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SRα Promoter: an Efficient and Versatile Mammalian cDNA Expression System Composed of the Simian Virus 40 Early Promoter and the R-U5 Segment of Human T-Cell Leukemia Virus Type 1 Long Terminal Repeat

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We developed a novel promoter system, designated SRα, which is composed of the simian virus 40 (SV40) early promoter and the R segment and part of the U5 sequence (R-U5′) of the long terminal repeat of human T-cell leukemia virus type 1. The R-U5′ sequence stimulated chloramphenicol acetyltransferase (CAT) gene expression only when placed immediately downstream of the SV40 early promoter in the sense orientation. The SRα expression system was 1 or 2 orders of magnitude more active than the SV40 early promoter in a wide variety of cell types, including fibroblasts and lymphoid cells, and was capable of promoting a high level of expression of various lymphokine cDNAs. These features of the SRα promoter were incorporated into the pcD-cDNA expression cloning vector originally developed by Okayama and Berg.

High-level expression of any desired gene is of prime importance in various aspects of studies in molecular biology. These may include (i) biochemical study of the gene product in a purified form, (ii) gene transfer experiments designed for the functional study of the gene in certain cell types, and (iii) cloning of cDNA or genomic DNA using functional assays. Okayama and Berg (18, 19) originally described a pcD vector which allows the expression of a full-length cDNA insert under the control of the simian virus 40 (SV40) early promoter. Using this vector, we have developed a functional cloning protocol to identify various lymphokine cDNA clones, including mouse interleukin-2 (IL-2) (32), human granulocyte-macrophage colony-stimulating factor (GM-CSF) (11), and mouse IL-4 (12) cDNA clones which represented about 0.1 to 0.01% of the total cDNA library, based entirely on the expression of the active polypeptide in transiently transfected COS7 cells (33). We recognized, however, that improvement of the expression level of the original pcD vector would greatly increase the likelihood of identifying less-abundant cDNA clones in the library. Furthermore, by improving the original pcD vector, we hoped to obtain large amounts of recombinant protein from mammalian sources soon after identification of the cDNA clone of interest. Toward this end, we decided to explore the unique characteristics of the long terminal repeat (LTR) of human T-cell leukemia virus type 1 (HTLV-1) (5, 29).

HTLV-1 encodes within the pX region a transcriptional activator ($p40^x$) which is responsible for the *trans*-activation of the cognate LTR (4, 24, 25, 28, 29). The 21-base-pair (bp) direct repeat units in the U3 region of HTLV-1 can be activated by $p40^x$ regardless of the site and orientation of insertion, similar to enhancer sequences (5, 20, 27). However, this enhancer sequence in the HTLV-1 LTR is not the sole element responsible for transcriptional enhancement, since the level of chloramphenicol acetyltransferase (CAT) gene expression directed by the U3 region alone is four to five times less than that of the intact LTR (5). Therefore, the R and U5 sequences which are located downstream of the transcriptional start site appear to be required for the maximal expression from the HTLV-1 LTR (5, 17). A similar observation has been reported for the bovine leukemia virus LTR (3). In this report, we describe a novel SRα promoter system which uses the R-U5 segment of the HTLV-1 LTR. We found that fusion of the R segment and part of the U5 sequence (R-U5′) from the HTLV-1 LTR to a heterologous SV40 early promoter-enhancer unit increased the expression level more than 1 order of magnitude over that from the original SV40 early promoter-enhancer. The mechanism of increasing gene expression by use of the SRα promoter and the application of this new expression vector system for gene cloning and amplification of gene product synthesis are discussed.

