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Functional Interaction between Transcriptional Elements in the Long Terminal Repeat of Reticuloendotheliosis Virus: Cooperative DNA Binding of Promoter- and Enhancer-Specific Factors

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Transcription from reticuloenodotheliosis virus strain T (REV-T), an avian retrovirus unrelated to avian leukosis and sarcoma viruses, is modulated by sequences in at least five functional domains. A promoter containing a TATA and multiple CCAAT motifs in U3 of the long terminal repeat was absolutely required for transcription. Transcriptional efficiency was greatly augmented by an enhancer immediately upstream, which contained a 22-base-pair repeated sequence. Transcription was further influenced by a negative-acting domain in the 5′ region of U3 and two downstream domains in the transcribed non-protein-coding region. One of these latter domains contained a consensus enhancer core sequence and positively affected transcription in both mammalian and avian cells; the other acted negatively in a dog cell line. Transcription from REV-T in vivo required cellular factors which could be competed for specifically by the promoter or enhancer domain. The downstream domains competed with reporter genes containing these domains, but not directly with the U3 sequences. The promoter, enhancer, and the positive-acting downstream domains formed multiple complexes with distinct classes of cellular factors in both avian and mammalian cell extracts. Binding of factors to the promoter and enhancer domains was cooperative when these domains were joined in *cis*.

Retroviruses do not shut off host cell transcription. Instead, they rely on efficient elements in the proviral DNA which utilize the host transcriptional machinery to produce up to 10 to 20% of the total polyadenylated RNA in the infected cells (43). Retroviruses therefore provide a powerful tool for studying the transcriptional process in eucaryotic cells.

Reticuloendotheliosis viruses (REVs) and avian sarcoma and leukemia viruses represent two distinct families of retroviruses which naturally infect avian species (41). The transcriptional elements in avian sarcoma viruses (ASVs) have been localized in the U3 region of the long terminal repeat (LTR). These include a promoter absolutely required for transcription and an enhancer which greatly augments transcriptional efficiency (7, 14, 23, 24, 30, 44). The exact 5′ boundary of the enhancer sequence appears to vary among different ASV strains (7, 23, 24), and additional regulatory elements have been found downstream of the LTR in the protein-coding region (1). Sequences in the ASV LTR which interact with avian cellular factors in vitro have been identified (37). However, the functional relevance of this interaction remains to be established.

By comparison, the transcriptional function in REVs is largely unknown. Although sequences of several REV members have been determined (38, 40, 49), the functional significance of the sequences has not been determined. In this study, we used deletion mapping and RNase protection analysis to define the transcription initiation site, splice junction, and the promoter, enhancer, and two downstream regulatory regions in REV strain T (REV-T), a prototype of the REV group. By an in vivo competition assay, we showed that transcription from REV-T in both avian and mammalian cells required *trans*-acting cellular factors which recognized specific DNA elements. The promoter, enhancer, and one of the downstream regulatory regions formed specific complexes with distinct classes of cellular factors in vitro. Furthermore, the promoter- and enhancer-binding factors interacted cooperatively with their cognate domains when these domains were joined in *cis*.

MATERIALS AND METHODS

Cell culture and transfection. Primary cultures of chicken embryo fibroblasts (CEF) were prepared from gs-negative, chf-negative embryos of the C/O phenotype (Hyline Farms, Dallas Center, Iowa). Dog osteosarcoma D17 cells were obtained from the American Type Culture Collection. CEF were grown in growth medium containing 1% chicken serum (20). D17 cells were maintained in Eagle minimal essential medium containing 10% fetal bovine calf serum and nonessential amino acids. CEF (1.2×10^6) or D17 (1.8×10^6) cells were seeded onto 60-mm culture dishes 16 to 20 h before transfection. Constant amounts of DNA, as indicated in the figure legends, were applied to the cells by the calcium phosphate method (16, 48). Transfected cultures were shocked with 10% dimethyl sulfoxide for 4 min for CEF or 10 min for D17 cells at 4 to 6 h posttransfection. Cells were washed twice, maintained in appropriate growth medium overnight, and replenished with fresh medium several hours before harvest.

Plasmid construction. pREV-T3 containing the permuted provirus of REV-T (3) was obtained from the American Type Culture Collection. To construct pTZ-REV-T-LTR (pTZ-wt; see Fig. 3A), the *Avr*II-*Sal*I (−406 to +455) fragment containing the LTR region flanked by 3′ and 5′ viral sequences was subcloned into vector pTZ18R or pTZ19R (Pharmacia) (see references 38 and 49 for the related sequences in spleen necrosis virus [SNV] and REV-A, the helper virus of REV-T). To generate pREV-T-LTR-NEO (wt-NEO; see Fig. 1A) and pREV-T-LTR-CAT (wt-CAT; see Fig. 3A), the same *Avr*II-*Sal*I fragment was inserted into pSV2-NEO (39; provided by N. Davidson, California Institute of Technology) or pSV2-CAT (15; obtained from the American Type Culture

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Collection), respectively, to replace the *Nde*I-*Hin*dIII simian virus 40 (SV40) promoter region.

Deletion mutants and sequence analysis. A nested set of 3′ deletion mutants were generated by unidirectional exonuclease III (ExoIII) digestion (19) of the pTZ18R or pTZ19R subclone described above. The deleted LTR regions were excised from the vector by digestion with *Sst*I (−349 in U3) and *Hin*dIII (in pTZ vector) and inserted between the corresponding *Sst*I and *Hin*dIII sites in wt-NEO to generate 3′Δ1-NEO, 3′Δ2-NEO, 3′Δ3-NEO, and 3′Δ4-NEO (Fig. 1A). 5′ deletion mutants were generated similarly, starting with a pTZ19R subclone containing the *Sst*I fragment (−349 to +313) (5′Δ1-NEO, Fig. 1A). The truncated LTR regions were excised by digestion with *Eco*RI and *Hin*dIII (both in the pTZ vector) and inserted into pSV2-NEO to replace the SV40 promoter region between the *Nde*I and *Hin*dIII sites, generating 5′Δ2-NEO and 5′Δ3-NEO (Fig. 1A). All the deletion mutants as well as the entire REV-T LTR and flanking regions were sequenced in both directions by the dideoxynucleotide chain-termination technique (34). The sequence was similar but not identical to the corresponding regions in SNV, chicken syncytial virus (CSV), and REV-A (38, 40, 49) and is described in the text.

In vivo competition. Constant amounts (1 μg) of wt-CAT or 3′Δ2-CAT were cotransfected into CEF or D17 cells with a 10-fold molar excess of competitors which contained the various domains of interest as specified in the figure legends. For comparison, parallel cultures were transfected with the same reporter gene and an equivalent molar excess of a mock competitor consisting of the same vector without the viral sequences. Transfection efficiency was comparable among the different competitors, as shown by quantitating the actual input DNA recovered from the transfectants by CsCl gradient centrifugation (see below).

RNA preparation and quantitation. Approximately 28 h posttransfection, cells were washed twice with ice-cold buffer A (25 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 5 mM KCl, 5.5 mM glucose) and lysed in 4 M guanidium isothiocyanate (4). Total cell lysates were centrifuged through CsCl with a specific gravity of 1.72 in 50 mM sodium acetate–50 mM EDTA (pH 5.0) in an SW50.1 rotor at 120,000 × *g* for 16 to 20 h (12). The RNA pellets were dried at room temperature, suspended in TSE (10 mM Tris hydrochloride [pH 7.0], 1 mM EDTA, 0.2% sodium dodecyl sulfate [SDS]), precipitated in ethanol at −70°C overnight, redissolved in TSE, and stored at −70°C. RNA samples were resolved by electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde, transferred onto nitrocellulose paper (25), and hybridized with riboprobes specific for the *neo* or *cat* gene prepared by in vitro transcription from vector pGEM-3 (Promega Biotec) containing these genes by using T7 or SP6 polymerase (28). Relative RNA levels were quantitated by an LKB model 2202 laser densitometer with multiple X-ray films exposed to the hybridized blots to a nonsaturated intensity. To ascertain transfection efficiency, the input DNA was recovered from the interface of the CsCl cushion, dialyzed and extracted with phenol, resolved by electrophoresis, and quantitated by hybridization with the *neo*- or *cat*-specific probe.

