MOLECULAR AND CELLULAR BIOLOGY, June 1986, p. 2068–2079
0270-7306/86/062068-12$02.00/0
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Vol. 6, No. 6

Lymphoid and Other Tissue-Specific Phenotypes of Polyomavirus Enhancer Recombinants: Positive and Negative Combinational Effects on Enhancer Specificity and Activity

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Heterologous enhancer recombinants and deletions of the polyomavirus (Py) noncoding region were constructed and analyzed for tissue specificity of DNA replication and transcription in a number of lymphoid and other cell lines. The simian virus 40 72-base-pair repeat, mouse immunoglobulin heavy-chain enhancer, and Moloney murine leukemia virus enhancer were inserted into the *Pvu*II-D locus (nucleotides 5128 through 5265) of Py. The ability of these recombinants and the parental *Pvu*II-D deletion mutant to replicate in permissive 3T6 cells and MOP-6 cells as well as in nonpermissive mouse B lymphoid, T lymphoid, mastocyte, and embryonal carcinoma cells was determined. Wild-type Py DNA was not permissive for replication in most lymphoid cell lines, except one hybridoma line. Simply deleting the Py *Pvu*II-D region, however, gave Py an expanded host range, allowing high-level replication in some T lymphoid and mastocytoma cell lines, indicating that this element can be a tissue-specific negative as well as positive element. Substitution of the murine leukemia virus enhancer for Py *Pvu*II-D yielded a Py genome which retained the ability to replicate in 3T6 cells but also replicated well in B lymphoid cells. Substitution with the immunoglobulin heavy-chain enhancer allowed replication in B lymphoid cells but interfered with replication in 3T6 cells and mastocytomas. Surprisingly, substitution with the simian virus 40 72-base-pair enhancer repeat gave a recombinant which would not replicate in any cell line tried, including MOP-6 cells, even though other recombinants with this enhancer would replicate. Thus, we observed both cooperation and interference in these combinations between enhancer components and the Py genome and that these combined activities were cell specific. These results are presented as evidence that there may be a positional dependence, or syntax, for the recognition of genetic elements controlling Py tissue specificity.

The study of the tissue specificity of gene expression is one in which papovavirus models have been most useful. Papovavirus studies have led to the identification of enhancer sequences and subsequently to their implication in regulating tissue specificity (for reviews, see references 11 and 16). The mouse virus, polyoma (Py), has been particularly useful due to the ability to select variants with altered tissue specificity (1, 13, 22, 36, 40). Selection for growth in nonpermissive embryonal carcinoma (EC) cell lines and other studies have led to the identification of two separate transcription elements in the Py noncoding region, the A enhancer and B enhancer, which appear to display different tissue specificities (18). It has been proposed that the A enhancer, with the core sequence at nucleotides (nt) 5107 through 5117 (30), shows homology to the core sequence of the adenovirus type 5 E1A enhancer (17) and may be a relatively nonspecific enhancer, while the B enhancer, with a core sequence at nt 5189 through 5198 (30), shows homology to the core sequences of both the mouse immunoglobulin heavy-chain (IgG-H) enhancer (3) and the simian virus 40 (SV40) enhancer sequence (20, 50) but appears to be a tissue-specific enhancer and, when modified in selected variants, allows expression in F9 EC cells. Besides their activity as transcriptional enhancers, these elements are also closely involved in viral DNA replication. Two regions, α (nt 5021 through 5128) and β (nt 5128 through 5265), on the late side of the Py replication origin have also been identified as important for DNA replication (34). One or the other of these regions is required for DNA replication, implying a functional redundance for these two elements (27, 45, 47). In the absence of both the α and β regions, Py DNA does not replicate. Although the α region, which includes the A enhancer, and the β region, which includes the B enhancer, are both required in *cis* for full levels of DNA replication in 3T6 cells, it has recently been shown that both the α and β region can be replaced by the cellular IgG-H enhancer to give a DNA which replicates in mouse B lymphoid cells but not in 3T6 cells (9). This indicates that a heterologous enhancer can provide the *cis* requirement for DNA replication of the α-β region in a tissue-specific manner. Mixed infection or transfection experiments indicated that this and other tissue-specific variants and recombinants of Py show a *cis*-dependent advantage over the wild type for tissue-specific replication (7, 9, 14, 51). It is clear, therefore, that both Py transcription and DNA replication are affected by these tissue-specific genetic changes.

It seems possible that these apparently redundant and adjacent elements might interact to determine final tissue specificity. The genetic rearrangements that occur in the tissue-specific variants of Py support this idea in that nonrandom patterns of genetic changes are observed. Mostly these changes are rearrangements of the enhancer cores, in which duplications of the A enhancer into either a tandem position or to a position adjacent to the nearby B enhancer element are often observed (7, 30, 33). Specific deletions of enhancer elements as well as specific point mutations within enhancer core regions also occur. It is assumed that this pattern of enhancer duplication and deletion is a basic feature of the cellular mechanism which controls tissue-specific gene expression. Other papova-

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TABLE 1. Characteristics of murine lymphoid cell lines

Cell line	Characteristics (reference)
CTLL-20	T cell: IL-2 dependent, Lyt-1⁻, Lyt-2⁺, Lyt-3-4⁻ (2)
PSW	T cell: Lyt-1⁻, Lyt-2⁺, Lyt-3-4⁻, IL-2 independent[a]
3DT52.5	T cell hybridoma: fusion of helper T cell with BW5147 thymoma. IL-2 producer, Lyt-1[b], Lyt-2⁻, Lyt-3 -4⁺ (12)
P815	Mastocytoma (10)
A20	B cell lymphoma: produces surface immunoglobulin, not IgG, IgM, or IgA (23)
P3X	B cell myeloma: produces IgG1 (24)

[a] David W. Talmage, unpublished.
[b] Lyt-1 reactivity is not known.

viruses (28, 43) as well as leukemogenic variants of mouse mammary tumor virus (31, 35) show similar patterns of genetic rearrangement. It appears, therefore, that this tendency to duplicate or rearrange enhancer elements may be a general feature of tissue-specific selection. An issue which has not been addressed, however, is how these apparently separate and functionally redundant enhancer components might act in combination to yield the overall tissue specificity.

In this study, we observed both cooperative and interfering effects between enhancer components. We examined the ability of three heterologous enhancers to compliment a β region deletion mutant of Py and to alter the tissue specificity of transcription and DNA replication when adjacent to the Py α region. Using several lymphoid cell lines, we showed that while the inserted enhancers allowed replication in some of the expected nonpermissive tissues, other effects, such as restriction in permissive tissues and an unpredicted expansion of tissue specificity, were also observed, indicating negative as well as positive regulation of tissue specificity. In addition, an unexpected loss of SV40 enhancer activity was observed, apparently due to the adjacent Py α region. From these expected and unexpected tissue specificities, we propose that overall tissue specificity is determined not only by the presence or absence of these *cis* elements but also by their combined interactions and that this combination is an additional determinant of tissue specificity.

MATERIALS AND METHODS

Cell lines. Murine 3T6 cells were grown in Dulbecco modified Eagle medium containing 7% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. The MOP-6 line of 3T6 cells (34) and the PCC4-Aza and F9 EC cell lines were cultured under the same conditions. The MOP-6 cell line constitutively produces Py T antigens and was generously provided by John A. Hassel, McGill University, Montreal, Canada. Several murine lymphoid cell lines were provided by J. John Cohen, University of Colorado Health Sciences Center, Denver (Table 1). Lymphoid cells were grown in RPMI 1640 medium containing 5% fetal bovine serum and 50 μg of gentamicin per ml.

Construction of Py mutants. The Py *Pvu*II D fragment was replaced with an *Xho*I linker essentially as described by Tyndall et al. (45). Briefly, the Py *Bam*HI (nt 4632) to *Pst*I (nt 484) fragment was cloned into plasmid PiAN7 (39) at the *Bam*HI and *Pst*I sites within the polylinker. This recombinant has only two *Pvu*II sites located at Py nt 5128 and 5265. After digestion with *Pvu*II, the plasmid was gel purified and self-ligated with *Xho*I linkers. Recombinants were screened for loss of the *Pvu*II D fragment and acquisition of an *Xho*I site. The origin of replication of the desired mutant was subcloned as a *Bam*HI (nt 4632) to *Bgl*I (nt 109) fragment into a full-length Py genome cloned at the *Eco*RI site in plasmid pAT153 (45). Plasmids were grown in *Escherichia coli* HB101 and purified by cesium chloride density gradient centrifugation.

