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In Vitro Transcription Analysis of the Viral Promoter Involved in c-*myc* Activation in Chicken B Lymphomas: Detection and Mapping of Two RNA Initiation Sites Within the Reticuloendotheliosis Virus Long Terminal Repeat

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Chicken syncytial virus, a member of the reticuloendotheliosis virus family, induces B-cell lymphomas in chickens that arise by transcriptional activation of the chicken c-*myc* gene. In vitro transcription studies on cloned tumor DNA containing a deleted chicken syncytial virus provirus integrated upstream from, and in the same transcriptional orientation as, the chicken c-*myc* coding region were utilized to map possible transcriptional promoters and initiation sites. In vitro transcripts extending into c-*myc* sequences were initiated at two sites within the downstream long terminal repeat (LTR) closest to c-*myc* coding sequences. Both initiation sites have been precisely mapped by S1 nuclease and DNA sequencing methods. One site (I_1) lies at the U3-R junction of the LTR, and the other site (I_2) lies approximately 160 nucleotides upstream. Transcriptional control signals, including TATA- and CAAT-like sequences are present at appropriate distances upstream from the initiation sites. Both initiation sites are utilized to a similar extent. The upstream chicken syncytial virus LTR was also shown to be transcriptionally active in vitro. Two strong transcriptional initiation sites were also found in the LTR of spleen necrosis virus, a related member of the reticuloendotheliosis virus family; therefore, it seems likely that the existence of two transcriptional initiation sites is a common feature of the reticuloendotheliosis virus LTR, in contrast to other previously studied retroviral LTRs that exhibit one such site. The possible implications of these findings are discussed.

Experimental studies on lymphoid leukosis induced by avian leukosis virus (ALV) have been instrumental in establishing that the oncogenic activity of nonacute retroviruses is mediated by the activation of host cell genes (6, 12, 13, 17, 30, 31, 35, 36). The predominant forms of neoplasia induced by ALV are lymphomas of the B-cell type, such as lymphoid leukemia, which begin to develop approximately 12 to 16 weeks after infection of young chickens with virus (7, 52). Studies have shown the neoplastic event to be intimately associated with integration of retrovirus in the immediate vicinity of the chicken c-*myc* gene (12, 17, 30, 31, 34, 35), the cellular homologue of the transforming gene (v-*myc*) of MC29 virus (40). We have previously reported that chicken syncytial virus (CSV), a member of the reticuloendotheliosis (REV) group of retroviruses, can also induce B lymphomas that are histologically indistinguishable from those induced by ALV (32, 52). Although ALV and CSV are genetically unrelated viruses, the mechanism by which they induce lymphoid leukosis in chickens appears to be similar.

Studies on DNA isolated from ALV-induced chicken B-cell lymphomas have indicated that, in most cases, proviral DNA was integrated upstream of the c-*myc* gene in the same transcriptional orientation as c-*myc*. These findings suggest that a promoter insertion mechanism was involved in the activation of the c-*myc* gene, utilizing a strong transcriptional promoter located in a viral long terminal repeat (LTR) element (12, 17, 30, 31). Other arrangements of integrated ALV have been observed in which viral LTR-containing sequences were located downstream from c-*myc* or upstream from c-*myc* in the opposite transcriptional orientation (35). In these instances, activation of the c-*myc* gene is thought to be mediated by enhancer sequences in the viral LTRs, which are known to increase transcription from promoters located either upstream or downstream, regardless of transcriptional polarity (20, 35, 36). In the case of CSV-induced B-cell lymphomas, all insertion events characterized to date have involved the integration of CSV proviral sequences upstream from, and in the same transcriptional orientation as, c-*myc*. In most cases, an internally deleted provirus has been found to be integrated within the first intron, between exon one, which encodes most of the mRNA leader sequence, and the first part of exon two, which contains the translational start codon for c-*myc* and the beginning of the region of sequence homology with v-*myc* (41, 47; R. A. Swift, E. Schaller, R. L. Witter, and H.-J. Kung, manuscript in preparation).

To examine the detailed mechanism through which the CSV provirus activates the c-*myc* gene in CSV-mediated B-cell lymphomas, we isolated from tumor DNA an activated c-*myc* molecular clone in which a CSV provirus is integrated between the first and second c-*myc* exons. We employed in vitro transcription experiments using the HeLa cell lysate system of Manley et al. (23) which has been shown to accurately transcribe a variety of genes and has been used extensively to characterize transcriptional control signals, including those of several retroviral LTRs (11, 33, 53). In particular, we wished to define the location and nature of transcriptional initiation and control sites that were likely to be involved in the CSV LTR-mediated activation of the c-*myc* gene.

Results of our studies indicate that transcripts that extend into c-*myc* coding sequences can be initiated from within the

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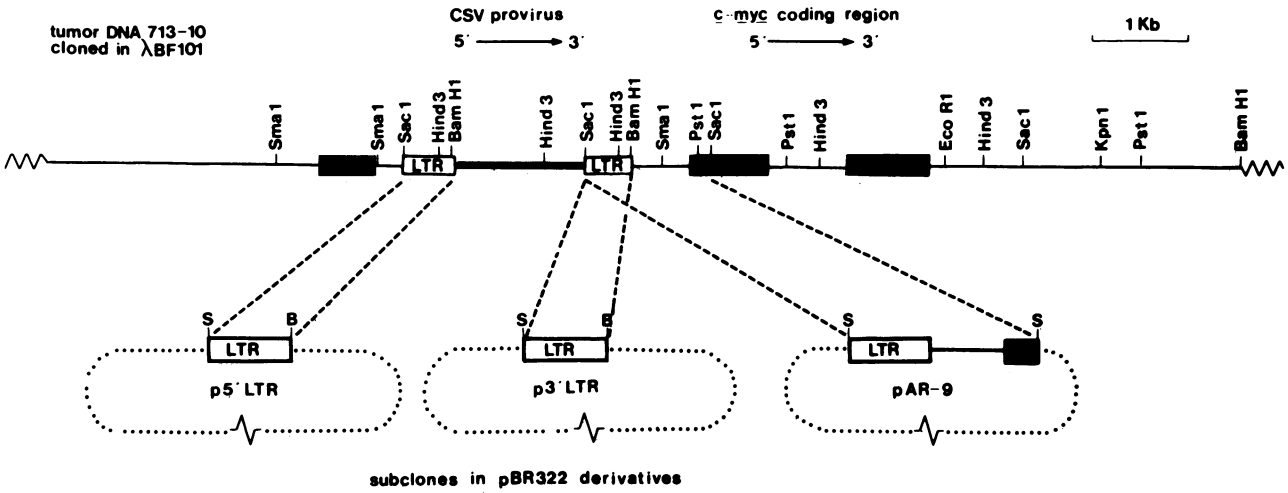


FIG. 1. Map of cloned DNA derived from chicken bursal lymphoma 713-10, showing an internally deleted CSV provirus integrated upstream from the c-*myc* coding region. Also shown are DNA fragments subcloned into pBR322-derived vectors for use in in vitro transcription studies, as described in the text. Black boxes, c-*myc* exons; S, *Sac*I; B, *Bam*HI; Kb, Kilobase pairs. Further details regarding the map will be described (Swift et al., in preparation).