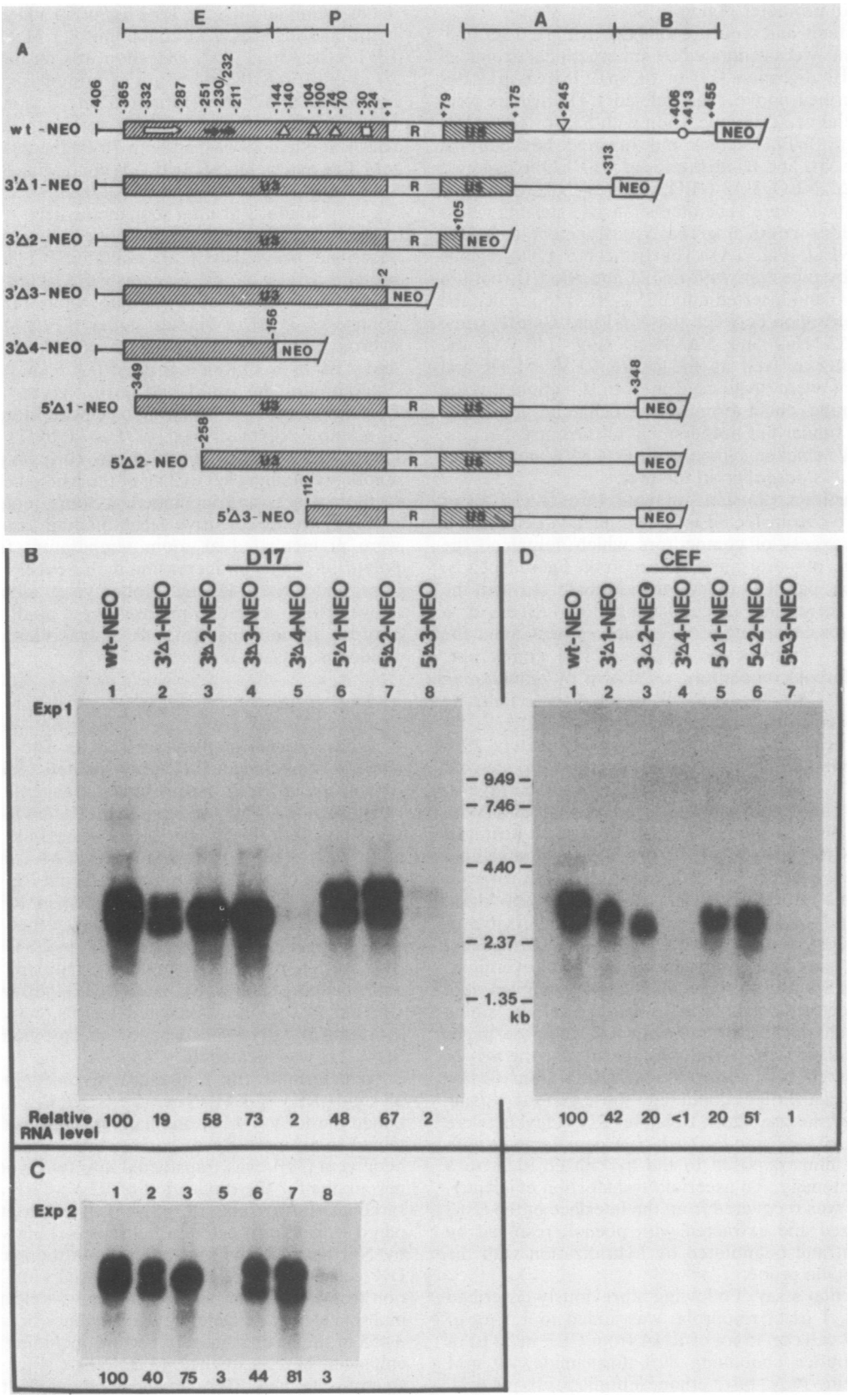
RNase protection assay. Following a previously described procedure (28), 1 μl of riboprobe was added to 1.5 μg of RNA from D17 cells or 15 μg of RNA from CEF in 30 μl of hybridization buffer containing 80% formamide, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.7], 0.4 M NaCl, and 1 mM EDTA. After denaturing at 85°C for 5 min, the samples were incubated overnight at 50°C. Following hybridization, the samples were digested with RNase A (40 μg/ml) and RNase T_1 (2 μg/ml) in 300 μl of 10 mM Tris hydrochloride (pH 7.5) containing 0.3 M NaCl and 5 mM EDTA for 1 h at 30°C. Digestion was terminated by adding 20 μl of 10% SDS and 50 μg of proteinase K, followed by incubation at 37°C for 15 min. The reaction products were extracted with phenol, precipitated with 20 μg of carrier tRNA in ethanol, washed with 70% ethanol, dissolved in 10 mM Tris hydrochloride (pH 7.5) containing 80% formamide, 1 mM EDTA, and dyes, and analyzed by electrophoresis in 5% acrylamide–8 M urea sequencing gels.

Gel retardation analysis. Whole-cell extracts, prepared as described previously (27), containing 1 μg of D17 cell proteins or 2 μg of CEF proteins were incubated with 1 μg of poly(dI:dC) (Sigma) at room temperature for 5 min in a final volume of 25 μl of binding buffer (7.5 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 8], 2.5 mM Tris hydrochloride [pH 7.5], 0.8 mM EDTA, 1 mM dithiothreitol, 62.5 mM NaCl, 10% glycerol). Gel-purified DNA fragments were end labeled with either [α-^{32}P]dATP by Klenow polymerase or [γ-^{32}P]ATP by T4 polynucleotide kinase to a specific activity of ca. 10^7 cpm/μg (25). A 1-μl amount containing 0.3 to 2 ng of the labeled DNA was added to the extracts and incubated at room temperature for 30 min. For the competitive DNA-binding assay, the extracts were preincubated at room temperature for 5 min with poly(dI:dC) plus an increasing molar excess of the competitors, and the end-labeled probes were added as described above. DNA-protein complexes were analyzed by electrophoresis in nondenaturing 4% polyacrylamide gels (acrylamide-bisacrylamide, 30:1).

RESULTS

Transcriptional domains in REV-T. The LTR of REV-T (Fig. 1A) contained a TATA box located about 30 base pairs (bp) upstream from the presumed transcriptional start site (+1), preceded by three sets of CCAAT motifs spaced precisely 30 or 40 bp apart (−144 to −140, −104 to −100, and −74 to −70). Upstream of the CCAAT motifs were two sets of sequences repeated only in some REV members: an overlapping 22-bp sequence repeated in REV-T (between −251 and −211), and a 46-bp sequence (between −332 and −287) repeated in SNV (38) but not in CSV (40) or REV-T (this study). A sequence perfectly matching the consensus enhancer core sequence (GTGGTTTG [46]) was found not in U3, but in the 5′-untranslated region (between +406 and +413). A putative splice donor site previously mapped in SNV (11) was located at +245.

To determine the sequences involved in transcription, plasmid pREV-T-LTR-NEO (wt-NEO; Fig. 1A), which contained the REV-T LTR and flanking regions (−406 to +455) joined to a reporter gene, *neo* (which confers resistance to neomycin [39]), was transfected into two types of host cells permissive for the replication of REV: CEF and dog osteosarcoma (D17) cells (10, 42, 45). Transcription was compared with that in deletion mutants lacking various regions by Northern (RNA) blot analysis. Although expression in D17 cells was about 15-fold higher than in CEF, transcription from REV-T in both cell types was clearly affected by multiple elements. Deleting the sequence between +313 and +455 in the 5′ untranslated region, including the consensus enhancer core sequence (3′Δ1-NEO, Fig. 1A), reduced steady-state *neo* RNA levels to about 20 to 40% of the wild-type level in both D17 cells (Fig. 1B and C, lane 2) and CEF (Fig. 1D, lane 2). Surprisingly, deleting all the 5′


A
E
P
A
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wt-NEO
3'Δ1-NEO
3'Δ2-NEO
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3'Δ4-NEO
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5'Δ2-NEO
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R
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5'Δ1-NEO
5'Δ2-NEO
5'Δ3-NEO
Exp1
9.49
7.46
4.40
2.37
1.35
kb
Relative RNA level
100 19 58 73 2 48 67 2
D
CEF
100 42 20 <1 20 51 1
C
Exp 2
100 40 75 3 44 81 3

untranslated and most of the U5 sequences (3'Δ2-NEO, Fig. 1A) only reduced the *neo* RNA level to about 58 to 75% of the wild-type level in D17 cells (Fig. 1B and C, lane 3), but further decreased RNA levels to about 20% in CEF (Fig. 1D, lane 3). The host-dependent modulating effects of the 5' untranslated sequences were observed in three independent experiments. This result suggests that the region between +314 and +455 harbors a positive-acting element, and an additional element(s) which acts negatively in D17 cells might reside between +106 and +313 (Fig. 1A).

