Multiple enhancer domains in the 3' terminus of the Prague strain of Rous sarcoma virus

Laimonis A.Laimins, Philip Tsichlis[1] and George Khoury

Laboratory of Molecular Virology, National Cancer Institute, National Institutes of Health, Building 41, Suite 200, Bethesda, MD 20205, and [1]The Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA 19111, USA

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ABSTRACT

The 3' terminal region of the Prague strain of Rous sarcoma virus (PrRSV) contains at least three distinct domains that comprise two functional enhancer elements. Two of these domains (designated B and C) are found in the U3 region of the 3' long terminal repeat (LTR) while the third (designated A) is located in the sequences immediately preceding the LTR termed XSR sequences. Combinations of adjacent domains [e.g., (A + B or B + C)] are capable of activating the expression of the SV40 early promoter (21 bp repeats and TATA box) coupled to coding sequences from the prokaryotic gene chloramphenicol acetyltransferase (CAT) while a single domain is inactive. Furthermore, duplication or triplication of the central domain B restores activity. The related, Schmidt-Ruppin, strain of RSV, contains an almost identical 3' LTR element, but differs in the enhancer sequences immediately preceding the 3' LTR. A model is presented in which the sequence differences may contribute to the difference in disease spectrum of transformation defective (td) variants of these viruses.

INTRODUCTION

Enhancers induce high levels of expression of viral and chromosomal genes in a orientation and distance independent manner (1). These elements have been found associated with the many DNA tumor viruses such as SV40 (2, 3), BKV (4), polyoma virus (5, 6), adenovirus (7-9, 39), and BPV (10, 42) as well as several retroviruses such as MSV (11-14) and the Schmidt-Ruppin strain of Rous sarcoma virus (15). In addition, enhancers have been identified in association with the human and mouse immunoglobulin genes (16-18) and insulin genes (19, our unpublished observations). One of the most intriguing properties of enhancers is their cell-type or tissue specificity (12, 16-18, 20-21). This specificity suggests a role for enhancers in determining the host range of viruses, as well as in the tissue-specific expression of certain cellular genes. Previous studies have suggested that sequences in the long terminal repeats of retroviruses, presumably the enhancer elements play an important role in determining the viral disease spectra, especially in those viruses which do not carry

their own transforming proteins (22, 23).

While some retroviral enhancers like that of the murine sarcoma virus (MSV) (24) have been localized within the LTR sequences, studies with the Schmidt-Ruppin strain of RSV have identified enhancer elements associated with sequences both preceding and including the 3' LTR (15). Although the 3' LTR does not appear to be essential for proviral gene expression, it has been implicated in activation of adjacent cellular genes such as c-myc resulting in cellular transformation by a model referred to as "promoter insertion" (25-27). In the case of SR-RSV, sequences immediately preceding the 3' LTR have been implicated in the activation of a downstream thymidine kinase (tk) gene (15).

We have examined the 3' region of the related Prague strain of Rous sarcoma virus to see if analogous enhancer sequences are present in this genome. The sequences immediately preceding the 3' LTR in the Prague strain have previously been shown to differ from those present in the Schmidt-Ruppin strain (28, 29). A DNA segment (F1), which constitutes a direct repeat located on either side of the src gene of both viral strains, immediately precedes the 3' LTR of Schmidt-Ruppin RSV; in PrRSV a 158 bp sequence designated XSR precedes the 3' LTR (see Fig. 1). The XSR sequence (29), while also present in the Schmidt-Ruppin strain of RSV, is situated upstream of the src gene (see Fig. 1). The U3 regions of the two RSV strains are identical except for the presence of seven extra nucleotides in Prague RSV (see Fig. 5).

A transient expression assay (CAT) (30) was used to measure enhancer and promoter activities. An enhancer element analogous to the one identified in Schmidt-Ruppin RSV, was found to overlap the 5' end of the PrRSV LTR and the adjoining XSR sequences. Additional elements, located entirely within the U3 region of the LTR, were sufficient by themselves to enhance the expression of CAT. Our data is compatible with the existence of at least three independent enhancer domains at the 3' end of the PrRSV proviral DNA. Two of the three appear to be required to form a functional enhancer element. The sequences immediately upstream of the 3' LTR of Rous associated viruses (RAV) and the td mutants of Rous sarcoma viruses have been implicated in the disease spectrum of these viruses (29). We suggest that the unique enhancer domains preceding the 3' LTR regions of the Schmidt-Ruppin and Prague strains of RSV may be involved in the disease spectra associated with each virus.