DNA transfection. Mutant Py genomes were excised from plasmid sequences by digestion with *Eco*RI and separated by electrophoresis in 1% low-melting-temperature agarose (SeaPlaque; FMC Corporation, Rockland, Maine) gels (40 mM Tris-acetate, pH 7.5, 1 mM EDTA). After electrophoresis, bands containing Py sequences were visualized by staining with ethidium bromide and cut from the gel, and the Py DNAs were purified by passage through an Elutip-d column (Schleicher & Schuell, Keene, N.H.) as described by Schmitt and Cohen (38). The purified Py sequences were circularized with T4 DNA ligase at substantial dilution to yield mostly unit-sized, circular molecules. Transfections were done with DEAE-dextran and chloroquine phosphate as described by Luthman and Magnusson (26). Briefly, 3T6 or MOP-6 cell monolayers grown in 100-mm tissue culture plates to approximately 70% confluence were washed twice with TSM (30 mM Tris hydrochloride, pH 7.6, 150 mM sodium chloride, 1.5 mM magnesium chloride) and overlaid with 1 ml of TSM containing 2 μg of DNA and 500 μg of DEAE-dextran. After 30 min at 37°C, the monolayers were washed once with TSM and overlaid with 5 ml of 0.1 mM chloroquine phosphate in Dulbecco medium containing 10% fetal bovine serum. After 4 h at 37°C, the chloroquine solution was replaced with 10 ml of growth medium, and the monolayers were incubated at 37°C. Murine lymphocyte cell lines were transfected by a modification of the above protocol. About 2×10^7 cells were pelleted by centrifugation (200 × *g*, 5 min). The cell pellet was suspended in 1 ml of TSM containing 2 μg of DNA and 500 μg of DEAE-dextran. After 30 min at 37°C, the cells were mixed with 10 ml of TSM and pelleted. The cells were suspended in 5 ml of 0.1 mM chloroquine phosphate in growth medium and incubated at 37°C. After 4 h at 37°C the cells were pelleted by centrifugation, suspended in growth medium to a density of 2×10^5 cells per ml, and grown at 37°C. EC cells were dispersed into single cell suspension with trypsin and transfected as described for the lymphocytes. The cells were then suspended to a density of 2×10^5 cells per ml, seeded into 100-mm tissue culture plates, and grown at 37°C.

Isolation of cytoplasmic RNA. After 40 to 48 h of incubation at 37°C, transfected cells were washed twice with cold TSM and lysed by adding 1.25 ml of cold TSM containing 0.5% Nonidet P-40 and aspirating the suspension 15 times with a Pasteur pipette. Nuclei were pelleted by centrifugation at 2,000 × *g* for 3 min at 4°C. Low-molecular-weight DNAs were extracted from this pellet as described below. The supernatant fraction was extracted once with phenol, and the nucleic acids were precipitated with 2 volumes of ethanol. Precipitated nucleic acids were pelleted by centrifugation and suspended in DNase buffer (20 mM Tris hydrochloride, pH 7.6, 10 mM magnesium chloride, 10 mM sodium chloride, 1 mM dithiothreitol). DNA was degraded by adding 60 U of RNasin (Promega Biotech, Madison, Wis.) and 10 U of RNase-free DNase I. After incubation for 20 min at 37°C the cytoplasmic RNAs were extracted once with

phenol and precipitated by adding 2 volumes of absolute ethanol to the aqueous phase.

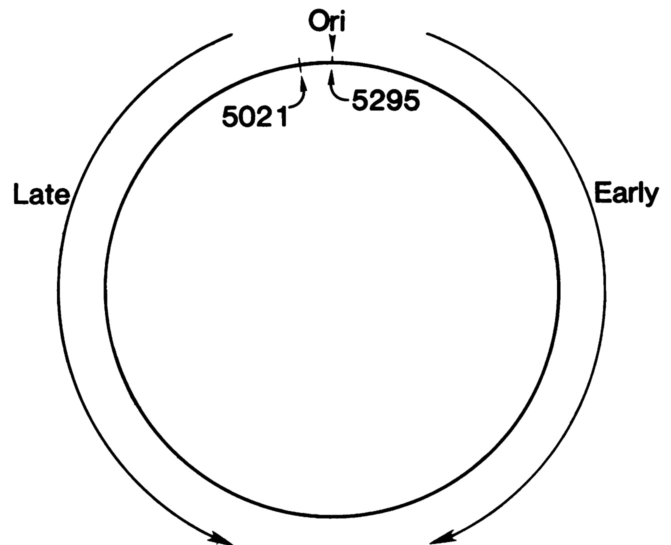
S1 nuclease analysis. Py-specific RNAs were detected by hybridization in 80% formamide to a 5′-end-labeled Py A2 *Hin*dIII A fragment (5) essentially as described previously (48). Briefly, 25 μg of cytoplasmic RNAs was coprecipitated with 10^6 cpm of probe. The precipitated nucleic acids were suspended in 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.4]–400 mM sodium chloride–1 mM EDTA–80% formamide. After 4 h at 49°C the hybridization reactions were quickly cooled on ice, and cold S1 nuclease cocktail (30 mM sodium acetate, pH 4.4, 168 mM sodium chloride, 2.7 mM zinc acetate, and 2,500 U of S1 nuclease [Boehringer Mannheim Biochemicals, Indianapolis, Ind.] per reaction) was added. Hybrids were digested for 45 min at 37°C. After incubation nucleic acids were precipitated by addition of 2 volumes of absolute ethanol, and labeled probe fragments protected from S1 nuclease digestion were separated by electrophoresis in alkaline gels (2% agarose in 30 mM sodium hydroxide–1 mM EDTA). Following electrophoresis the gels were neutralized by soaking in 40 mM Tris-acetate (pH 7.4)–1 mM EDTA and dried, and the nuclease-resistant probe fragments were detected by exposure to Kodak XAR-5 film in the presence of a Cronex Lightning-Plus intensifying screen (Du Pont).

Analysis of RNA-RNA hybrids. Early Py transcripts were detected by hybridization to high-specific-activity, single-stranded, complementary RNA probes basically as described previously (52). Briefly, cytoplasmic RNAs (25 μg) were hybridized with 5×10^7 cpm of probe. After 10 h at 49°C, RNase cocktail (10 mM Tris hydrochloride, pH 7.6, 300 mM NaCl, 5 mM EDTA, 0.8 U of RNase T_1, 1.65 μg of RNase A per ml) was added, and hybrids were digested for 30 min at 37°C. The hybrid digestions were treated with proteinase K and analyzed on 5.8% acrylamide–8 M urea gels. Following electrophoresis, probe fragments protected from RNase digestion were detected by directly exposing the gels to XAR-5 film in the presence of a Cronex Lightning-Plus intensifying screen.

Extraction of nuclear DNA and replication analysis. The nuclear pellet resulting from the isolation of cytoplasmic RNAs was suspended in an equal volume of TE (10 mM Tris hydrochloride, pH 7.6, 1 mM EDTA). Low-molecular-weight DNAs were extracted with sodium dodecyl sulfate and sodium chloride by standard methods (21). The DNAs were treated with RNase A and proteinase K, extracted once with phenol, and precipitated with ethanol, and samples were digested for 4 h with endonuclease *Mbo*I. Digested and undigested samples were electrophoresed in gels (1% agarose in 89 mM Tris-borate, pH 8.2, 2 mM EDTA) and transferred to nitrocellulose membranes (42). Py-specific sequences were detected by autoradiography after probing with nick-translated ^{32}P-labeled Py DNA and exposure to Kodak XAR-5 film in the presence of a Cronex Lightning-Plus intensifying screen.

