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Genetics

Tissue specificity of a glucocorticoid-dependent enhancer in transgenic mice

(tyrosine aminotransferase/transcriptional regulation/glucocorticoid receptor/hepatocyte nuclear factor 3)

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ABSTRACT **The glucocorticoid-responsive units (GRUs) of the rat tyrosine aminotransferase were associated with the regulatory sequences of a cellular gene expressed ubiquitously—that coding for the largest subunit of RNA polymerase II. In transient expression assays, glucocorticoid responsiveness of the hybrid regulatory regions depends on the spatial relationship and number of regulatory elements. Two parameters affect the ratio of induction by glucocorticoids: the basal level of the hybrid promoter that is affected by the RNA polymerase II regulatory sequences and the glucocorticoid-induced level that depends on the distance between the GRUs and the TATA box. A fully active glucocorticoid-responsive hybrid gene was used to generate transgenic mice. Results show that a composite regulatory pattern is obtained: ubiquitous basal expression characteristic of the RNA polymerase II gene and liver-specific glucocorticoid activation characteristic of the tyrosine aminotransferase GRUs. This result demonstrates that the activity of the tyrosine aminotransferase GRUs is cell-type-specific not only in cultured cells but also in the whole animal.**

Future applications of gene therapy will require that the expression of a therapeutic gene with respect to cell type, amount, timing, and duration of delivery be precisely controlled. This control would rely on the availability of regulatory sequences capable of providing specificities that may not correspond to an already established regulatory pattern. To obtain novel patterns, artificial regulatory units that associate different types of regulations can be engineered. However, one needs to understand the subtleties of regulatory sequences to know how to assemble the desired traits.

Gene expression is regulated, at least in part, by protein–DNA interactions. Regulatory sequences have a modular structure that consists of the arrangement of the binding sites for ubiquitous and tissue-enriched transcription factors (1). The limited cellular distribution of these factors provides, at least in part, the cell-specificity of the gene expression. In addition, some of these specific interactions are controlled by hormonal stimuli. The glucocorticoid receptor (GR) is an example of a hormone-responsive transcription factor (for review, see ref. 2). Upon binding of the hormone, the GR interacts with DNA at a specific site termed glucocorticoid-responsive element (GRE). The GREs are generally clustered with other transcription factor-binding sites, and together these clusters form the so-called glucocorticoid-responsive units (GRUs, ref. 3). The GR is expressed in most cell types, but the set of glucocorticoid-regulated genes varies in each cell type. The cell specificity of the response of a given gene is provided, at least in part, by an interplay between the GR and tissue-enriched transcription factors, and some of these interactions occur at the GRUs (for examples, see refs. 4–6).

Tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase; EC 2.6.1.5) gene expression is restricted to the parenchymal cells of the liver, where its transcription is stimulated by glucocorticoids and cAMP (for review, see ref. 7). Multiple regulatory elements spread over >10 kbp of the 5′-flanking sequence are involved in both the liver-specific and hormone-dependent transcriptional control of the gene. The presently identified elements are a hormone-independent proximal promoter (−0.1 kbp) that shows some preferential activity in liver cells (8), a liver-specific hormone-independent enhancer at −10.5 kbp (9), a cAMP-responsive unit at −3.6 kbp (10), and, finally, two cooperatively interacting GRUs at −5.5 and −2.5 kbp (11, 12). The former is inactive by itself but potentiates the latter, which alone provides only 30% of the induction (12). In transient expression, the GRUs are active in hepatoma cells but are not active in fibroblasts (6). In addition to the GR, the transcription factors that interact with the GRUs are members of the C/EBP, HNF3 (formerly designated HNF5), and Ets families (6, 13). Most activity of the −2.5 GRU in hepatoma cells results from the combined action of the GR, Ets, and HNF3 factors (13). Dynamic interplay between the GR and HNF3 is believed to account for the cell-type specificity of this GRU (6, 14).