MATERIALS AND METHODS

Plasmid construction

The recombinant plasmids used in this study were constructed according to previously described methods (24) using standard recombinant DNA techniques. The parental vectors $pA_{10}cat_2$ and pCAT3M have been described previously (24). Briefly, $pA_{10}cat_2$ contains the SphI-BamHI fragment from pSV2cat (30) inserted at corresponding sites of plasmid pA_{10} (a pBR322 plasmid with a deletion from BamHI to EcoRI). The SphI/BamHI fragment contains 23 nucleotides of the SV40 enhancer plus the 21 bp repeats, the Goldberg-Hogness box, the coding sequences for CAT, and the SV40 early polyadenylation signal. The SphI site has been converted to BglII. The plasmid, pCAT3M, contains no enhancer, but only an AUG for translational initiation, the CAT coding sequences and SV40 polyadenylation signals. A BglII site immediately precedes the initiation codon.

The clone pATV-8 contains the entire PrRSV genome (28). New plasmids containing segments of Prague RSV are shown diagrammatically in Figs. 2 and 3 and are:

pPr10 The 591 bp SphI fragment containing 90 nucleotides of the 3' LTR U3 region and the immediate upstream sequences preceding the LTR (including the XSR sequences, the direct repeat sequences (F1), and the src gene), was cloned into the BglII site of $pA_{10}cat_2$ in both orientations. The sense orientation plasmid was called pPr10A, while the anti-sense plasmid was called pPr10B (Fig. 2).

pPr8 and pPr7 The 323 bp fragment from the PvuII site to the BalI site, (containing all but 40 nucleotides of the sequences between the 3' LTR and the src gene of the PrRSV clone pATV-8) and the 343 bp fragment from the PvuII site to the AluI site were isolated and their ends converted by the addition of BglII synthetic linkers. These fragments were then cloned into plasmid $pA_{10}cat_2$ at the BglII site to generate plasmids pPr8 and pPr7, respectively (Fig. 2).

pPr6A, B and pPr5 The SphI/BalI and the SphI/AluI fragments containing the 90 nucleotides at the end of the U3 region and the adjoining XSR sequences were isolated, modified by the addition of synthetic BglII linkers and cloned into the BglII site of $pA_{10}cat_2$ to generate pPr6A,B (sense, anti-sense) and pPr5, respectively (Fig. 2).

pPr-LTR13 A segment of the 3' LTR, including the entire U3 region, the R region, and a portion of the U5 region to the BstEII site was isolated on a fragment that also contained the XSR sequences and the direct repeat

sequence (F1) to the PvuII site (726 bp) (Fig. 3). Following addition of BglII linkers to the ends, this fragment was cloned into the BglII site of pCAT3M to generate the plasmid pPr-LTR13 (sense orientation).

pPr-LTR14 The BalI site of plasmid pPr-LTR13 was converted to a BglII site and the 403 bp fragment containing the converted BstEII end was re-cloned into pCAT3M (Fig. 3).

pPr-LTR15 The 381 bp AluI to BglII fragment of pPr-LTR13 containing the entire U3 region with associated R and U5 sequences was cloned into pCAT3M in the sense orientation following the addition of BglII linkers to the AluI site.

pPr-LTR2 and pPr-LTR16 A series of Bal31 deletions from the 3' end of the PrRSV sequences of plasmid pPr-LTR14 were constructed as follows. The plasmid was cleaved at the single XbaI site preceding the BglII site and treated with Bal31 for various periods of time followed by the addition of XbaI linkers. Resultant plasmids were analyzed by restriction enzyme analysis and two were chosen for further study. pPr-LTR2 was sequenced using the M13 dideoxy sequencing methodology (32) while pPr-LTR16 was analyzed only by restriction enzyme analysis.

pPr-LTR17 The SphI site of plasmid pPr-LTR2 was converted to an XbaI site by the addition synthetic linkers. Deletion of the sequences between the converted SphI site and the XbaI site in the U3 LTR of pPr-LTR2 generated pPr-LTR17.

pPr12A, B The XbaI/EcoRI fragment containing the U3 sequences of the PrRSV LTR was isolated from plasmid pPR-LTR2 and modified by the addition of BglII synthetic linkers. This fragment was then cloned into the BglII site of plasmid, $pA_{10}cat_2$ (A = sense; B = anti-sense).

pA7 The SphI site of pPr-LTR14 was converted to a BamHI site by the use of T4 polymerase and the addition of synthetic linkers. The presence of an additional BamHI site at the 3' end of the CAT gene allowed the deletion of all CAT coding sequences as well as all U3 and U5 sequences to the SphI site. The SphI/BalI fragment now contains BamHI and BglII ends, respectively.

pPr1, pPr2, pPr3 A series of Bal31 deletions was generated from the BamHI site of plasmid pA7. Following the addition of BamHI linkers, the individual BglII/BamHI fragments were isolated and cloned into $pA_{10}cat_2$. Restriction analysis was used to determined the approximate size of the deletions, in plasmids pPr3, pPr2, pPr1.

pPr4 The XbaI/SphI fragment from pPr-LTR2 was isolated, modified by

addition of BglII linkers and cloned into the BglII site of $pA_{10}cat_2$.