3′ LTR. Interestingly, we found that the CSV LTR and the LTR of the related spleen necrosis virus (SNV), another member of the REV family, contain two strong transcriptional promoters, instead of a single strong promoter that is characteristic of other retrovirus LTRs that have been examined previously (11, 33, 53). These promoters, and their transcriptional initiation sites have been precisely mapped by S1 nuclease analyses and DNA sequencing.

MATERIALS AND METHODS

Plasmid preparation. A recombinant DNA clone (713-10) isolated from a phage genomic library of a chicken B-lymphoma DNA (Swift et al., manuscript in preparation) was the source of DNA for construction of plasmid subclones (Fig. 1). Plasmids p5′LTR and p3′LTR were prepared by insertion of the appropriate *Sac*I-*Bam*HI DNA fragments into DNA of pDH24 (a pBR322-derived vector containing a *Sac*I site), after removal of the *Sac*I-*Bam*HI fragment from the vector DNA (Fig. 1). Plasmid pAR-9 was prepared with a different pBR322 derivative containing heterologous DNA of known sequence, including a *Sac*I site (A. Tanaka, unpublished data; Fig. 1). Clones of hybrid plasmids were selected on the basis of ampicillin resistance and tetracycline sensitivity, after introduction into *Escherichia coli* HB101 by standard methods (21). A DNA clone pLTR(SNV), containing the spleen necrosis virus LTR and derived from pDH24, was kindly provided by H. Temin. A pBR322 clone containing two Rous sarcoma virus (RSV) strain SR-A LTRs (*Pvu*II-D, -G; circle junction) (9) was kindly provided by J. M. Bishop, H. E. Varmus, and W. DeLorbe. Plasmid DNA was prepared and purified by standard procedures (21).

In vitro transcription. HeLa cell extracts were prepared as described by Manley et al. (23). The quality of our lysate preparations was tested by comparison with commercial preparations (Bethesda Research Laboratories; New England Nuclear Corp.) using various DNA templates in addition to those under study. Addition of purified RNA polymerase II (pol II; Bethesda Research Laboratories) to reactions containing our lysate preparations did not increase the transcriptional signals. Therefore, our lysate preparations contained RNA pol II in functional excess. All data shown were obtained with lysates prepared in our laboratory; no difference in transcription patterns was observed with commercial preparations. In vitro transcription reactions (final volume, 25 μl) contained 15 μl of extract; 1.25 μg of template DNA; 500 μM each of ATP, CTP, and UTP; and 50 μM GTP, including 5 to 10 μCi of [α-^{32}P]GTP (600 Ci/mmol; New England Nuclear), 0.2 mM EDTA, and 1 mM phosphocreatine. In nonradioactively labeled reactions, the GTP concentration was increased to 500 μM. After a 1-h incubation at 30°C, nucleic acids were extensively purified as described previously (23). Reaction products were denatured by treatment with glyoxal (27) and analyzed by electrophoresis on 1.4% agarose gels with 10 mM sodium phosphate buffer. Alternatively, reaction products were suspended in 4 μl of 80% formamide–0.1% (wt/vol) xylene cyanol–0.1% (wt/vol) bromophenol blue–1× TBE (0.05 M Tris-borate [pH 7.3], 1 mM EDTA); heated at 90°C for 2 min; and analyzed by electrophoresis on 0.4-mm-thick, 5% polyacrylamide, 8 M urea gels in TBE buffer. Polyacrylamide gels or dried agarose gels were exposed to X-ray film (Kodak XAR-5) with Cronex intensifying screens (Du Pont Co.) at −70°C. If in vitro transcripts were to be hybridized to radiolabeled probe, purified, unlabeled reaction products were treated with 200 μg of RNase-free, DNase I (Worthington Diagnostics) per ml for 10 min at 37°C in 100 μl of 100 mM NaCl–10 mM Tris (pH 7.4)–10 mM $MgCl_2$ to destroy template DNA. Samples were then adjusted to 0.45 M NaCl–40 mM EDTA–0.1% sodium dodecyl sulfate–400 μg of proteinase K per ml, incubated for 30 min at 68°C, and then extracted with phenol-chloroform and ethanol precipitated.

S1 nuclease mapping. Appropriate restriction endonuclease fragments were dephosphorylated and radiolabeled at their 5′ ends as described by Weaver and Weissman (49) with the following modifications: (i) the dephosphorylation reaction contained 4 to 60 pmol of the 5′ end of the fragment of interest in a final volume of 10 μl; (ii) after inactivation of calf intestinal phosphatase, the reaction buffer was adjusted to 10 mM dithiothreitol–10 mM $MgCl_2$ for the labeling reaction which contained 10 U of polynucleotide kinase (New England Biolabs) and 1 μM [γ-^{32}P]ATP (2900 Ci/mmol; New England Nuclear) in a volume of 20 μl.

Radiolabeled DNA was separated from unlabeled nucleotides with a spun column of Sephadex G-50 (Pharmacia Fine

Chemicals) (21), precipitated from ethanol, and digested with an appropriate restriction endonuclease. The desired end-labeled fragment was separated from other fragments by gel electrophoresis, elution from a gel slice, and ethanol precipitation. A typical hybridization experiment included 50,000 cpm of labeled DNA probe (ca. 50 to 100 ng) and unlabeled in vitro transcripts from two reactions in 30 μl of 80% formamide–0.4 M NaCl–0.04 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 6.5)–1 mM EDTA. After 15 min at 68°C, the nucleic acids were incubated for 4 h at 47°C. Hybridization reactions were then adjusted to 400 μl with chilled S1 nuclease buffer (0.03 M sodium acetate [pH 4.5], 0.25 M NaCl, 1 mM $ZnSO_4$, 5% glycerol, 20 μg of denatured calf thymus DNA per ml) containing 40 U of S1 nuclease (Calbiochem). Samples were incubated for 60 min at 45°C and then ethanol precipitated with 5 μg of tRNA as carrier, suspended in 4 μl of 80% formamide–10 mM NaOH–1 mM EDTA–0.1% (wt/vol) xylene cyanol–0.1% (wt/vol) bromophenol blue, heated for 2 min at 90°C, quenched on ice, and analyzed by electrophoresis in TBE buffer on 0.4-mm thick, 5% polyacrylamide, 8 M urea gels. On similar 8% polyacrylamide gels, DNA sequencing ladders generated by chemical cleavage (26) were loaded onto lanes adjacent to those containing S1 nuclease-digested probe. Radiolabeled bands were visualized by exposure of X-ray film as described above.

