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Genetics

Developmental and tissue-specific expression directed by the α_2 type I collagen promoter in transgenic mice

(developmental regulation/type I collagen genes)

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ABSTRACT **Eight transgenic mice were generated in which the promoter of the mouse α_2(I) collagen gene (nucleotides −2000 to +54), linked to the bacterial gene for chloramphenicol acetyltransferase (CAT), is stably integrated in the germ line. These strains contain from 1 to 20 copies of the α_2(I) collagen-CAT chimeric gene per haploid genome. In seven of the eight strains, the CAT gene is expressed, although the levels of CAT enzyme activity vary considerably from one strain to the other. In six of these strains, the expression of the CAT gene follows the expected tissue distribution pattern of expression of the α_2(I) collagen gene. In these six strains, the level of CAT activity is much higher in extracts of tail, a tissue that is very rich in tendons, than in any other tissue that was tested. This distribution parallels the much higher levels of α_2(I) collagen RNA that are found in the tail as compared to other tissues. Expression of the chimeric gene is detected in the embryo after 8.5 days of gestation, at approximately the same time that the endogenous type I genes become active. We conclude that the α_2(I) collagen promoter sequences present in the recombinant plasmid used for our experiments contain sufficient information to ensure stage- and tissue-specific activity of this promoter.**

The generation of transgenic mice by introduction of genes or segments of genes into fertilized mouse oocytes adds a novel dimension to the study of the control of gene expression (1). One can examine whether microinjected genes respond to the same temporal and spatial developmental controls that regulate the corresponding endogenous genes and analyze which DNA sequences constitute the signals that determine developmental and tissue-specific gene expression.

There are at least 10 different collagen types present in higher vertebrates (2–7). As a group, these collagens constitute the most abundant protein of the extracellular matrix. Each of these different collagen types is preferentially synthesized by certain tissues. Type I collagen is found mainly in tendon, bone, skin, and smooth muscles, whereas type II collagen is found in cartilage, and type III is found in similar tissues as those where type I is found, yet its relative proportion to type I collagen varies in different tissues (2).

The synthesis of several of these collagens has been shown to be regulated during development (8, 9). We have chosen the type I collagen genes as a model to study these developmental controls.

Since the type I collagen genes are very large (10, 11) and, therefore, difficult to manipulate *in vitro*, we have fused a segment containing 2 kilobases (kb) upstream of the start of transcription of the α_2(I) collagen gene to a marker gene, the bacterial gene for chloramphenicol acetyltransferase (CAT) (12). Although such a construction may not contain all the regulatory elements that control the expression of the endogenous α_2(I) collagen gene, DNA transfection experiments with tissue culture cells have shown that the 5′ flanking sequences of the α_2(I) collagen gene are sufficient for cell-specific expression (30) and for regulation by oncogenes (12).

The cell-specific expression of the α_2(I) collagen-CAT chimeric gene, observed in tissue culture cells, prompted us to examine the expression of the same gene in intact mice. We introduced this gene via microinjection into one-cell mouse embryos and established a number of transgenic mouse strains. Our results show spatial and temporal specificity of expression of the α_2(I) collagen-CAT chimeric gene in these animals.

MATERIALS AND METHODS

Gene Transfer into Embryos. Unless specified, the manipulation of embryos and animals followed published procedures (13). One-cell embryos were obtained from 3-week-old superovulated inbred FVB/N females that had been mated with males of the same strain or with C_3H males. An aliquot of a 1-μg/ml solution of plasmid pAZ1003 (Fig. 1), linearized with *Pvu* I, in 10 mM Tris·HCl, pH 7.6/ 0.1 mM EDTA was injected into the male pronuclei (1). After injection, embryos were surgically transferred to the oviducts of pseudopregnant NIH N:GP females which carried them to term.