To unambiguously prove this cell specificity, several conditions must be met: the GRUs must be isolated from interference by other cell-specific regulatory elements of the *Tat* gene; the gene regulated by the GRUs must be active in every tissue; and, finally, identical copy number and integration site of the hybrid reporter gene must be used to analyze its expression in each tissue. This result can be achieved by using transgenic mice technology. The tyrosine aminotransferase GRUs were associated with the regulatory sequences of a ubiquitously expressed gene, those of the largest subunit of RNA polymerase II (RPII). Using transient expression assays, we have determined the conditions in terms of relative arrangements of the regulatory sequences to render the promoter responsive to the tyrosine aminotransferase GRUs. The resulting hybrid gene showed the predicted composite regulatory pattern in transgenic mice: ubiquitous expression and liver-specific glucocorticoid-responsiveness.

MATERIALS AND METHODS

Construction of Expression Vectors. Standard recombinant DNA procedures were used (15). A detailed description of plasmid construction is available upon request. All hybrid genes contain the two GRUs of the *Tat* gene encompassing sequences −5857 to −5180 and −2630 to −2341 (12). The expression plasmids are derived from pKT531 (12), where the

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Abbreviations: GR, glucocorticoid receptor; GRE, glucocorticoid-responsive element; GRU, glucocorticoid-responsive unit; CAT, chloramphenicol acetyltransferase; RPII, largest subunit of RNA polymerase II; UAS, upstream activating sequence; HSV tk, herpes simplex virus thymidine kinase.
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herpes simplex virus thymidine kinase (HSV tk) promoter was replaced by various fragments of the mouse RPII regulatory sequences (ref. 16; a gift of J. L. Corden, The John Hopkins University School of Medicine). These fragments were obtained either as restriction fragments or using *in vitro* synthesis by PCR. Their boundaries are −621 to +301 in plasmid pTP621, −133 to +93 in pTP133, −79 to +93 in pTP79, and −51 to +93 in pTP51. The DNA sequence of the PCR-generated fragments was verified by DNA sequencing. pTPXY was obtained by the insertion of the GRUs into the *Sac* II site downstream of the polyadenylylation site of the chloramphenicol acetyltransferase (CAT) reporter in pTP51. pWa and pWb correspond to the insertion of a fragment of the RPII regulatory sequences (−621 to −37) upstream of the GRUs in pTP51. The construction used to generate the transgenic mice corresponds to pWb, except that the 3′-untranslated region of the HSV tk gene was replaced by that of the rabbit β-globin gene.

Transfection Experiments. Cell culture of rat hepatoma cells (H4II), transfection using calcium phosphate coprecipitation, and measurement of enzymatic activities were done as described (12).

Production and Analysis of Transgenic Mice. The DNA fragment for microinjection was separated from the vector sequence and purified on an agarose gel followed by an Elutip column (Schleicher & Schuell). About 400 copies of the purified fragment were microinjected into the male pronuclei of fertilized C57BL/6J × DBA/2J eggs, and the microinjected eggs were subsequently transferred to the oviduct of pseudopregnant mice by using standard procedures (17). Transgenic mice were identified by slot-blot analysis. To define the structure of each integrated transgene, Southern blottings were done by using *Pvu* II-restricted genomic DNA and a CAT probe.

The progeny of transgenic animals (6 weeks old) was used for expression analyses. Pairs of animals from the same litter were separated into two groups: mice of the first group were injected with 2 μg of dexamethasone per g of body weight, whereas mice of the second group were injected with a 9 g/l NaCl solution as controls. Five hours after i.p. injection, protein extracts were prepared and analyzed as described (18).