CAT assay Transfection of DNAs into the CV-1 line of monkey kidney cells, mouse L cells, and chicken embryo fibroblast (CEF) cells was performed as previously described (33). Following incubation at 37° for 48 hours, cell lysates were prepared and assayed for CAT activity by previously described methods (24, 30).

Primer Extension At 40 to 48 hours following transfection, RNA was extracted from cell cultures by the hot phenol method (18). Briefly, the cell monolayer in 100 mm petri dishes was lysed by the addition of 3 ml of 50 mM NaAc pH 5.2, 1% SDS. After incubation for 3 minutes at 23°, 3 ml of phenol equilibrated in 50 mM NaAc at 60° was also added. Following incubation at 60° for 15 minutes and at 0°C ice bath for 15 minutes, the lysate/phenol mixture was centrifuged for 15 minutes at 1000 xg, the aqueous layer removed, and the RNA was precipitated with ethanol to be used for primer extension analysis. A primer was constructed from a 102 bp EcoRI/PvuII CAT coding fragment which had been labeled with $\gamma[^{32}P]$ ATP at the RI site (see Fig. 4). The fragment was treated with ExoIII for 30 minutes and then coprecipitated with the RNA (20-30 µg) from one petri dish. The RNA/DNA pellet was resuspended in 10 µl of hybridization buffer (80% formamide, 0.4 M NaCl, 40 mM Pipes pH 6-8, 1 mM EDTA) and incubated for 5 minutes at 75°C followed by 3 hours at 37°C. The hybrids were then precipitated and resuspended in 50 mM Tris pH 8.3, 100 mM KCl 6 mM $MgCl_2$, 10 mM DTT, and 2.5 mM of each dXTP. Following the addition of actinomycin D and reverse transcriptase (Life Sciences), the solution was incubated at 4°C incubated for 60 minutes followed by the addition of 2 µl 250 mM EDTA and 2 µl 2 M NaOH. After additional incubation for 30 minutes at 37°C, the pellet was precipitated, resuspended in a buffer containing 80% formamide, 1 mM EDTA, 10 mM NaOH and analyzed on a 6% polyacrylamide urea gel.

RESULTS

The 3' LTR and adjacent viral sequences from Prague Rous sarcoma virus (PrRSV) were examined for their ability to activate the expression of a heterologous gene, a property characteristic of enhancer elements. DNA fragments containing various portions of the direct repeat (F1) downstream from the src gene, the exogenous virus-specific region (XSR) and the U3 region of the LTR (see Fig. 1) were placed upstream of the SV40 early promoter (consisting of 21 bp repeats and the Goldberg-Hogness box) coupled

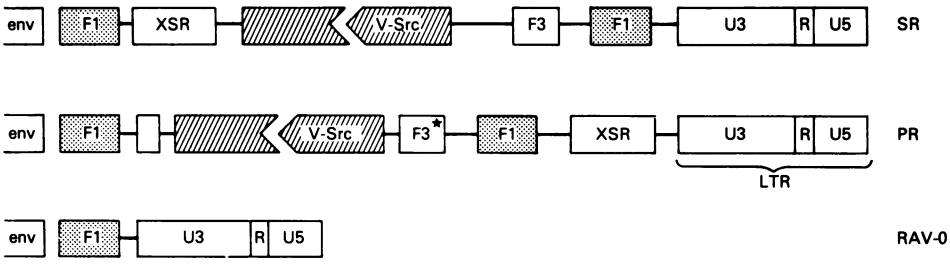

FIG. 1. The arrangement of sequences at the 3' termini of the Schmidt-Ruppin (SR) and Prague (Pr) strains of RSV and the endogenous RSV-associated virus RAV-0 (36). The LTR region of the SR and PrRSV differ only by the presence of seven additional nucleotides in PrRSV (see Fig. 5). The 148 bp XSR sequence immediately precedes the U3 region of PrRSV; in SR, the F1 direct repeat immediately precedes U3. The XSR sequence which is adjacent to src in SR, is deleted in the td variants of this strain by a recombination event occurring between the two F1 direct repeats. The F1 repeats are identical in the two strains. RAV-0 LTR contains numerous deletions in U3 region of its LTR by comparison with the RSV strains.