To elucidate the mechanism underlying the activation of the HTLV-1 promoter by the R-U5 segment, we constructed a series of CAT fusion plasmids (Fig. 1). pSRα-CAT and pSRβ-CAT (Fig. 1A) were constructed by inserting a 267-bp fragment containing R and part of the U5 sequence, from the exact 5′ end of R (position 354) to the *Sau*3AI site in the U5 sequence (position 620) (21) (Fig. 1B), with a sense or antisense orientation, respectively, at the *Hin*dIII site immediately downstream of the SV40 early promoter of pSV2-CAT (6). The SRα construct activated CAT gene expression 40-fold relative to the pSV2-CAT plasmid, whereas pSRβ-CAT, in which the R-U5′ segment was inserted in the antisense orientation, was unable to activate CAT gene expression efficiently. This activation was not modified by cotransfection with the $p40^x$ expression plasmid (data not

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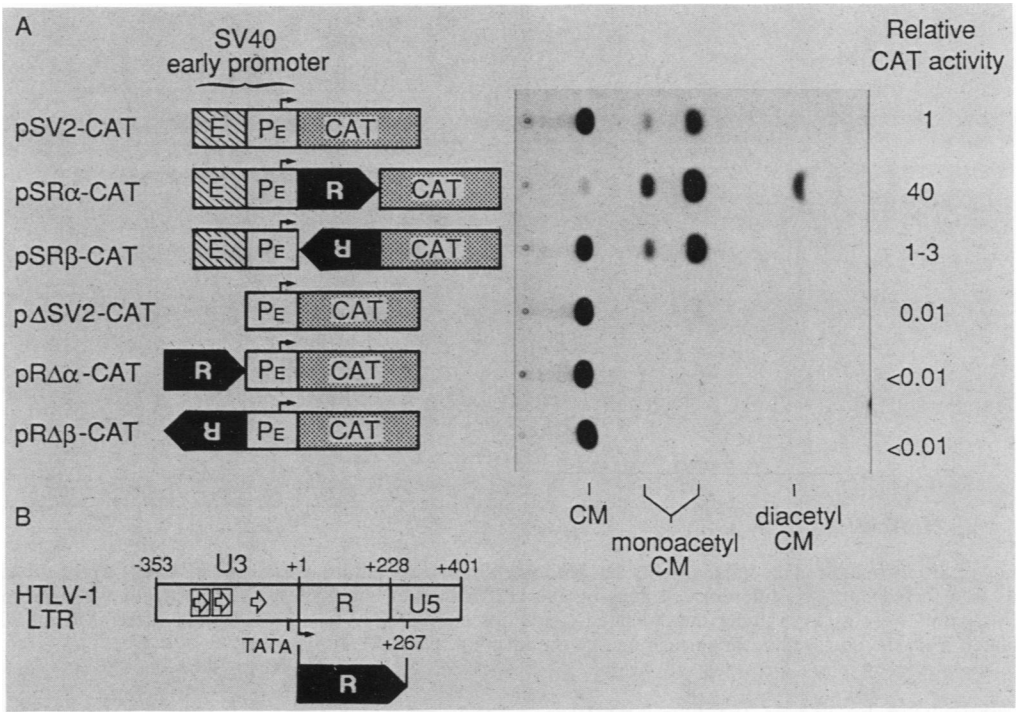

FIG. 1. Position- and orientation-dependent activation of SV40 early promoter by an R-U5′ segment of the HTLV-1 LTR. (A) Schematic representation of various CAT constructs carrying an R-U5′ segment with a heterologous SV40 early-region promoter-enhancer unit. The *Sph*I-*Hin*dIII segment of the SV40 early promoter was used as a core promoter (P_E) lacking the SV40 enhancer (E). R-U5′ segments with sense (R) or antisense (inverted R) orientation and the bacterial CAT gene are shown. Each construct (3 μg) was transfected into HOS, a human osteosarcoma cell line (10), by the DEAE-dextran method (32), and CAT activity (6) was assayed after a 30-min incubation with 50 μg of protein at 37°C and is expressed relative to that of pSV2-CAT. The positions of chloramphenicol (CM) and its acetylated forms are indicated. (B) Structure of HTLV-1 LTR. The LTR is composed of U3, R, and U5 sequences. The locations of the 42-bp repeat (open box), 21-bp repeat (open arrows), and TATA box are shown. The numbers are positions relative to the mRNA cap site shown by a bent arrow. The location and orientation of the R-U5′ segment which is incorporated in the SRα-CAT construct are indicated by a solid arrow with a highlighted R.

shown). To examine the possibility that this segment acts as an enhancer, the R-U5′ fragment was inserted into the *Sph*I site of enhancerless pΔSV2-CAT in two orientations (pRΔα-CAT and pRΔβ-CAT). Regardless of the orientation of the insert, the R-U5′ segment failed to activate the SV40 core promoter (Fig. 1A). The inability to activate a heterologous promoter in a position- and orientation-independent manner indicates that the R-U5 segment does not function as an enhancer.