DNA Analysis. Skin or tail tissue was excised from newborns at the age of 3–6 weeks, and DNA was isolated as follows. The tissue was homogenized in 50 mM Tris·HCl, pH 7.6/10 mM EDTA/1% $NaDodSO_4$ and incubated for 2 hr at 44–45°C with proteinase K at 100 μg/ml. NaCl was added to 0.5 M and homogenates were extracted with phenol/chloroform/isoamyl alcohol (25:24:1). Total nucleic acids were precipitated by addition of two volumes of 95% ethanol and resuspended in 50 mM Tris·HCl, pH 7.6/10 mM EDTA/10 mM NaCl. For dot blot analysis, 10-μg samples of heat-denatured DNA were transferred to nitrocellulose filters. Samples for Southern blot analysis were treated with DNase-free RNase (50 μg/ml) and reextracted in phenol/chloroform/isoamyl alcohol, precipitated with ethanol and resuspended in 10 mM Tris·HCl, pH 7.6/0.1 mM EDTA.

Southern blot analysis was performed after digesting the DNA with restriction enzymes purchased with Bethesda Research Laboratories and New England Biolabs, using assay conditions prescribed by the suppliers. DNA fragments were separated by electrophoresis in 1% agarose gels and transferred to nitrocellulose filter paper (14). Southern blots were hybridized with a nick-translated 500-base-pair (bp)

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Abbreviations: CAT, chloramphenicol acetyltransferase; kb, kilobase(s); bp, base pair(s).

CAT probe as described (12). Dot blots were hybridized with nick-translated pRSVCAT DNA (15) by use of procedures described before (16).

RNA Analysis. Total RNA was prepared according to Chirgwin *et al.* (17). Serial dilutions (in 3 M NaCl/0.3 M sodium citrate, pH 7, with bacterial rRNA at 250 μg/ml) of RNA were applied to nitrocellulose filters using a dot blot template. The hybridization conditions were the same as those used for Southern analysis.

Assay for CAT Enzyme. Tissue extracts, prepared by homogenization in 0.25 M Tris·HCl (pH 7.5) were analyzed for CAT as described (18), using 50 μg of protein for each CAT assay. Assay mixtures were preincubated for 5 min at 65°C (19) in the absence of acetyl-CoA to neutralize factors present in the extracts that might interfere with the assay. Incubations were for 4–12 hr at 37°C. Enzyme activity was expressed in units as defined by Shaw (20).

RESULTS

The recombinant plasmid (pAZ1003) that was used in these experiments is illustrated in Fig. 1. It contains a segment of the mouse α_2(I) collagen gene from 2000 bp upstream of the start of transcription to 54 bp downstream of this site. This segment is fused to the bacterial gene for CAT. The plasmid is described in more detail in the legend to Fig. 1.

On the average, about 150 molecules of pAZ1003 plasmid DNA, made linear by digestion with *Pvu* I, were injected into the male pronuclei of fertilized one-cell mouse embryos. Among the 77 animals that were born as a result of the transfer of these injected embryos into pseudopregnant females, 13 were positive for CAT DNA as assayed by dot blot hybridization of DNA extracted from a segment of the tail (data not shown). Eight of these animals survived and gave rise to transgenic strains. Fig. 2 tabulates the results of the dot blots of tail DNA of the 8 positive animals. The relative intensities of the dots on the autoradiographs compared to known standards showed that these animals carried 1–20 copies of the plasmid per haploid genome. Fig. 2 also shows the levels of CAT enzyme activity as assayed in tail extracts. CAT activity was found in 7 of the 8 mice, but it is clear that there was considerable variation in the levels of CAT enzyme activity from one strain to another. Further, there is no apparent correlation between copy number and CAT enzyme activity. For instance, strain 6, which contains only 1–2 copies of the α_2(I) collagen promoter-CAT DNA unit, and strain 7, which carries 10 copies of the chimeric gene, display similar levels of CAT activity. Other strains carrying 8–10 copies of the gene show much lower CAT activity. No CAT activity was detected in extracts of strain 8. This strain apparently carries only 1 copy of the gene and it is possible that the gene may have lost some regulatory sequences prior to its integration into the mouse genome. A similar observation was made after stable introduction of similar plasmids into tissue culture cells (5).