Completely eliminating the R region (3'Δ3-NEO, Fig. 1A) did not reduce transcriptional activity further (Fig. 1B, lane 4), indicating that the U3 region was sufficient for transcription. However, further deletion to −156 (3'Δ4-NEO) completely abolished transcriptional activity in both cell types (Fig. 1B and C, lane 5; Fig. 1D, lane 4). The trace amounts of remaining *neo* RNA in D17 cells did not originate from within the LTR but from an unidentified promoter in the upstream vector sequences (see below). Therefore, sequences between −155 and −2, which contain the TATA and CCAAT motifs, are absolutely required for transcription in both CEF and D17 cells.

Effects of 5' deletions were compared with transcription in a plasmid lacking the downstream positive-acting region (5'Δ1-NEO, Fig. 1A) in order to avoid complications due to that domain. This plasmid expressed only about 40 to 50% as much RNA as wt-NEO in D17 cells (Fig. 1B and C, lane 6) and about 20% as much as wt-NEO in CEF (Fig. 1D, lane 5), presumably due to the loss of the positive-acting domain (although minor effects due to a small deletion at the 5' terminus of U3 have not been completely ruled out). Deleting the 5' region which contained the 46-bp sequence (5'Δ2-NEO) consistently increased expression in both D17 cells (Fig. 1B and C, lane 7) and CEF (Fig. 1D, lane 6). Further deleting the region which contained the 22-bp repeats and one CCAAT motif (5'Δ3-NEO) drastically reduced expression in both cell types (Fig. 1B and C, lane 8; Fig. 1D, lane 7), but the remaining RNA (1 to 3% of wt-NEO level) was still correctly initiated (see below). Therefore, sequences between −258 and −113 are crucial for transcriptional efficiency but not for accurate initiation. We have found that sequences upstream of −156 could substitute for the 72-bp repeats of SV40 to provide an enhancer function in a host cell-dependent manner (manuscript in preparation).

These experiments show that transcription from REV-T requires a promoter between −155 and −2 (P region, Fig. 1A) and is augmented by an enhancer between −258 and −156. Transcription from these minimal sequences is further modulated by upstream and downstream sequences. At least one negative-acting element resides in the upstream region. However, since the entire region upstream of −156 has an overall positive effect on transcription, we shall simply refer to that region as the E region until the exact number of regulatory elements within that region is defined (Fig. 1A). The downstream domain, which acts negatively in dog cells (+105 to +313), is called the A domain, and the positive-acting region between +314 and +455, including the consensus enhancer core, is called the B domain (Fig. 1A).

A and B downstream domains affect both splicing and RNA levels. The downstream A domain contained a putative splice donor (+245, Fig. 1A). To distinguish the effects of the 3' deletions on splicing and RNA levels and to map the transcriptional start site, we quantitated spliced and total RNA by an RNase protection assay with two riboprobes: 3'Δ1, which spanned the putative splice donor site, and 3'Δ2, which did not (Fig. 2A).

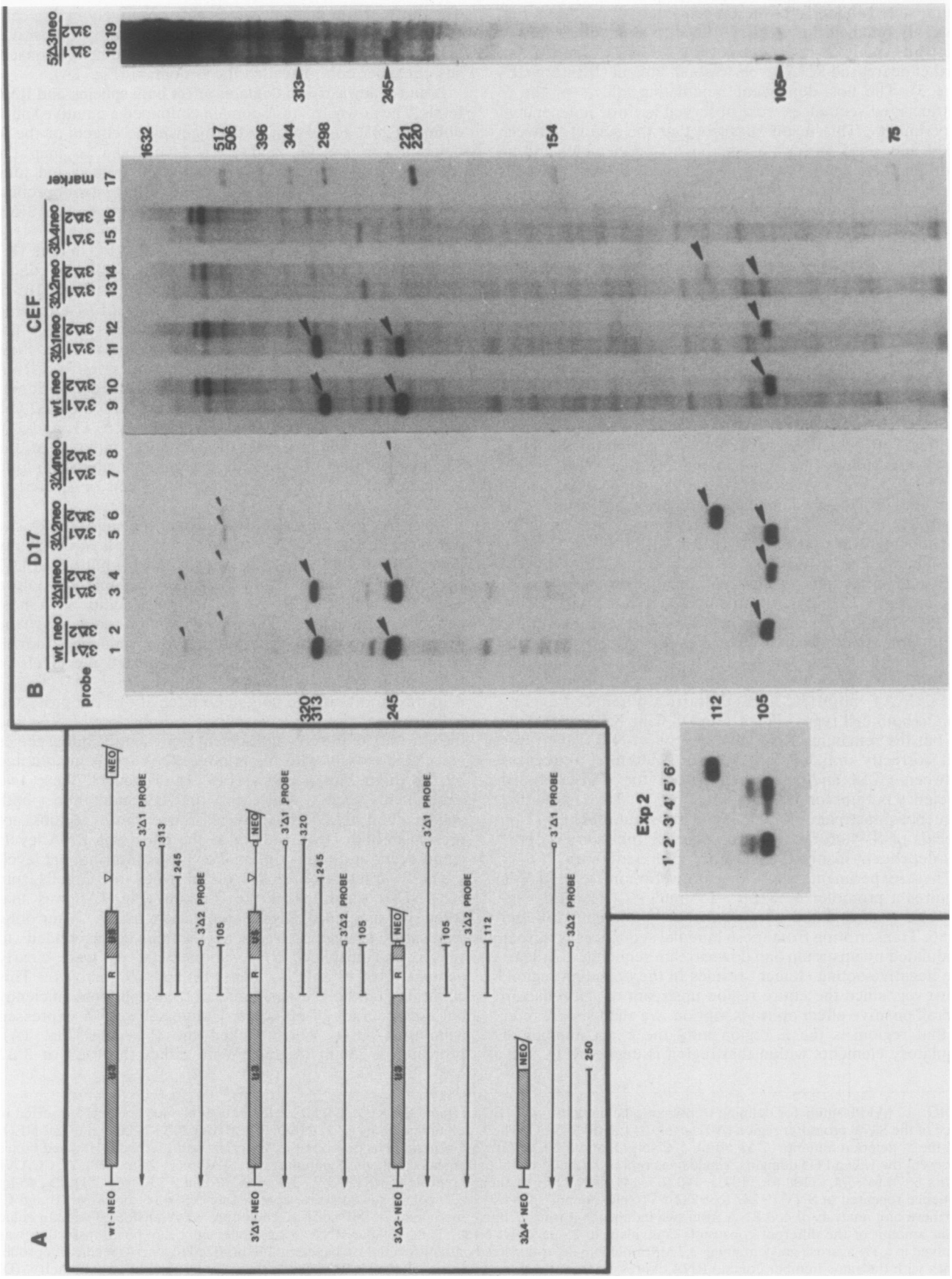
RNA expressed from wt-NEO and 3'Δ1-NEO in both D17 cells and CEF protected a 313- to 320-nucleotide (nt) and a 245-nt fragment with the 3'Δ1 probe and a single 105-nt fragment with the 3'Δ2 probe (Fig. 2B, lanes 1 to 4 and 9 to 12). (Extraneous bands in the CEF samples which do not vary in size and intensity are background digestion products due to the low expression in CEF.) In contrast, RNA expressed from 3'Δ2-NEO, which lacked the splice donor, protected a single 105- or 112-nt fragment with the 3'Δ1 and 3'Δ2 probe, respectively (Fig. 2B, lanes 5, 6, 13, and 14). These results indicate that transcription initiates at the beginning of R (+1) in both dog and chicken cells and produces RNAs which are either unspliced or spliced at +245 (Fig. 2A).