RESULTS

Relative replication in 3T6 cells of DNA from wild-type and substituted *Pvu*II-D regions. As it has been proposed that the B enhancer (within the *Pvu*II-D sequence, nt 5128 to 5265) may be the tissue-specific enhancer for Py (18), mutants were made with deleted and substituted *Pvu*II-D regions to examine this possibility. The genetic structure of these mutants is shown schematically in Fig. 1. The parental genome (mutant *dl*D) contained a unique *Xho*I site in place of the deleted *Pvu*II-D sequence to allow insertion of the heterologous enhancers (Fig. 1B, *dl*D). The enhancers from Moloney murine leukemia virus (M-MuLV), mouse IgG-H intron, and SV40 72-base-pair (bp) repeat were inserted in both sense and antisense orientations into the *Xho*I site (Fig. 1B). Detailed restriction enzyme analysis and, for the SV40 enhancer recombinant, direct DNA sequencing by the



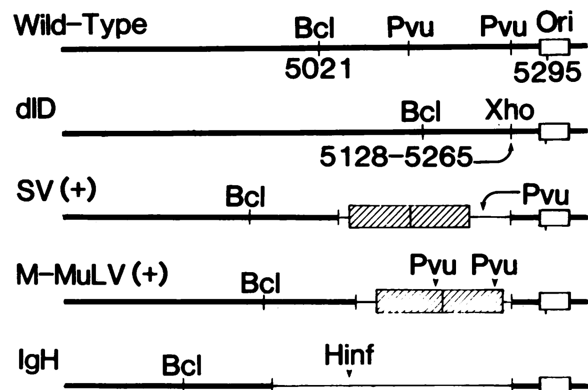


FIG. 1. Structures of the Py enhancer recombinants. (A) Py genome, showing *Bcl*I site (nt 5021 according to the A2 numbering system of Tyndall et al. [45]). Directions of early and late transcription are shown with arrows (ORI, nt 5295). (B) Structures of Py recombinant genomes. The genomes are aligned at the origin of replication (Ori). Wild type is Py A2. *dl*D has an *Xho*I linker inserted into the Py genome in place of the 137-bp *Pvu*II D fragment. SV has the SV40 72-bp repeats as a 200-bp fragment (SV40 nt 100 to 300) (5, 8). A *Pvu*II site (nt 270 of the SV40 sequence) is shown, and the 72-bp repeats are designated by hatched boxes. M-MuLV has the enhancer from the U3 region of the M-MuLV LTR cloned into *dl*D at the *Xho*I site. The M-MuLV enhancer is a 160-bp fragment stretching from nt −340 to −180 (relative to the mRNA cap site) within the U3 region of the LTR (6, 25, 46). Two *Pvu*II sites are shown (nt −330 and −250) within the 75-bp repeats (hatched boxes). The IgG-H enhancer (IgH) has about 310 bp from the intron of the mouse immunoglobulin heavy-chain gene (3). These sequences are spanned by the *Pst*I (nt 376) and the *Eco*RI site (nt 684) and have enhancer activity. The inserted enhancer elements are shown in the positive orientation relative to the Py genome.

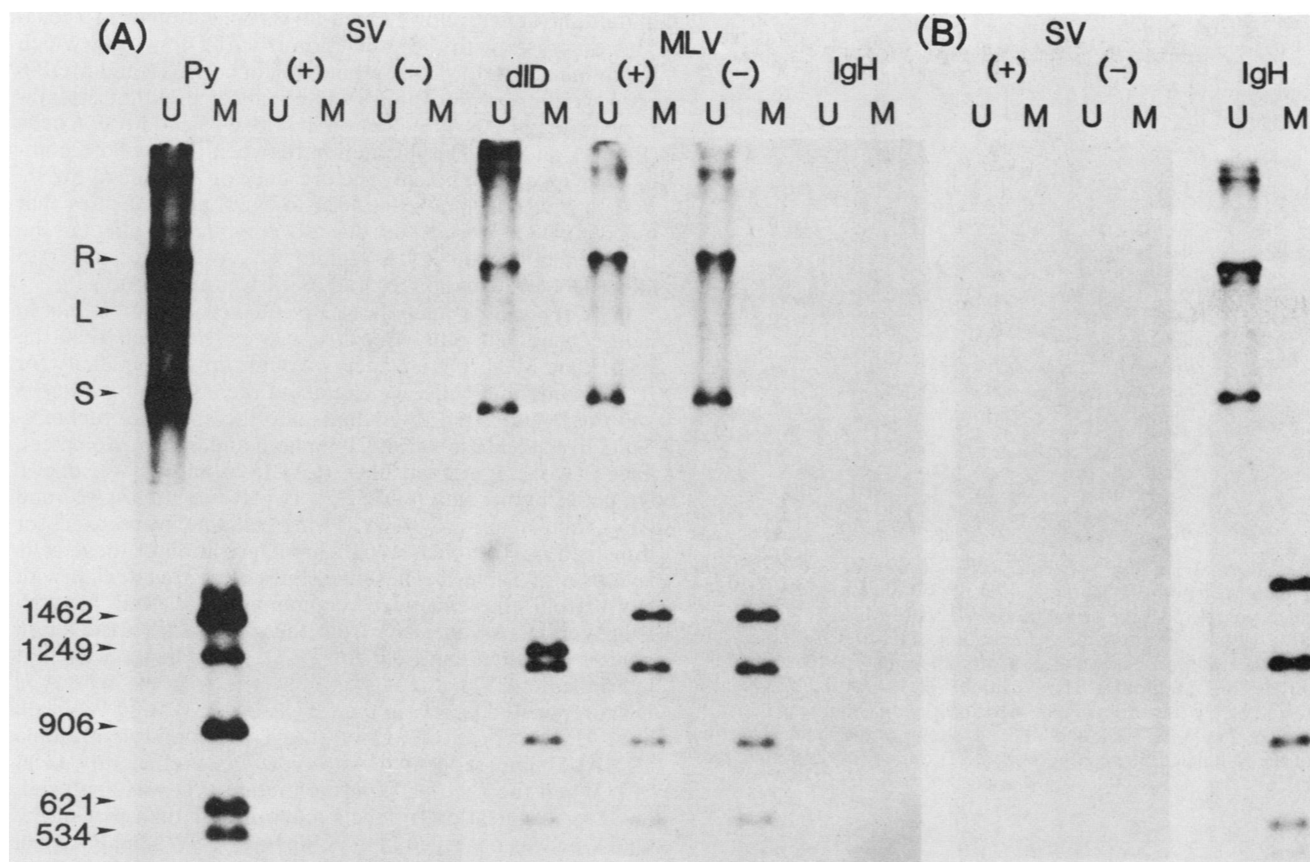


FIG. 2. Replication of Py recombinants in 3T6 cells. *dl*D contains a deletion of the *Pvu*II D fragment. The following enhancers were inserted at the *dl*D *Xho*I site. SV has the SV40 enhancer, and MLV has the M-MuLV enhancer in either the positive (+) or negative (−) orientation. IgH has the IgG-H enhancer in the positive orientation. Py is wild-type Py. Shown is a Southern analysis for Py-specific sequences of cells transfected with recombinant DNAs. Lanes: M, *Mbo*I digested; U, undigested (6, 8). Autoradiography was for 10 h (A) or 6 days (B). The positions of the linear (L), relaxed circular (R), and supercoiled (S) forms of viral DNA are indicated, with the sizes of the *Mbo*I fragments shown (in base pairs).

method of Maxam and Gilbert (29) were done to confirm the structure of the recombinants (data not shown). Following transfection into mouse 3T6 fibroblasts, Hirt extracts were prepared, digested with *Mbo*I, and analyzed by Southern hybridization for Py DNA replication. Wild-type Py DNA replicated to very high levels, as expected (Fig. 2A). Deletion of the Py *Pvu*II D fragment resulted in a genome which would replicate, albeit to levels which were approximately one-tenth those of the wild type (Fig. 2A, *dl*D). This is consistent with the previous observation of others (27, 33, 34, 45, 47) that although this β region is not essential, it does stimulate DNA replication in 3T6 cells. Substituting the M-MuLV enhancer in either orientation for the *Pvu*II D fragment did not change the levels of Py DNA replication (Fig. 2A) above that of the parental deletion mutant. Furthermore, replicated DNA was the predominant Py DNA in these extracts, with little of the transfection inoculum remaining, as seen by the absence of genome-length DNA in the digested tracks. Interestingly, the IgG-H enhancer recombinant replicated very poorly in 3T6 cells (about 1/100 that of the parental deletion mutant) and was apparent only after prolonged exposure of the autoradiogram (Fig. 2B). This implies that a restriction of DNA replication was due to the presence of this IgG-H enhancer. Insertion of the SV40 enhancer in either orientation at this *Pvu*II locus gave a recombinant DNA which would not replicate to detectable levels in 3T6 cells even after prolonged exposure of the autoradiograms (Fig. 2B). This analysis was repeated numerous times to ensure confidence in the assay. This result was most unexpected, as both we (5) and de Villiers et al. (9) had previously been successful in complementing the DNA replication and early transcription of an α-plus-β deletion mutant of Py with the same SV40 enhancer. To summarize these results, it appears that in 3T6 cells, the wild-type *Pvu*II D fragment activated Py replication. Both the IgG-H enhancer and the SV40 enhancer repressed Py DNA replication, although the SV40 enhancer repression was more complete. The M-MuLV enhancer had no effect on the level of Py DNA replication. These results were observed when these enhancer components were positioned at the *Pvu*II-D locus.