RESULTS

The GRUs Do Not Activate a Silent Gene. In transient expression analyses of cultured hepatoma cells and fibroblasts, the tyrosine aminotransferase GRUs linked upstream of the HSV tk promoter confer cell-type-specific glucocorticoid inducibility to a reporter CAT gene (6, 12). To verify the capacity of the GRUs to fully function in a cell-specific manner, we used this construction to create transgenic mice. The analysis of two out of four lines obtained is presented in Table 1. A weak activity was found in the lungs and brain but was absent in the liver. Injection of dexamethasone had no effect on expression of the transgene in any tissue examined. The two other lines likewise showed no detectable expression in liver after dexamethasone treatment and were not analyzed further. Therefore, the pattern of expression directed by the HSV tk promoter precludes any conclusion about the tissue-specific activity of the GRUs in transgenic animals.

Table 1. CAT activity driven by the tyrosine aminotransferase GRUs linked to the HSV tk promoter in transgenic mice

	CAT activity, pmol of acetylated chloramphenicol/min per mg of protein		
	Liver	Lung	Brain
Line 19			
− Dex	Bgd	3.9 ± 1.5	34.5 ± 10.8
+ Dex	Bgd	7.3 ± 1.5	28.9 ± 2.3
Line 22			
− Dex	Bgd	10.8 ± 1.7	19.5 ± 1.4
+ Dex	Bgd	9.3 ± 1.1	20.5 ± 2.8

Values are the means ± SEMs of three independent measures. Dex, dexamethasone; Bgd, background level (<0.4).

Activity of the GRUs Depends on Their Spatial Relationship with Other Regulatory Sequences. To circumvent the problem raised by the viral HSV tk regulatory sequences, we used those of a ubiquitously expressed cellular gene. The RPII gene was selected for two reasons. (*i*) Although it is a housekeeping gene, its promoter has a conventional polymerase II-dependent gene architecture with a TATA box (16). (ii) The DNA segment allowing expression in many tissues of transgenic mice, including liver, has been determined (from −621 to +93; J. L. Corden, personal communication; see also ref. 19).

The tyrosine aminotransferase GRUs were inserted at the 5′ end of the RPII fragment, and their activity was studied in transient expression assays using hepatoma cells where the GRUs can stimulate the HSV tk and the tyrosine aminotransferase promoters (11). Fig. 1 shows that with this arrangement (plasmid pTP621), dexamethasone does not significantly increase CAT activity.

The level of transcription from the RPII promoter is up to one order of magnitude higher than that driven by the tyrosine aminotransferase and HSV tk promoters (data not shown). This result suggests that the inactivity of the GRUs could be due to the fact that the RPII promoter is already maximally activated by its own regulatory sequences, thus precluding any additional activation. To test this hypothesis, we attempted to lower the transcriptional activity of pTP621 by gradual 5′ → 3′ deletions of the RPII regulatory sequences. The results obtained with three representative deletions are shown in Fig. 1 (pTP133, pTP79, and pTP51). The progressive deletions lead to a progressive increase of the ratio of stimulation by glucocorticoids that can reach 50-fold with the shortest RPII 5′-flanking region (−51 to +93, pTP51). This increase in the ratio of stimulation results from the combination of a decrease in the basal level and an increase in the induced level (compare pTP621, pTP79, and pTP51). These constructions were also tested without GRUs. This removal abolishes glucocorticoid inducibility but does not affect the basal level of the constructions (data not shown).

The progressive deletions not only remove known or putative regulatory sequences such as the GC boxes (ref. 16; represented on Fig. 1) but also change the distance between the GRUs and the TATA box. To estimate the role of the distance on the observed efficiency of the GRUs, they were separated from the −51 promoter by insertion of a 2-kbp fragment of plasmid DNA (pTPXY, Fig. 1). This change leads to a marked decrease in the induced level that becomes even slightly lower than that of pTP621. Because the basal level remains low (compare pTPXY with pTP51), the construct still shows glucocorticoid inducibility (8-fold stimulation).