to chloramphenicol acetyltransferase (CAT) coding sequences ($pA_{10}cat_2$). The ability of these fragments to enhance expression from the SV40 early promoter was examined in a transient assay in which plasmids were transfected into chicken embryo fibroblasts (CEF) by the calcium-phosphate method. At 48 hours after transfection, cell lysates were prepared and examined for CAT enzyme activity. The CAT assay (24, 30) permits rapid and quantitative screening of sequences for enhancer activity.

Identification of an enhancer overlapping the U3 and XSR regions. A 591 bp fragment containing 99 nucleotides of the U3 region, the entire XSR region (148 bp), the F1 direct repeat (101 bp), 96 nucleotides of F3, and approximately 156 nucleotides of the src coding sequences was initially examined for enhancer activity. This fragment was inserted into the BglII site in the $pA_{10}cat_2$ plasmid in both orientations (pPr10A, B) relative to the SV40 early promoter (Fig. 2). $pA_{10}cat_2$, which contains a portion of the SV40 early region from SphI to HindIII coupled to CAT coding sequences as well as the polyadenylation signals, has been described previously (see 24). This plasmid lacks an enhancer element. Following transfection into CEF cells, plasmids pPr10A and B induced CAT activity at approximately 60 times the level observed with the recipient plasmid, $pA_{10}cat_2$, (29.5 U versus 0.5 U, see Fig. 3). This is twice the level of activity observed when plasmid pSV2cat, which contains the SV40 enhancer (30) was transfected in the same cells. The ability of this 591 bp frag-

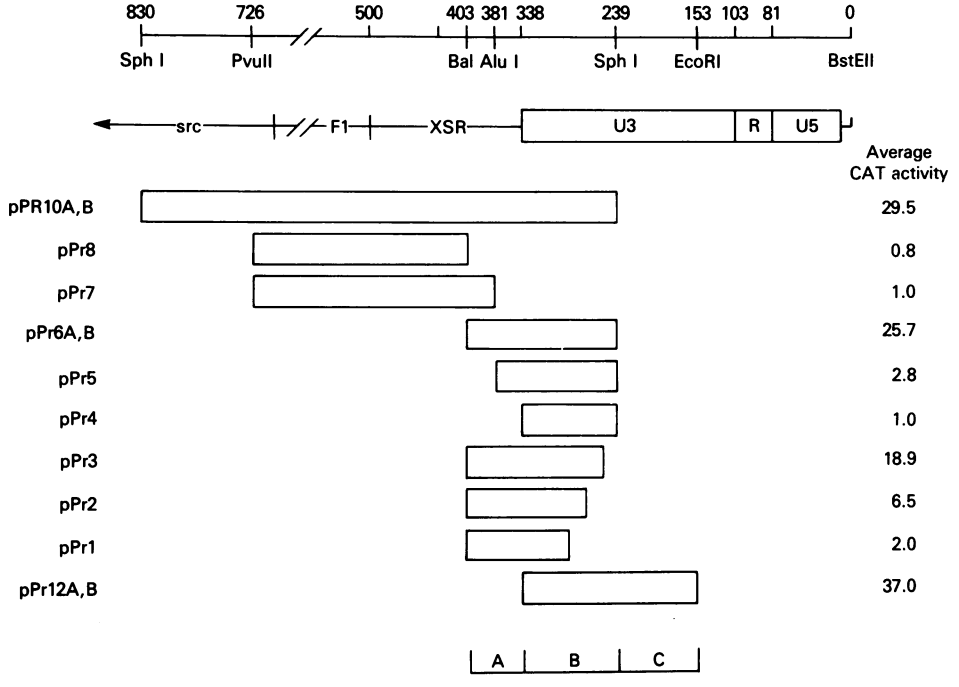

FIG. 2. Identification of enhancer regions in 3' terminus of Prague Rous sarcoma virus. The fragments shown above were isolated as described in Methods and inserted at the BglII site in the test plasmid $pA_{10}cat_2$ to evaluate enhancer activity. The plasmid, $pA_{10}cat_2$, contains the SV40 21 bp repeat sequences, the Goldberg-Hogness box and the SV40 polyadenylation signals coupled to chloramphenicol acetyltransferase coding sequences. The CAT activity is the average of at least three experiments and is compared to the expression of pPrRSV (LTR13) which is set at 100. The assays were conducted on extracts from 5 x 10^5 cells for 30 min. The three enhancer domains (A, B and C) are shown at the bottom.

ment to activate expression of the SV40 early promoter at high levels suggests that it contains an enhancer function.