DNA sequencing. Appropriate DNA fragments containing one radiolabeled 5′ end were generated as described above and subjected to chemical cleavage and resolved on thin, denaturing, polyacrylamide gels as described by Maxam and Gilbert (26). Radiolabeled bands were visualized by exposure of X-ray film as described above.

Restriction endonuclease digestions. Restriction endonuclease digestions were carried out under conditions specified by the manufacturers (New England Biolabs; Bethesda Research Laboratories; Boehringer-Mannheim Biochemicals), except that a two- to sixfold excess of enzyme was usually used.

RESULTS

Transcriptional analysis in vitro of cloned tumor DNA. A lambda phage clone (713-10) containing tumor DNA prepared from a CSV-induced chicken B-cell lymphoma was the original source of DNA used in this study. Clone 713-10 contains a CSV provirus that harbors an internal deletion and is integrated upstream from c-*myc* coding sequences (Fig. 1) described elsewhere (Swift et al., in preparation). The CSV provirus and the c-*myc* gene are in the same transcriptional orientation. To facilitate in vitro transcription studies, we subcloned regions of this DNA insert into plasmid vectors (Fig. 1). Clone pAR-9 contains approximately 1.2 kilobase pairs of tumor DNA, extending from the *Sac*I site at the left end of the 3′-integrated LTR to the *Sac*I site in c-*myc* exon 2. The clones p5′LTR and p3′LTR contain *Sac*I to *Bam*HI fragments of the integrated CSV LTRs and are missing only 9 nucleotides and 30 nucleotides from the left and right ends, respectively, of each LTR.

Transcription initiated by RNA pol II in vitro will, under appropriate conditions, continue until the polymerase "runs off" the DNA template (22, 23). Therefore, transcriptional initiation sites can be mapped within defined restriction fragments of DNA by measuring the size of radiolabeled run-off transcripts by gel electrophoresis. When *Sac*I-cleaved pAR-9 DNA was used as template for in vitro transcription, two major radiolabeled bands of approximately 1100 and 940 nucleotides were observed (Fig. 2, lane C). These transcripts were dependent on the tumor DNA template because they were not synthesized in control reactions containing no DNA or vector DNA alone (Fig. 2, lanes A and B). The transcripts were not seen in reactions containing α-amanitin (1 μg/ml) (Fig. 2, lane D) and are therefore pol II-dependent. They were synthesized in all HeLa lysates tested, including those prepared commercially (Bethesda Research Laboratories, New England Nuclear). These results suggest that there are two initiation sites for RNA transcription located within the tumor DNA fragment examined. To test this possibility further, and to determine the direction of transcription, we altered the DNA template by restriction enzyme cleavage. We took advantage of a *Pst*I site which lies 42 nucleotides upstream of the *Sac*I site near the beginning of the second exon of the chicken c-*myc* gene (Fig. 1) (47). When pAR-9 DNA cleaved with both *Sac*I and *Pst*I was used as template, two transcripts were generated, the migration of which after agarose gel electrophoresis reflected a size reduction of approximately 40 nucleotides, in comparison with those observed with the *Sac*I-cleaved template (Fig. 3). Because only one end of the template DNA was altered by *Pst*I cleavage, these results demonstrate that both transcripts are initiated within the CSV LTR and extend into c-*myc* coding sequences before running off the template. The sizes of transcripts generated from these template DNAs indicate that the shorter transcript in each

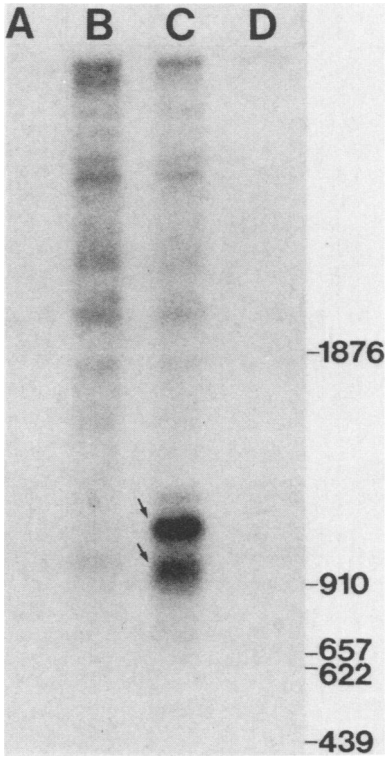


FIG. 2. Electrophoretic analysis of [α-^{32}P]GTP-labeled transcription products generated in vitro with HeLa cell lysates. Template DNAs were: lane A, no DNA; lane B, *Sac*I-cleaved vector; lanes C and D, *Sac*I-cleaved pAR-9. The reaction in lane D contained 1 μg of α-amanitin per ml. The numbers indicate the size, in nucleotides, of end-labeled *Hae*II-digested or *Alu*I-digested pBR322 marker fragments. Transcripts and markers were denatured in glyoxal, subjected to electrophoresis through 1.4% agarose, and detected by autoradiography. Arrows indicate the pol II-specific transcripts derived from cloned tumor DNA.

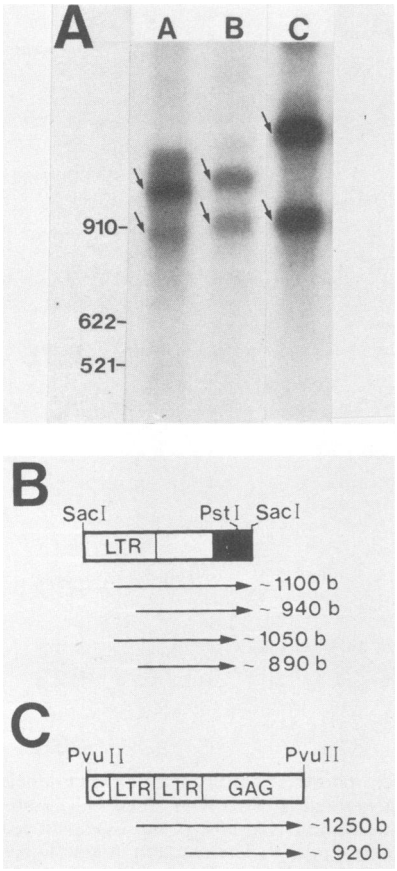


FIG. 3. Determination of the origin and direction of RNAs transcribed in vitro. (A) In vitro transcription, size markers, radioactive label, and conditions for electrophoresis were as described in the legend to Fig. 2. Template DNAs were: lane A, *Sac*I-cleaved, *Pst*I-cleaved pAR-9; lane B, *Sac*I-cleaved pAR-9; lane C, *Pvu*II-cleaved circle junction plasmid of RSV DNA that has two LTRs arranged in the same transcriptional orientation. (B and C) Schematic representations of transcripts derived from pAR-9 DNA and from the RSV circle junction plasmid, respectively. Arrows indicate the pol II-specific transcripts derived from cloned DNAs. b, bases.