These observations suggest that the key parameter is the relative position occupied by the different regulatory sequences. Thus, the deleted RPII sequences were reinserted upstream of the GRUs in pTP51, giving rise to plasmids pWa and pWb, with the RPII sequences in the correct and inverted orientation, respectively. Results show that the basal level is essentially restored—slightly better when the relative orientation of the RPII fragments is conserved (compare pWa and pWb with pTP621; Fig. 1). Both constructions are glucocorticoid-inducible (7- and 19-fold induction, respectively) because the induced levels remain high. Thus, insertion of the 1-kbp fragment containing both GRUs does not alter the basal RPII transcription rate, but their proximal location allows them to function.

Tyrosine Aminotransferase GRUs Are Active Essentially in the Liver of Transgenic Mice. In transient expression assays,

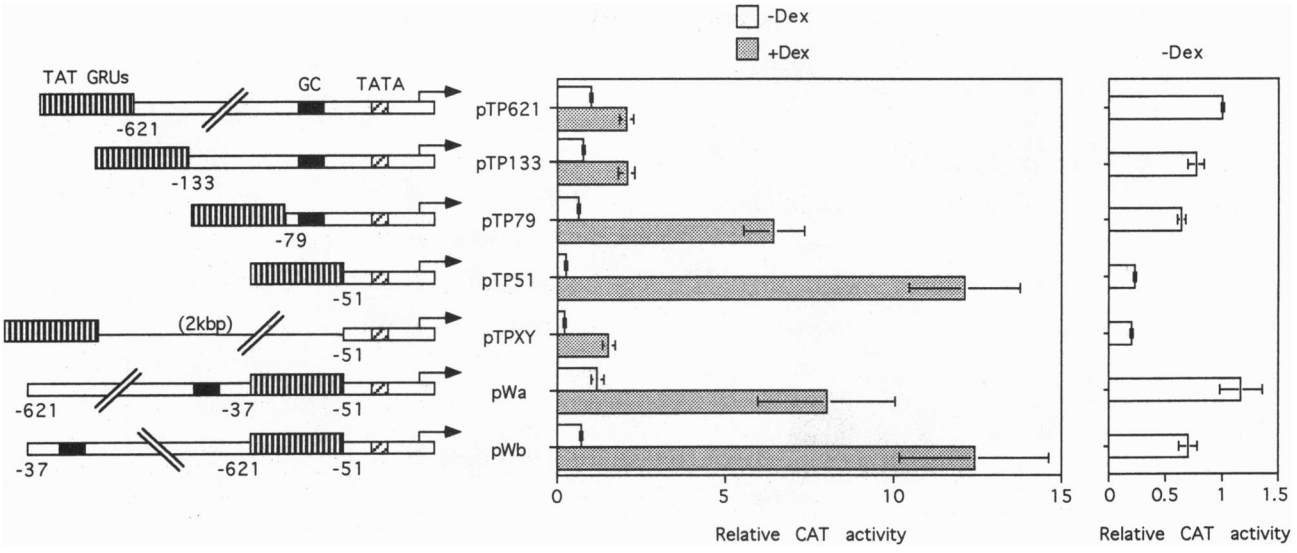

FIG. 1. Spatial relationship between the tyrosine aminotransferase (TAT) GRUs and the RPII promoter elements determines glucocorticoid inducibility. Regulatory sequences of the chimeric gene are schematically represented at left as follows: the striped box represents the two tyrosine aminotransferase GRUs; the RPII promoter is represented as a box where the GC and TATA boxes are indicated by black and stippled rectangles, respectively, as indicated above pTP621; numbers refer to positions relative to the cap site of the RPII transcript that is represented by an arrow; the line in pTPXY represents 2 kbp of plasmid sequence. CAT activities are from transient transfection of rat hepatoma cells and are expressed relative to basal activity of pTP621. Values correspond to means ± SEMs of at least three independent experiments. Glucocorticoid induction (+Dex) was for 24 hr with 10^{-6} M dexamethasone. Basal CAT activity is enlarged at right.