To determine whether the SRα promoter is efficient in many different cell types, we compared the CAT activity directed by the SRα promoter or SV40 early promoter in five commonly used cell lines derived from different tissues and species (Fig. 2). These included lymphocytes (Raji cells [an Epstein-Barr virus-transformed human B-cell line] and Jurkat cells [a human T-cell leukemia line]) and adherent cells (CV1 African green monkey kidney cells, mouse Ltk^- fibroblasts, and COS7 cells [an SV40-transformed derivative of CV1 cells]). In general, the SRα promoter was 10- to 30-fold more active than the SV40 early promoter regardless of the species and the origin of the tissue. Especially in Jurkat cells, the difference in the efficiency of the two promoters was striking; the SV40 early promoter-enhancer unit was unable to promote efficient CAT expression, whereas the SRα promoter could direct a significant enhancement of the CAT expression level. The broader host range and higher level of expression of the SRα promoter will be especially advantageous for gene transfer experiments. We also compared the efficiency of the SRα promoter with the Rous sarcoma virus LTR promoter and the immediate-early promoter of human cytomegalovirus, which are known to be strong promoters, by using CAT gene expression as an assayable indicator. The SRα promoter was about 10- to 20-fold and 4-fold more active, respectively, than the Rous sarcoma virus LTR promoter and the immediate-early promoter of cytomegalovirus in L cells (data not shown).

To evaluate whether the SRα promoter can direct the efficient expression of a mammalian cDNA clone, we constructed an SRα promoter-based pcD plasmid carrying various lymphokine cDNAs as indicator genes. The construction of the SRα plasmid containing mouse IL-4 cDNA (pcD-SRα205) is illustrated in Fig. 3A. pcD-137 is a derivative of 2AE3, previously isolated mouse IL-4 cDNA clone (12), in which the *Nde*I site is converted to a *Sal*I site. 2AE3 and pcD-137 gave the same level of mouse IL-4 activities in the transient expression system. To preserve compatibility with the pcD vector developed by Okayama and Berg (18, 19), the restriction sites in pSRα-CAT were modified as shown in Fig. 3A, yielding pSRα-CAT196. pcD-SRα205 was then constructed by the trimolecular ligation reaction of the indicated DNA fragments derived from pcD-137 and pSRα-CAT196 (Fig. 3A). Starting from pcD-SRα205, a series of pcD-SRα lymphokine cDNA recombinants (Table 1) were constructed by replacement of the *Xho*I segment harboring the mouse IL-4 cDNA with the *Xho*I fragments containing various lymphokine cDNA sequences from the cDNA