To examine whether the injected collagen promoter-CAT DNA unit was stably integrated in the genome of the positive mice, a Southern hybridization analysis was performed on several pedigrees. Fig. 3*A* shows the results of a *Bam*HI digestion of the genomic DNA of samples obtained from three strains. In these three cases, we examined the DNA of the parent and of one F_1 offspring of each parent. *Bam*HI cleaves once in plasmid pAZ1003. In addition to the major hybridizing species of 6.8 kb, which has the size of a full-length pAZ1003 plasmid, each strain contains at least one additional band of lesser intensity whose size is unique to each strain. These less abundant DNA species presumably contain both plasmid DNA and host DNA sequences flanking the inserted DNA. Similar observations were made after Southern analysis of *Pvu* II digestions and of *Eco*RI digestions of the same samples (data not shown). Fig. 3*B* shows a Southern analysis of genomic DNAs from representatives of the five other strains digested with the enzyme *Apa* I. This enzyme cleaves twice in pAZ1003, but the CAT probe that was used hybridizes to only one of these fragments, a species of 2 kb. Four of the five strains contain this species. In the fifth strain, this species is absent, suggesting that some rearrangement occurred in the 2-kb segment. This is the only strain that is positive for CAT DNA but in which no CAT

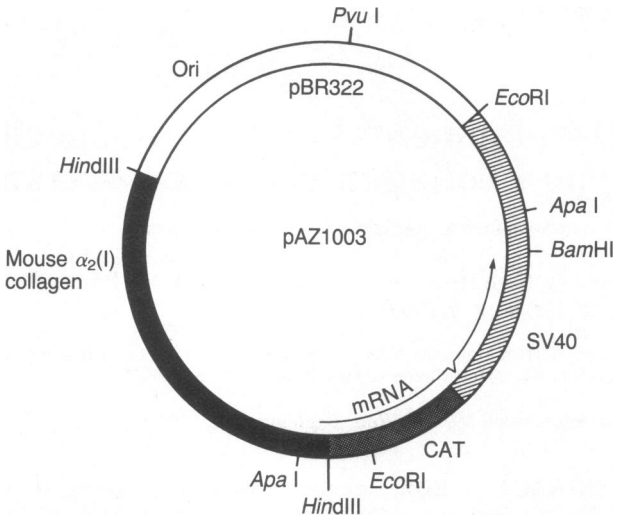

FIG. 1. Structure of plasmid pAZ1003. In this plasmid, a segment of the mouse α_2(I) collagen gene, from 2000 bp upstream of the start of transcription to 54 bp downstream of this site, is fused to the bacterial gene for CAT. The plasmid is a derivative of pAZ1005, which has been described (12). In addition to the mouse α_2(I) collagen (solid region) and CAT (stippled region) DNA sequences, it also contains a segment of simian virus 40 (SV40) DNA (hatched region) that includes the intron and splice sites for the small-tumor-antigen gene and the polyadenylylation site of the SV40 early region. The plasmid also contains the origin of replication (Ori) and ampicillin-resistance gene of plasmid pBR322 (open region).