case was initiated at or near the U3-R boundary at a site we have called I_1, and the longer transcripts were initiated at a site, designated I_2, that is located about 160 nucleotides upstream, near the middle of the U3 region (Fig. 3B). The intensity of the labeled bands suggests that both initiation sites were utilized at approximately equal efficiencies (Fig. 2 and 3). These conclusions are also supported by run-off transcription experiments with pAR-9 DNA cleaved with both *Sac*I and *Sma*I as a template (data not shown).

As a control, we utilized *Pvu*II-cleaved plasmid DNA containing two RSV LTRs tandemly arranged in the same transcriptional orientation (9). Two run-off transcripts were produced, the sizes of which (ca. 1250 and 950 nucleotides) indicate that one major transcriptional initiation site was utilized in each RSV LTR and that the initiation sites were located at or near the U3-R junction of each LTR (Fig. 3C), as previously determined for RSV (53).

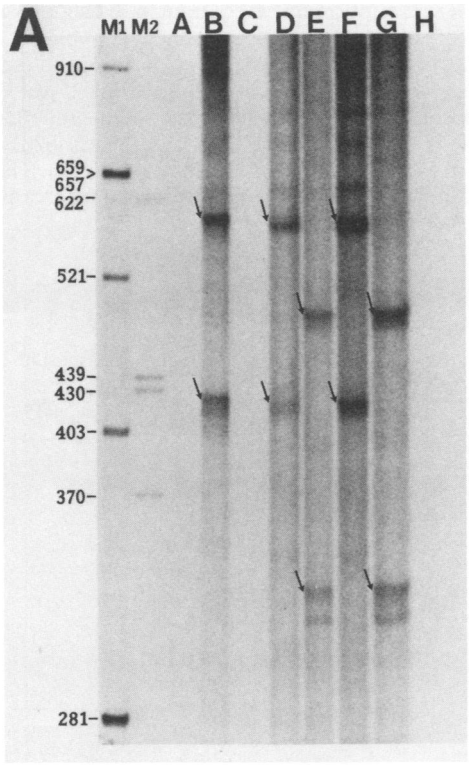
In addition to specific transcripts described above, we detected some nonspecific transcripts (Fig. 1) which presumably originated at promoter-like sequences in vector DNA or resulted from end-to-end transcription of DNA fragments. Some of these were due to transcription by polymerase III and were insensitive to low concentrations of α-amanitin. Similar types of artifacts have been described in detail elsewhere (23, 50).

5′ LTR is transcriptionally active. Results of restriction endonuclease and DNA sequencing analyses of phage BF101-713-10 DNA suggest that the CSV provirus integrated in the c-*myc* locus has retained an intact 5′ LTR (Swift et al., in preparation). In contrast, in previous studies involving ALV-induced B-cell lymphomas, the proviral 5′ LTR was often found to be deleted, and its loss or inactivation has been suggested as a mechanism that might contribute to, or facilitate c-*myc* overexpression (8, 31, 32, 34–36). Therefore, we tested to determine whether the CSV 5′ LTR present in the 713-10 tumor DNA was still transcriptionally competent. Plasmid subclones of the 5′- and 3′-integrated CSV LTRs in tumor DNA were constructed (Fig. 1) and compared for their ability to support transcription in vitro. A plasmid clone containing the LTR of the related REV, SNV, was included for comparison. The plasmid DNAs were digested with either *Sal*I or *Sph*I, which cleave each construct once at a site within the vector sequence (Fig. 4). Transcripts initiated within the viral LTRs would be expected to run off the templates at the *Sal*I or *Sph*I sites. The results of this experiment (Fig. 4) indicate that the 5′ LTR was indeed transcriptionally active in vitro in this construction at a level comparable to that observed with the 3′ LTR. The sizes of the transcripts indicate that the same initiation sites were used in both the 5′ and 3′ LTRs; these sites are identical to those described above (Fig. 2 and 3). In addition, these experiments demonstrate that the SNV LTR also contains two transcriptional promoters which function in vitro to produce transcripts with similar efficiency and from equivalent regions of the LTR as those of the CSV LTR.

Location of transcriptional initiation sites determined by S1 nuclease mapping. S1 nuclease mapping (49) allowed us to locate more precisely the initiation sites of transcription. Probes derived from the LTR sequences of the CSV p3′LTR clone were used. A *Sac*I to *Bam*HI restriction fragment, 5′ end-labeled at the *Bam*HI end (Fig. 5A and B) was hybridized to RNA transcribed in vitro from tumor DNA templates which contained the CSV LTR and extended beyond the *Bam*HI site. As expected from our transcription results, two major DNA fragments of approximately 143 and 295 nucleotides were protected from S1 nuclease digestion (Fig. 5A, lane B). The smaller fragment is only one or two nucleotides larger than a size marker generated by digestion of the CSV LTR *Sac*I to *Bam*HI fragment by *Ava*I. The results obtained with an analogous probe derived from the SNV LTR were similar; the two major DNA fragments were approximately 145 and 305 nucleotides (Fig. 5A, lane F; Fig. 5C). The size differences betwee the CSV and SNV probe fragments reflect the slight differences in DNA sequence which occur between I_2 and the *Bam*HI site in each LTR (42; R. A. Swift, J. Dogson, and H.-J. Kung, manuscript in preparation). The large, nearly full-sized, labeled probe fragments remaining after S1 digestion presumably arose from self-renatured probe DNA (see legend of Fig. 5). Other faint bands visible between the two major transcript-protected bands (Fig. 5A, lanes B and F) are artifacts of the S1 mapping technique, because corresponding transcripts that would protect such probe fragments are not observed as labeled products of in vitro transcription reactions (Fig. 2 through 4). Similar artifacts have been noted previously (16, 24, 25).