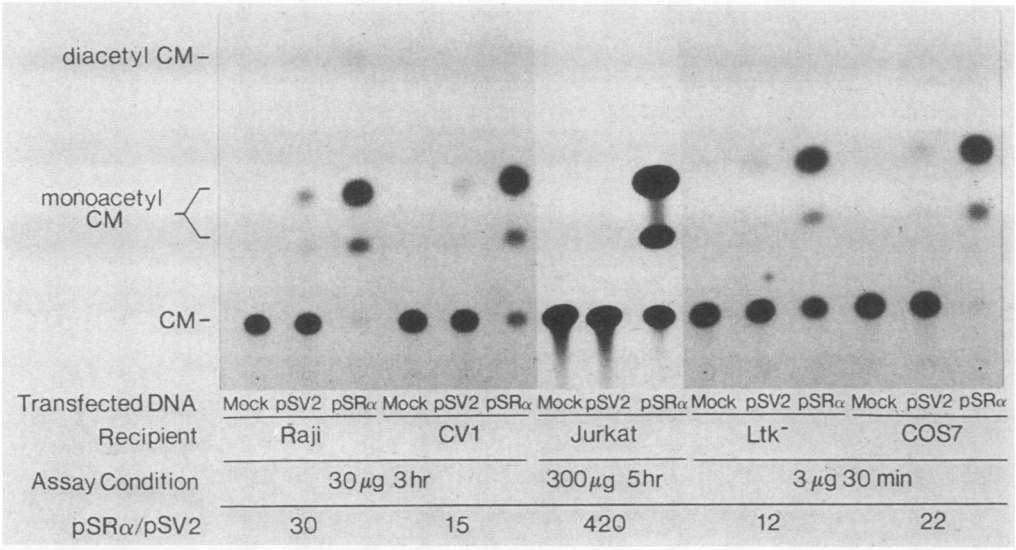

FIG. 2. Efficiency of SRα promoter in mammalian cells derived from various species and tissues. pSV2-CAT or pSRα-CAT plasmid DNA (5 μg of each) was transfected into five different cell lines by the DEAE-dextran methods (7, 32), and cells were harvested 48 h after transfection. CAT activities were assayed under the indicated conditions at 37°C. The relative CAT activities (pSRα/pSV2) shown at the bottom were calculated from the data within the linear range of the reaction. CM, chloramphenicol.

clones, previously isolated by use of the Okayama-Berg vector (Table 1, Fig. 3B). The generalized structure of pcD and pcD-SRα cDNA recombinants is shown in Fig. 3B. The DNA segment, flanked by *Hin*dIII and *Pst*I sites, which consists of the SV40 late-gene (16S) splice junction and either an SV40 early or SRα promoter, can be used as an alternative plasmid linker for the construction of a cDNA library (Fig. 3B).

The SRα promoter was capable of increasing the expression of various lymphokine cDNA clones, including mouse and human IL-2, mouse IL-3, mouse and human IL-4; mouse and human GM-CSF and human G-CSF, about 10- to 100-fold higher than that directed by the SV40 early promoter in the COS7 transient expression system.

These features of the SRα promoter were incorporated into the pcD vector for cloning of low-abundance cDNA clones by functional assays. The structure of the SRα promoter-based cDNA expression-cloning vector pcDL-SRα296 is shown in Fig. 3C. This vector is designed to function as the source for a plasmid linker and as a vector-primer (Fig. 3C; 18, 19) for the construction of a cDNA library, in addition to having the convenient structural features of the Okayama-Berg vector. Although the SV40 early promoter-based pcD vector was used to isolate several lymphokine cDNA clones on the basis of their biological activities, the total cDNA library had to be divided into subpools which contained less than 100 clones in order to detect the activity. In contrast, when the SRα promoter-based pcD vector was used, the mouse IL-3 or IL-4 cDNAs, which represented 0.1% of the library, could be detected in the supernatants of COS7 cells transfected with the total (undivided) cDNA library. Using the SRα promoter-based vector, we have recently identified a mouse cDNA clone which represented about 0.02% of the library on the basis of immunoglobulin A-enhancing and eosinophil colony-stimulating activities (T. Yokota, R. L. Coffman, H. Hagiwara, D. M. Rennick, Y. Takebe, K. Yokota, L. Gemmell, B. Shrader, G. Yang, P. Meyerson, J. Luh, P. Hoy, J. Pene, F. Briere, J. Banchereau, J. De Vries, F. D. Lee, N. Arai, and K. Arai, Proc. Natl. Acad. Sci. USA, in press).

Interestingly, IL-4 activity in culture supernatants of COS7 cells transfected with pcD-SRα205 was greatly reduced by cotransfection with an excess amount of pcDL-SRα296, which has no cDNA insert, whereas it was not affected by cotransfection with an excess amount of the plasmid carrying the SV40 early promoter (data not shown).