Deleting the B region downstream of the splice donor site reduced the proportion of spliced to unspliced RNA by about 50% in CEF (Fig. 2B, lanes 9 and 11) but not in D17 cells (Fig. 2B, lanes 1 and 3), suggesting that the intron sequences influence RNA splicing or stability in a host cell-dependent manner. This effect might be analogous to the previous observation that altering the sequences downstream of the splice donor in SNV affected the levels of subgenomic spliced RNA (29).

More important, the combined intensity of the protected fragments representing the spliced and unspliced RNAs and the intensity of the 105-nt fragment representing both species correlated closely with the relative RNA levels quantitated by Northern blot analysis (Fig. 1). Together, these two experiments suggest that the A and B regions affect both splicing and total RNA levels. Since these regions are present in both DNA and RNA, the effects on RNA levels could occur at a transcriptional or posttranscriptional level.

The 5' structure of the RNA transcribed in D17 cells from 5'Δ3-NEO, which lacked the E region (Fig. 1A), was analyzed by a similar RNase protection assay. After long exposure, protected fragments which corresponded to spliced and unspliced RNAs initiated at +1 were clearly demonstrable (Fig. 2B, lanes 18 and 19, arrows). This confirms that the E domain affects transcriptional efficiency but not accuracy of initiation. Conversely, RNA expressed from 3'Δ4-NEO, which lacked the P region (Fig. 1A), protected a 250-nt fragment with either the 3'Δ1 or 3'Δ2

FIG. 1. (A) Plasmids for defining transcriptional domains. wt-NEO contains the REV-T LTR region between −406 and +455 inserted in place of the SV40 promoter region (*Nde*I-*Hin*dIII) in pSV2-NEO. The 3' deletion mutants (3'Δ1-NEO, 3'Δ2-NEO, 3'Δ3-NEO, and 3'Δ4-NEO) and the 5' deletion mutants (5'Δ1-NEO, 5'Δ2-NEO, and 5'Δ3-NEO) were generated as described in Materials and Methods. Hatched boxes represent the U3 and U5 domains; single lines represent the 3' and 5' untranslated regions. Symbols: □, TATA box (−24 to −30); △, CCAAT motifs (−70 to −74, −100 to −104, −140 to −144); ▶, 22-bp sequence repeated in REV-T (−211 to −232 and −230 to −251); ▷, 46-bp sequence repeated in SNV (−287 to −332); ▽, splice donor site (+245); ○, consensus enhancer core sequence (+406 to +413). (B and C) Northern blot analysis of *neo* RNA from two independent transfection experiments in D17 cells. Each culture was transfected with an equal molar amount of the different constructs equivalent to 25 μg of wt-NEO. Total cellular RNA (3 μg) collected 1 day posttransfection was resolved in a 1% agarose gel containing 2.2 M formaldehyde, transferred onto nitrocellulose paper, and hybridized to a *neo*-specific riboprobe. Lanes with the same number contain RNA expressed from the same construct. Relative RNA levels were determined by densitometry. (D) Northern blot analysis of *neo* RNA from transfected CEF. A 25-μg amount of RNA per CEF culture was analyzed.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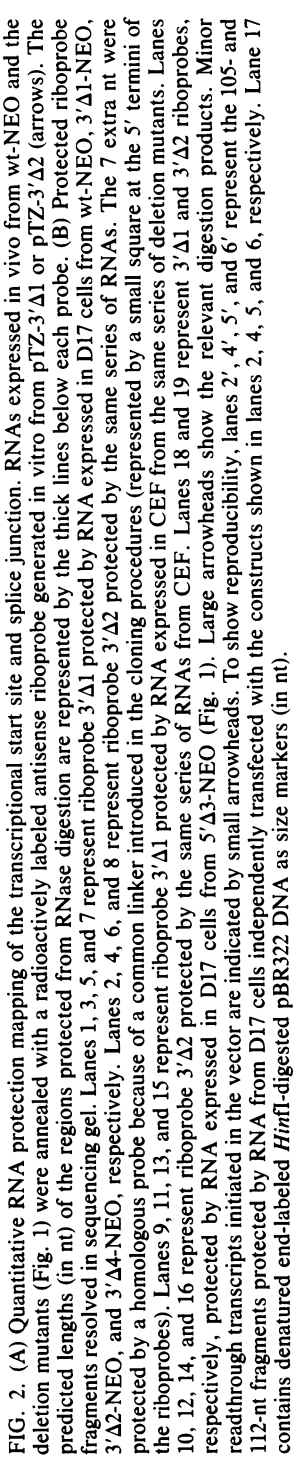

FIG. 2. (A) Quantitative RNA protection mapping of the transcriptional start site and splice junction. RNAs expressed in vivo from wt-NEO and the deletion mutants (Fig. 1) were annealed with a radioactively labeled antisense riboprobe generated in vitro from pTZ-3′Δ1 or pTZ-3′Δ2 (arrows). The predicted lengths (in nt) of the regions protected from RNase digestion are represented by the thick lines below each probe. (B) Protected riboprobe fragments resolved in sequencing gel. Lanes 1, 3, 5, and 7 represent riboprobe 3′Δ1 protected by RNA expressed in D17 cells from wt-NEO, 3′Δ1-NEO, 3′Δ2-NEO, and 3′Δ4-NEO, respectively. Lanes 2, 4, 6, and 8 represent riboprobe 3′Δ2 protected by the same series of RNAs. The 7 extra nt were protected by a homologous probe because of a common linker introduced in the cloning procedures (represented by a small square at the 5′ termini of the riboprobes). Lanes 9, 11, 13, and 15 represent riboprobe 3′Δ1 protected by RNA expressed in CEF from the same series of deletion mutants. Lanes 10, 12, 14, and 16 represent riboprobe 3′Δ2 protected by the same series of RNAs from CEF. Lanes 18 and 19 represent 3′Δ1 and 3′Δ2 riboprobes, respectively, protected by RNA expressed in D17 cells from 5′Δ3-NEO (Fig. 1). Large arrowheads show the relevant digestion products. Minor readthrough transcripts initiated in the vector are indicated by small arrowheads. To show reproducibility, lanes 2′, 4′, 5′, and 6′ represent the 105- and 112-nt fragments protected by RNA from D17 cells independently transfected with the constructs shown in lanes 2, 4, 5, and 6, respectively. Lane 17 contains denatured end-labeled *Hin*fI-digested pBR322 DNA as size markers (in nt).

probe (Fig. 2B, lanes 7 and 8, small arrow). Since this fragment spanned the entire remaining U3 region, the RNA produced from 3′Δ4-NEO was a readthrough transcript initiated in upstream vector sequences and not in the U3 region. Similar aberrant readthrough RNA was produced from all the constructs in D17 cells (Fig. 2B, lanes 1 to 6, small arrows). These results fail to confirm a previous in vitro transcription study which concluded that a second promoter resided in the U3 region of CSV (32), even though CSV and REV-T have extensive sequence homology in the LTR.

Transcription from REV-T in vivo requires *trans*-acting cellular factors which recognize specific viral sequences. To elucidate the mechanism by which the various domains affect transcription, we tested whether cellular factors were involved by using an in vivo competition assay (35) (see Materials and Methods). A constant amount of a reporter gene driven by the REV-T LTR (wt-CAT, Fig. 3A) was cotransfected into duplicate cultures of D17 cells with a 10-fold molar excess of a mock competitor plasmid (pTZ) which lacked viral sequence to serve as a standard (100%) (Fig. 3B, lanes 1 and 2). When wt-CAT was cotransfected with a competitor which contained the same LTR and flanking regions (pTZ-wt, Fig. 3A), *cat* RNA was reduced to 8 to 10% of the standard level (Fig. 3B, lanes 3 and 4). We have ruled out differential DNA uptake as a possible cause for the competition effects, since equivalent amounts of plasmid DNA were recovered from all the transfectants (data not shown). This suggests that the LTR and flanking sequences compete for cellular factors essential for transcription from REV-T.