To map sites of initiation more precisely, appropriate probe fragments protected from S1 nuclease by in vitro-transcribed RNA were electrophoresed on polyacrylamide gels

next to DNA sequencing ladders generated by base-specific chemical cleavage (26) of the corresponding intact probe fragment. At this higher resolution, the bands which appear to be single in Fig. 5A are resolved into several bands (Fig. 6 and 7). Comparison with the sequencing ladder allowed the determination of the 3′-terminal base of each fragment and therefore of the complementary initial nucleotides transcribed into RNA. The transcripts originating at site I_1 within the CSV LTR appear to have been initiated at any of three adjacent guanine residues (by their complementarity with the cytosine residues which terminate the 3′ end of the negative sense DNA probe fragments) which form part of



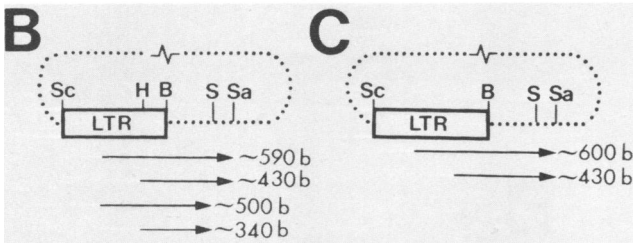


FIG. 4. Comparison of in vitro transcripts from both integrated CSV LTRs and SNV LTR. [α-^{32}P]GTP-labeled transcripts and ^{32}P-end-labeled markers were analyzed on a denaturing 5% polyacrylamide gel and detected by autoradiography. (A) Lanes M1 and M2, end-labeled markers generated from pBR322 digested with *Alu*I and *Hae*II, respectively. Lanes A, C, and H contain RNA synthesized in the presence of 1 μg of α-amanitin per ml. Template DNAs were: lanes A and B, *Sal*I-cleaved pLTR(SNV); lanes C and D, *Sal*I-cleaved p5′LTR (CSV); lane E, *Sph*I-cleaved p5′LTR (CSV); lane F, *Sal*I-cleaved p3′LTR (CSV); lanes G and H, *Sph*I-cleaved p3′LTR (CSV). Arrows indicate pol II-specific transcripts derived from cloned DNA. (B and C) Schematic representations of transcripts derived from either CSV LTR or pLTR(SNV), respectively. Sc, *Sac*I; H, *Hin*dIII; B, *Bam*HI; S, *Sph*I; Sa, *Sal*I; b, bases.

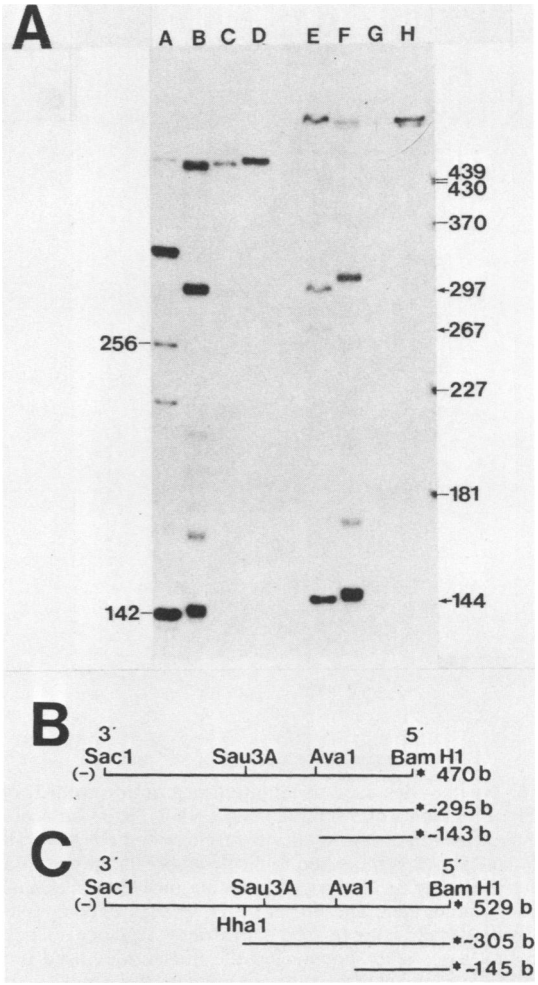


FIG. 5. S1 nuclease analysis of RNAs transcribed in vitro. Probe fragments were separated on a denaturing 5% polyacrylamide gel and detected by autoradiography. (A) Lanes A and E contain size markers derived by restriction endonuclease digestion of *Sac*I to *Bam*HI fragments of the CSV and SNV LTRs, respectively. Other numbers on the right side represent the positions of end-labeled *Hae*II restriction endonuclease fragments of pBR322 included as size markers. The 5′-end-labeled *Sac*I to *Bam*HI fragments of the CSV LTR (lanes B, C, and D) and the SNV LTR (lanes F, G, and H) were used as probes. Lanes B and F, fragments remaining after hybridization to RNA translated in vitro from CSV and SNV LTR templates, respectively, and digestion with S1 nuclease; lanes C and G, fragments remaining after hybridization to tRNA and digestion with S1 nuclease; lanes D and H, portions (1/100) of material loaded onto lanes B and F, respectively, before digestion with S1 nuclease. (B) CSV LTR probe and the probe fragments protected from S1 nuclease digestion in lane B in (A). (C) Diagram of the SNV LTR probe and the probe fragments protected from S1 nuclease digestion in lane F. Stars denote location of radioactive label. b, bases. The protected probe fragments of almost full size that are visible in (A), lanes B and F, probably arise from artifacts in which probe molecules with labeled ends protected by RNA transcripts renature with complementary DNA strands on dilution of the hybridization buffer with S1 nuclease digestion buffer (49).

the *Ava*I recognition site CTCGGG (Fig. 6). This may reflect microheterogeneity in the choice of the first base transcribed or may be due to steric hindrance of S1 nuclease digestion by the cap structure (49). The transcripts originating from I_2 within the CSV LTR appear to initiate at any of four

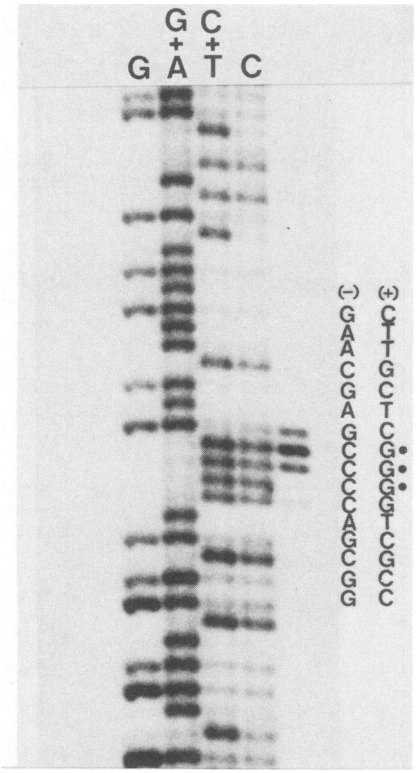


FIG. 6. Nucleotides used to initiate transcription from I_1 of the CSV LTR. Fragments of 5'-end-labeled CSV LTR probe protected from S1 nuclease digestion by in vitro-transcribed RNA (as shown in Fig. 5, lane B) were subjected to electrophoresis, next to Maxam and Gilbert sequencing ladders of the same probe, on a denaturing 8% polyacrylamide gel. The nucleotide sequence encompassing I_1 derived from the gel is shown. The plus-strand sequence deduced by complementarity is also shown, and the nucleotides used to start transcription are indicated by dots. In aligning the bands, it should be noted that the base present at the 3' terminus of the RNA-protected probe fragment is the one that was destroyed in producing the Maxam and Gilbert fragment that migrated approximately one base faster.