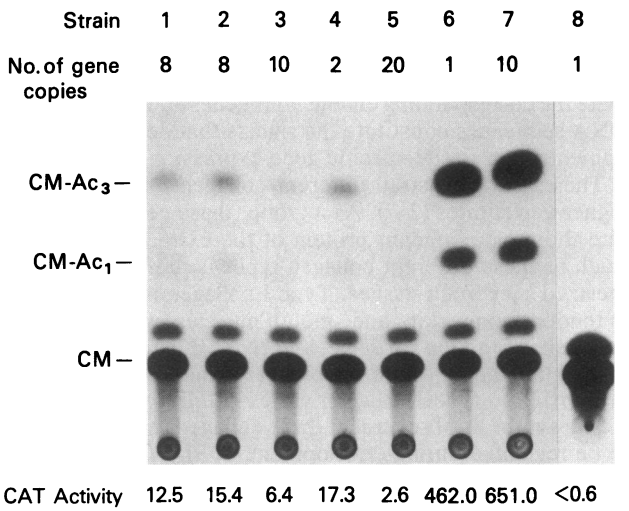

FIG. 2. Copy number and CAT expression in various strains of transgenic mice. The copy number of plasmid pAZ1003 in strains 1–8 was determined by transfer of 10 μg of tail DNA to nitrocellulose paper followed by hybridization of this DNA to ^{32}P-labeled pRSVCAT DNA (15). The autoradiograph shows the chromatographic separation of radiolabeled chloroamphenicol (CM) and its acetylated derivatives (CM-Ac_1 and CM-Ac_3) produced in CAT assays, which were performed using tail extracts as described in *Materials and Methods*. Each assay contained 50 μg of protein. CAT activity is expressed as microunits/mg of protein.

activity was found in extracts of tails (see Fig. 2). We interpret the results of Fig. 3 as follows. (*i*) In strains where several copies of the injected DNA have integrated into the host genome, these copies appear to have integrated in a head-to-tail configuration. (*ii*) Because the unit size of the injected DNA is maintained (in seven of eight strains) no major rearrangements of this DNA have occurred after microinjection of the zygotes. We assume, therefore, that the integrity of the α_2(I) collagen promoter segment present in the plasmid has also been conserved. (*iii*) The bands of weaker intensity most likely represent the sites of integration in the mouse genome. (*iv*) Both the number of copies of the collagen promoter-CAT DNA unit and its site of integration are stably inherited from one generation to the next.

