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A pancreas specificity results from the combination of polyomavirus and Moloney murine leukemia virus enhancer

(polyomavirus/enhancer recombinant/tissue-specific DNA replication/mouse)

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ABSTRACT **An infectious recombinant polyomavirus was constructed in which a regulatory region of its genome, the B enhancer region (nucleotides 5128–5265) has been replaced with the 72- or 73-base-pair repeat enhancer from the Moloney murine leukemia virus genome. We show that this recombinant polyomavirus displays a strong tissue specificity for the pancreas of mice. This organ was not permissive for either the parental polyomavirus, which is predominantly kidney and salivary gland specific, or the Moloney murine leukemia virus, which is lymphotropic. This result indicated that tissue specificity can be achieved by a combination of apparently modular elements. Some of the implications of a modular mechanism of tissue specificity are considered.**

The enhancer regions, which have been identified in numerous viral and cellular genes, are important genetic determinants of the tissue specificity for gene expression in mammalian cells. These enhancer regions are often, though not always, located 5′ of the promoter sites for RNA polymerase II initiation and display a long-range ability to activate transcription of cis-linked genes, often in a tissue-specific manner (1–3). One prototypic enhancer, that of simian virus 40 (SV40), has been analyzed in the greatest detail and is observed to display an enhancer function that is surprisingly refractory to genetic changes, such as the introduction of small deletions (4–7). A similar resilience to mutation has been observed with the polyomavirus (Py) enhancer region (8–10) and has led to proposals that both SV40 and Py enhancers are composed of multiple elements, which are apparently redundant but can act together in a synergistic fashion to give an enhancer effect (8, 11, 12).

Two adjacent enhancers have been observed in the Py noncoding DNA (8, 11) and have been designated as the A and B enhancer. It appears that alterations in the B enhancer can display a tissue specificity for expression in certain lines of embryonal carcinoma cells (13–15). In addition to transcriptional regulation, the Py enhancers and also the heterologous immunoglobulin heavy-chain core enhancer regulate permissivity or tissue specificity of Py viral DNA replication (11, 16), indicating that some linkage exists between tissue-specific DNA replication and transcription. Although there appears to be an overall lack of sequence specificity for enhancer function, short core sequences have been observed in many enhancers and appear to be functionally important, as shown by reversion analysis (17, 18) and tissue-specific selection (19). These core sequences, such as the immunoglobulin heavy-chain core element (TGTGGTTT) (20) present in the Py B enhancer and the adenovirus E1A core (CAGGAAG) (18) present in the Py A enhancer, are, however, also observed in various other enhancer regions that share no apparent tissue specificity (18, 21), making it difficult to envision how they might be involved in mediating tissue specificity.

We have constructed a recombinant Py virus in which the B enhancer (*Pvu* II D fragment, nucleotides 5128–5265) (9) was replaced with the 72- or 73-base-pair (bp) repeat region in the long terminal repeat (LTR) (nucleotides −340 to −180) from Moloney murine leukemia virus, Mo-MuLV (22). This recombinant will grow as an infectious though less efficient virus on 3T6 cells following DNA transfection (23). As the lymphotropism of Mo-MuLV is known to be associated with its LTR sequence (24), it was rationalized that the enhancer region within this LTR may function as a lymphotropic element. However, it was found that this recombinant virus had a tissue specificity for replication in the pancreas. The implications of this result on possible mechanisms of enhancer function are discussed.

MATERIALS AND METHODS

Cells and Viruses. Propagation of Py(A2) and Py–MuLV viruses on NIH 3T6 mouse fibroblast cells was done as described (25). Infectious Mo-MuLV was obtained from the supernatant of infected 3T3 mouse fibroblast cells also as described (26).