positions; A, T, C, and A (Fig. 7). However, with longer autoradiographic exposure, a few additional bands appear, suggesting that a somewhat higher degree of heterogeneity exists at the 5' end of I_2 transcripts than I_1 transcripts.

The patterns of bands produced by fragments of SNV LTR probes protected from S1 nuclease digestion were identical to those for CSV, and the same bases were implicated as starting sites for transcription (data not shown; Fig. 8).

Transcriptional control sequences in the CSV and SNV LTRs. Sequence homology between the CSV and SNV LTRs is extensive; the regions of each LTR that encompass the putative transcriptional control signals are shown and compared in Fig. 8 (the complete CSV LTR sequence appears elsewhere; Swift, Dogson, and Kung, in preparation). Site I_1 in SNV corresponds to the transcriptional initiation site predicted by Shimotohno et al. (42) on the basis of nucleotide sequence data. As a reference point, we have designated as nucleotide +1 the guanine residue that was implicated by S1 nuclease mapping as that most frequently used to start transcription from I_1 (Fig. 6). This is the base equivalent to that previously proposed as the start site for transcription in the SNV LTR (42) and is the second guanine residue, or fifth base, of the six-base *Ava*I restriction endonuclease recognition signal that occurs at the U3-R junction of both the CSV and SNV LTRs.

The sequence TATATAA lies 30 nucleotides upstream (−30) from the initiation site I_1 in the CSV LTR. The sequence CCAATG lies another 40 nucleotides further upstream (−70). These sequences and their location relative to I_1 suggest that they function as transcriptional control signals (for a review, see reference 2). Other CAAT boxes which may influence transcription from I_1 occur at positions −96 and −136 in the CSV LTR sequence (Swift, Dogson, and Kung, in preparation). A role for similar control sequences that affect transcription of SNV DNA has been suggested previously, based on sequence analysis of the SNV LTR (42). Transcription initiated in vitro in the region −153 to −156 of the CSV LTR sequence (I_2) may be influenced by a tandem arrangement of TATA-like sequences (CATAAACCATAAA) that extend from 25 to 37 nucleotides upstream of I_2 (Fig. 8). The equivalent sequence in the SNV LTR is CACAAACCACAAA (−189 to −201), which is also 25 to 37 nucleotides upstream of I_2 (Fig. 8). The CAAT-like sequence CATT, which may influence transcrip-

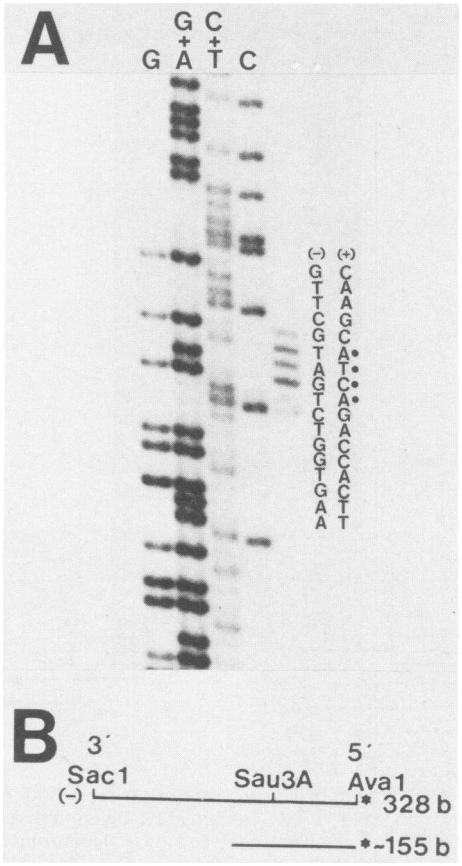


FIG. 7. Nucleotides used to initiate transcription from I_2 of the CSV LTR. (A) Fragments of a *Sac*I to *Ava*I, 5'-end-labeled, CSV LTR probe, protected from S1 nuclease digestion by in vitro-transcribed RNA, were subjected to electrophoresis next to Maxam and Gilbert sequencing ladders of the same probe. The nucleotide sequence encompassing I_2 is shown, and the nucleotides used to start transcription are indicated by dots. Details are as described in the legend to Fig. 6. (B) Schematic of the probe and the probe fragments protected from S1 nuclease digestion. Stars denote the location of the radioactive label.