Transcription in 3T6 cells correlates with DNA replication. In addition to measuring the levels of DNA replication in 3T6 cells, we also analyzed transcription by S1 nuclease analysis using the 5′-end-labeled Py *Hin*dIII A fragment as probe (5). With this probe both early and late transcripts can be detected among the cytoplasmic RNAs isolated from 3T6 cells transfected with all the Py mutant genomes. Cytoplasmic RNAs were therefore prepared from the same transfected cells used for the DNA replication analysis and characterized by a nuclease S1 assay (Fig. 3). The 16S late transcripts were seen as an S1-protected band of 204 nt.

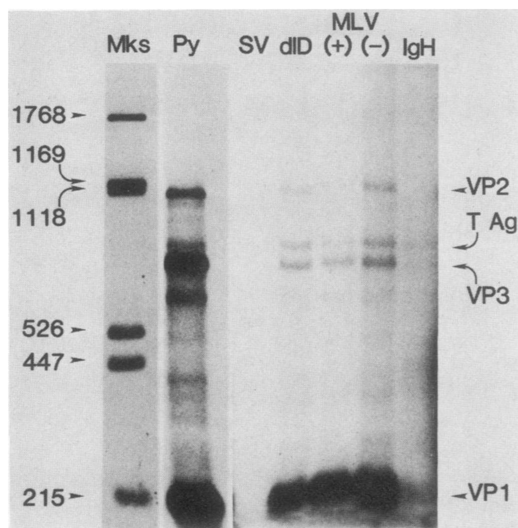


FIG. 3. Analysis of Py-specific RNAs in 3T6 cells transfected with mutant genomes. Mutants: *dl*D, *Pvu*II deletion; SV, SV40 enhancer insert (positive orientation); MLV, M-MuLV enhancer in positive (+) or negative (−) orientation; IgH, IgG-H enhancer insert; Py, wild type. Shown is an S1 nuclease analysis (described in Materials and Methods). The positions of the probe fragments protected by Py transcripts from the early region (T antigen [T Ag]) and late region (VP1, VP2, and VP3) are shown at the right. Sizes (in nt) of the 5'-labeled SV40 *Hin*dIII markers (lane Mks) are indicated at the left.

These were the predominant species observed. The 19S and 18S late transcripts appeared as bands of 1,102 and 789 nt, respectively. Early transcripts are seen as one band of about 850 nt. In all cases the amounts of transcripts that were seen were lower than those seen in cells transfected with wild-type Py DNA (Fig. 3). No Py transcripts were detected in 3T6 cells transfected with the SV40 enhancer recombinant. Small amounts of Py transcripts were detected in 3T6 cells transfected with the IgG-H enhancer recombinants. The *dl*D and M-MuLV constructs expressed both early and late RNA at moderate levels. Considered together with the replication data, this suggested that the *Pvu*II-D deletion mutant and the M-MuLV recombinant should be viable and propagate as virus in 3T6 cells. We have subsequently grown these two mutant genomes as virus by transfection of 3T6 cells (manuscript in preparation).

SV40 enhancer represses Py DNA replication in combination with, but not without, the Py α region. Because the recombinants containing the SV40 72-bp repeat inserted in either orientation into the *Pvu*II-D locus were inactive for DNA replication and transcription in 3T6 cells, we investigated further the nature of this expression. MOP-6 cells, which constitutively express the Py early proteins (34), were transfected with this recombinant to determine whether the observed repression of DNA replication could be complemented with Py early genes. In addition, we compared the replicative activity of a Py recombinant in which the Py α region in addition to the Py β region had also been deleted (nt 5021 through 5265) and replaced with the same SV40 enhancer region (7, 9). The levels of DNA replication of this α-plus-β parental deletion mutant and with the inserted SV40 enhancer are compared in Fig. 4A. The α-plus-β deletion mutant did not replicate to detectable levels in any cell line tried (Fig. 4A). Insertion of the SV40 enhancer into this mutant, however, allowed high-level replication in 3T6 cells. This contrasts with the β deletion (*Pvu*II-D) mutant, which was quite active for DNA replication in both 3T6 and MOP-6 cells, and insertion of the SV40 enhancer in either orientation abolished DNA replication in both 3T6 and MOP-6 cells (Fig. 4A and B). The distinction between these two recombinants appears to be only the presence or absence of the Py *Bcl*I to *Pvu*II region (α, nt 5021 to 5128) and indicates that this repression is somehow due to a combination effect of the SV40 enhancer and Py α region, which operates in *cis* to affect DNA replication as well as early transcription.

DNA replication analysis of Py enhancer recombinants in mouse lymphoid cells. Because it was expected that the IgG-H and M-MuLV enhancers would show a specificity for B or T lymphoid cells, we examined the ability of wild-type and *Pvu*II-D-deleted Py mutants and the enhancer recombinants to replicate in various lymphoid and mastocytoma cell lines. Two T lymphoid lines (CTLL-20 and PSW), one T lymphoid hybridoma (3DT52.5), two B lymphoid (A20 and P3X), and a mastocytoma (P815) cell line were used for transfection. The phenotypic characterization of these cells is shown in Table 1. These cell lines were transfected with DNA from the enhancer recombinants as well as with wild-type DNA and DNA from the parental *Pvu*II deletion mutant and then analyzed for Py DNA replication by blot hybridization (Fig. 5). It was observed that the wild-type DNA replicated poorly in the CTLL-20, PSW, and P815 cell lines (Fig. 5A to C). Only the T lymphoid hybridoma (3DT52.5) line replicated wild-type DNA efficiently (Fig. 5F). When the *Pvu*II-D deletion mutant *dl*D was analyzed, however, replication to levels much higher than that of the wild type was observed in the CTLL-20, PSW, and P815 cell lines (Fig. 5A to C). The most dramatic result was obtained in the PSW cell line, in which wild-type Py did not replicate at all and *dl*D replicated very well (Fig. 5A). Lack of wild-type DNA replication was clear since the wild-type Py DNA detected in PSW cells was resistant to *Mbo*I and no supercoiled Py DNA was present (Fig. 5A). It was, however, observed that most of these lymphocyte lines appeared to retain the DNA from the transfection inoculum. This was apparent from the blot hybridization, which showed significant levels of unreplicated, *Mbo*I-resistant, relaxed circular DNA remaining 48 h after transfection. This retention of input DNA was not seen with any of the nonlymphoid lines that we examined. A similar result was obtained in the P815 cell line, in which wild-type Py DNA replicated to low levels (Fig. 5C). But again *dl*D replicated to very high levels (Fig. 5C). We also examined CTLL-20 cells to determine whether the derepression seen with *dl*D also occurred in an interleuken-2 (IL-2)-requiring T lymphoid cell line. In CTLL-20 cells we observed again that wild-type DNA replicated to low levels, while deletion of the *Pvu*II D fragment allowed Py replication to much higher levels (Fig. 5B). The replicative efficiency of the IgG-H and M-MuLV enhancer recombinants in these T lymphoid and mastocytoma cell lines is also shown in Fig. 5. The IgG-H and M-MuLV enhancers had neutral effects on DNA replication in PSW cells, since the Py genomes containing these enhancers replicated to levels equal to that observed for the parental *Pvu*II-D deletion mutant (Fig. 5A). The P815 cells showed a distinction from the PSW cells in that the IgG-H and M-MuLV enhancers appeared to repress DNA replication in P815 cells, since the Py genomes containing these enhancers replicated poorly relative to the parental deletion mutant and at levels equal to that of nonpermissive wild-type Py (Fig. 5C). The effects of the IgG-H and M-MuLV

