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A Negative Regulatory Element with Properties Similar to Those of Enhancers Is Contained within an *Alu* Sequence

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A negative regulatory element has been found within a member of the African green monkey *Alu* family of interspersed repeated sequences. This "reducer" element decreased transcription in both directions from a cellular simian virus 40-like bidirectional promoter independently of both orientation and position. The reducer was not promoter specific since it also decreased expression from the simian virus 40 early and human β-globin promoters. In addition, the reducer decreased transcription from a polymerase III promoter. The reducer was contained in 38 base pairs of an *Alu* family member and interacted specifically with a monkey cell nuclear protein.

At the level of transcription, both general and sequence-specific mechanisms for repression of gene expression complement the methods of positive control. An example of a general mechanism that represses transcription is formation of a condensed chromatin structure as modulated by histone H1 (42). Sequence-specific repression is exemplified by the effect of the adenovirus E1a gene product on papovaviral enhancers (4, 41). Negative regulation by sequences upstream of the promoter has also been observed in several cellular genes. A negative regulatory element has been found approximately 220 base pairs (bp) upstream of the mouse p53 gene (3); the 5′ end of the hamster HMG coenzyme A reductase gene also includes negative regulatory sequences whose effect is mediated by cholesterol (28).

Of particular interest are negative regulatory elements that have the enhancerlike properties of orientation and position independence as well as the ability to affect heterologous promoters. The first such sequence to be described was the "silencer" element in yeast cells (5). The silencer sequence eliminates transcription from the *HMR* locus promoter and also represses heterologous yeast promoters. This element functions in either orientation and can act at least 2.5 kilobases (kb) away (5). Similar control elements have been found upstream of the murine c-*myc* promoter (33) and within the human T-cell lymphotrophic virus type III long terminal repeat (35). Another silencerlike element has been found between 2 and 4 kb upstream of the rat insulin 1 gene promoter (20). This sequence, which represses transcription 5- to 15-fold, is located within a member of a long interspersed repetitive family. A cell-type-specific negative regulatory element, 3.5 kb from the rat α-fetoprotein promoter, also has been shown to repress heterologous promoters in an orientation-independent manner (24).

We have been studying simian virus 40 (SV40) homologous sequences in the African green monkey genome as potential targets for SV40 T-antigen-induced changes. Previously, we described one such cellular SV40-like region (termed 7.02) that is a bidirectional promoter (38). Although we have not yet identified the product(s) encoded by the adjacent gene(s), we have found that SV40 infection of cells stimulates these genes at the mRNA level (Thurston and Saffer, manuscript in preparation) and have begun to identify the associated regulatory elements. This report describes the properties of a sequence located in *cis* to 7.02 which acts as a negative regulatory element. The element reduces, but does not eliminate, expression from 7.02 and heterologous promoters, including a polymerase III promoter, in a manner independent of orientation and position. The negative control element, named a "reducer" to distinguish it from the yeast silencer which completely eliminates transcription, is fully contained within a member of the *Alu* family of interspersed repeated sequences.

MATERIALS AND METHODS

The expression vector used in these studies, pAngpt.LR (Fig. 1), has been described previously (38). It contains the *Escherichia coli* xanthine-guanine-phosphoribosyltransferase gene (*gpt*) and sequences required for processing of the message and propagation of the plasmid in the bacterial host. This vector is a derivative of pSV0gpt (25), which includes an SV40 polyadenylation signal immediately upstream of the promoter insertion site to decrease readthrough transcripts. A unique *Hin*dIII restriction site at the 5′ end of the *gpt* gene has been used for insertion of various promoter elements. Monkey genomic segments 7.02, 7.12, 7.11, and 7.22 (see Fig. 1 and 2) were cloned by digestion with the appropriate restriction enzymes, filling in the overlapping ends, and addition of a 12-bp *Hin*dIII linker (Collaborative Research). The 5′ end of the 7.22 fragment is at an *Alu*I site 56 bp to the left of the *Acc*I site shown in Fig. 1. A segment containing the reducer was made in an analogous manner, using the *Ava*I to *Acc*I restriction fragment shown at the left in Fig. 1. This segment was inserted into a *Hin*dIII partial digest of the expression vector containing sequence 7.12, and clones with both orientations of the reducer upstream of the promoter were chosen (clones 7.11H and 7.11HR). Clone 7.12B was prepared with a 900-bp *Hin*dIII fragment from the coding sequence of the mouse glycerolphosphate dehydrogenase gene (gift of L. Kozak); this segment is not known to regulate transcription. The human β-globin promoter region, kindly provided by D. Bodine, was modified with *Hin*dIII linkers and inserted into pAngpt.LR. The 907-bp *Hin*dIII reducer fragment from clone 7.11H was inserted into a partial *Hin*dIII digest of the pAngpt.LR/β-globin construct. A clone containing the β-globin promoter and the 56-bp reducer segment was constructed by combining the appropriate regions of the 7.22 and pAngpt.LR/β-globin plasmids. The potential Z-DNA-