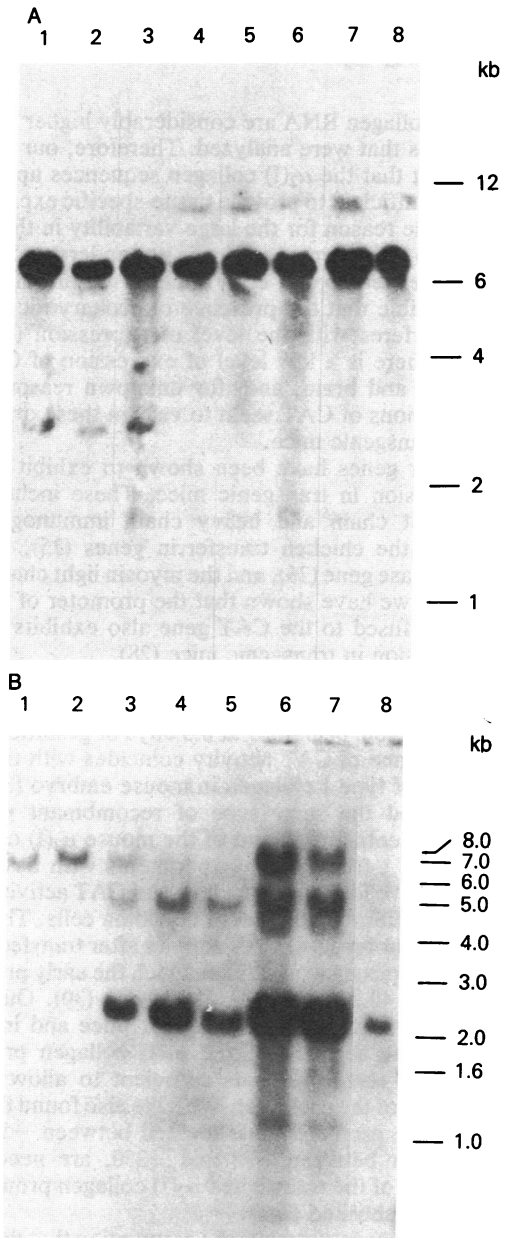

FIG. 3. Southern hybridization of genomic DNAs of representative samples of the different transgenic strains. (*A*) *Bam*HI digestion of DNAs of strains 1, 2, and 3. Lanes 1 and 2: tail DNA from two F_1 mice of strain 1. Lane 3: tail DNA of F_0 mouse of strain 1. Lane 4: tail DNA of F_1 offspring of strain 3. Lane 5: tail DNA of F_0 mouse of strain 3. Lane 6: skin DNA of same F_0 mouse as in lane 5. Lane 7: tail DNA of F_1 offspring of strain 2. Lane 8, tail DNA of F_0 mouse of strain 2. (*B*) *Apa* I digestions of tail DNAs from strains 4–8. Lanes 1 and 2: DNAs from two F_1 mice of strain 8. Lane 3: DNA from F_1 mouse of strain 4. Lanes 4 and 5: DNAs from two F_1 mice of strain 7. Lanes 6 and 7: DNAs from two F_1 mice of strain 5. Lane 8: DNA from F_1 mouse of strain 6.

To further assess the mode of transmission of the α_2(I) collagen promoter-CAT chimeric gene, the eight positive animals were mated with wild-type (wt) mice and then examined for the presence of CAT DNA sequences. About 50% of the F_0 × wt offspring of seven of the eight transgenic lines were positive for CAT DNA (data not shown). This is consistent with the idea that the α_2(I) collagen promoter-CAT gene has integrated at a single site in the mouse genome and is transmitted following a classical Mendelian segregation pattern.

Two positive F_1 animals from the same parent (strain 2) were also mated, and their offspring were examined for CAT DNA and CAT activity in a segment of tail. Fig. 4 shows that among the six F_2 mice generated by this cross, two were negative for CAT DNA. Among the four that were positive for CAT DNA, one showed a CAT DNA signal approximately twice as intense as the others. The levels of CAT activity in tail extracts followed the same distribution as those of CAT DNA among the six members of this family. The two mice that were negative for CAT DNA were negative for CAT activity, whereas the mouse that showed a DNA signal that was twice as intense as the other three also exhibited twice as much CAT enzyme activity. When this animal was mated to a wild-type mouse, every sibling in the offspring was positive for CAT, confirming that this mouse is homozygous.

Tissue-Specific Expression of CAT DNA. To find out whether the α_2(I) collagen promoter sequences are sufficient to direct tissue-specific expression of the CAT gene, various tissues from 3-week-old F_1 positive mice were examined for CAT activity. In parallel, we also determined the levels of α_2(I) collagen RNA in several of these tissues in order to compare the levels of expression of the α_2(I) collagen promoter-CAT chimeric gene with those of the endogenous α_2(I) collagen gene. Table 1 summarizes these results. In six strains, the extracts of tails contained a much higher level of CAT activity than those of any other organ. Although a detectable level of CAT activity was found in extracts of skin as well as in some other organs, such as intestine, lung, and brain, the CAT activity in these tissues was much less than that found in tail. To determine the expression of the endogenous α_2(I) collagen gene, we isolated RNA from various tissues. Fig. 5 compares the levels of endogenous α_2(I) collagen RNA in tail, skin, liver, and brain. It is clear that these levels are much higher in tails than in the other tissues, suggesting that in the 3-week-old mouse the tail is the tissue in which the endogenous α_2(I) collagen gene is most active. We conclude, therefore, that there is a good correlation between the levels of CAT activity and the levels of endogenous α_2(I) collagen RNA in various tissues. This suggests that the activity of the microinjected α_2(I) collagen