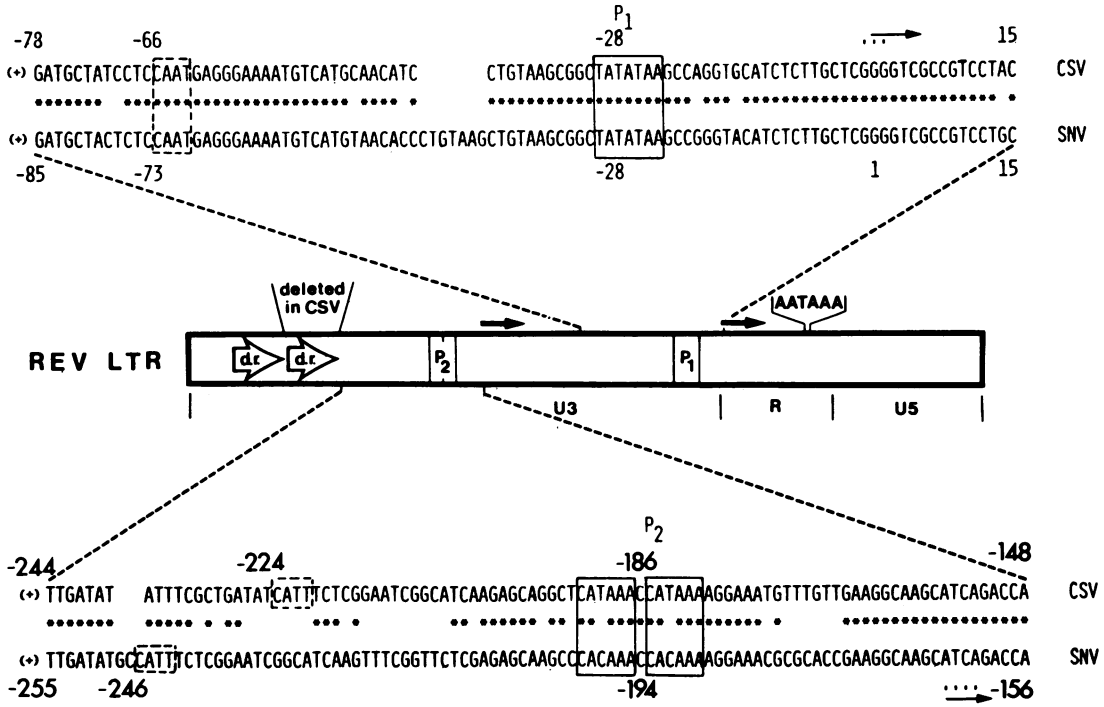


FIG. 8. Transcriptional control regions of the REV LTR. The schematic representation of the REV LTR is based on data presented in Fig. 1 to 7 and on sequencing data from reference 42 and Swift, Dogson, and Kung (in preparation). The DNA sequences (plus strand) adjacent to I_1 and I_2 are shown with the putative transcriptional control signals present immediately upstream (boxed). The CSV (upper) and SNV (lower) sequences are directly compared; stars denote nucleotide identity. Nucleotides used to start transcription in vitro are indicated by dots. Bold arrows denote positions of I_1 and I_2 and the direction of transcription. d.r., Direct repeat.

tion from I_2, occurs at positions −224 and −252 in the CSV LTR sequence and at positions −246 and −263 in the SNV LTR sequence (Fig. 8) (42; Swift, Dogson, and Kung, in preparation).

DISCUSSION

We analyzed a c-*myc* locus cloned from chicken tumor DNA which was altered and transcriptionally activated by insertion of CSV proviral DNA upstream from and adjacent to the c-*myc* locus. In vitro transcription and S1 nuclease mapping studies have resulted in the following conclusions. (i) The downstream CSV LTR is capable of acting as a strong promoter for RNA transcripts extending into c-*myc* sequences. (ii) Both CSV LTRs are present and transcriptionally competent. (iii) Each CSV LTR contains two separate transcriptional start sites with appropriate control signals. (iv) The SNV LTR also exhibits two transcriptional promoters; therefore, this is probably a general feature of REV. These findings and their implications regarding viral replication and leukemogenesis are discussed below.

REV LTRs contain two transcriptional start sites. To ensure the presence of the terminal repeat, or R region, at both ends of retroviral genomic RNA, it is necessary that the transcriptional initiation site be upstream from the R region of the 5′ viral LTR in proviral DNA (46). In several retroviral LTRs examined to date, transcriptional initiation sites have been mapped at or near the U3-R boundary (11, 33, 42, 53), and transcriptional control signals have been found in a region that suggests that transcription initiates at or near the U3-R boundary in most or all retroviral LTRs (46). In the present study, results of in vitro transcription (Fig. 2 through 4) and S1 nuclease mapping (Fig. 5) experiments have shown that two sites within the CSV LTR are used for initiation of transcription: I_1 at the U3-R junction and I_2 approximately 160 nucleotides upstream in the U3 region. Similar results were obtained for the LTR of SNV, another member of the REV family (Fig. 4 and 5).

Usage of the I_2 transcriptional start site is not dependent on a contiguous arrangement of LTR DNA with either cellular (Fig. 4) or plasmid vector DNA (Fig. 2 and 3) and is therefore a function of the LTR DNA sequences alone. Furthermore, the I_2 site is used as frequently as the I_1 site in vitro. The relative strength of the transcriptional signal from I_2 was not due to trivial considerations such as the proportion of guanine residues in each transcript or to the I_1 requirement for guanine as the first base under our labeling conditions which utilized labeled GTP and relatively low overall concentrations of GTP. The radioactive signals were also similar for both I_1- and I_2-derived transcripts when [α-^{32}P]UTP was used as precursor (data not shown), and the DNA sequence between I_1 and I_2 indicates that transcripts would not be disproportionately rich in guanine or uridine residues (42; Swift, Dogson, and Kung, in preparation).

We are unaware of any other reports that suggest the presence of a second, strong transcriptional promoter within a retroviral LTR. Evidence for two additional, relatively weak initiation sites in the U3 region of RSV has been presented based on S1 nuclease mapping. However, these transcripts could not be detected by direct in vitro transcription, and no recognizable promoter sequences were present in the appropriate regions of the RSV LTR (28).

Transcriptional control signals in the CSV and SNV LTRs. Sequence analysis of the CSV LTR revealed a canonical TATA box approximately 30 nucleotides upstream from I_1

(Fig. 8). We called this promoter element P_1. A CAAT box occupies a position 66 nucleotides upstream from I_1 (Fig. 8), a common site for such a structure (2), and two other CAAT boxes, the positions of which may influence transcription from I_1, occur at positions −96 and −136. For comparison, the first CAAT box upstream from the U3-R junction of the RSV LTR occurs at position −175 (28).

A putative transcriptional promoter element for I_2 (P_2), located approximately 20 to 30 nucleotides upstream from I_2, is a tandemly arranged, somewhat poor homologue of the TATA consensus sequence; the sequence CATAAA occurs twice, separated by a cytosine residue. This six-base sequence is identical to a sequence that serves as part of the promoter for the rabbit β-globin gene (15), and the sequence TACATAA has been implicated as part of the weak promoter for the Hepatitis B virus core antigen gene (37).

Based on S1 nuclease analyses, both I_1 and I_2 showed an apparent microheterogeneity in the use of the first base transcribed into RNA. At I_1, the first three in a run of four guanine residues served as the first base transcribed (Fig. 6); at I_2 any of the four adjacent bases A, T, C, and A were preferentially used (Fig. 7). This may be partially due to an artifact involving steric hindrance of S1 nuclease digestion by the cap structure at the 5′ end of mRNA (49). Although the presence of the cap structure was not directly demonstrated, only one gene so far studied has been found to produce a significant proportion of uncapped transcripts in vitro (22). Microheterogeneity resulting from transcription initiation at one of several adjacent nucleotides is, however, common in many genes. Greater degrees of heterogeneity are often associated with a run of identical nucleotides at the cap site, or with a poor homologue of the TATA consensus sequence or its absence (22).