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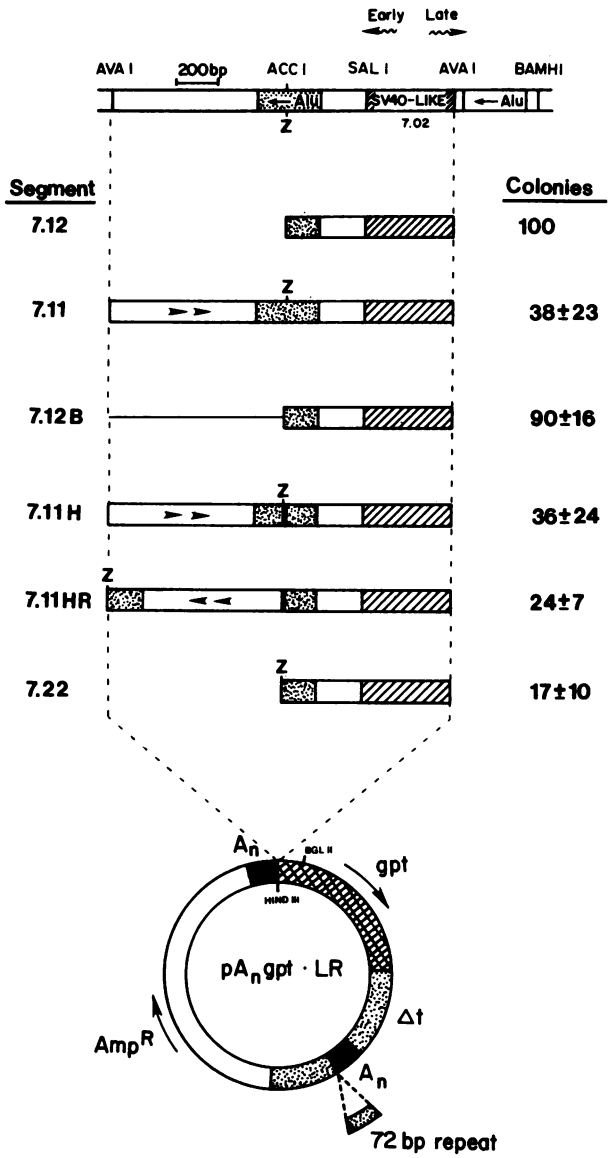

FIG. 1. Clones used to demonstrate reducer activity and transformation assay results. This African green monkey genomic region contains the SV40-like promoter 7.02, indicated by a hatched area. The positions of two *Alu* family members (one stippled and one open) are shown, with the arrows indicating their orientation. The position of a potential Z-DNA segment $(dG\text{-}dT)_9$ is indicated by "Z." Relevant restriction sites are indicated. "Late" and "Early" refer to the direction of transcription analogous to transcription from SV40; i.e., the C-rich strand (32) is the coding strand for late transcription. The cloned segments used in the expression vector are depicted below the map. Arrowheads are shown to clarify the orientation of the *Aval*-*Acc*I restriction fragment. The single line in 7.12B represents a 900-bp fragment of coding sequence from the mouse glycerol-phosphate dehydrogenase gene. The bold lines in clones 7.11H and 7.11HR represent a *Hin*dIII linker insertion. The expression vector used, pAngpt.LR (38), is shown at the bottom. Potential promoter and regulatory sequences are inserted at the unique *Hin*dIII restriction site just upstream of the *E. coli gpt* gene indicated as a cross-hatched area. The stippled and black regions are segments from SV40; A_n represents the SV40 polyadenylation signal, Δt is a fragment that contains the splice site for the small t-antigen gene, and "72-bp repeat" represents the viral enhancer (nucleotides 92 to 272). The unlabeled region contains pBR322 sequences. The results from transformation assays used to verify the S1 mapping results are shown to the right. At 48 h after transfection with the indicated vector constructs, CV-1 cells were replated in selection medium at a density of 10^5 cells per 100-mm plate. The medium was changed regularly for 2 weeks, and surviving colonies were stained with crystal violet. The number of colonies has been normalized to 100 for 7.12, and the standard deviations are shown. The actual number of colonies for 7.12 ranged from 500 to 1,000 per plate in different experiments.