To define more specifically the location of enhancer elements, a series of deletions within the 591 bp fragment were constructed (Fig. 2). Plasmids pPr8 and pPr7, contain the major portion of the XSR and F1 direct repeat sequences (from the PvuII site to the BalI or AluI sites, respectively, see Fig. 2) coupled to the SV40 early promoter. Neither of these plasmids enhanced expression of the CAT gene in CEF cells. In contrast, plasmids pPr6A and B, which contain a fragment consisting of 65 nucleotides at the 3' terminus of the XSR sequence and the adjoining 99 nucleotides of the U3 region of the LTR in both orientations, activated CAT expression to

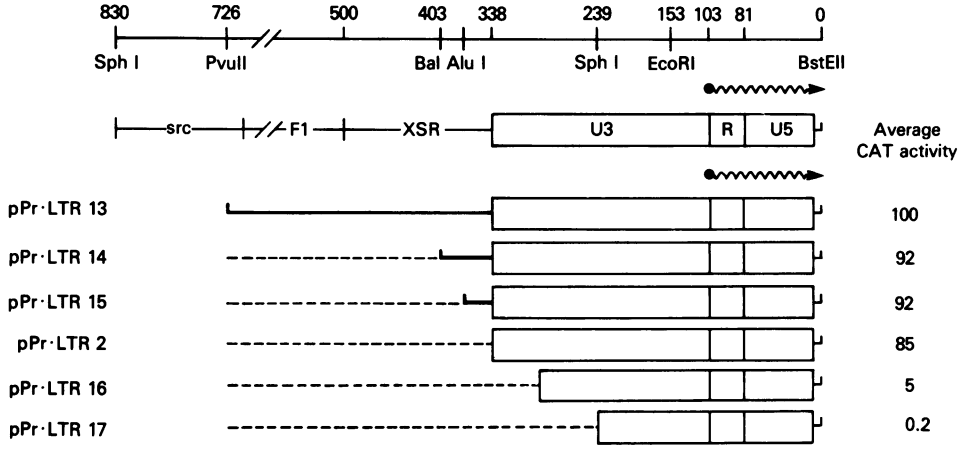

FIG. 3. Determination of sequences required for PrRSV LTR expression. Deletions of LTR and flanking sequences were constructed as described in Methods. The endogenous PrRSV promoter sequences and cap site were used in conjunction with the plasmid pCAT3M which contains the coding sequences for CAT, but no promoter elements. CAT activities for the various constructs placed immediately adjacent to the CAT gene are the average of at least three experiments. The bold lines indicate retained sequences while the dashed lines indicate deleted sequences. The wavy line represents the mRNA transcript.

the same levels observed with plasmids pPr10A and B. The deletion of 22 nucleotides in the 5' to 3' direction from the XSR region, present in Pr6 plasmid (plasmid pPr5, Fig. 2) resulted in a 90% reduction of CAT gene activity compared to pPr10. Removal of all the XSR sequences (plasmid pPr4) reduced CAT expression to background levels. The 3' boundary of the enhancer was defined following a series of Bal31 nuclease deletions from the SphI site. The sequential removal of nucleotides upstream from the SphI site in the pPr6 fragment (pPr3, pPr2, pPr1) resulted in a step-wise decrease in the induction of CAT activity. The deletion of approximately 26 nucleotides from this SphI site (pPr1) eliminated virtually all CAT activity. Thus, sequences both within and immediately upstream of the 3' LTR of the PrRSV are required for the function of this enhancer element. This is in agreement with the findings of Luciw et al. for the SR RSV enhancer element (15).

Deletions within the PrRSV. The observation that sequences outside the 3' LTR contribute to enhancer activity on a heterologous promoter (SV40) raises the question of how these sequences contribute to expression from the LTR, itself. To examine this question, a series of plasmids were