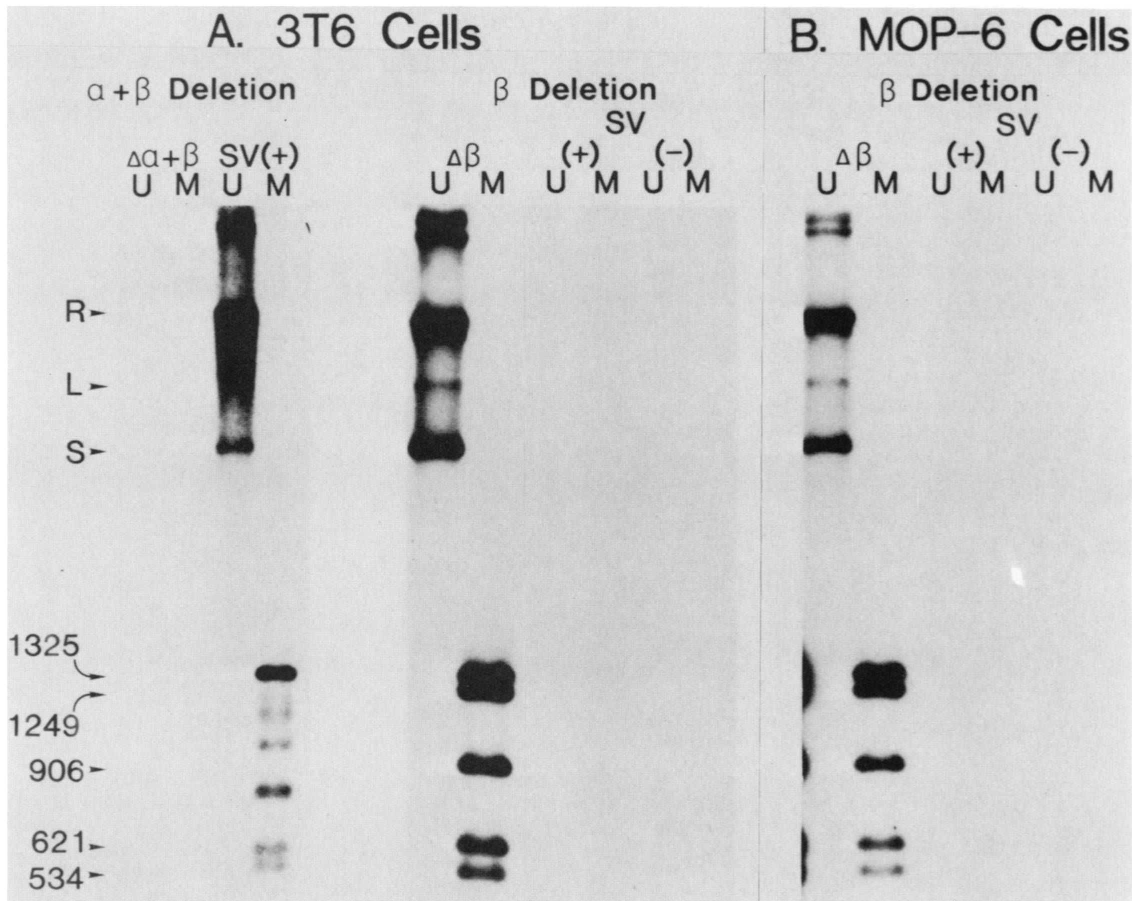


FIG. 4. Repression of Py replication by the SV40 72-bp repeats in combination with the Py α region. (A) 3T6 fibroblasts transfected with Py genomes with a deletion of the 244-bp *Bcl*I (nt 5021) to *Pvu*II (nt 5265) fragment (lanes Δα+β) or with the SV40 enhancer inserted in the positive orientation at the position of the α-plus-β deletion [lanes SV(+)]. In addition, 3T6 cells were transfected with DNA with a deletion of the *Pvu*II D fragment (lanes Δβ) or with the SV40 enhancer (lanes SV) in either the positive (+) or negative (−) orientation at the position of the deletion. (B) MOP-6 cells, expressing Py T antigens, transfected with DNA from mutant *dl*D (lanes Δβ) or one containing the SV40 enhancer (lanes SV) in the positive (+) or negative (−) orientation at the site of the deletion. Shown is a Southern analysis for Py-specific DNA. All lanes: U, undigested; M, digested with *Mbo*I. Autoradiography was for 15 h. The positions of the linear (L), relaxed circular (R), and supercoiled (S) forms of viral DNA are indicated at the left, with the sizes of the Py *dl*D *Mbo*I fragments shown (in base pairs).

enhancers on Py replication in CTLL-20 cells were not examined due to limiting supplies of the IL-2 required for growth (2). Thus it appears that the IgG-H and M-MuLV enhancers at the *Pvu*II locus show little if any activation of Py DNA replication in T lymphoid cells and may repress DNA replication in P815 cells. The clear exception to this conclusion was the hybridoma line, in which both wild-type and M-MuLV recombinant DNA replicated efficiently (Fig. 5).

It was expected that the IgG-H enhancer should confer a B-lymphoid tissue specificity to the recombinant Py genome. The M-MuLV enhancer was felt to probably be a lymphotropic enhancer due to the type of lymphomas caused in mice, but a B cell-versus-T cell specificity of enhancer function has not been established. Recombinant DNA replication in two B lymphoid cell lines (A20 and P3X) was therefore examined. In the control transfection experiment, it was noted that both the wild-type Py genome and the *Pvu*II-D deletion replicated at low but roughly equivalent levels (Fig. 5D and E), indicating relative inactivity for the Py β region in both these B lymphoid cell lines. Thus, unlike in the T lymphoid lines and the mastocytoma, the presence of the wild-type *Pvu*II D fragment did not alter the level of Py DNA replication in B cells, indicating that a T lymphoid cell specificity may exist for the repression that was due to the presence of *Pvu*II D fragment.

Insertion of either the IgG-H or M-MuLV enhancer allowed high-level DNA replication in both B lymphoid cell lines, consistent with the expected B lymphoid cell specificity for the IgG-H enhancer and also indicating a B lymphoid cell specificity for the M-MuLV enhancer in the context of the Py genome. In addition, it is worthwhile to note that the presence of the Py α element (nt 5021 to 5128) did not appear to affect the specificity of the adjacent IgG-H enhancer in that the specificity of our recombinant (i.e., β deletion) was the same as that reported for the α-plus-β deletion/IgG-H recombinant (9). This is in contrast to the SV40 recombinant, in which the presence of the Py α element had a strong combinational effect on activity.

DNA replication analysis of Py enhancer recombinants in EC cell lines. In addition to these lymphoid lines, both the F9 and PCC4-Aza EC cell lines were examined to determine