TABLE 1. Effects of SRα promoter and SV40 early-region promoter on production of various lymphokines in COS7 cells

Lymphokine	SV40 early promoter		SRα promoter		SRα activity/ SV40 activity	Reference
	pcD construct[a]	Activity (U/ml)[b]	pcD-SRα construct[a]	Activity (U/ml)[b]		
Mouse IL-2	pcD-2 (MT1)	1.1×10^3	pcD-SRα285	1.3×10^5	118	32
Human IL-2	pcD-306 (HT5)	8.3×10^3	pcD-SRα287	3.1×10^5	37	32
Mouse IL-3	pcD-1 (b9)	1.4×10^5	pcD-SRα266	1.2×10^6	8.6	33
Mouse IL-4	pcD-137	3.9×10^2	pcD-SRα205	4.1×10^4	105	12
Human IL-4	pcD-176	1.2×10^3	pcD-SRα224	2.8×10^4	23	34
Mouse GM-CSF	pcD-290 (E1-11)	7.2×10^3	pcD-SRα290	1.2×10^5	17	15
Human G-CSF	pcD-234	1.5×10^4	pcD-SRα246	2.6×10^5	17	16

[a] The structures of pcD and pcD-SRα constructs are shown in Fig. 3B; except for human IL-4 cDNA the d(G-C) stretch was deleted (34).

[b] Lymphokine activities of culture supernatants were assayed 3 days after transfection. The cell lines used for assays were as follows: mouse and human IL-2 and mouse IL-4, HT-2 T-cell clone; mouse IL-3, MC/9 mast-cell clone; mouse GM-CSF and human G-CSF, NFS60 line; human IL-4, human peripheral blood. One unit of lymphokine activity is defined as the amount required to produce the half-maximal proliferation response, using the indicated cell line.