constructed which utilized the endogenous transcriptional regulatory sequences (i.e., promoter elements) within the LTR including the TATA box and mRNA cap site. The plasmid, pPr-LTR13, was constructed which contains the entire 3' LTR of PrRSV, the F1 direct repeat and XSR regions (Fig. 3) coupled to CAT coding sequences present in plasmid pCAT3M (24). The latter contains no promoter elements. CAT expression of pPr-LTR13 was due entirely to endogenous LTR and XSR regulatory sequences. A derivative of pPr-LTR13 that contains only 65 nucleotides (BalI) of the XSR sequence in addition to the 3' LTR and CAT coding sequences (pPR-LTR14) was constructed. The activity of both pPr-LTR14 and pPr-LTR13 were approximately equal suggesting that the sequences between the PvuII site and the BalI site (Fig. 3) do not contribute significantly to the transcriptional activity of the LTR promoter. Deletions extending into the 5' end of the LTR were generated by Bal31 nuclease digestion from the BalI site in plasmid pPr14. Surprisingly, the transcriptional activity of the LTR did not diminish until sequences between nucleotides 338 and 290 were removed (pPr-LTR16). Upon further deletion of 51 additional nucleotides to the SphI site (pPr-LTR17), expression was completely abolished. The reduction in LTR activity was not observed until nucleotides well inside the region defined above for enhancer activity on the heterologous SV40 promoter were deleted. A portion of the 164 bp Pr-RSV enhancer element (from BalI to SphI) as defined in the CAT assay employing SV40 components (Fig. 2) appears not to be required for transcriptional activity of the LTR transcriptional unit.

Identification of a second enhancer element in the U3 region of the PrRSV LTR. As demonstrated above, the promoter function of the PrRSV LTR does not require adjacent viral sequences. If transcription from this LTR promoter is similar to that described for other retroviruses, one might expect an enhancer element to reside entirely within the U3 region. Yet, enhancer activity defined above for PrRSV, and by Luciw et al. for SR RSV (15) extends beyond the 5' boundary of the 3' LTR. To determine whether the U3 region of the RSV-LTR contained another enhancer element located entirely within the LTR, which is responsible for the function of the LTR itself, a 175 bp fragment from the polypurine stretch (nucleotide 338) to the EcoRI site (nucleotide 153; see Fig. 2) was isolated and cloned into the BglII site of the test plasmid, $pA_{10}cat_2$. The resulting plasmids, pPr12A, B, clearly enhanced the activity of the SV40 early promoter in an orientation independent manner to levels equal to or greater than that observed with plasmid, pPr6 containing the SphI to BalI fragment (Fig. 2).

Based on the results with, pPr-LTR16, the 5' boundary of this enhancer element probably lies within 30 nucleotides of the polypurine stretch at the 5' end of the 3' LTR. As both plasmids pR6 (A, B) and pPR12(A, B) contain a common set of sequences (referred to here as domain B) from the polypurine stretch at the end of the U3 region to the SphI site inside U3, it seems likely that this may be a common domain for the two (overlapping) enhancers. Plasmids pPr6 (A, B) and pPr12 (A, B) contain different sets of sequences. As indicated diagrammatically in Fig. 2, pPr6 contains segments A and B while pPr12 contains segments B and C. The 3' LTR and the upstream XSR sequences of PrRSV therefore appear to contain at least three sequential enhancer domains. Combinations of adjacent domains (A + B or B + C) are sufficient for functional enhancer activity.

Multimeric forms of a central enhancer domain. It appears from the data presented above that the PrRSV enhancer element consists of at least 3 domains which do not function independently. To determine whether each of these putative domains provides a unique complementary function to the adjacent domains or whether tandem repetition of a domain is sufficient to create a functional enhancer element, the following experiment was performed. The central enhancer domain (B) from the SphI site to the polypurine stretch, (pPr4), was isolated and recloned singly or multiply into $pA_{10}cat_2$. By itself, this fragment does not function as an enhancer (pPr4, see Fig. 2). CAT activity in plasmids carrying a duplication (pPr13) or a triplication (pPr14) of the B domain were 30% and 100%, respectively, of the level observed with the plasmids pPR6 containing domains A + B (8 U, 25 U and 25.7 U). This suggests that an important feature of the RSV enhancer element is the multimeric nature of its domains, rather than a functional complimentarity between the individual segments present in the native sequence.

Mapping the 5' ends of CAT transcripts. To confirm that the fragments from PrRSV LTR act as enhancers by augmenting transcription at the correct start sites, the 5' ends of the CAT transcripts were mapped using the primer extension technique (see Methods). A band of 307 nucleotides was expected if initiation occurs at the same site observed with plasmid pSV2cat (see lane a, Fig. 4). Similar size bands were observed in transfections with plasmids pPr6A (lane b) and pPr12a (lane c). We therefore conclude that correct initiation of CAT RNA occurs under the influence of the SV40 enhancer and both PrRSV enhancers (domains A + B and B + C). Although primer extension does not necessarily represent a quantitative RNA assay, the intensity of the bands observed correlates with the relative