the cell-type specificity of the GRUs (6) is also observed with the RPII promoter: all constructions that are glucocorticoid-inducible in hepatoma cells show no response in fibroblasts (data not shown). To assess the cell-type specificity of the GRUs in the animal, transgenic mice were created. The pWb construction was selected because it shows slightly better glucocorticoid inducibility than the pWa construction while retaining most basal activity of the RPII promoter (Fig. 1). To improve detectability of the reporter gene product, the β-globin 3′ untranslated region was substituted for that of the HSV tk, as the former can increase RNA stability (20). Transient expression assays showed that this substitution leads to a 2- to 5-fold increase in CAT activity but, as expected, to no difference in the ratio of stimulation (data not shown).

Five transgenic mice, with one to five intact copies of the transgene, were obtained. These mice showed a Mendelian transmission of the transgene. Their progeny have been analyzed for CAT activity in various tissues derived from the three embryonic layers. Three lines, 29, 33, and 41, harboring, respectively, 2, 5, and 2 gene copies, presented a high level of CAT activity in all tissues tested, whereas the two other transgenic lines showed no activity regardless of tissue.

To demonstrate hormonal regulation of CAT expression, transgenic mice from each expressing line were separated into two groups. The first group was injected with saline; the second group was injected with dexamethasone. Fig. 2*A* shows the results of a representative CAT assay measured from tissue extracts originating from a pair of mice of line no. 29. This assay shows that CAT activity is present in all tissues tested, demonstrating that the hybrid gene retains the ubiquitous pattern of expression of the RPII gene. This CAT activity is greatly elevated in the liver of the glucocorticoid-treated mouse, to a lesser extent in the pancreas, and only slightly in the lung and stomach. No induction is observed in six other tissues tested. Fig. 2*B* displays the ratio of induction of CAT activity measured in several tissues of three pairs of mice of line no. 33 treated or not treated with dexamethasone. Again, induction of CAT activity is highest in the liver, high in pancreas, and low in lung and stomach. A similar pattern of expression and glucocorticoid response is also seen in mice of line no. 41 (data not shown). Because the tyrosine aminotransferase GRUs are preferentially active in the liver of all three transgenic lines tested, we conclude that these units contain enough information to provide both glucocorticoid responsiveness and liver specificity.

DISCUSSION

In higher eukaryotes, regulatory regions generally consist of clusters of transcription factor-binding sites (1). The precise relative location of these binding sites can be important for the cooperation of transcription factors. For example, the distance separating a GRE and a CACCC box is critical for cooperation between these elements (21). Once a cluster has reached a certain critical size that can vary depending on the identity of the various factors interacting and on their affinity for the site, it can function at a distance and in both orientations. For example, a GRE can be active in the proximity of a TATA box, but action at a distance requires combination with other binding sites (4). Such clusters of binding sites can then be associated in a more flexible manner, either spread over long distances or concentrated in smaller regions. For example, the *Tat* gene has at least five regulatory clusters interspersed over 10 kbp of the 5′-flanking region (8–12), whereas the elastase gene has all regulatory information for proper developmental control concentrated in a 213-bp fragment (22).

Each cluster can have an independent regulatory potential, and the overall pattern of regulation results from the combination of all these regulatory potentials. As shown here, the relative spatial relationship of the clusters can be important for the outcome of the integration of the different types of regulation. The RPII regulatory sequences have been separated here into two clusters (even though it is likely that they can be separated in a higher number of smaller units): the "basal" promoter region (−51 to +93) and the upstream activating sequences (UASs). The tyrosine aminotransferase GRUs correspond to two independent clusters (12) that have been fused in this analysis. The UASs of the RPII promoter are fairly flexible in their regulatory potential because they can act on the basal promoter even when separated from it by the tyrosine aminotransferase GRUs (compare the basal level of pWa and pWb constructs with that of pTP621, Fig. 1). In