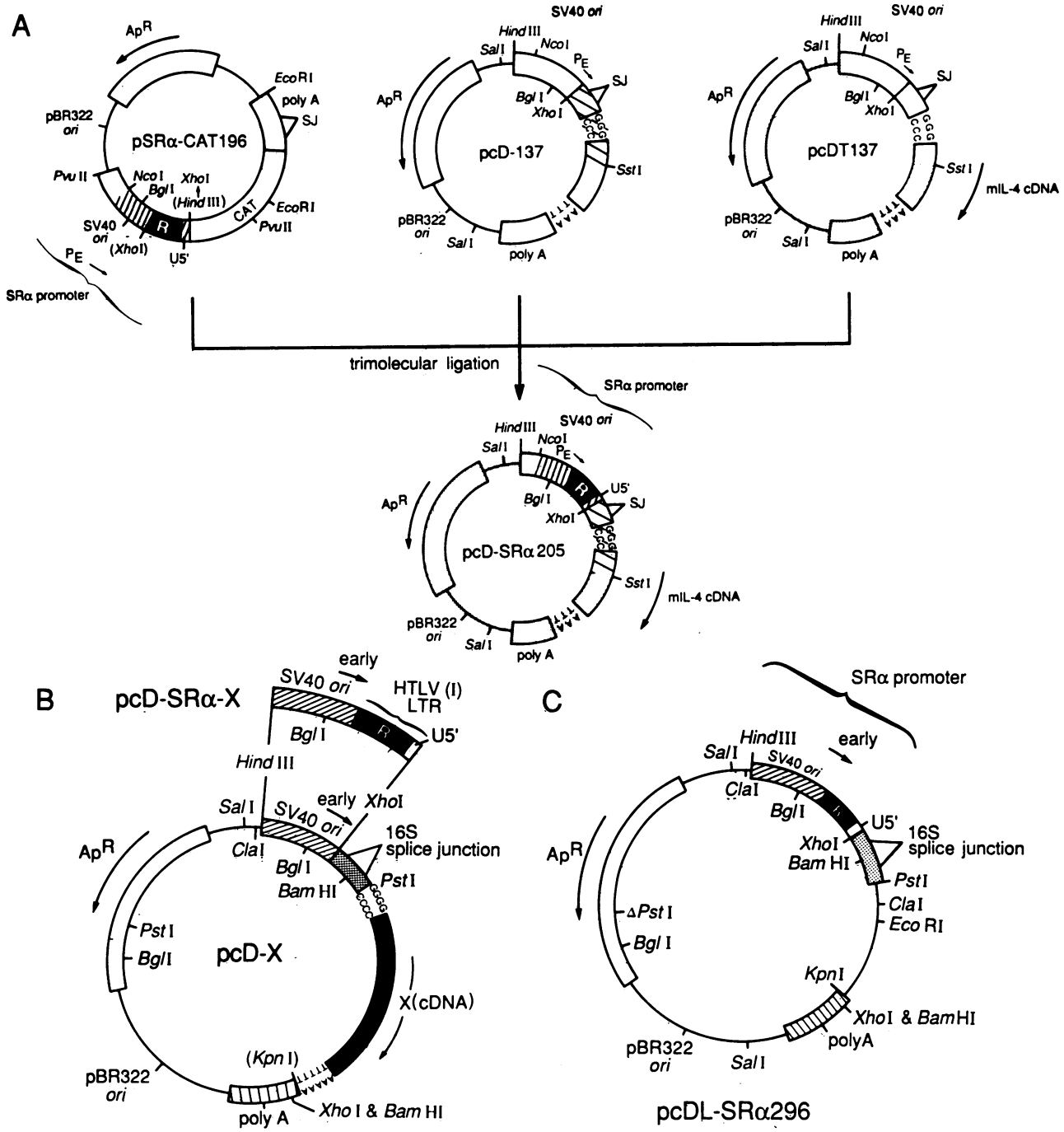

FIG. 3. SRα promoter-based cDNA expression-cloning vectors. (A) Schematic diagram of the construction of pcD-SRα205 carrying mouse IL-4 cDNA under the control of the SRα promoter. In the original pSRα-CAT plasmid, the *Xho*I site was disrupted by filling in and the *Hin*dIII site was converted to an *Xho*I site by the ligation of an *Xho*I linker to its filled-in termini. (*Xho*I) and (*Hin*dIII) in pSRα-CAT196 show the original location of the indicated restriction sites in pSRα-CAT. pcD-SRα205 was constructed by the trimolecular ligation reaction of the 373-bp *Nco*I-*Xho*I fragment of pSRα-CAT196 and the 434-bp and calf intestine alkaline phosphatase-treated 3,221-bp *Xho*I-*Sst*I fragments of pcD-137. The locations of each fragment and the R segment of the HTLV-1 LTR are indicated. (B) Generalized structure of pcD-cDNA recombinants pcD-X and pcD-SRα-X. Shown are the two alternative linker segments (*Hin*dIII-*Pst*I) composed of either an SV40 early promoter or an SRα promoter (*Hin*dIII-*Xho*I segment) and the SV40 late-gene splice junction (*Xho*I-*Pst*I segment), the d(G-C) bridge between the linker fragment and the cloned cDNA (X), the d(A-T) stretch derived from the oligo(dT) primer and the poly(A) tail of RNA, and the segment carrying the SV40 late region polyadenylation signal [poly(A)]. (C) Structure of the SRα promoter-based cDNA cloning-expression vector pcDL-SRα296. This vector (3.7 kilobase pairs long) is composed of (i) the plasmid linker (18, 19) (*Hin*dIII-*Pst*I) segment (802 bp) which provides the SRα promoter and SV40 late-gene splicing junction; (ii) the vector-primer (18, 19) (*Kpn*I-*Hin*dIII) segment (2,606 bp) which comprises the *Kpn*I-oligo(dT) priming site, SV40 late-region polyadenylation signal [poly(A)], and pUC plasmid-derived vector sequence (the pBR322 replication origin and β-lactamase [Ap^R] gene lacking a *Pst*I site [Δ*Pst*I] are shown; the *Nde*I site in the vector sequence of this particular construct is converted to a *Sal*I site for the convenience of excision of an entire cDNA transcriptional unit by *Sal*I cleavage); and (iii) the intervening DNA piece (*Pst*I-*Kpn*I) (336 bp) between the plasmid linker and vector-primer segments, consisting of the SV40 DNA fragment flanked by the *Eco*RI (SV40 map position 0.754) and *Kpn*I (map position 0.715) sites from plasmid pcDV1 (19) and the short DNA (*Pst*I-*Eco*RI) fragment from plasmid pL1 (19). Details of the construction will be described elsewhere.