Whole Mouse Hybridization. Three litters of newborn mice were injected by intraperitoneal inoculation with 10^7 plaque-forming units of wild-type (A2) or recombinant Py virus and with 2.5×10^4 plaque-forming units of Mo-MuLV. Six days after infection with the Py viral stocks and 16 days after infection with Mo-MuLV, mice were sacrificed and either frozen for sectioning or organs were removed for DNA blot analysis of tissue. Adjacent sagittal thin sections were cut and transferred to either nitrocellulose for DNA hybridization (27) or to book-binding tape for hemotoxylin and eosin tissue staining. Py-specific DNA in transferred sections was detected by hybridization and autoradiography essentially as described (27). For the detection of Mo-MuLV-specific RNA, the sections were transferred to nylon membranes (GeneScreen), fixed by blotting on filter paper wet with a solution of 4% paraformaldehyde/0.1% glutaraldehyde/100 mM PO_4, pH 7.5, then digested for 5 min in a solution of proteinase K (0.1 mg/ml)/1% Nonidet P-40/10 mM sodium acetate/1 mM EDTA. The hybridization was done according to the manufacturer's (Dupont) specifications. Dried filters were exposed to Kodak XRP film at room temperature for the times indicated in the figure legends.

Immunofluorescence. Infected mice were sacrificed and frozen as described for whole mouse hybridization. Sections (20 μm thick) were transferred to book-binding tape and fixed in a solution containing 5% glacial acetic acid, 3% formalin, and 70% ethanol. Fixed sections were then treated with

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Abbreviations: Py, polyomavirus; Mo-MuLV, Moloney murine leukemia virus; SV40, simian virus 40; LTR, long terminal repeat; bp, base pair(s).

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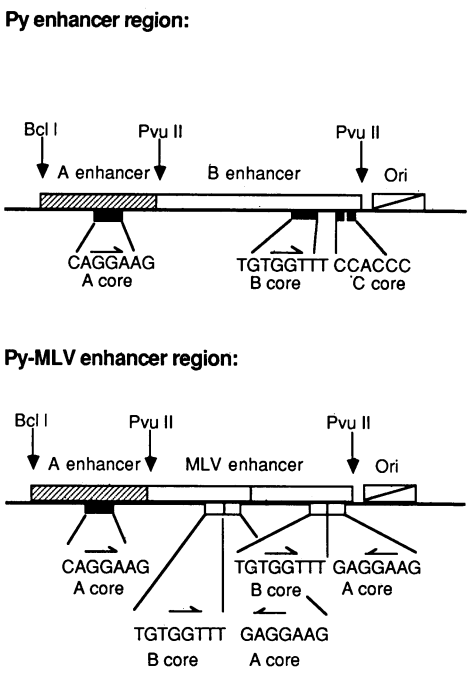

FIG. 1. Schematic diagram of the enhancer region of Py(A2) and the recombinant Py–MuLV genomes. The numbering system of Tyndall *et al.* (9) was used. The locations of the A and B enhancer elements (11) are shown. The location and orientation of core sequences are also shown. The CAGGAAG element is homologous to the adenovirus type element described by Hearing and Shenk (18). The TGTGGTAA element is homologous to the immunoglobulin heavy-chain element described by Banerji *et al.* (4). The CCACCC element corresponds to the consensus sequences found in bovine papillomavirus (28). The inserted Mo-MuLV enhancer element is shown in a positive orientation relative to the Py genome.

rabbit anti-Py capsid polyclonal antibody, followed by addition of goat anti-rabbit fluorescein isothiocyanate-conjugated antibody. Sections were mounted on glass slides with 10% glycerol, and fluorescence was visualized with a Leitz fluorescence microscope.

Southern Blot Analysis of Tissue DNA. Extraction of DNA from tissues and DNA blot hybridization were done as described (25). Tissue DNA (10 μg) was digested with *Bam*HI or *Hpa* II and run on a 1% agarose gel. Reconstructions for the determination of viral DNA copy numbers were included as controls. The relative intensities of bands on the autoradiograms were determined by tracing the grain absorbance. The quantity of Py viral DNA copies per cell in extracted tissue DNA samples was then determined by comparison to the control bands.