Interestingly, competitor pTZ-3′Δ1, which lacked the positive-acting downstream B domain (Fig. 1), competed less effectively than pTZ-wt, reducing *cat* RNA to only 41 to 43% (Fig. 3B, lanes 5 and 6). Competitor pTZ-3′Δ2, which lacked both the positive B domain and the negative A domain, competed almost as effectively as pTZ-wt (Fig. 3B, lanes 7 and 8). Similar results were obtained with wt-NEO as a reporter gene (data not shown). Therefore, the positive and negative effects of the downstream domains on transcription and factor competition are coordinated. This suggests that the downstream domains modulate transcription by interacting with positive and negative cellular factors.

To delineate the cellular factors which interact with the U3 domains, a competitor carrying either the promoter or enhancer region was used in competition against wt-CAT (Fig. 3C). The promoter region (pTZ-Pr, Fig. 3A) reduced *cat* RNA to about 38% (Fig. 3C, lane 3). The enhancer region (pTZ-3′Δ4) also reduced *cat* RNA to about 33 to 47% (Fig. 3B, lanes 9 and 10; Fig. 3C, lane 4). This indicates that cellular factors which recognize the promoter and enhancer regions are required for transcription from REV-T. Furthermore, the entire U3-R region clearly competed more effectively than the individual promoter or enhancer region (Fig. 3C, lane 2). This additive effect has also been observed in competition experiments with the promoter and enhancer elements of SV40 (35, 47).

To investigate whether the downstream domains competed for factors utilized by the U3 domains, we tested the A and B domains against the reporter gene 3′Δ2-CAT, which was driven only by the U3-R region (Fig. 3A). As shown in Fig. 3D, neither the A nor the B domain competed effectively with 3′Δ2-CAT (Fig. 3D, lanes 3 and 4). In contrast, transcription from this reporter gene was strongly competed with by the U3-R region (pTZ-3′Δ2, Fig. 3D, lane 5), as well as by the individual promoter (pTZ-Pr, Fig. 3D, lane 6) or

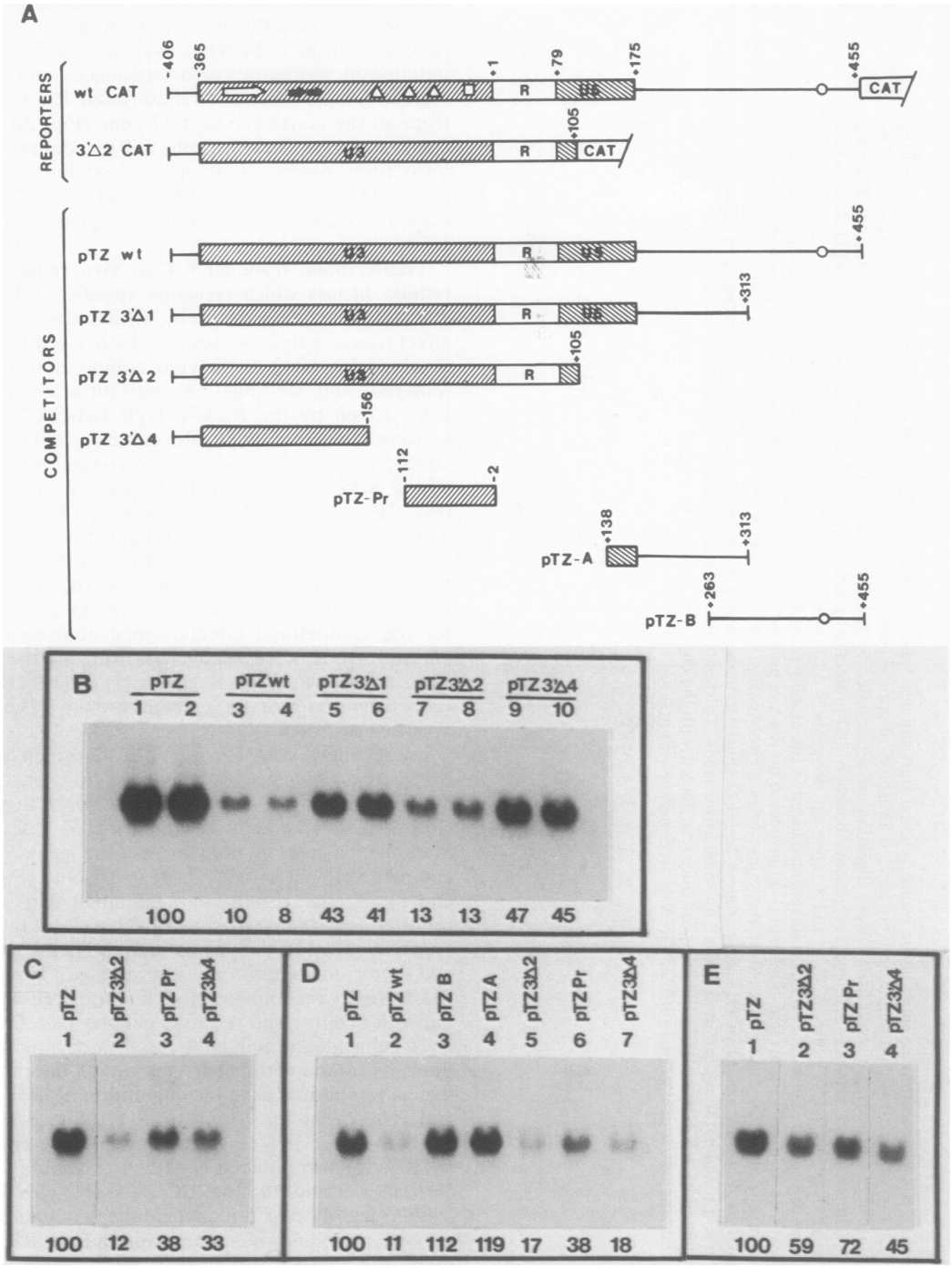

FIG. 3. (A) In vivo competition for cellular factors essential for transcription from REV-T. Constant amounts (1 μg) of a reporter gene (wt-CAT or 3′Δ2-CAT) were cotransfected into D17 or CEF cultures with a 10-fold molar excess of competitors, consisting of different REV-T sequences in a common pTZ vector. Expression from the reporter genes was quantitated by Northern blot analysis with a *cat*-specific riboprobe. pTZ-wt, pTZ-3′Δ1, pTZ-3′Δ2, and pTZ-3′Δ4 contain the same viral sequences as in the expression clones with the same prefixes shown in Fig. 1. pTZ-Pr contains the promoter region (−112 to −2), including the TATA box and two CCAAT motifs. pTZ-A contains the A domain between a *Bam*HI site (+138) and +313. pTZ-B contains the region between a *Kpn*I site (+263) and +455, including the B domain plus 50 bp overlapping with the A domain. Symbols are as explained in the legend to Fig. 1. (B) *cat*-specific RNA expressed from wt-CAT in duplicate D17 cell cultures cotransfected with a mock competitor (lanes 1 and 2), pTZ-wt (lanes 3 and 4), pTZ-3′Δ1 (lanes 5 and 6), pTZ-3′Δ2 (lanes 7 and 8), and pTZ-3′Δ4 (lanes 9 and 10). (C) *cat*-specific RNA expressed from wt-CAT in D-17 cells cotransfected with pTZ, pTZ-3′Δ2, pTZ-Pr, and pTZ-3′Δ4 (lanes 1, 2, 3, and 4, respectively). (D) *cat*-specific RNA expressed from 3′Δ2-CAT in D17 cells cotransfected with pTZ, pTZ-wt, pTZ-B, pTZ-A, pTZ-3′Δ2, pTZ-Pr, and pTZ-3′Δ4 (lanes 1 to 7, respectively). (E) *cat*-specific RNA expressed from 3′Δ2-CAT in CEF cultures cotransfected with pTZ, pTZ-3′Δ2, pTZ-Pr, and pTZ-3′Δ4 (lanes 1 to 4, respectively). All competition experiments were reproduced at least three times. Numbers below lanes indicate the percent RNA level compared with the standard transfection with pTZ as a competitor.