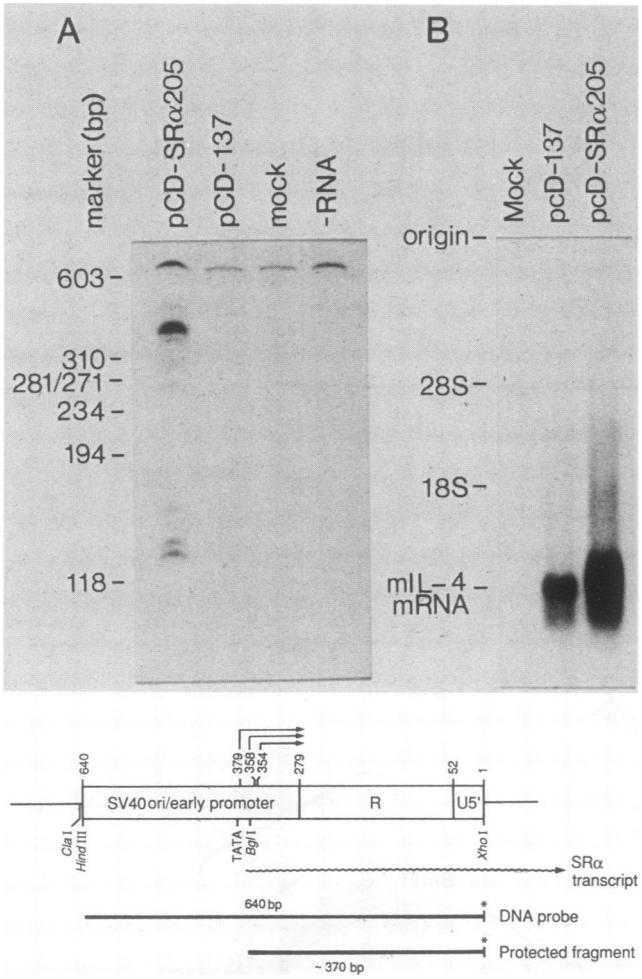

FIG. 4. Analysis of mouse IL-4 cDNA transcripts directed by SRα promoter. (A) S1 nuclease mapping of the 5′ end of the SRα transcript. Total RNA (2) prepared from COS7 cells transfected with a mouse IL-4 cDNA plasmid carrying either an SRα promoter (pcD-SRα205) or an SV40 early promoter (pcD-137) was hybridized with a 640-bp ^{32}P-labeled *Xho*I-*Hin*dIII fragment of pcD-SRα205 and was digested with S1 endonuclease. The protected products were fractionated on a 4% polyacrylamide gel containing 7 M urea. Each lane represents about 10 μg of total RNA hybridized with 10^4 cpm of the probe ^{32}P-labeled at the *Xho*I site. Markers are the *Hae*III digest of φX174 replicative-form DNA. The relationship between the SV40 early promoter start site and the 5′ end of the SRα transcript is diagrammed at the bottom. Arrows represent SV40 early-promoter start sites (1). (B) Quantitation of the steady-state levels of mouse IL-4 cDNA transcripts directed by SV40 early and SRα promoters in a COS7 transient expression system. pcD-137 or pcD-SRα205 (5 μg of each) was transfected into COS7 cells by the DEAE-dextran method. Total cellular RNA (2) was prepared at 48 h after transfection. Equal amounts of RNA (5 μg per lane) were run on a denaturing 1% agarose gel, transferred to a nitrocellulose filter, and hybridized (14) with a nick-translated 224-bp *Sac*I-*Rsa*I fragment of 2AE3 (12). The positions of 18S and 28S rRNA and the mouse IL-4 mRNA (mIL-4-mRNA) from concanavalin A-stimulated mouse cloned T cells C1. Ly1$^+$2$^-$/9 (12) are indicated.

These results may imply that the COS7 cell has only a limited amount of certain factor(s) required for efficient expression from the SRα promoter. The DNA segment carrying an SRα promoter or RNA transcript directed by the SRα promoter may have a stronger affinity for such factor(s) than do the corresponding regions of the SV40 early promoter and thus promote the efficient initiation of transcription or translation or both.