RESULTS

The construction of the recombinant Py–MuLV virus has been described (23). The genetic structure of this virus along with various sequence landmarks is shown in Fig. 1. Both orientations of the inserted sequence were made and the recombinant viruses were sequenced to verify the structure of the inserted DNA (29).

If the Mo-MuLV enhancer can act as a separate and complete unit of lymphotropic tissue specificity [as our previous *in vitro* results had implied (23)], then replacement of the tissue-specific Py B enhancer with the Mo-MuLV enhancer may yield a Py virus that is lymphotropic in mice. To determine the tissue specificity of the recombinant virus, newborn mice inoculated i.p. with the Py-MuLV virus were analyzed by whole mouse hybridization. Newborn mice were also inoculated i.p. with either Py(A2) or Mo-MuLV to demonstrate the *in vivo* tissue specificity of the wild-type viruses. The results are shown in Fig. 2. As expected, the Mo-MuLV-infected mice show a clear lymphotropic pattern of Mo-MuLV RNA synthesis in which the thymus and spleen are the predominant sites of hybridization. No hybridization was observed in the kidneys or salivary glands of the Mo-MuLV-infected mice. The analysis of mice at earlier times after Mo-MuLV infection shows a similar, though fainter, pattern of hybridization (not shown). The Py-infected mice show a distinctly different and nonoverlapping pattern of tissue-specific hybridization. Here the kidneys and salivary glands are the prominent sites of DNA hybridization with less, but substantial, hybridization to the epidermis, sorotal surfaces of the visceral organs and bone surfaces, and also with notable hybridization to the liver. No hybridization