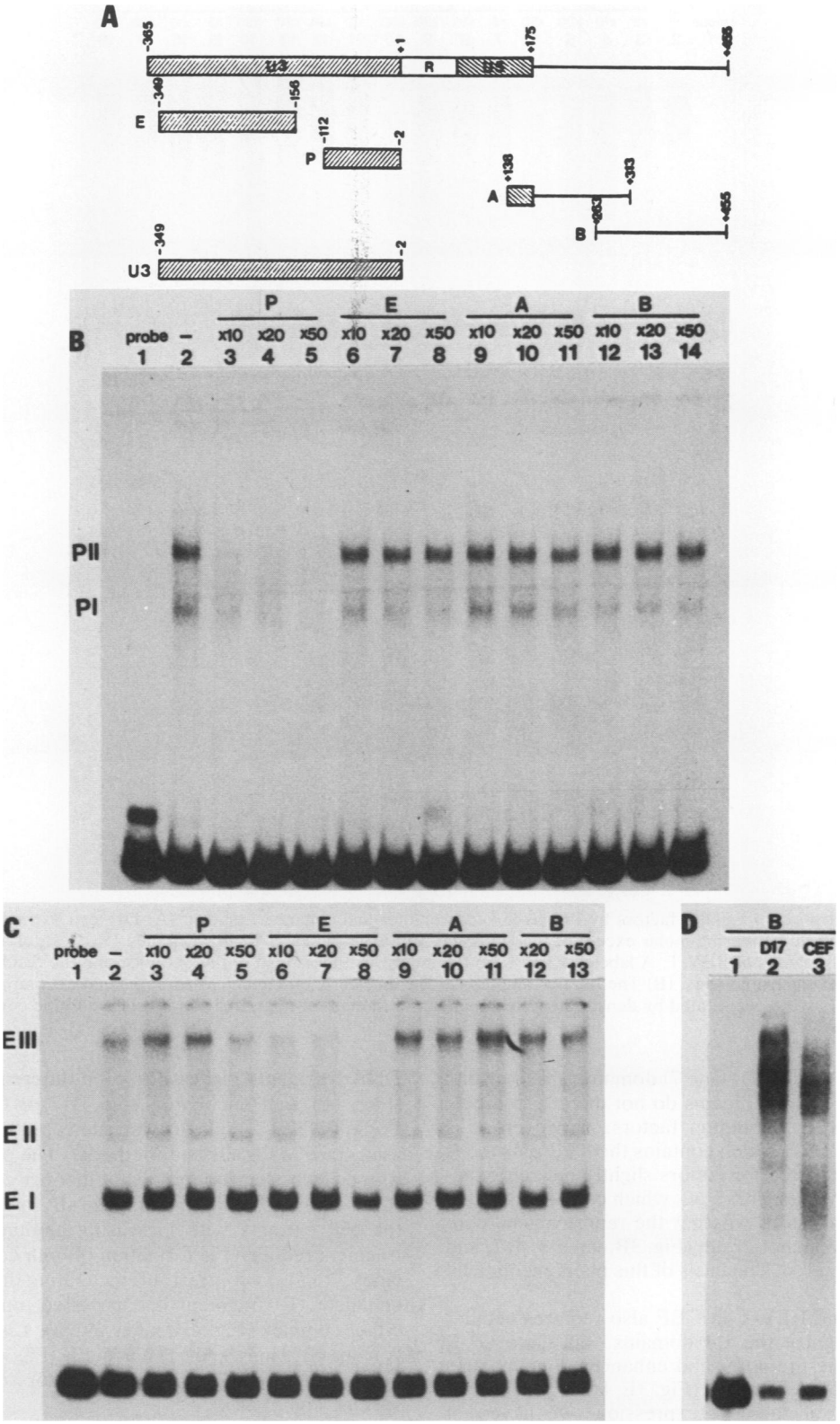

FIG. 4. Binding of dog and chicken cell factors to REV-T sequences in vitro. (A) The E, P, A, B, and U3 fragments purified by gel electrophoresis were used to detect cellular factors binding to the enhancer, promoter, A, B, and U3 domains, respectively. (B) Titration of promoter-specific factors in D17 cells. D17 cell extract (1 μg) was preincubated for 5 min with 1 μg of poly(dI:dC) and a 10-, 20-, or 50-fold molar excess of competitor representing the P (lanes 3, 4, and 5), E (lanes 6, 7, and 8), A (lanes 9, 10, and 11), or B (lanes 12, 13, and 14) region of REV-T. A labeled P fragment was then added to these extracts and further incubated for 30 min. Formation of complexes was analyzed by electrophoresis in a nondenaturing gel. (C) Titration of REV-T enhancer-specific factors in D17 cells. The same competitors as in panel B were used to titrate a labeled E fragment with D17 cell extracts. (D) Formation of complexes with the B domain in D17 and CEF cell extracts. D17 (lane 2) or CEF (lane 3) cell extract preincubated with poly(dI:dC) was incubated with a labeled B fragment for 30 min and analyzed as described for panel B. Lane 1 in each panel contained the same amount of probe incubated without cell extract.

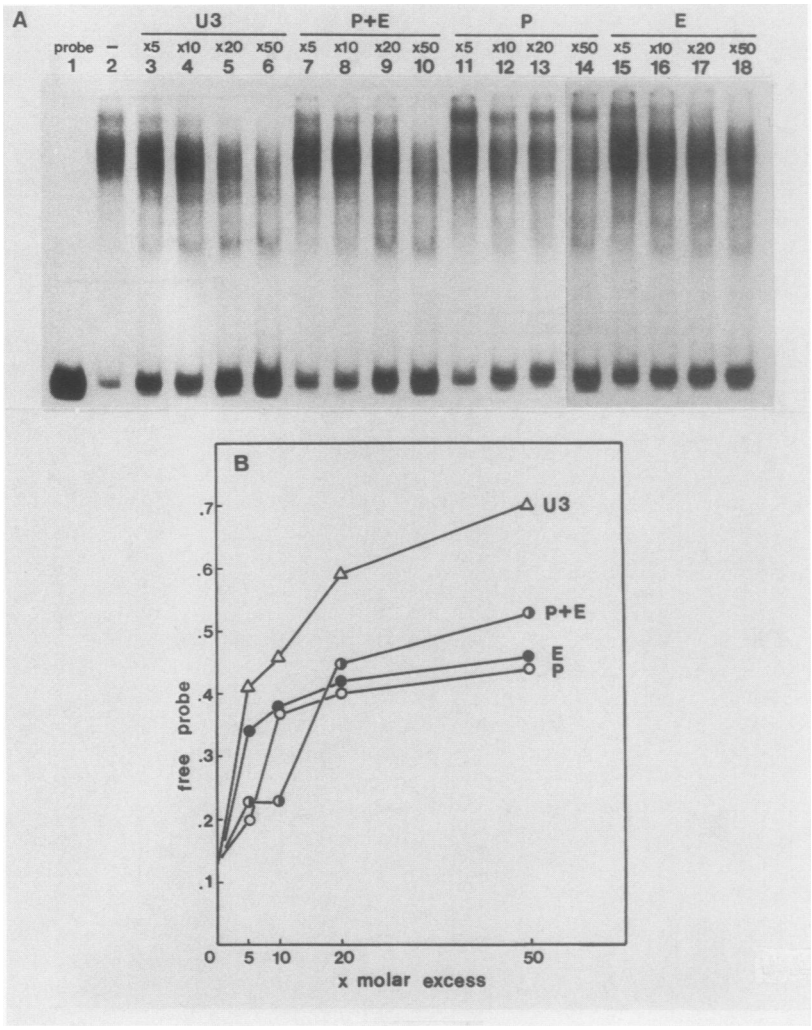

FIG. 5. Efficient titration of cellular factors by the *cis*-linked promoter and enhancer regions. (A) D17 cell extracts were preincubated without (lane 2) or with an increasing molar excess of the U3 (lanes 3 to 6), P (lanes 11 to 14), E (lanes 15 to 18), or a mixture of both the P and E (lanes 7 to 10) regions of REV-T. A labeled REV-T U3 fragment was then added to the extracts, and the resulting complexes were examined by native gel electrophoresis. (B) The relative amounts of the labeled U3 fragment which remained uncomplexed in the presence of different competitors were quantitated by densitometric comparison of the fastest-migrating bands relative to the control sample (lane 1).

enhancer (pTZ-3′Δ4, Fig. 3D, lane 7) domain. These results indicate that the A and B domains do not directly compete with the U3 region for common factors. However, it is noteworthy that pTZ-wt, which contains the LTR plus the A and B domains, competed for factors slightly but reproducibly more effectively than pTZ-3′Δ2, which contains only the U3-R region, regardless of whether the reporter gene contained the A and B domains or not (Fig. 3B, lanes 3, 4, 7, and 8; Fig. 3D, lanes 2 and 5). The basis of this phenomenon will be discussed later.