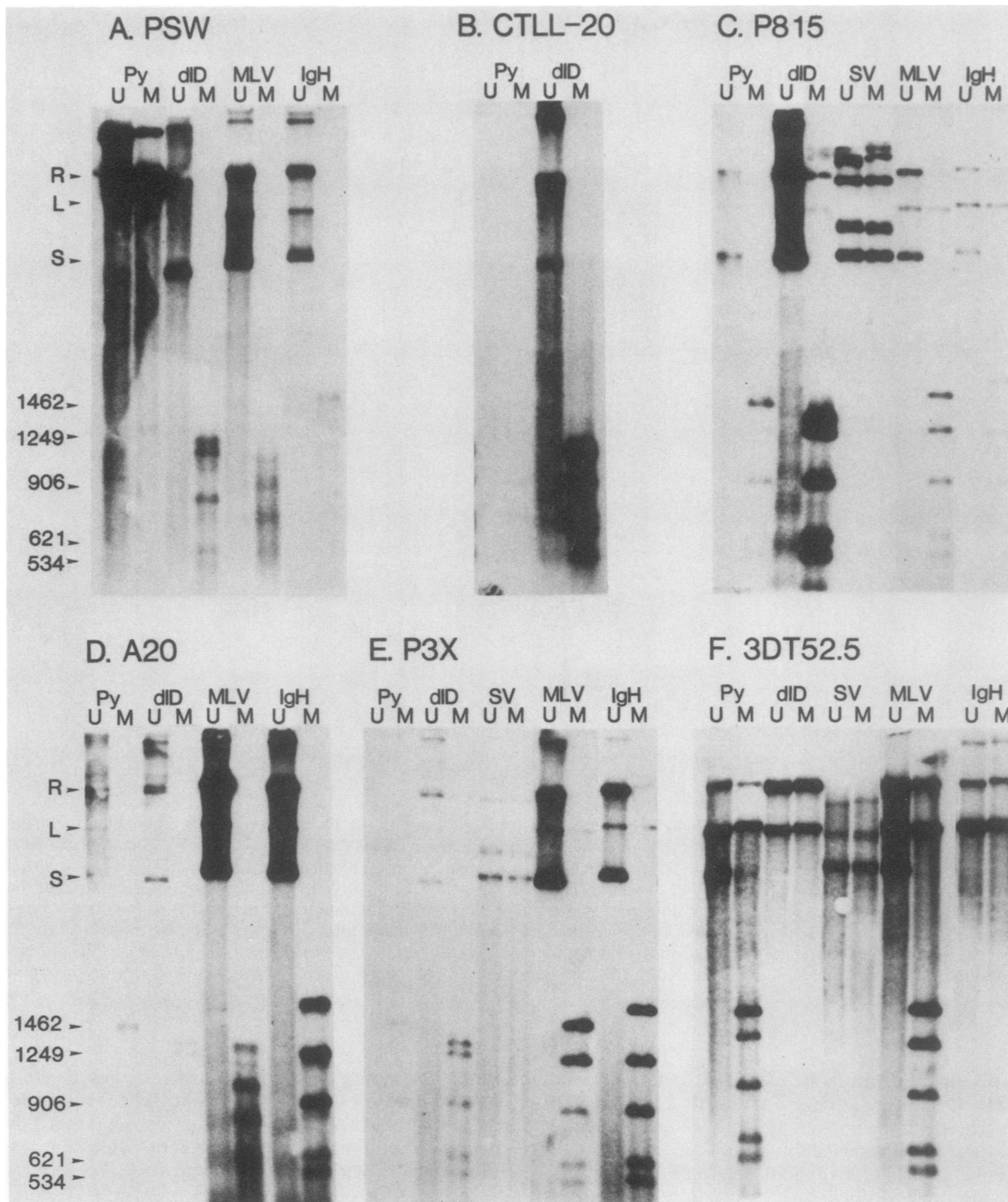


FIG. 5. Replication of recombinant Py genomes in murine lymphoid cell lines. Transfected DNAs: Py, wild type; *dl*D, *Pvu*II deletion; SV, SV40 enhancer insert; MLV, M-MuLV enhancer insert; IgH, IgG-H enhancer insert. Enhancers were in the positive orientation. Lanes: U, undigested; M, digested with the methylation-sensitive *Mbo*I endonuclease. Shown is a Southern analysis for Py-specific DNAs. Autoradiography was for 18 h. The positions of the linear (L), relaxed circular (R), and supercoiled (S) forms of viral DNA are indicated at the left, and the sizes of the Py A2 *Mbo*I fragments are indicated (in base pairs).

whether the alterations in tissue specificity that were observed with the enhancer recombinants were restricted to the lymphoid lines or represented a more generalized alteration of tissue specificity. As a positive control, an enhancer recombinant was made which contained the *Pvu*II D fragment from the Py F101 variant inserted at the *Xho*I site of our *Pvu*II deletion mutant. The DNAs were transfected into the F9 and PCC4-Aza cell lines and analyzed for replication by blot hybridization of *Mbo*I-digested DNA. Only the *Pvu*II D fragment from the Py F101 variant replicated DNA efficiently in these EC cells, although a very low level of replication was also seen with the IgG-H recombinant in

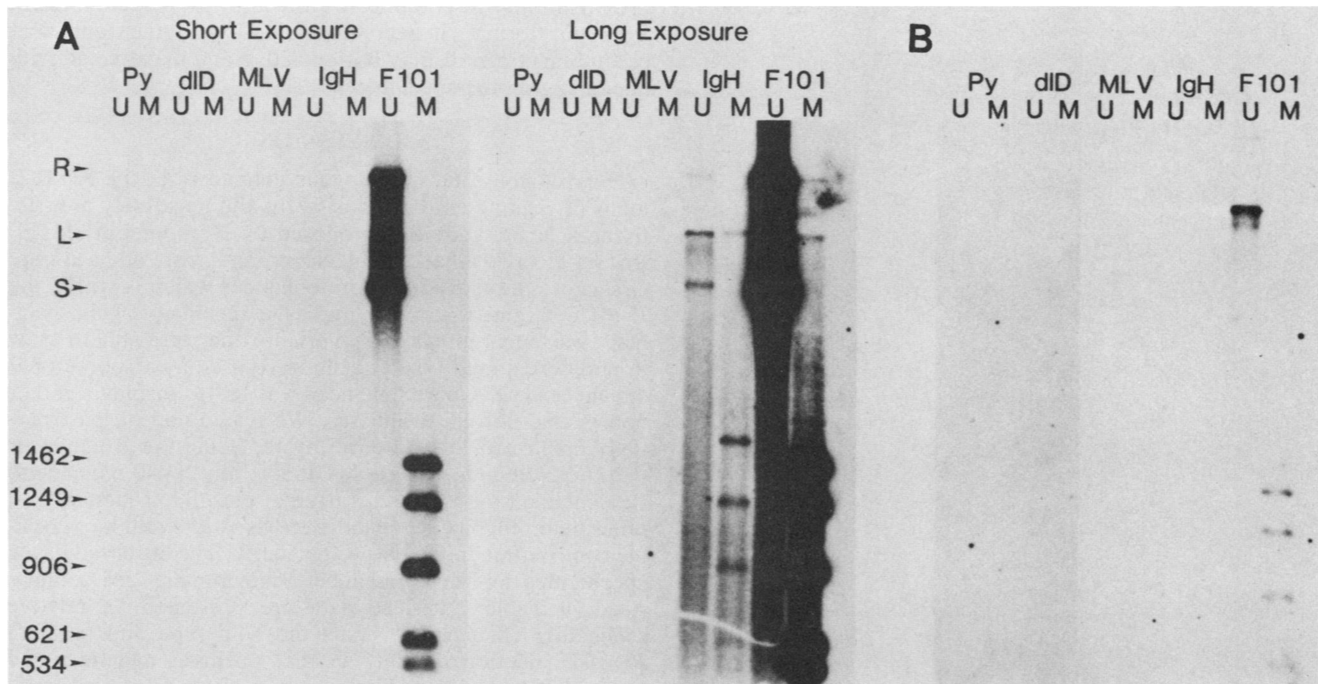


FIG. 6. Replication of recombinant Py genomes in EC cells. The PCC4-Aza (A) and F9 (B) lines of EC cells were transfected with Py (wild-type A2), *dl*D (*Pvu*II-D deletion), MLV (M-MuLV enhancer insert), IgH (IgG-H enhancer insert), or F101 (Py A2 genome containing the Py F101 [13] *Pvu*II D fragment inserted at the *Xho*I site of *dl*D). Inserted elements were all in the positive orientation. Lanes: M, *Mbo*I digested; U, undigested. Autoradiography was for 20 h (A, short exposure), 5 days (A, long exposure), or 10 days (B). The positions of linear (L), relaxed circular (R), and supercoiled (S) forms of viral DNA are indicated, along with sizes of the Py A2 *Mbo*I fragments (in base pairs).

PCC4-Aza cells (Fig. 6A, long exposure). It therefore appears that the tissue-specific effects displayed by our enhancer deletion and recombinants may not be completely generalized, because replication in these EC cells was for the most part restricted.

Tissue-specific restriction of DNA replication correlates with tissue-specific restriction of early transcription. To confirm that the tissue restriction or derepression in DNA replication correlated with a restriction in early transcription, the P815 mastocytoma line was examined for levels of early transcripts following transfection with the recombinant and parental viral genomes. The P815 cell line was chosen because of its ability replicate *dl*D efficiently. SP6 polymerase was used to direct the synthesis of labeled single-stranded RNA complementary to early mRNA (Fig. 7). Following hybridization to transfected cellular RNA and digestion with RNase, protected probe fragments were analyzed by electrophoresis in acrylamide-urea gels (Fig. 7). As expected, major bands corresponding to the large T antigen mRNA exon from the transfection with *dl*D were seen (Fig. 7, lane 3). Furthermore, a prominent band (B, 259 nt) indicated that the normal early-early initiation site was used (44). Clearly, the derepressed mutant was using the usual start sites for the synthesis of early mRNA at high levels; moreover, the large T antigen mRNA appeared to be properly spliced (44). With the wild-type transfection, little mRNA was present, consistent with the previously observed lack of DNA replication (Fig. 7, lane 1). The M-MuLV and IgG-H recombinants, which showed low levels of DNA replication, also showed a low level of early transcription, which was consistent with the notion that both DNA replication and early transcription are restricted in a tissue-specific manner.

Tissue-specific restriction in B lymphoid cells is a *cis*-dependent restriction. We next examined the possibility that the lymphoid specificity displayed by some recombinants was *cis* dependent. The B lymphoid P3X cell line was used for mixed transfection experiments to determine whether complementation would affect DNA replication. In these cells the IgG-H enhancer recombinant replicated to high levels, whereas *dl*D replicated at 10-fold lower efficiency. If the lower efficiency of DNA replication is *cis* dependent, cotransfection with the IgG-H recombinant should not increase the relative level of replication for *dl*D. P3X cells were transfected with one or both of these DNAs. The DNA replication of *dl*D was not increased by cotransfection with the IgG-H recombinant (Fig. 8). The relative levels of replication for these two molecules, which could be distinguished by *Mbo*I digestion, were seen to remain the same in mixed and separate transfections. Thus, it appears that the IgG-H recombinant had a *cis*-dependent advantage for DNA replication in P3X cells. This agreed well with the MOP-6 cell results (Table 2) in that all of the recombinant genomes which were restricted for replication in 3T6 cells were also restricted for replication in MOP-6 cells, indicating that expression of the Py early proteins is not sufficient to overcome this tissue-specific restriction.