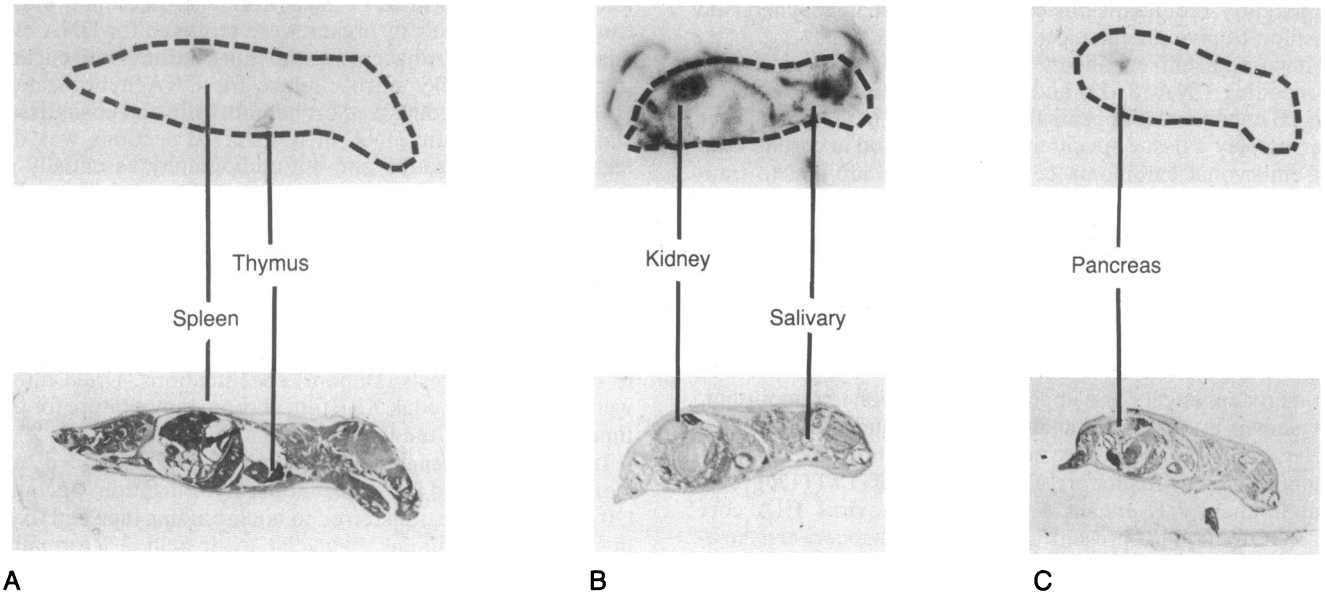

FIG. 2. (*A*) Whole mouse hybridization of a Mo-MuLV-infected mouse 16 days postinfection. (*B*) Whole mouse hybridization of a Py(A2)-infected mouse 6 days postinfection. (*C*) Whole mouse hybridization of a Py–MuLV-infected mouse 6 days postinfection. Upper panels correspond to typical autoradiographs exposed for 4 days on low-sensitivity XRP (Kodak) x-ray film. Lower panels correspond to hemotoxylin and eosin stained sections that are adjacent to the ones used for hybridization.

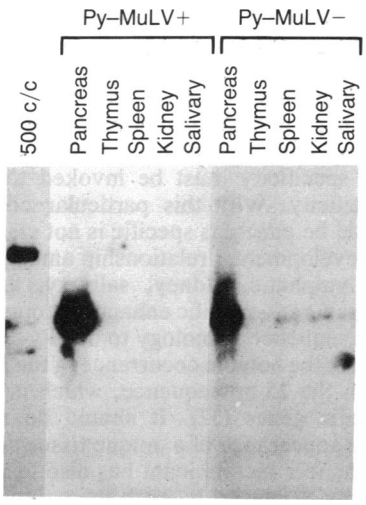

FIG. 3. Southern blot hybridization of extracted tissue DNA from mice infected with Py containing either orientation of the Mo-MuLV enhancer insert. Mice were sacrificed 6 days postinfection. Tissue DNA (10 μg) was digested with *Bam*HI. Copy/cell control lane (500 c/c) was undigested. Exposure time was for 16 hr on high-sensitivity XAR (Kodak) x-ray film.

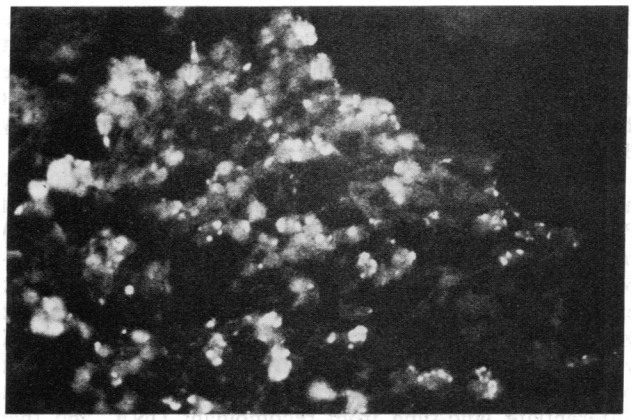

FIG. 4. Immunofluorescence of the pancreas of a Py–MuLV-infected mouse 6 days postinfection. Focal points of fluorescence are observed in the acinar cells.

was detected in the lymphatic organs of Py-infected mice. When mice were infected with the Py–Mo-MuLV enhancer recombinant in which the Mo-MuLV enhancer is in the positive orientation with respect to the Py early transcription unit, a most unexpected pattern of tissue-specific replication was observed. Only the pancreas was observed to support substantial levels of viral DNA replication. Little if any replication was apparent in the kidneys or lymphatic organs of these mice, although some low level replication could be detected on the sorotal surfaces and epidermis with long exposures of the autoradiograms (not shown).