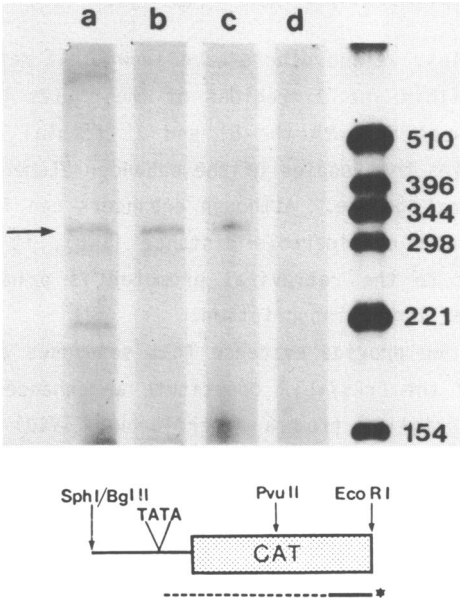

FIG. 4. Primer extension analysis of RNA induced by the PrRSV enhancer elements. Primer extension analysis was performed as described in Methods on RNA harvested from a 10 cm plates of CEF cells transfected with 30 μg of plasmid DNA. The expected 307 nucleotide band indicating the use of correct start from the SV40 promoter (see diagram below) is present by RNA from cells transfected with the CAT plasmids. a) pSV2cat; b) pPR12A; c) pPR6A; d) mock transfection. Molecular weight markers are from a HinfI digest of labeled pBR322.

ratios of CAT expression as monitored by enzyme activity (Fig. 4 and data not presented).

Species-specificity of PrRSV enhancer elements. Earlier experiments suggested that the enhancer element of pPR10 was more active in CEF cells and less active than the 72 bp repeats of SV40 (pSV2cat) in CV-1 cells. The expression of plasmids pPr6A and pPr12A, containing the A + B and B + C RSV enhancer domains, respectively, was compared with pSV2cat expression in both cell types. The RSV enhancers were one-half to one-third as active as the SV40 72 bp repeats in CV-1 cells twice as active as the SV40 enhancer in CEF cells. Thus, the overlapping RSV, like the parental pPR10, appear to exhibit a relative species (cell-type) preference for expression, similar to the activity observed for a number of viral and cellular enhancers (11, 12, 20).

DISCUSSION

In recent studies, we and others have shown that retroviruses contain enhancer elements within the U3 regions of their LTRs (11-15). The presence of LTR sequences at both the 5' and 3' termini of the integrated proviral DNA signifies that copies of the enhancer elements reside at both ends of the retroviral genome. Although enhancers can function in either orientation and over a considerable distance (1-6), it is presumed that the 5' LTR adjacent to the retroviral promoter is primarily responsible for the activation of viral transcription.

In this study, we provide evidence that sequences entirely localized to the U3 region of the PrRSV LTR constitute an enhancer element capable of activating transcription from a heterologous (SV40) promoter and the endogenous LTR promoter. In addition, a second enhancer overlapping the U3 region and the upstream viral sequences was localized in the Prague strain of RSV. A similar enhancer element has also been detected in the Schmidt-Ruppin strain of RSV, although the viral sequences upstream from U3 differ considerably between the two RSV strains (28, 29). Based on the data presented above, we suggest that at least three enhancer domains exist at the 3' end of the PrRSV viral genome; two of these domains are located entirely within the U3 region of the LTR (domains B and C), while the third resides adjacent to the 3' LTR (domain A). Two of the three adjacent domains (i.e., either A + B or B + C) appear to be capable of functioning together as an enhancer (we have not tested the combination of A + C). Further data to suggest that the enhancer consists of multiple domains is derived from the observation that a single domain can activate expression of an adjacent gene when it exists in multiple copies. In fact, the dimeric and trimeric forms of the central B domains provide levels of the enhancer activity comparable to those found with the intact viral sequences (30% and 100%, respectively). The observation that duplication of an otherwise non-functional domain is sufficient to reconstitute enhancer function suggests that an important feature of some enhancers is the presence of at least two copies of a basic sequence unit. An examination of the nucleotide sequence of domains A, B and C revealed several interesting features. Despite the lack of extensive sequence homology, one set of nucleotides appears to be present in all three domains (AGGGAGG in domain A, AGGAAAG in domain B, and AGGAAGG in domain C). Each of these sequences resembles the E1A enhancer repeats (AGGAAGGTGA) observed by Hearing and Shenk (8). A core sequence, which is common to a number of enhancer ele-