forming sequence was deleted from the plasmids, using "slow" BAL31 (International Biotechnologies, Inc.). Plasmid pSV2Agpt, the gift of T. Kadesch, was modified by inserting the 907-bp reducer fragment with *Eco*RI linkers into the vector *Eco*RI restriction site. Plasmids, amplified in *E. coli* RR1 or HB101, were purified by Triton lysis, with final purification by chromatography on Sepharose 4B (Pharmacia).

Monkey kidney fibroblast cell lines CV-1 and COS1 (12) were maintained in OPTI-MEM (GIBCO Laboratories) supplemented with 2% fetal calf serum and penicillin-streptomycin in an 8% CO_2 atmosphere. Transfection procedures were as described before (36), using calcium phosphate precipitates (13) with glycerol shock (29).

gpt selection assays were carried out as described previously (26) but using an OPTI-MEM-based selection medium. Cells were placed in this selection medium 48 h after transfection, and surviving colonies were counted after an additional 14 days.

RNA for S1 nuclease mapping was isolated 48 h after transfection. Cytoplasmic RNA was prepared (11) in the presence of vanadyl riboside complex and oligo(dT) selected by using Hybond-mAP (Amersham Corp.). Total RNA was prepared with guanidine hydrochloride (16). S1 mapping probes were end labeled at a *Bgl*II restriction site 120 bp into the xanthine-guanine phosphoribosyltransferase sequence, allowing detection of vector-specific transcripts. Double-stranded probes were prepared directly from the expression vectors. Single-stranded probes were prepared from M13mp7 clones of the probe sequence (6). Equal amounts of RNA were used in experiments comparing different constructs. Hybridizations with double-stranded probes were carried out in 80% formamide (2), and those with single-stranded probes were carried out in 0.6 M NaCl–0.06 M sodium citrate–0.1% sodium dodecyl sulfate, pH 7 (6); S1 nuclease (Bethesda Research Laboratories) digestion was at 37°C for 45 to 60 min, using 660 U/ml. Following extraction with phenol and precipitation by ethanol, the nuclease digestion products were analyzed on 8% acrylamide–8 M urea sequencing gels. Quantitation of band intensities on autoradiographs was done with a Bio-Rad model 620 densitometer, using additional exposures not shown when necessary for accuracy.

Gel electrophoresis DNA-binding assays were carried out by the methodology of Singh et al. (39). Binding reactions (20 μl) contained 4,000 dpm of end-labeled probe (0.5 ng), 200 ng of salmon sperm DNA, and 4 μg of CV-1 nuclear extract. Assays contained up to a sevenfold excess of competing plasmids. After binding for 30 min at 22°C, the material was analyzed on a low-ionic-strength 4% acrylamide gel (39).

RESULTS

A distant regulatory element reduces expression from a cellular SV40-like bidirectional promoter. Restriction fragments which contain the cellular SV40-like promoter 7.02 with adjacent sequences of differing lengths were cloned into the expression vector pAngpt.LR (38) (Fig. 1, bottom). In