A quantitation of the level of Py-MuLV DNA in the pancreas by Southern blot hybridization (Fig. 3) indicates that viral DNA was present at ≈2500 copies per cell in both the plus and minus orientations of the MuLV enhancer insert. This level is somewhat greater than that previously seen in kidneys with the fully permissive wild-type (A2) virus after i.p. inoculation (25) and therefore represents very efficient levels of *in vivo* DNA replication. Viral DNA was also detected in the kidneys, spleen, and salivary glands of mice infected with the minus orientation of the recombinant but at levels of <100 copies per cell. Examination of infected pancreas by immunofluorescence of whole mouse sections using polyclonal antisera to polyoma capsid antigens shows clear immunofluorescence in scattered cells throughout the pancreas (Fig. 4).

A possible interpretation of these results is that the pancreas specificity of the Mo-MuLV–Py recombinant results from the loss of a negative element in the Py B enhancer, which restricts the replication of wild-type virus, and that the Mo-MuLV enhancer is not contributing to the observed pancreas specificity. To test this possibility, mice were infected with a mixed virus stock that contained an equal mixture of genomes with a deletion of the Py B enhancer (PydlD; deletion of nucleotides 5128–5265) as well as some wild-type revertant genomes (Fig. 5*A*). This was necessitated because of the unstable nature of the PydlD virus, which quickly reverts to wild type when passaged as a virus

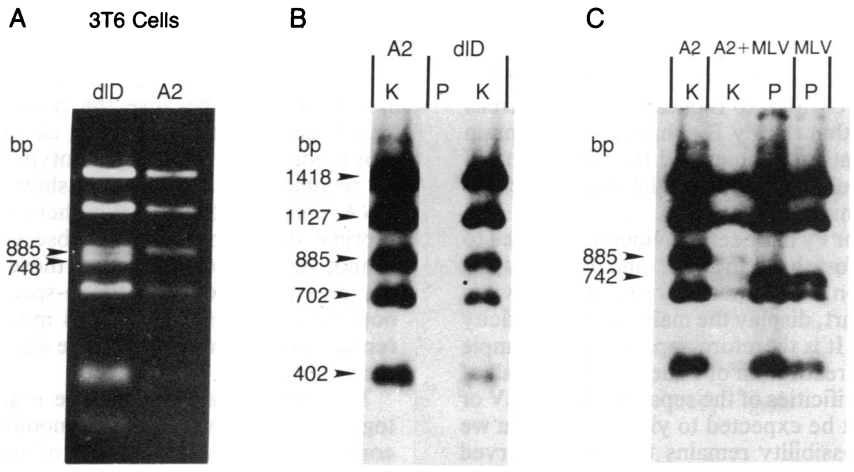

FIG. 5. (*A*) Restriction digest pattern of Hirt-extracted DNA from 3T6 mouse fibroblast cells infected with the dlD virus stocks containing dlD (748 bp) and revertant (885 bp) virus mix. Restriction pattern is compared to the Py(A2) restriction pattern. DNA (1 μg) was digested with *Hpa* II and run on a 2% agarose gel. (*B*) Southern analysis of kidney (K) and pancreas (P) DNA from mice infected with the dlD virus stock and sacrificed 6 days postinfection. Kidney or pancreas DNA (10 μg) was digested with *Hpa* II and run on a 1% agarose gel. The molecular sizes (bp) of the fragments are indicated. Exposure time was 24 hr on high-sensitivity XAR (Kodak) x-ray film. (*C*) Southern analysis of extracted tissue DNA from mice infected with Py(A2) (lane A2), Py(A2) plus Py–MuLV (lanes A2+MLV), or Py–MuLV (lane MLV) viruses and sacrificed 6 days postinfection. Kidney (K) or pancreas (P) DNA (10 μg) was digested with *Hpa* II restriction enzyme and run on a 1% agarose gel. Digestion of Py–MuLV with *Hpa* II yields a 742-bp fragment instead of the 885-bp fragment [Py(A2)] due to the presence of *Hpa* II restriction sites in the Mo-MuLV enhancer insert. Exposure time was 72 hr on high-sensitivity XAR film.