The identity of the bases implicated as those used to initiate transcription were the same in CSV and SNV LTRs (data for SNV not shown). The DNA sequences in the immediate regions of P_1, I_1, and I_2 are the same in the two viral LTRs (Fig. 8). P_2 in SNV comprises the sequence CACAAA repeated twice, separated by a cytosine residue (Fig. 8). This sequence is devoid of tyrosine residues and bears little homology to the TATA consensus sequence. It differs by only one base, however, from the comparable P_2 sequence component of CSV (CATAAA). It also differs by only one base from the sequence TACAAA, which provides a promoter function for late transcription of the adenovirus E2A gene (5). Longer autoradiographic exposure of the gel shown in Fig. 7 revealed a number of other adjacent nucleotides less frequently used to initiate transcription at I_2, and a similar result was observed for SNV (data not shown). Because the P_2 promoter elements of CSV and SNV differ quite significantly from the classical TATA sequence, the greater heterogeneity observed in both at I_2 may be a consequence either of these sequence differences or of the tandem duplication of these promoter sequence elements. Because of the tandem arrangement, a spacing of seven nucleotides between preferred initiation sites may have been expected, but we did not observe this. The tandem arrangement may, however, contribute to the unexpected high frequency of initiation at I_2 relative to that at I_1.

The CAAT-like sequence CATT occurs at two sites upstream from I_2 in both the CSV and SNV LTRs. The more distant CATT falls within a region of sequence identity between CSV and SNV (−252 in the CSV LTR) (data not shown; Swift, Dogson, and Kung, in preparation). The other CATT occurs slightly closer to I_2 in CSV than in SNV and in a region of nonhomology (Fig. 8). The conservation of this structure at this location, despite surrounding sequence divergence, suggests that these CAAT-like sequences are functionally important.

Possible implications of two transcriptional promoters in the REV LTR, and in certain cellular genes. Several eucaryotic structural genes have been found to possess two or more promoters, including the zein gene of maize (20), the mouse α-amylase gene (38), the myosin gene (29) and several estrogen-regulated genes in chickens (3), various human globin genes (14), and the human c-*myc* gene (1, 48). Interestingly, the two promoters for the human c-*myc* gene are about 170 nucleotides apart, a distance that is very similar to the spacing of promoters in the REV LTR. The existence of alternate promoters might perhaps allow a more delicate transcriptional regulation and provide a mechanism for the production of significantly different mRNAs. These differences may relate to the nature of untranslated 5′ leader sequences, or perhaps to alternate splicing events, and may be tissue or cell-type specific (3, 14, 20, 29, 38, 48).

One further consequence of these promoter arrangements in normal cellular genes, or in genes activated by an appropriate viral LTR, is the production of mRNAs which, if transcribed from the upstream promoter, would harbor the sequences for the downstream promoter (provided they were not spliced out). If such transcripts were then copied into DNA by a reverse transcription mechanism, the DNA copies would carry the signals for a functional promoter. If such DNA copies were reintegrated into the genome, then transcription of these sequences would not be dependent on fortuitous integration adjacent to or near other cellular promoters. Although such a series of events is rather speculative at this time, it is conceivable that the presence of two promoters in certain LTRs or structural genes could facilitate, or play important roles, in processes such as the evolution of mobile genetic elements, retroviruses, and processed genes, and perhaps even in novel mechanisms of gene amplification (18, 39, 44, 45, 51).

Similar mechanisms have been proposed to account for the possible evolution, mobility, and spread of human *Alu* family sequences that carry RNA polymerase III promoters internally within the region of DNA transcribed by polymerase III into RNA (18, 45).

In relation to the REV LTR, the presence of P_2 may have important effects on REV replication or pathogenesis: (i) the presence of a second promoter may increase overall levels of transcription; (ii) there may be some beneficial specialization between the two promoters for the production of genomic versus subgenomic mRNAs; (iii) the two promoters may possess different cell or tissue-specific patterns of regulation; and (iv) P_2-directed transcripts may have evolved a regulatory role governing P_1-directed transcription, as has been proposed for minor transcripts detected during transcriptional analysis of the RSV LTR (28). In this regard, it is interesting that an ATG translation initiation codon occurs approximately 70 nucleotides downstream from I_2 in the CSV LTR and an open reading frame extends to the TGA triplet at +90; this region has the potential to code for 61 amino acids or 6 kilodaltons of protein (Swift, Dogson, and Kung, in preparation). Although the function, if any, of this open reading frame is unknown, an equivalent open reading frame does not exist in the related SNV LTR (42).

Similarly, in respect to leukemogenesis, it is possible that the existence of two promoters within the right-hand (or 3′) CSV LTR might result in enhanced expression of a downstream oncogene, possibly in a tissue- or stage-specific manner. Recently, transcriptional factors have been identi-

fied that discriminate between different RNA pol II promoters, and certain LTR regions have been shown to enhance or modulate the ability of certain retroviruses to replicate and cause diseases in specific hosts and tissues (4, 10, 43).

A CSV provirus retains both 5′ and 3′ LTRs in tumor DNA. In ALV-induced bursal lymphoma, the 5′-integrated LTR is frequently missing. It has been proposed that transcription initiated in a 5′ LTR and terminated in a 3′ LTR may interfere with the efficiency of initiation of transcription in the 3′ LTR and hence of transcription into downstream sequences (8, 31). DNA constructs of the form 5′-(ALV LTR)-(gene A)-(ALV LTR)-(gene B)-3′ support this hypothesis when transfected into cells; transcription of gene B is more efficient if the first LTR is inactivated (8). However, in nearly all CSV-induced bursal lymphomas so far analyzed by restriction endonuclease digestion and Southern blotting, each provirus within the c-*myc* locus appears to have both 5′ and 3′ LTRs present (Swift et al., in preparation). In clone 713-10 analyzed in this report, the 5′ LTR was found to be transcriptionally competent in vitro (Fig. 4). It is possible that in this tumor other mechanisms prevent the transcription of viral DNA sequences into stable mRNAs in vivo. One possibility is that, as a result of internal deletions, most in vivo transcripts initiated from the 5′ LTR were prematurely terminated, thus diminishing the interference effects of upstream transcripts that were demonstrated by Cullen et al. (8). Alternatively, it is conceivable that the interference events postulated to affect the efficiency of ALV-mediated promoter insertion events might be less important in CSV-mediated promoter insertion events. This may be somehow related to certain structural aspects of the CSV LTR. For example, the termination events that are postulated to disrupt initiation events within the 3′ integrated ALV LTR (8) might not affect both transcriptional sites in the integrated 3′ CSV LTRs, because the two sites are separated by 160 nucleotides.

In conclusion, the LTRs of the REVs studied here exhibit two strong transcriptional promoters, a characteristic which distinguishes them from other viral LTRs. Experiments designed to determine whether both transcriptional initiation sites are utilized in vivo and the mechanisms of regulating their use are in progress.

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