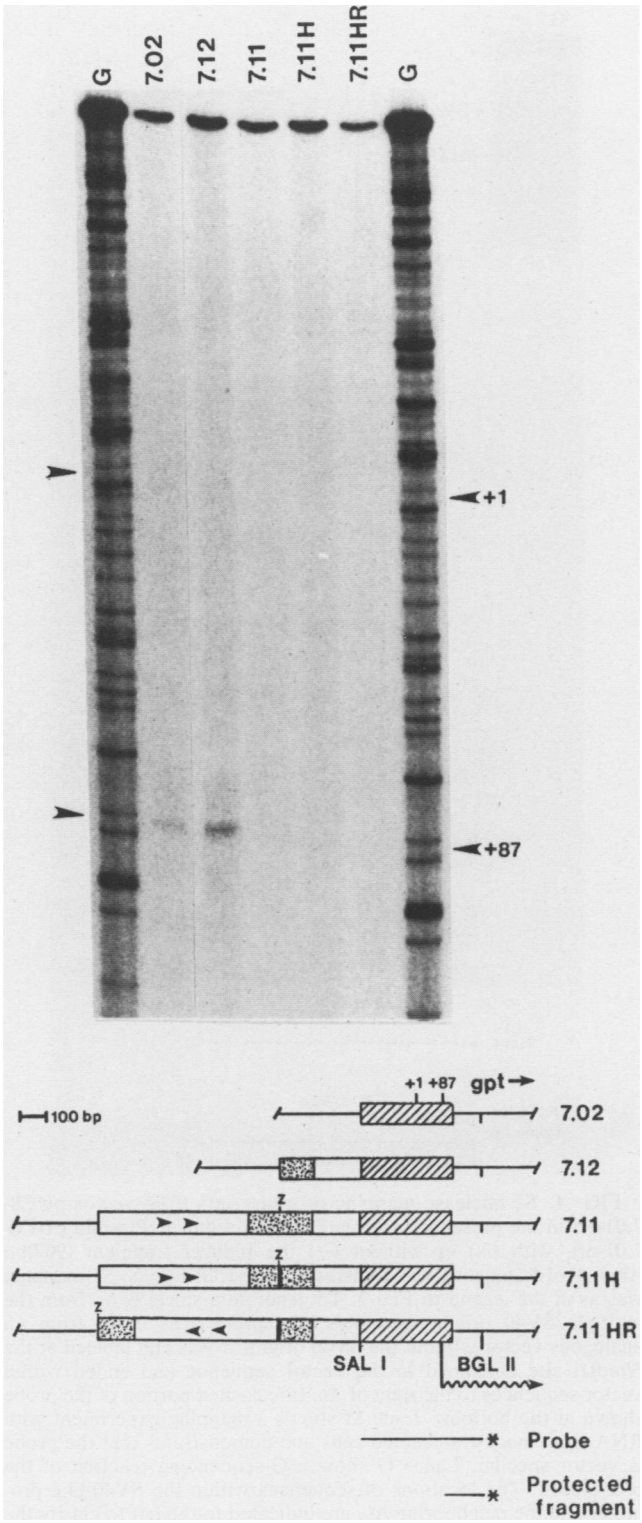

FIG. 2. S1 nuclease mapping of transcripts from the SV40-like promoter in the presence and absence of upstream sequences. Oligo(dT)-selected cytoplasmic RNA was prepared from CV-1 cells 48 h after transfection with vector pAngpt.LR containing the segments indicated at the bottom. Single lines represent vector sequences. Boxed areas are monkey sequences as described in detail in the legend to Fig. 1. The cellular SV40-like promoter (hatched) and a flanking *Alu* sequence (stippled) are indicated. The arrow heads in clones 7.11, 7.11H, and 7.11HR indicate the orientation of the sequences in 7.11 not contained in 7.12. Equal amounts of RNA were hybridized with a vector-specific probe (see bottom) end labeled at a *Bgl*II site 120 bp into the *gpt* sequence and extending to the *Sal*I site at the left of the promoter (hatched region). After digestion with S1 nuclease, the protected DNA fragments were analyzed on a denaturing acrylamide gel. The positions of nucleotides +1 and +87 (numbering as in reference 38) are shown. Lanes G are G-sequencing reactions (21) of the probe used. The bands at the top are full-length probe.

these clones, transcription from the 7.02 promoter in the "late" direction (to the right in Fig. 1 and 2) will result in expression of the bacterial marker gene *gpt*. These plasmids were transfected into the monkey kidney fibroblast cell line CV-1, and expression from the 7.02 late promoter was then assayed by S1 nuclease mapping of transient plasmid-specific transcripts (Fig. 2).