(B.A.C., unpublished observation). As shown in Fig. 5*B*, by DNA blot hybridization, these infected mice did not support replication of the Py B enhancer deletion or wild-type revertant in their pancreas, which argues, therefore, against the presence of a pancreas-specific negative element in the Py B enhancer.

It has previously been observed that, in addition to tissue-specific transcription, most tissue-specific variants of Py also show a cis-dependent activation at the level of DNA replication in their corresponding tissues and cannot complement a mixed infection with wild-type virus (30, 31). To examine this issue, newborn mice were infected with mixed virus stocks containing equal amounts of Py wild-type A2 virus and the Py–MuLV enhancer recombinant. Kidney and pancreas DNA was then extracted, digested with *Hpa* II to differentiate wild-type from recombinant DNA, and then analyzed by Southern hybridization for the presence of Py DNA. As shown in Fig. 5*C*, only the Py–MuLV recombinant DNA was observed to replicate in the pancreas. No complementation of Py A2 DNA replication was observed in the pancreas.

DISCUSSION

We have developed a polyoma-based episomal system for introducing infectious recombinant Py genomes into mice that allows us to test the cis activity of regulatory DNA on tissue-specific Py DNA replication and gene expression. This simplified approach will allow us to avoid possible complexities of chromosomal integration. This report is our first *in vivo* success at retargeting Py DNA replication via regulatory DNA rearrangements. The use of the whole mouse hybridization procedure was clearly useful in locating nonpathogenic and unexpected sites of viral DNA replication. A limitation of this approach, however, is that the recombinant genomes must propagate as an infectious virus *in vitro* and therefore may restrict the genetic analysis that can be done. We have examined the tissue specificity of a Py recombinant containing a substitution of its B enhancer for Mo-MuLV enhancer repeat region. It is apparent that this combination of the Mo-MuLV enhancer in the Py genome containing the A enhancer has yielded a pancreatic tissue specificity for Py infection of mice. This specificity is observed even when the Mo-MuLV enhancer is inserted in the opposite orientation and cannot, therefore, be due to a fortuitous *de novo* generation of an enhancer sequence at the fusion of Mo-MuLV and Py sequences. Furthermore, the inability of the Py B enhancer deletion (PydlD) to replicate in the pancreas argues against the idea that the Py B enhancer may contain a pancreas-specific negative element and that the observed pancreas specificity results simply from the loss of such an element in the recombinant.

Neither Mo-MuLV nor Py has been previously observed to show any specificity for the pancreas, and our analysis confirms this observation. In addition, our recombinant virus does not, for the most part, display the main tissue specificity of either parental virus. It is therefore apparent that a simple addition, expansion, or restriction of either a subset or all of the expected tissue specificities of the separate Mo-MuLV or Py enhancers would not be expected to yield the pattern we have observed. The possibility remains that this observed specificity is not a consequence of an interaction between the Py A enhancer and the MoMuLV enhancer but rather to an interaction of the Mo-MuLV enhancer with some other component of the Py genome such as the origin for DNA replication or the early promoter. As all the previously characterized tissue-specific variants of Py and some Mo-MuLV have been observed, however, to undergo enhancer rearrangements with duplications and deletions of the A and B core sequences (13–15) and with no changes in the origin of DNA replication or early promoter, we feel that an interaction between the Py A enhancer and the Mo-MuLV enhancer is a likely cause of the pancreatic tissue specificity we observe. With either of these possibilities, however, it remains clear that combinational principles in which distinct genetic elements can act together to yield a different and unique tissue specificity must be invoked to explain this pancreas specificity. Why this particular combination of elements should be pancreas specific is not clear, as there is no apparent developmental relationship amongst the tissues in question: lymphatic, kidney, salivary, and pancreas. Inspection of pancreas-specific enhancer sequences does not show a strong sequence homology to our Py–MuLV recombinant except for the notable occurrence of the Py B-like core element within the 25-bp sequence, which is conserved in pancreas-specific genes (32). It should be noted that in addition to the appearance of a unique tissue specificity, the Py–MuLV enhancer recombinant has also lost much of the tissue specificity displayed by both the parental Mo-MuLV and Py genomes. Although this appears to be a new observation, it is consistent with the *in vivo* phenotype of other Py enhancer variants. The PyF101 virus, for example, was selected for growth in the F9 embryonal carcinoma cell line, yet it grows rather well in NIH 3T6 cells and appears to have an expanded *in vitro* tissue specificity. In mice, however, this virus grows poorly in kidneys (a major target tissue for wild-type Py) and therefore also displays a loss of parental tissue specificity (R.R., unpublished data). A similar loss of *in vivo* tissue specificity was observed with a Mo-MuLV recombinant virus in that loss of leukemogenesis results from the insertion of the PyF101 B enhancer into its LTR region (33). In addition to these *in vivo* effects, *in vitro* tissue specificity can also display negative effects as a consequence of enhancer combinations. The substitution of the Py B enhancer with either the SV40 72-bp repeat or the IgG enhancer results in loss of the ability to replicate in several cell lines permissive for either the wild-type or B enhancer deletion. Furthermore, in the case of the SV40 enhancer recombinant, the resulting loss of replicative activity is a consequence of an interaction with the Py A enhancer, as the deletion of this A enhancer restores replication in several cell lines (23). Thus, this lost tissue specificity may also result from a combinational interaction of elements.