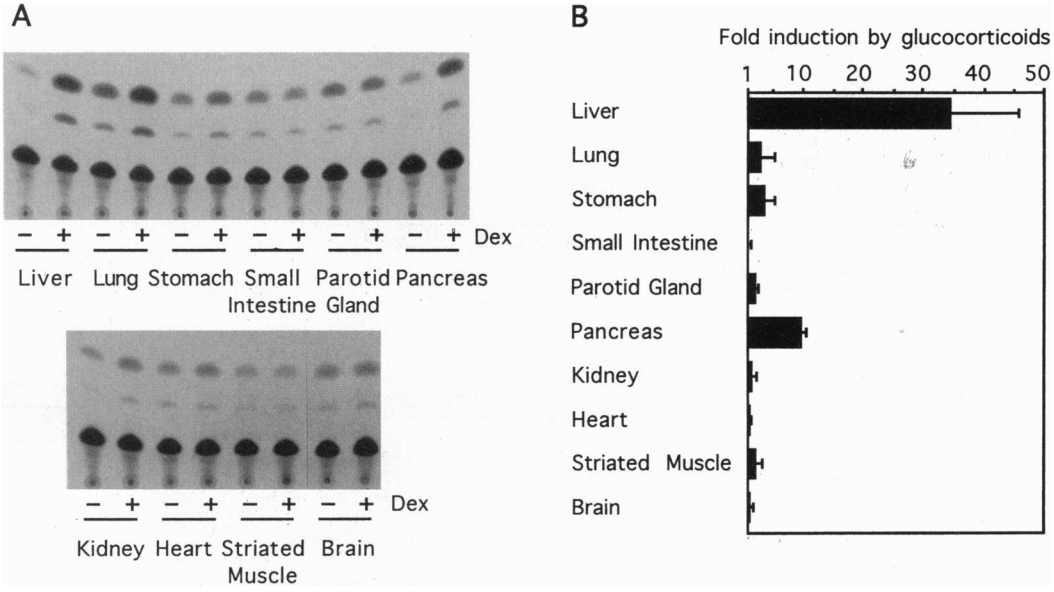

FIG. 2. Tyrosine aminotransferase GRUs confer liver-specific glucocorticoid responsiveness to the ubiquitously expressed RPII promoter. (*A*) CAT assay of a representative pair of mice from a litter of line 29, injected with either saline (−) or dexamethasone (Dex) (+). The same amount of extract (20 μg) from the indicated tissues was used, except for brain and muscle, where 80 μg was used. The reaction was incubated for 90 min at 37°C. In the liver of this pair of mice, tyrosine aminotransferase activity was 7.7-fold higher in the dexamethasone-treated mouse. (*B*) Ratio of induction of CAT activity by glucocorticoids in line 33. Three pairs of transgenic mice from the same litter were analyzed; values correspond to the means ± SEMs. In liver, the ratio of induction of tyrosine aminotransferase activity was 8.6 ± 1.

contrast, the tyrosine aminotransferase GRUs are more sensitive to the arrangement; they are not active when separated from the basal promoter by the UAS (pTP621). Furthermore, they show great sensitivity to the distance that separates them from the basal promoter. When separated by 2 kbp of an apparently neutral plasmid sequence, they activate transcription less efficiently (compare pTPXY with pTP51). This distance effect can also be observed in the context of *Tat* gene sequences because removal of the 2.5-kbp-long region that separates the proximal GRU from the basal promoter leads to a 4-fold increase in the glucocorticoid response (unpublished results). Another observation made here is that the activities of these clusters are not multiplicative. At similar distances separating the GRUs from the basal promoter, the hormone-induced level is almost unaffected by the presence of the RPII UAS (compare pWa and pWb with pTP51, and pTPXY with pTP621). As a consequence, the addition of sequences that stimulate the basal level leads to a reduction of the fold induction by glucocorticoids.