Transcription from 7.02 occurred with a start site at nucleotide +87 (numbering as in reference 38) (Fig. 2). With clone 7.12, the level of transcription was greater than that from clone 7.02, as reported previously (38). However, transcription was not detectable from clone 7.11, which contains an additional 907-bp sequence beginning at a distance of 750 bp from the start site. Since the additional sequences in clone 7.11 not contained in clone 7.12 are upstream of the transcription unit, the resulting difference in transcript levels cannot be due to posttranscriptional events such as messenger processing or stability. This inhibitory sequence in clone 7.11 (Fig. 1 and 2) is named the reducer. The lanes labeled 7.11H and 7.11HR will be discussed below.

In the experiments in Fig. 2, the vectors used did not replicate after transfection; consequently, transcript yields were low. We have carried out analogous experiments with vectors containing an SV40 viral origin with transfection into COS1 cells. Those experiments (data not shown) confirm the presence of an upstream negative regulatory element but are complicated by replication-dependent changes in promoter activity (Saffer and Thurston, in preparation).

Additional controls confirm the negative effect. A control gene on the expression vector has not been included in the experiments shown in Fig. 2 because the reducer element functions at a distance and is promoter independent (see below). Also, the initial experiments did not include a cotransfected control plasmid to rule out competition of a limiting factor as the basis for reduced expression. Instead, in the initial experiments, transfection efficiencies were monitored by examining Hirt extracts (14) from transfected cells, and Southern blots showed equal amounts of vector for each plasmid used (data not shown). However, we do consider a cotransfected control to be essential and show extensive analysis done with a contransfected control vector (see Fig. 6 and 8). In all of the experiments, the quality of the transfected plasmids has been controlled by using several independent preparations of plasmids grown in two different strains of *E. coli*. In each case, similar results were obtained.

Transformation assays were performed in which CV-1 cells transfected with the indicated expression vectors were placed in medium in which cell survival requires expression of the *gpt* marker gene. Since no other manipulations are required (such as RNA isolation or a multistep assay), this transformation assay serves as a useful control for the S1 mapping experiments. The number of surviving colonies, while not necessarily related linearly to transcriptional levels, does reflect the relative promoter strength. The results from the transformation assays (Fig. 1, right) confirm the

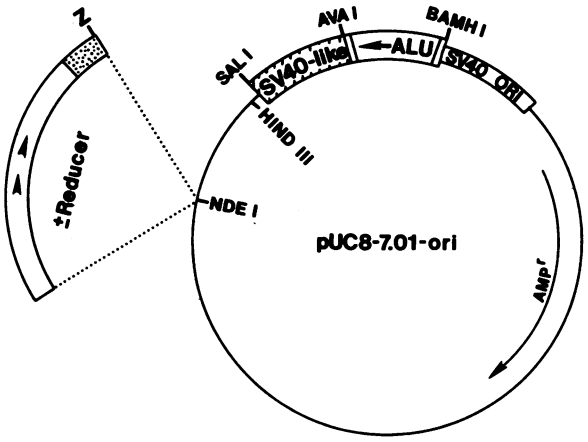

FIG. 3. Vector pUC8-7.01-ori for testing effect of the reducer on early direction transcription from the SV40-like sequence. The cellular SV40-like promoter (hatched area), a neighboring *Alu* sequence (to the right in Fig. 1), and the SV40 viral origin of replication were cloned into plasmid pUC8. The 907-bp reducer element was inserted at the *Nde*I restriction site. As in Fig. 1, the stippled region is from the *Alu* sequence at the left of the SV40-like promoter. "Z" shows the position of a potential Z-DNA-forming sequence, and the arrowheads are used to clarify the orientation of the reducer.

presence of a negative regulatory element in clone 7.11 and show that this effect was not duplicated by heterologous DNA of the same length (clone 7.12B).

The reducer functions independently of orientation and position. As described above, several negative regulatory elements have been found that exert their influence independently of orientation and position. To test whether the reducer sequence has these properties, a clone was constructed analogous to clone 7.11 with the entire 907-bp *Ava*I to *Acc*I fragment in reversed orientation (7.11HR). As a control for the potential effect of inserting a *Hin*dIII linker at the junction of 7.12 and the reducer, a clone (7.11H) was also made that contains this linker with the reducer in its natural orientation.