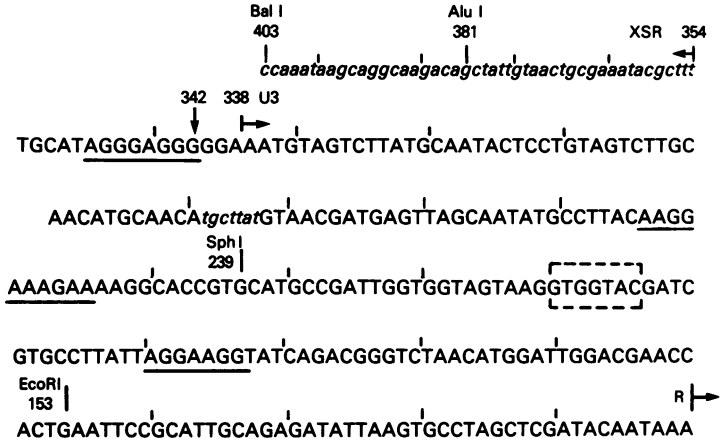

FIG. 5. Nucleotide sequence of the PrRSV enhancer region. The sequence of the region containing the PrRSV enhancer domains (see Fig. 2) and restriction enzyme landmarks is from (28, 29). The 5' end of U3 begins at nucleotide 337. Sequences homologous to the E1A enhancer core (8) are underlined. The end point of pPr-LTR2 is designated with an arrow at nucleotide 342. The nucleotides in italic print (the XSR region plus a seven nucleotide insertion, n.t. 288-295) represent sequences unique to the PrRSV strain and absent from the SR RSV strain.

ments, especially those of papovaviruses (TGTGG$\overset{AAA}{TTT}$G), is found only in the C domain (see Fig. 5). Finally, each of the three PrRSV enhancer domains contains a stretch of seven alternating purines and pyrimidines, a predisposing factor to the formation of zDNA (34). The potential role of zDNA in the function of enhancer elements has been discussed in detail by Nordheim and Rich (34).

In this study, we have performed experiments to evaluate RSV enhancer function using both heterologous (SV40) and homologous (LTR) promoter element. As has previously been demonstrated, the intact RSV LTR is a highly efficient transcriptional unit that is active in a variety of cells (37, 38). The relative decrease in activity seen when the RSV enhancer functions with heterologous promoter elements may reflect an intimate relationship required among the sets of nucleotides which constitute the transcriptional initiation signals. While the 5' retroviral LTR appears to be essential for transcription of proviral DNA, the function of the 3' LTR and its associated enhancer sequences are unclear. However, the 3' LTR appears to play a critical role in the ability of the virus to induce neoplastic disease through activation of adjacent cellular sequences (25-

27). This activation may occur either by transcription initiated from the promoter in the 3' LTR or through activation of proximal cellular promoters by enhancer elements located in the U3 region. The nature of the activated cellular sequences may determine the spectra of diseases induced by different retroviruses. This could be affected either by selection of the cellular DNA site for provirus integration or by the ability of control elements in the viral genome to induce transcription of cellular sequences in a tissue-specific manner (22, 23, 29, 35, 40, 41). Tissue-specific enhancer activity is consistent with the host cell specificity of activity by retroviral enhancers (11, 12, 20). In addition, several cellular enhancers, such as those associated with immunoglobulin genes (16-18) or insulin genes (19, Laimins et al., unpublished) exhibit strong tissue-specificity for expression.

Avian retroviruses that lack a transforming gene can be classified in at least two distinct groups depending on the spectra of disease they induce (29,35). Members of the first group (RAV-1, RAV-2 and td mutants of the Schmidt-Ruppin strain of RSV) induce mostly bursal lymphomas. Members of the second group (td PrRSV and certain recombinants between td PrRSV and RAV0) induce bursal lymphomas only with low frequency (35). A distinct genetic difference between the two groups lies in the sequences immediately upstream from the proviral LTR. Previous studies by Tsichlis et al. (29) have suggested the XSR region preceding the 3' LTR of PrRSV as an element responsible for differences in oncogenicity. In a related study, Luciw et al. (15) examined the ability of the 3' LTR and adjacent viral sequences of the Schmidt-Ruppin strain of RSV to enhance expression of a downstream tk gene. The direct repeat (F1) sequences, immediately preceding the 3' LTR, were found to be important for enhancement of tk expression. This region of SR RSV corresponds in location to the A enhancer domain present in XSR sequences of PrRSV. One explanation for the different disease spectrum of the td mutants of these two viruses would suggest differential activation of downstream genes by enhancer domains immediately preceding the 3' LTR.

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