Transcription from REV-T in CEF also requires cellular factors which recognize the U3 domains, since the U3-R region as well as the promoter and enhancer domains also competed with 3′Δ2-CAT in CEF (Fig. 3E, lanes 2, 3, and 4). However, because of the low expression, we have not quantitated the in vivo competition effects by the downstream domains in chicken cells. As will be shown later, the B domain also interacts with chicken cell factors in vitro.

Promoter, enhancer, and B domains bind distinct classes of dog and chicken cell factors in vitro. To directly demonstrate the interaction with cellular factors, radioactively labeled DNA fragments representing the different REV-T domains (Fig. 4A) were incubated with D17 or CEF cell extracts. Protein-DNA complexes were detected by a gel retardation assay (see Materials and Methods). The promoter (P) region formed two electrophoretically distinct complexes (P-I and P-II) with D17 cell extracts (Fig. 4B, lane 2). Preincubating the cell extracts with increasing amounts of unlabeled P fragment reduced the formation of both complexes (Fig. 4B, lanes 3 to 5). In contrast, preincubating the extracts with the enhancer (E) fragment had no effect on the formation of either complex (Fig. 4B, lanes 6 to 8). Likewise, neither the A (Fig. 4B, lanes 9 to 11) nor the B (Fig. 4B, lanes 12 to 14) domain titrated out the cellular factors in these promoter-specific complexes.

Similarly, the enhancer region formed three complexes (E-I, E-II, and E-III) with D17 cell extracts (Fig. 4C, lane 2). All three complexes were titratable by preincubation with excess unlabeled E fragment (Fig. 4C, lanes 6 to 8) but not with the P fragment (Fig. 4C, lanes 3 to 5) or with the two downstream domains (Fig. 4C, lanes 9 to 13). Identical

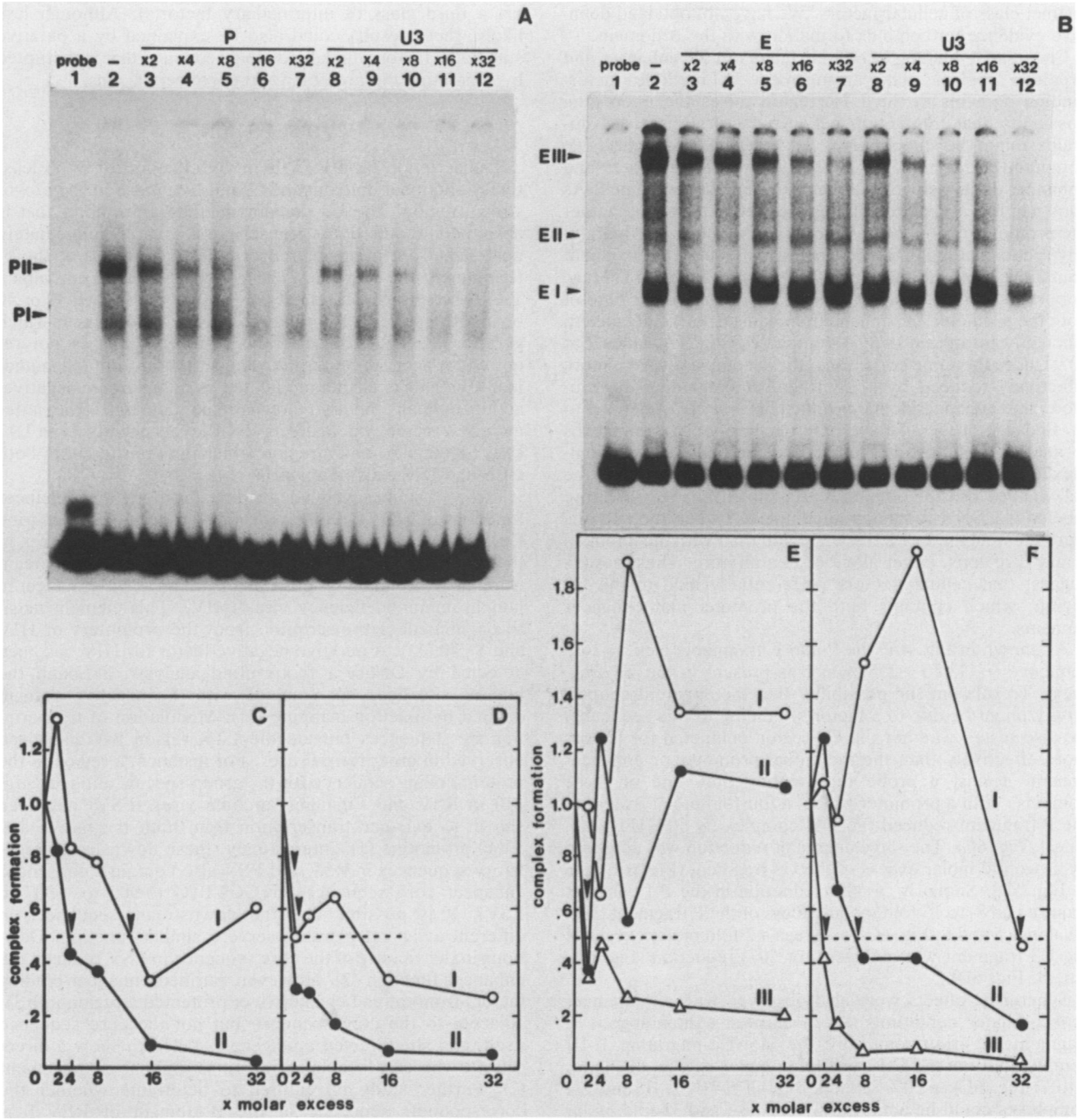

FIG. 6. Cooperative binding of cellular factors to the enhancer and promoter domains. (A) D17 cell extracts were preincubated without (lane 2) or with an increasing molar excess of the P (lanes 3 to 7) or U3 (lanes 8 to 12) region of REV-T. A labeled REV-T P fragment was then added to the extracts, and the resulting complexes were analyzed by electrophoresis. (B) D17 cell extracts were preincubated without (lane 2) or with the E (lanes 3 to 7) or U3 (lanes 8 to 12) region of REV-T. A labeled REV-T E fragment was then added to the extracts, and the resulting complexes were similarly analyzed. Lane 1 in both panels contained the same amount of probe incubated without cell extract. (C) Titration of the P-I and P-II complexes by the homologous P region revealed by densitometric quantitation of the corresponding bands in lanes 2 to 7 in panel A. (D) Titration of the P-I and P-II complexes by the U3 region (lanes 8 to 12 in panel A). (E) Titration of the E-I, E-II, and E-III complexes by the homologous E region as shown in lanes 3 to 7 in panel B. (F) Titration of the E-I, E-II, and E-III complexes by the U3 region (lanes 8 to 12 in panel B). Arrows indicate the dosage of competitors needed to reduce complex formation by 50%.

results were obtained with CEF extracts, except that the E region formed only two complexes (data not shown).

Even though the B domain failed to compete for factors with the P or E domain, a labeled B fragment formed a series of heterogeneous complexes with both D17 and CEF extracts (Fig. 4D, lanes 2 and 3). These complexes were titratable only by a homologous B fragment (data not shown). These results are consistent with the lack of competition in vivo between the downstream and U3 domains (Fig. 3) and suggest that the B domain interacts with a

distinct class of cellular factors. We have not obtained definitive evidence for complex formation with the A domain.