The range of tissue-specific phenotypes displayed by these enhancer recombinants is summarized in Table 2. The activity of a given enhancer region (*Pvu*II-D, M-MuLV, or IgG-H) in each of the cell lines is expressed as either positive, which indicates a substantial increase in replication

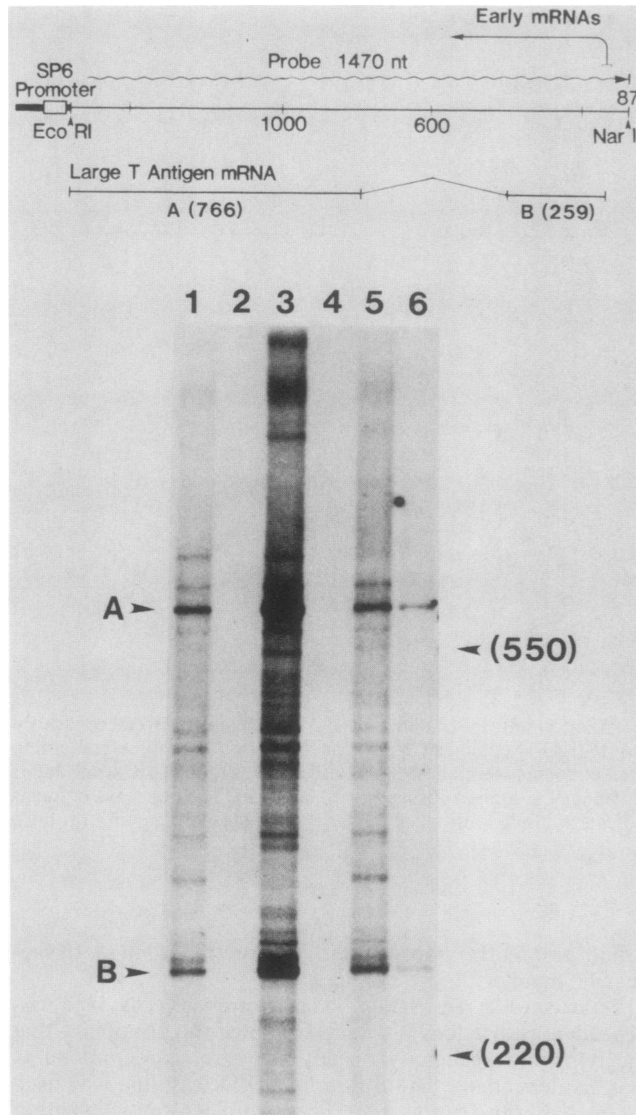


FIG. 7. Py large T antigen mRNAs in P815 cells. The strategy for detection of Py early mRNAs by hybridization to complementary RNA probes is diagrammed at the top. Py early mRNAs are initiated at nt 150 and transcribed from right to left. The cRNA probe was 1,470 nt long, from the Py *Eco*RI site (nt 1561) to the *Nar*I site (nt 87). Below the probe diagram is shown the structure of the Py large T antigen mRNAs. The probe fragments protected by the Py mRNA were 766 nt (arrow A) and 259 nt (arrow B). P815 cells were transfected with the (lane 1) wild-type Py DNA, (lane 2) no DNA (mock transfection), (lane 3) *Pvu*II-D deletion mutant, (lane 4) SV40 enhancer insert, (lane 5) M-MuLV enhancer insert, or (lane 6) IgG-H enhancer insert. Elements were all inserted in the positive orientation, and autoradiography was for 24 h. Positions of the probe fragments protected by Py mRNAs are indicated by arrows A and B. The sizes (in nucleotides) of the labeled RNA size markers (not shown) are shown in parentheses at the right.

activity; zero, which indicates replication was not affected and was equal to that of the parental deletion mutant; or negative, which indicates that replication was inhibited compared with the parental deletion. Only the hybrid T-cell line 3DT52.5 (class I major histocompatibility complex [MHC] restricted but Lyt-3-4$^+$) was able to replicate wild-type viral DNA but not that of the parental deletion mutant *dl*D. This line also replicated the M-MuLV recombinant. With this format, it becomes clear that a given enhancer recombinant can display both positive and negative activity depending on the specific cell.

DISCUSSION

Heterologous and homologous enhancers at the *Pvu*II-D locus (β region) can both positively and negatively alter Py tissue specificity. It was proposed by Herbomel et al. (19) that Py has two adjacent enhancers, an A core and a B core enhancer, and that the B core enhancer which is within the *Pvu*II D fragment can display tissue specificity. This specificity was apparent when Py variants that were able to grow in undifferentiated F9 EC cells were used as a source of B enhancer. The A core enhancer was felt to display a rather nonspecific fibroblast activity. We tested and at least partially confirmed this proposal by replacing the B enhancer with heterologous IgG-H, M-MuLV, and SV40 enhancers. Recombinant viral genomes, free of plasmid sequences and other heterologous elements, were used to avoid any possible contribution to the tissue specificity. The observed tissue specificities for these enhancer recombinants are summarized in Table 2. These data are presented as relative replication efficiencies versus the wild type and parental *Pvu*II-D deletion mutant, so that positive, negative, and neutral tissue-specific effects are apparent. These recombinant viral genomes had clear alterations in their tissue specificity. The IgG-H enhancer recombinant allowed Py to replicate and express transcripts in normally nonpermissive

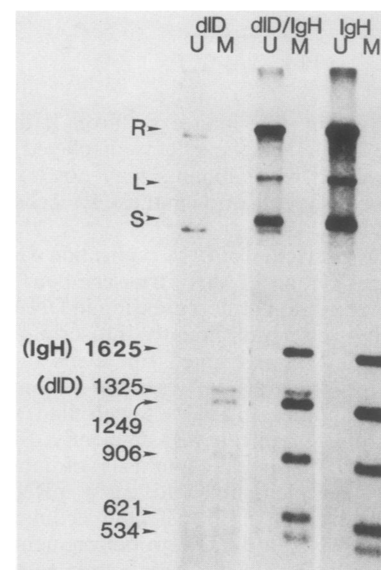


FIG. 8. Cotransfection of permissive and semipermissive genomes into P3X B lymphoid cells. P3X cells were transfected with DNA from *dl*D (semipermissive), the IgG-H enhancer insert (IgH; permissive) in the positive orientation, or with equal amounts of both DNAs (*dl*D/IgH). Shown is a Southern analysis for Py DNAs. Lanes: M, *Mbo*I digested; U, undigested. Autoradiography was for 10 h. The positions of the linear (L), relaxed circular (R), and supercoiled (S) forms of viral DNA are indicated at the left. The sizes of the Py A2 *Mbo*I fragments are indicated (in base pairs). The *Mbo*I A fragment (nt 5022 to 1192) spans the Py origin of replication, and the size of this fragment reflects deletions or insertions within the *Pvu*II-D region. Positions of the *Mbo*I A fragments corresponding to the *dl*D and IgG-H (IgH) genomes are shown at 1,325 and 1,625 bp, respectively.

TABLE 2. Effects of enhancer elements on Py DNA replication in various cell types

Cell line	Relative replication efficiency[a] with enhancer element:			
	*Pvu*II-D	IgG-H	M-MuLV	F101 *Pvu*II-D[b]
3T6	+	−	0	+
MOP-6	+	−	0	+
CTLL-20	−	ND	ND	ND
PSW	−	0	0	ND
3DT52.5	+	−	+	ND
P815	−	−	−	ND
A20	0	+	+	ND
P3X	0	+	+	ND
PCC4-Aza EC	−	±[c]	−	+
F9 EC	−	−	−	+

[a] Symbols: +, 10-fold increase in replication relative to that of *dl*D; 0, replication equal to that of *dl*D; −, replication repressed relative to *dl*D; ND, not determined.

[b] F101 *Pvu*II-D is the *Pvu*II D fragment from Py F101 (13, 14).

[c] Py recombinants containing the IgG-H enhancer replicated at low levels in PCC4-Aza EC cells.