The mixed infection experiment in which mice were inoculated with both wild-type and the Py–MuLV recombinant indicates that the pancreas-specific DNA replication is operating in cis to restrict the replication of wild-type DNA in the pancreas, because the replicating recombinant Py–MuLV genome should supply all necessary trans factors. This is consistent with the phenotypes of other tissue-specific Py recombinants, which also show this cis restriction for DNA replication (30, 31). Whether or not transcription is also a prime determinant of the observed pancreas specificity cannot yet be determined from these experiments, but given that previously examined tissue-specific variants of Py affect both these processes, it seems most likely that both DNA replication and transcription are also tissue specific with our recombinant (23, 31).

The apparent ability of these regulatory elements to act together is consistent with a modular mechanism that can control tissue specificity. Some of the modular features of the SV40 and Py enhancer regions have been previously noted and usually encompass the core sequences common to several enhancers, such as the immunoglobulin heavy chain and adenovirus E1A enhancer cores, which are found in both Py A and B enhancers as well as in the Mo-MuLV enhancer (Fig. 1). These cores display little if any enhancer activity by themselves, but they do show significant activity when combined with either themselves or other cores, implicating a redundant feature (8, 11, 12). With the Mo-MuLV enhancer

insert, it should be noted that its A and B enhancer cores are juxtaposed but with an inverted A core. It appears then that the relative arrangement of modular elements may be an important determinant of tissue specificity. Nervous tissue specificities have been observed to result from the fusion of the metallothionein promoter and growth hormone regulatory element in transgenic mice (34), but only with a Mo-MuLV recombinant-containing enhancer from Py F101 virus have enhancer–enhancer fusions displayed alterations of tissue specificity as seen by a loss of leukemogenesis (33). A modular mechanism for tissue specificity might simplify the mechanisms and increase the versatility of the cellular machinery needed to accomplish cis-regulated tissue specificity by eliminating the apparent requirement for a large array of specific enhancers with corresponding trans-activating factors. Furthermore, such a mechanism would allow the formation of regulatory networks, possibly based on the relative arrangements of these putative modular core elements, and could suggest a possible explanation for the occurrence of such similar core elements in different enhancers with such different tissue specificities. In addition, we have recently shown that Py tissue specificity can be modified by repositioning the Py enhancers without introducing heterologous enhancer or promoter elements and thus show that the simple syntax of endogenous elements within an enhancer region can also strongly affect the tissue specificity of Py replication (23).

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