Once the proper arrangement of the regulatory clusters had been established in transient expression assays, we characterized the specific regulatory potential of the hybrid gene in transgenic mice. The gene shows the ubiquitous pattern of expression of the parental RPII promoter and allows us to analyze the modality of the glucocorticoid responsiveness conferred by the tyrosine aminotransferase GRUs. Transient expression analyses of gene construction associating the GRUs and a heterologous promoter in hepatoma cells and fibroblasts show that the GRUs function as hepatoma cell-specific regulatory elements (6). We have also observed such a cell-type specificity in stable transfection experiments. In these experiments, the tyrosine aminotransferase GRUs can stimulate transcription from a simian virus 40 early gene promoter located several thousand base pairs away. This stimulation is observed only in hepatoma cells and is extinguished simultaneously with the expression of the *Tat* gene in hepatoma cell × fibroblast hybrids (23). Altogether, these observations suggest that the GRUs are autonomous glucocorticoid regulatory modules containing all the information necessary for cell specificity.

Our results with the transgenic mice agree with this hypothesis. Dexamethasone injection induces a strong enhancement of expression in the liver, whereas the expression is almost unchanged in other tissues except in the pancreas. The induction in these two tissues may be related to their common embryonic origin: the liver and the pancreas anlagen develop from the same region of the primitive gut (the hepatopancreatic ring; ref. 24) between 14 and 20 somites (25). Furthermore, members of the HNF3 family are expressed in both tissues (26), and these factors have been implicated in the activity of the tyrosine aminotransferase GRUs in hepatoma cells (6, 14, 27, 28). However, glucocorticoid inducibility is not observed systematically in all tissues that express HNF3 members: in lung and stomach, the reporter gene shows only weak glucocorticoid inducibility, and no inducibility is observed in the small intestine (for a detailed description of the tissue distribution of HNF3 members, see refs. 26 and 29). This result suggests that either HNF3 members do not activate transcription similarly in all tissues where they are present or the other DNA-binding proteins interacting with the GRUs (6, 13) further restrict their tissue-specific activity.

The tissue-specific enhancing activity observed with the tyrosine aminotransferase GRUs linked to a ubiquitously expressed promoter appears somewhat less strict than that of the intact *Tat* gene. The other *Tat* regulatory clusters that show tissue-specific activity (8–10) could participate in the stringency of the liver specificity of the glucocorticoid response. For example, one of the remote regulatory clusters could modify the chromatin structure of the entire gene domain specifically in liver cells, such as to render the GRUs accessible to their interacting transcription factors. We have proposed such a mechanism to account for the absence of interaction of Ets-related transcription factors with the −2,500 GRU that is observed in fibroblasts (13).

Our results with the HSV tk promoter suggest that the tyrosine aminotransferase GRUs are not able to activate a silenced gene even in liver. When integrated in the genome, the HSV tk promoter does not contain enough information to be expressed at a large proportion of the integration sites (30). This information can be provided by some enhancers, such as

those of Rous sarcoma virus or simian virus 40, which allow expression at many integration sites (30). In the absence of this information, the expression pattern of the HSV tk promoter is extremely sensitive to regulatory signals provided by the integration locus (31). In contrast to Rous sarcoma virus or simian virus 40 enhancers, the tyrosine aminotransferase GRUs do not appear to be able to reverse this dependency. Thus, they correspond to modulators rather than to activators of transcription.

These results show that, by using RPII regulatory sequences, GRUs, and probably other types of complex regulatory elements, still function in transgenic animals once transferred in a regulatory context other than their own. The magnitude of the effect on the expression of the recipient gene is the result of many factors, including the spatial relationship between the various regulatory sequences. Because complex regulatory units can be manipulated independently from each other, it should be possible to generate additional specific regulatory patterns adapted to therapeutic or basic purposes. Hormone-responsive units could be particularly useful for such applications.

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