Cooperative binding of cellular factors to the enhancer and promoter regions. The arrangement of multiple factor-binding domains in the LTR region raises the interesting possibility that interaction between factors and these domains might be cooperative. To test this possibility, we compared factor binding by the individual promoter and enhancer domains with that by both domains combined. As shown in Fig. 5A, preincubating D17 cell extracts with either the promoter (P) or the enhancer (E) fragment only marginally reduced complex formation with a labeled U3 fragment containing both domains (Fig. 5A, lanes 11 to 18). More important, competitors consisting of an equal molar ratio of both the promoter and enhancer domains (P+E) still failed to efficiently titrate out the U3 complexes (Fig. 5A, lanes 7 to 10). Under the same conditions, these complexes were more effectively reduced by a U3 fragment containing the promoter and enhancer domains linked in *cis* (Fig. 5A, lanes 3 to 6). Because of the size and heterogeneity of the complexes formed by the entire U3 region, we quantitated the uncomplexed DNA fragment (prominent bands at the bottom of the gel) instead of the complexes. As shown in Fig. 5B, more labeled U3 DNA remained uncomplexed when the extracts were preincubated with the U3 region than with the unlinked P and E regions, either alone or in a mixture. These results suggest that cellular factors preferentially bind to the U3 region, which contains both the promoter and enhancer domains.

As shown in Fig. 4A, the P and E fragments lack a 42-bp sequence (−113 to −155) which is present in the U3 fragment. To rule out the possibility that the apparent cooperativity might be due to a factor(s) binding to this sequence, we also tested whether the U3 region competed for factors more effectively than the individual promoter or enhancer domain against a probe representing only one of these domains. With a promoter probe, a fourfold molar excess of the P fragment reduced the P-II complex by 50% (Fig. 6A, lane 4; Fig. 6C). The same degree of reduction was achieved by a twofold molar excess of the U3 fragment (Fig. 6A, lane 8; Fig. 6D). Similarly, a 50% reduction in the P-I complex required an 8- to 16-fold molar excess of the P fragment (Fig. 6A, lanes 5 and 6; Fig. 6C), whereas a 2-fold molar excess of the U3 fragment was sufficient for 50% reduction (Fig. 6A, lane 8; Fig. 6D).

Synergistic effects were also observed with an enhancer probe. Under conditions which allowed a homologous E fragment to titrate out only the slowest-migrating E-III complex but not the E-I and E-II complexes (Fig. 6B, lanes 3 to 7; Fig. 6E), a 50% reduction in all E-III, E-II, and E-I complexes could be achieved by a 2-, 8-, and 32-fold molar excess of the U3 region, respectively (Fig. 6B, lanes 8 to 12; Fig. 6F).

These results suggest that binding of factors to the promoter domain is facilitated by the *cis*-linked enhancer domain and that binding of factors to the enhancer domain is facilitated by the promoter domain. Since the promoter- and enhancer-binding factors only recognize their cognate domains (Fig. 4B and C), the enhanced binding cannot be due to interaction of the same factors with both domains. Moreover, synergy in factor binding is more than simply additive, since it requires that the target domains be physically joined in *cis*. These results strongly suggest that binding of the promoter- and enhancer-specific factors to their target sequences might be facilitated by cooperative protein-protein interactions either between these two classes of factors or via a third class of intermediary factor(s). Although less likely, these results could also be explained by a putative stabilizing factor which binds to a sequence that is disrupted by separating the promoter and enhancer regions.

DISCUSSION

Transcription from REV-T in vivo is affected by at least three functional domains in U3 and two more in the downstream region. The U3 domains include a promoter that is absolutely required for transcription located immediately upstream from a single transcriptional start site at the 5′ terminus of the R region. The promoter domain contains a TATA box and three sets of CCAAT motifs spaced 30 or 40 bp apart. Upstream from the promoter is an enhancer which greatly augments transcriptional efficiency but is not required for accurate initiation. Transcription from these minimal elements is further modulated by an upstream negative-acting domain and two downstream domains. The latter include a region which affects RNA levels negatively in D17 cells (domain A) and a region which acts positively in both CEF and D17 cells (domain B).

Previous studies which defined the promoter and enhancer functions of Rous sarcoma virus (RSV) and Moloney murine leukemia virus did not reveal negative-acting sequences in the U3 region (7, 17, 24). However, a negative-acting domain is found in an analogous position upstream of the enhancer in human immunodeficiency virus (HIV). This element negatively influences transcription from the promoters of HIV and SV40 (33). A putative negative factor for HIV has been detected by DNase I footprinting analysis, although the binding site does not coincide with the negative domain defined by deletion mapping (51). Modulation of transcription by sequences outside the LTR region has also been observed in other retroviruses. For instance, a region in the protein-coding sequence for the group-specific antigen (*gag*) p10 in RSV and Fujinami sarcoma virus (FSV) has been shown to enhance transcription from both the native and SV40 promoters (1). Interestingly, these downstream regulatory sequences in RSV and FSV also contain a consensus enhancer core sequence, GTGGTTTG (+900 to +907 in FSV). It is possible that the downstream sequences in different avian retroviruses serve a similar function. Deletions in the vicinity of the core sequence in FSV reduced the enhancer function (2). However, purified enhancer-binding factors from rat and chicken liver protected a region in FSV adjacent to the core sequence but not the core sequence itself, and site-directed mutagenesis failed to show a direct role for the core sequence in transcriptional enhancement (2). Further study is required to determine whether the corresponding sequence in the B domain of REV-T is involved in transcriptional activation.

The present data strongly suggest that the transcriptional domains in REV-T are targets for multiple classes of cellular factors which can be competed for by the cognate DNA sequences in vivo and which form specific complexes with these sequences in vitro. The modulating effects by DNA sequences on transcription and factor competition are coordinated. Thus, a competitor containing a positive-acting transcriptional domain competes for expression more effectively than a competitor containing a negative-acting domain (Fig. 3). These results suggest that the DNA elements in REV-T modulate transcription by interacting with positive- and negative-acting cellular factors. Several potential targets for such interaction are found in the U3 region. For example, the CCAAT motifs might be targets for a member(s) of a

family of transcriptional factors which recognize similar sequences in a variety of viral and cellular genes (5, 6, 8, 18, 21, 22, 31, 50). The regular spacing between the CCAAT motifs in REV-T implies that these elements lie on the same face of the DNA helix, a feature conducive for interaction between multiple bound factors. We have also obtained preliminary footprint evidence for multiple factor-binding sites in the E region (unpublished results).

Functional cooperation between promoter and enhancer elements mediated by cellular factors has been suggested for several eucaryotic genes. For instance, the enhancer for an immunoglobulin gene encoding a kappa light chain augments transcription from the homologous promoter more efficiently than from a heterologous promoter (13). In that case, synergistic transcriptional activation might be due to interaction with the same factor, NF-kB, by a conserved octamer motif found in both the promoter and enhancer domains (13, 26). In REV-T, the promoter and enhancer do not have any obvious common sequences. Reciprocal competition experiments have ruled out a common factor which recognizes both the promoter and enhancer domains (Fig. 4B and C), yet the *cis*-linked promoter and enhancer regions compete for factors against the U3 region more effectively than a mixture of the individual domains (Fig. 5). Furthermore, the promoter or enhancer region competes against a homologous fragment more effectively when the competitor is linked in *cis* to an enhancer or promoter, respectively (Fig. 6). These results suggest a cooperative interaction between the promoter- and enhancer-binding factors, either directly or through a third class of factors. A similar mechanism has been proposed to account for the effective competition for in vivo expression by the *cis*-linked promoter and enhancer elements of SV40 (47), as well as the cooperativity between the glucocorticoid receptor-binding element and a CACCC motif upon induction by dexamethasone (36).

From a broader perspective, even though the sequences between −258 and −2 in the REV-T LTR are fully capable of transcription in vivo, transcriptional efficiency is modulated by sequences hundreds of base pairs upstream and downstream of these minimal sequences. It is possible that the distal sequences modulate transcription by interacting with accessory factors which strengthen or weaken the overall stability of the protein-DNA complex in the LTR region. Consistent with this hypothesis, the LTR plus the downstream domains reproducibly competed for expression slightly more effectively than the LTR alone, even against reporter genes without the downstream domains (Fig. 3D, lanes 2 and 5). Such interplay between positive and negative factors could explain the dependency between the positive- and negative-acting elements in a class I major histocompatibility gene (9). In that case, even though the two elements interact with distinct classes of factors, the negative element downregulates transcription only in the presence of the positive element (9).

In summary, the present study provides basic knowledge about the transcriptional elements in REV-T and sheds light on the functional interactions between these elements and host cell factors.

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