificity of CAT expression. Samples of liver (L), spleen (S), intestine (I), and heart (H) in addition to the spinal cord (SC) and brain (B) were assayed for CAT. In this experiment, a 50-fold excess of protein was used to assay the nonneuronal tissues relative to the brain and spinal cord samples. Offspring with the active VP16 integration site (offspring 2 and 9) exhibit CAT activity in the brain, spinal cord, and heart. Animals that did not inherit the VP16 gene (offspring 3) or that inherited the inactive integration site (offspring 6) exhibit no significant CAT activity in any of the tested tissues.

(Fig. 3*A*; lanes 2, 4, 7, and 9). A weak level of CAT is also apparent in the heart, but not the liver, spleen, or intestine (Fig. 4). These results demonstrate that mice expressing VP16 can be established, that VP16 expression in the central nervous system is not detrimental to the developing embryo, and that transactivation by VP16 is tissue specific.

DISCUSSION

The MGR system was created to assist in the analysis of developmental gene function. Previous use of transgenic mice to study gene function has regulated transgene expression by either relying on an inducible promoter, such as metallothionein (26–35), or using tissue-specific promoters to target transgene expression to specific, generally dispensable, organs or tissues (36–42). A more general use of transgenic mice to analyze developmental gene function would require both an inducible method of gene regulation, so that transgenic lines containing the gene of interest can be established, and a highly versatile means of transgene induction capable of matching the sometimes complex spatial patterns of developmental gene expression. The current single-tiered regulatory methods do not provide these capabilities since they necessarily have some degree of unregulated developmental expression (both inducible and tissue-specific promoters) or suffer a limited experimental ability to control the time and pattern of transgene expression. The MGR system we have described permits the establishment of transgenic lines containing IE-regulated genes. The IE-regulated transgene can be activated in any variety of tissues during development simply by mating with a second transgenic mouse that contains the VP16 transactivator, regulated

by a promoter with the appropriate specificity. In addition, the MGR system makes it possible to simultaneously induce two or more IE-regulated transgenes within a single embryo. This capability is unique to the two-tiered regulatory approach and should be very useful for studying the interactions between developmental regulatory gene products.

There are two important criteria that are necessary for the MGR process to be useful for the analysis of developmental gene function. The IE promoter element should have very low basal activity in the absence of VP16 and expression of VP16 should not be oncogenic or produce developmental abnormalities. We have demonstrated that the 360-bp IE promoter of ICP4 has very little basal activity in the adult eye (Fig. 2*B*) and in newborn liver, kidney, spleen, brain, spinal cord, heart, lung, and intestine (Fig. 2*A*). We have also been unable to detect CAT activity in transgenic embryonic tissues (data not shown). In contrast, transient transfection assays with the same IE promoter consistently exhibit a high basal level of expression in several different tissue culture cell lines (1, 19, 25). In these experiments, the basal level of IE expression is linearly related to the size and number of TAATGARAT sequences present in the promoter. It is likely that the high number of unintegrated plasmid molecules present in transfected cells can account for the observed difference in IE basal activity. We are aware of only one case in which stable cell lines with IE-regulated genes were produced. In this study, Mosca *et al.* (24) used a 1900-bp ICP4 promoter to regulate the human interferon and CAT reporter genes. Both of the IE-regulated constructs exhibited significant levels of basal expression. This high level of basal expression may be due to the size of the ICP4 promoter, which in this case included a HSV origin of replication, or may possibly result from the *in vitro* culture conditions. In any case, the results presented here strongly indicate that in transgenic mice the 360-bp IE promoter element has a very low level of basal activity in a wide spectrum of tissues.

In addition to a low basal state for the IE-regulated transgene, the MGR process requires that expression of the transactivator VP16 should not produce oncogenic or developmental abnormalities. We have demonstrated that expression of the VP16 gene product in the central nervous system does not affect the development or viability of the mouse. The NFT4 founder animal and his offspring with the active integration site are all apparently healthy, breed well, and exhibit no signs of tumor development. Other transactivator lines that we have derived express VP16 early during development in both neuronal and nonneuronal tissues and exhibit no deleterious effects (data not shown). While this does not preclude the possibility of subtle neurological changes, these results indicate that expression of VP16 may be well tolerated in a variety of tissues.

In earlier experiments, we were unsuccessful in deriving a transactivator line using a β-actin-regulated VP16 construct. There have been several recent insights into the mechanism of VP16 transactivation that may explain the lethality of the β-actin–VP16 transgene. Induction by VP16 requires an interaction with at least one other cellular factor, most likely the octamer binding protein (9, 10). Octamer binding sites have been detected in the regulatory elements of a number of genes including the small nuclear RNA genes (43), histone H2B (44), the immunoglobulin genes (45), and the simian virus 40 enhancer (46). Kemp and Latchman (47) have shown that expression of the endogenous U3 small nuclear RNA, but not U1, is enhanced by VP16. In this same study, the authors noted that high levels of VP16 expression depressed the level of U3 expression. Similar inhibitory effects have been observed by others (48, 49). Repression by high levels of VP16 may occur if VP16 diminishes the available pool of octamer binding protein, thus inhibiting the transcription of genes that normally require the octamer binding protein for expression. Since the NFT4 line and the other transactivator mice that we have made appear normal, we believe that the lethality of the β-actin-regulated VP16 construct could result not from the activation of endogenous genes, but from a general inhibition of the many cellular genes that require the octamer binding protein for their transcription.

The MGR system is a two-tiered method of gene regulation for transgenic mice. This system should be useful for engineering ectopic expression patterns for developmental regulatory gene products. An inherent advantage of the multiplex system is its combinatorial feature, which permits testing of all pairwise combinations of transactivator and transresponder mice. This combinatorial approach can potentially be used to produce multitiered networks of transresponder and transactivator pairs. In this report, we describe the simplest network consisting of a single transresponder and transactivator pair. More complex networks might contain several transresponder genes, the products of which might normally interact with each other during development, or multiple transactivator genes, with different spatial and temporal patterns of expression, to produce complex patterns of transresponder induction. By using different transactivating genes, such as the yeast GAL4 gene or GAL4–VP16 fusion genes (49), a network of transresponder and transactivator pairs might be assembled to control a cascade of transresponder gene expression. No other method of transgene regulation provides this degree of flexibility; thus, the MGR system should be useful for studying the interactions between two or more developmental regulatory genes. Finally, in addition to the developmental applications of the MGR system, mice containing the IE–CAT or other IE-regulated genes and mice expressing the HSV-1 VP16 gene product will be useful for studying HSV-1 infection and latency.

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