The unique position and orientation dependence of the effect of the R-U5′ segment (Fig. 1) suggests a stimulatory mechanism distinct from that of a prototype enhancer. Since the Southern blotting analysis of Hirt supernatants (9) showed no detectable difference in the copy number between plasmids carrying either the SV40 early promoter or the SRα promoter (data not shown), the enhancing effect of the R-U5′ segment is not due to a difference in the copy number. S1 nuclease protection assays showed that the major initiation start sites of transcription directed by the SRα promoter were located in the SV40 early promoter region (Fig. 4A). Based on the Northern blot analysis, the steady-state level of IL-4 cDNA transcripts increased about fourfold in the SRα promoter system compared with that in the SV40 early promoter system in COS7 cells (Fig. 4B). The result was further confirmed by quantitative slot blot analysis. No significant difference was found in the stability of the transcripts in either system after the addition of actinomycin D (data not shown). Therefore, the R-U5′ segment seems to enhance the initiation mechanism of transcription.

However, the role of the R-U5′ region appears to be more complex because, although the increase in the steady-state level of IL-4 transcripts was fourfold (Fig. 4B), IL-4 activity increased almost 100-fold (Table 1; the ratio [SRα/SV40] of IL-4 activities at 2 days after transfection was approximately the same as that for day 3). This discrepancy suggests that the enhancement of expression by the SRα promoter may also involve a translation effect. This seems to be consistent with the fact that the enhancing effect of the R-U5′ region occurred only when it was located downstream of the

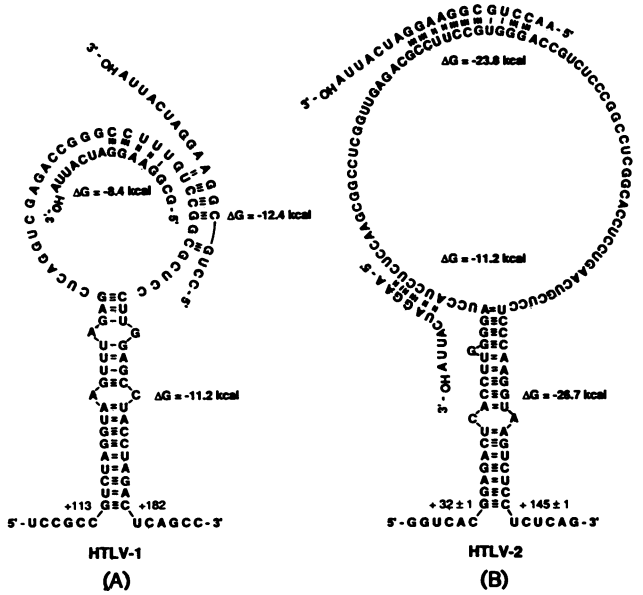

FIG. 5. Possible secondary structures of the 5′ regions of mRNAs in the HTLV family. Nucleotide sequences of the 5′-proximal regions of viral transcripts were deduced from the DNA sequence data described by Seiki et al. (21, 22) (A) and Shimotohno et al. (26) (B). The possible secondary structures of the 5′-proximal parts of viral mRNAs derived from the R regions of HTLV-1 and HTLV-2 have been noted (23). The predicted free energy values (ΔG) of the stem-loop structures and the association with 18S rRNA (31) were calculated by the method of Tinoco et al. (30). Nucleotides are numbered from the 5′ end of each viral transcript, which starts from the 5′ end of the R segment in the LTR.

transcription start site. It has been pointed out that the 5′ noncoding regions of the mature transcripts of several eucaryotic genes, including the rat preproinsulin and rabbit α- and β-globin genes, have the capability of forming a stable stem-loop structure just upstream of the AUG start codon and part of the open loop can theoretically base pair with the 3′ end of 18S rRNA (8, 13). Similar structural features were found in 5′-proximal parts of transcripts in the HTLV family (Fig. 5). It is tempting to speculate that such an interaction may play a role in efficient translation of the SRα promoter transcript. Insertion of the R-U5′ segment in the antisense orientation (pSRβ-CAT) weakly stimulated CAT activity (Fig. 1). In contrast to the 5′ flanking region of the SRα transcript, which does not contain any AUG codon, extra AUG codons appear twice downstream of the stem-loop structure of the SRβ transcript. It is plausible that this may reduce the frequency of correct initiation from the AUG codon of the CAT gene in the SRβ construct.

The analysis of the roles of sequence elements located downstream of the viral and eucaryotic promoters might give further insights into the regulatory mechanism of gene expression.

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