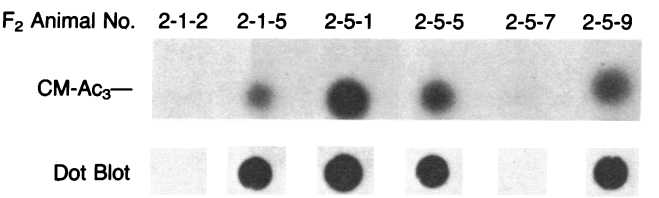

FIG. 4. Results of mating between two F_1 mice of transgenic strain 2. (*Upper*) Tail extracts were prepared from the six F_2 generation animals of a cross between male and female strain 2 mice and analyzed for CAT enzyme activity. Only the 3-acetylchloramphenicol (CM-Ac_3) spots are shown. (*Lower*) Tail DNA (10-μg samples) from the same animals were transferred to nitrocellulose paper and hybridized to a nick-translated pRSVCAT (15) probe.

Table 1. Expression of the chimeric α_2(I) collagen promoter-CAT gene in different tissues

Tissue	CAT activity, microunits/mg of protein							
	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6	Strain 7	Strain 8
Tail	12.5	15.4	6.4	17.3	2.6	462.0	651.3	<0.6
Skin	<0.6	<0.6	<0.6	<0.6	2.5	10.9	139.6	<0.6
Intestine	1.6	2.6	<0.6	<0.6	<0.6	48.5	<0.6	<0.6
Kidney	<0.6	<0.6	<0.6	<0.6	1.3	<0.6	<0.6	<0.6
Lung	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	29.8	<0.6
Brain	<0.6	<0.6	<0.6	<0.6	<0.6	33.7	20.1	<0.6
Testis/ovary	<0.6	<0.6	<0.6	<0.6	2.3	<0.6	<0.6	<0.6
Foot	ND	ND	ND	2.6	2.9	ND	ND	ND

Extracts containing 50 μg of protein from various tissues were used for CAT assay. No CAT activity was found in serum, thymus, eye muscle, tongue, heart, spleen, or sternum. ND, not done.

promoter responds to the same tissue-specific controlling mechanism as the endogenous α_2(I) collagen promoter.

We have also measured the levels of CAT activity in embryos of strain 7 at several times during development. Extracts were prepared from whole embryos at days 8.5 and 9.5 and from the caudal end of the embryo at days 10.5, 13.5, and 16.5. CAT activity is detectable beginning at day 8.5. At day 13.5 the levels of CAT activity have increased considerably. The time of appearance of CAT activity coincides with the time of appearance of type I collagen in mouse embryos (8, 9).

DISCUSSION

We have generated eight transgenic mouse strains by microinjection of a plasmid in which mouse α_2(I) collagen promoter sequences were fused to the coding sequences of the bacterial CAT gene. This chimeric gene is present in 1–20 copies per haploid genome in the different strains. The strains with multiple copies appear to have integrated the DNA in a tandemly repeated head-to-tail configuration. In one instance, strain 8, the microinjected DNA either has integrated within the chimeric gene or has undergone rearrangements. In all other strains, the integrity of the microinjected DNA appears to be conserved. In one strain, the chimeric gene is only transmitted to female offspring, although the F_0 mouse is a male. Presumably the gene has integrated in the X chromosome of the parent mouse. In all but strain 8, the CAT gene is expressed although there are large variations in the level of expression from one transgenic strain to another. The expression of the CAT gene follows a pattern similar to the tissue-specific expression of the α_2(I) collagen gene in six out of eight mice. Among the tissues that we screened, the CAT gene is preferentially expressed in the tail, a tissue where the levels of α_2(I) collagen RNA are considerably higher than in the other tissues that were analyzed. Therefore, our results strongly suggest that the α_2(I) collagen sequences upstream of the gene are sufficient to provide tissue-specific expression of this gene. The reason for the large variability in the level of expression in the different strains is not understood and is apparently unrelated to the copy number of the chimeric gene. It is possible that the presence of prokaryotic vector sequences interferes with the level of expression (31). In some strains, there is a low level of expression of CAT in intestine, lung, and brain, and, for unknown reasons, the relative proportions of CAT seem to vary in these organs in the different transgenic mice.