B lymphoid cells. This was consistent with the results of de Villiers et al. (9), who showed that substitution of the α-plus-β region with the IgG-H enhancer gave a recombinant in which DNA replication and transcription were specific for B lymphoid cells. We made the additional observation, however, that not only was host specificity extended to B lymphoid cells with this IgG-H enhancer, but replication in permissive 3T6 and MOP-6 cells was also restricted. Because the parental *Pvu*II-D deletion mutant had considerable, albeit reduced, replication activity in 3T6 cells, the loss of this activity with the IgG-H recombinant was clear. Thus, the IgG-H enhancer can not only activate Py replication in B lymphoid cells, it can also repress Py replication in 3T6 cells and apparently override endogenous activation by the remaining Py α enhancer. The M-MuLV enhancer was also able to retarget Py replication for B lymphoid cells, but unlike the IgG-H enhancer it did not interfere with Py replication in 3T6 cells. Furthermore, the M-MuLV enhancer was also active in a hybridoma T cell line which has a mixed T cell phenotype. This result is consistent with the reported ability of the M-MuLV enhancer to function in B cells (15) and of M-MuLV to infect both B and T cells (41). Thus, these two enhancer recombinants display phenotypes that for the most part represent the specificity and apparent restrictions of the inserted enhancers but in addition can display tissue-specific negative effects. That the parental *Pvu*II-D deletion mutant *dl*D itself had a clearly altered tissue specificity was unexpected. This mutant was able to replicate to high levels in two T lymphoid and one mastocytoma cell line in which the wild type replicated poorly or not at all (Fig. 5). Therefore, the *Pvu*II D fragment itself appears to be both a tissue-specific stimulatory (enhanced replication in 3T6 cells, 3DT52.5, and mouse embryos [51]) and a tissue-specific repressive element (decreased replication in T lymphoid and mastocytoma cells). We are unaware of any reason a priori to expect this Py element to have a tissue-specific repressive effect. In addition, there is currently no explanation of why T lymphoid cell lines and not B lymphoid cell lines show this derepression with *dl*D, given the close developmental relationship of these cells. It is possible that the observed restrictions and activations represent some basic feature of these regulatory elements, such as an ability to function in combinations as switches in different tissues. The current results do not allow us to distinguish between these and other possibilities. Also, we cannot now rule out the possibility that there may be two very closely spaced but distinct elements, one of which is a positive and the other a negative tissue-specific regulator.

Of possible relevance to this point is an observation made with the 3DT52.5 hybridoma T cell line. These cells have a mixed T cell phenotype in that they are MHC class I restricted yet Lyt-3-4$^+$ and therefore show characteristics of both helper cells and cytotoxic/suppressor cells. These cells replicated wild-type Py DNA but not *dl*D. The converse situation was seen with the two T cell lines which were MHC class I restricted and Lyt-3-4$^-$ (CTLL-20 and PSW), in that only *dl*D replicated and the wild type did not. This could suggest that the *Pvu*II-D element may in fact be acting as a cell-specific switch being turned on in the 3DT52.5 cells and off in the CTLL-20 and PSW cells. The apparent correlation with T cell phenotype and the switching activity of the *Pvu*II-D element will require further examination to establish. A neutral tissue-specific phenotype was also often displayed by the M-MuLV enhancer recombinant. With this recombinant, replication in several cell lines was equal to that of the parental *dl*D, for example, with either 3T6 or PSW cells, hence the neutral designation. Yet even this neutral phenotype appears to be cell specific. Three groups of tissue-specific phenotypes (positive, neutral, and negative) have therefore been observed and can occur in the same recombinant genome. Yet these alterations in specificity are not completely generalized in that for the most part they did not extend to the EC cell lines (F9 and PCC4-Aza), indicating that some cellular restrictions were not affected by our recombinants.

Tissue-specific restrictions affect both DNA replication and transcription. Consistent with the observation of de Villiers et al. (9), when we examined the level at which the tissue-specific restriction was occurring with our enhancer recombinants in several cell types, we also observed that DNA replication as well as early transcription were restricted in the nonpermissive tissue. The replication activity of our recombinants in MOP-6 versus 3T6 cells, analysis of early transcript levels in P815 cells, and mixed transfection experiments in P3X cells were all consistent with the tissue-specific restriction occurring at both the level of DNA replication and early transcription and with no apparent change in the position of initiation or splicing patterns of early transcription. This is similar to the situation seen with the Py F101 variant in F9 EC cells (14) and in Friend erythroleukemia cells with the Friend leukemia 78 virus variant (7). As noted by de Villiers et al. (9), there remains no a priori reason to expect heterologous enhancers to also be tissue-specific activators of Py DNA replication. The suspicion remains that perhaps activators of viral DNA replication and transcriptional enhancers may both belong to a single class of control elements. Our results are consistent with this idea.

SV40 enhancer displays combination-dependent activity at the Py *Pvu*II-D locus and is modified by an adjacent Py α region. A most surprising observation was the inability of the SV40 enhancer to complement either Py transcription or DNA replication when placed at the *Pvu*II-D locus. Yet the same enhancer was clearly active when placed in a Py genome which was also deleted for the adjacent α region (nt 5021 to 5128; Fig. 4). It appears, therefore, that the presence of this Py α region affects SV40 enhancer function and furthermore that in addition to being unable to drive Py DNA replication, the SV40 enhancer appeared to inhibit the

remaining replication activity displayed by the parental *Pvu*II-D deletion mutant. This interference by the SV40 enhancer could not be overcome by complementation with Py early proteins, as MOP-6 cells were also unable to replicate this DNA. This is particularly surprising when it is noted that in the numerous analyses of the SV40 enhancer performed to date, this enhancer appears to be highly nonspecific in that it will activate expression of many heterologous promoters in most cell lines, including mouse cells (32, 49).

No inhibitory activity has yet been observed with the SV40 enhancer, except perhaps with the concurrent expression of adenovirus E1A protein (4). Furthermore, the ability to stimulate the Py α-plus-β deletion mutant showed that the SV40 enhancer is not inherently incompatible with the Py genome or mouse cells. This combined activity is clearly not a simple addition of the two elements (Py α and SV40 72-bp repeat) but rather a combination which somehow gives a novel inhibitory phenotype. Such a result is difficult to accommodate with models in which tissue-specific factors binding to a specific enhancer element to give either an activated or repressed genome are the prime determinants of tissue specificity, because both the α element and the SV40 enhancer, when assayed separately, are positive elements for Py DNA replication in mouse cells at this locus of the Py genome. In addition, other enhancers of similar size, both viral and cellular, placed at this locus do not show this inhibitory effect, indicating that the effect is dependent on the specific heterologous enhancer. The inference then is that the specific arrangement of these elements, and not only their presence or absence, is important in determining their combined activity.

A possible contribution to this activity by the remainder of the Py genome has yet to be determined. A perhaps related observation of enhancer incompatibility has been made by Davis et al. (6), in which the presence of the Py F101 *Pvu*II-D element within the M-MuLV long terminal repeat was not additive with the M-MuLV enhancer and led to suppression of leukemogenesis by the M-MuLV recombinant.

With our recombinant, the SV40 enhancer replaced the Py β enhancer, and it is of interest that the Py B core, the SV40 72-bp repeat, and the IgG-H enhancer all conserved the IgG-H-like core consensus sequence (5'-TGTGGTTT-3') even though they differed substantially from each other in tissue specificity (50). The Py enhancer and SV40 enhancer, however, do not compete with each other for activity in HeLa cell extracts, although the SV40 and IgG-H enhancers do compete (37). It is unlikely, however, that these consensus sequences alone account for the tissue specificity of these enhancers; furthermore, it would be difficult to envision how such apparently common elements can account for the observed tissue specificity. Yet it is clear from the patterns of genetic alterations which occurred with the tissue-specific variants of selected Py strains that the conservation and relative location of these enhancer consensus elements are important for specificity (1, 13, 22, 30). These variants displayed tandem A core duplications, tandem B core duplications, displaced A core duplications, B core deletions, and substitution of the B core with the A core. Each of these changes conferred a distinct tissue-specific phenotype, clearly implying that the combination and positioning of these elements is an important determinant of tissue specificity.

Our results also support the notion that these enhancer elements (both homologous and heterologous) may act in combination to affect the actual tissue specificity. We propose, therefore, that in addition to the enhancer specificity which is due to the specific enhancer DNA sequence, there may exist additional components of the regulatory system that are capable of recognizing other features of these sequences. One such feature may be the arrangement, or syntax, of these elements.

ACKNOWLEDGMENTS

We thank Ruth Armendariz, Nita Driscoll, and Kathy Ruiz for secretarial assistance. Rick Duke (University of Colorado) provided assistance in the maintenance of lymphoid cell lines. Frank Fujimura (La Jolla Cancer Research Foundation) and John M. Lehman (University of Colorado) provided the PCC4-Aza and F9 cell lines, respectively.

L.P.V. was a Rita Allen Scholar for cancer research and is supported by Public Health Service Career Development Award 2532413 from the National Institutes of Health. B.A.C. is supported by a Cancer Research Fellowship from the Ladies Auxiliary to the Veterans of Foreign Wars.

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