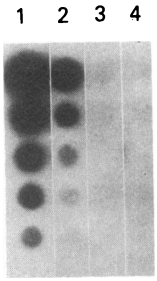

FIG. 5. Dot blot analysis of α_2(I) collagen-specific RNA in tail (strip 1), skin (strip 2), liver (strip 3), and brain (strip 4) of FVB/N mice. Three-fold dilutions (starting with 10 μg) of total RNA (in 3 M NaCl/0.3 M sodium citrate, pH 7, with *Escherichia coli* rRNA at 250 μg/ml) were spotted on a nitrocellulose filter and hybridized with an 850-bp *Eco*RI–*Sma* I DNA fragment of mouse α_2(I) collagen DNA (21), labeled by nick-translation. Hybridizations were carried out as described previously (13).

Several other genes have been shown to exhibit tissue-specific expression in transgenic mice. These include the rearranged light chain and heavy chain immunoglobulin genes (22–24), the chicken transferrin genes (25), the rat pancreatic elastase gene (26), and the myosin light chain gene (27). Recently, we have shown that the promoter of the αA crystallin gene fused to the CAT gene also exhibits tissue-specific expression in transgenic mice (28).

We have measured the levels of CAT activity in embryos of transgenic mice at several times during development. CAT activity is detectable beginning at 8.5 days of gestation. This time of appearance of CAT activity coincides with the time of appearance of type I collagen in mouse embryo (8, 9).

We have used the same type of recombinant plasmid containing an identical segment of the mouse α_2(I) collagen promoter in DNA-transfection experiments with tissue culture cells and have found that the levels of CAT activity were much higher in fibroblasts than in myeloma cells. This is in contrast to similar levels of CAT activity after transfection of these two cell types by a plasmid in which the early promoter of simian virus 40 controls the CAT gene (30). Our data, therefore, show that both in transgenic mice and in tissue culture cells, the segment of the α_2(I) collagen promoter between −2000 and +54 bp is sufficient to allow tissue-specific activity of the promoter. We have also found that two segments of the promoter, one located between −500 and −900, the other between −98 and −350, are needed for optimal activity of the transfected α_2(I) collagen promoter in fibroblasts (unpublished data).

In our experiments, we make the assumption that the levels of CAT activity are a measure of the activity of the α_2(I) collagen promoter. This assumption is supported by DNA-transfection experiments with tissue culture cells that demonstrated a direct correlation between the levels of CAT enzyme activity and the levels of correctly initiated CAT RNA, using different promoters to drive the CAT gene (15).

The level of CAT expression in the tail of transgenic mice is much lower than the level of expression of the endogenous α_2(I) collagen gene. Whereas the α_2(I) collagen RNA signal was strong, we had difficulties in detecting hybridization to CAT RNA (data not shown). One possible explanation for

this observation is that the α_2(I) collagen-promoter segment present in the recombinant plasmid pAZ1003 does not contain all the regulatory elements that are needed for optimal activity of this promoter. For example, an enhancing element, similar to those described in viral or other cellular genes (29), could be missing in our recombinant plasmid. An alternative explanation is that the CAT RNA is much less stable or that this RNA is not as efficiently spliced or not as efficiently transported from the nucleus to the cytosol. Runoff transcription experiments with isolated nuclei should help in the evaluation of the level of activity of the transgenic α_2(I) collagen promoter.

In summary, our data show that sequences upstream of the α_2(I) collagen gene that are present in our plasmid are sufficient to provide tissue-specific and stage-specific expression of this gene. This result implies that there are diffusible, *trans*-acting regulatory factors that interact with the control sequence of the α_2(I) collagen gene.

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