The results from S1 mapping of the transcripts in transient expression assays are shown in Fig. 2. No transcription is evident from clone 7.11H or 7.11HR in the CV-1 cells when compared with clone 7.12. This reduction in transcription is supported by the transformation assay data (Fig. 1). We conclude that the reducer functions independently of orientation.

Because the sequences responsible for reducer activity are localized to the rightmost end of the 907-bp restriction fragment (see below), reversing the orientation of the reducer fragment as in clone 7.11HR would move the reducer an additional 850 bp upstream for a total distance of 1,600 bp over which the element is active. Therefore, the reducer functions independently of position.

The reducer influences early direction transcription from the SV40-like cellular promoter. To determine the effect of the reducer on "early" direction (leftward) transcription from the 7.02 promoter, a pUC8 derivative, pUC8-7.01 (Fig. 3), was constructed, using the *Sal*I to *Bam*HI restriction fragment containing the promoter 7.02 and the right side flanking *Alu* sequence (Fig. 1). Insertion of the reducer at the *Nde*I site in the orientation shown in Fig. 3 results in a vector with the reducer located at the same distance from the 7.02 promoter and in the same orientation as found in vivo. To

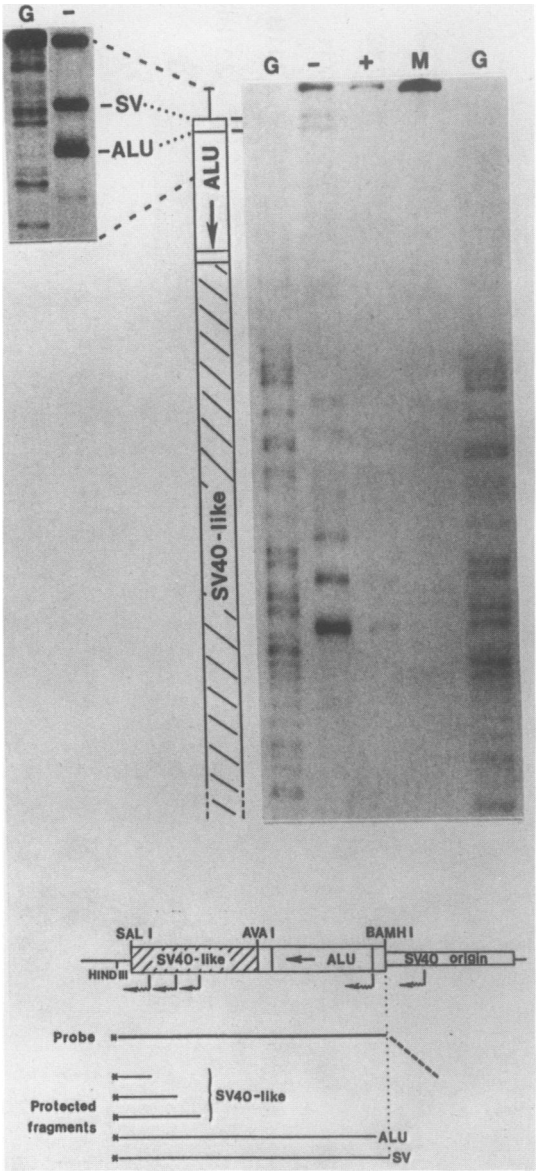

FIG. 4. S1 nuclease mapping of transcripts from vector pUC8-7.01-ori in the presence or absence of the reducer. Plasmid pUC8-7.01-ori, with (+) or without (−) the reducer fragment (907-bp *Ava*I-*Acc*I fragment), was transfected into COS1 cells. S1 mapping was as in the legend to Fig. 2. To generate a single band from the multiple SV40 origin start sites, the probe was made from an analogous vector without the SV40 origin. It was end labeled at the *Hin*dIII site contained in the vector sequence and ended within vector sequences to the right of *Alu* (the dashed portion of the probe shown at the bottom). Lane M shows a mapping experiment with RNA from mock-transfected cells and demonstrates that the probe is vector specific. Lanes G show a G-sequencing reaction of the probe used. The locations of sequences within the SV40-like promoter and the neighboring *Alu* are indicated to the left to clarify the identity of different bands. There are seven detectable initiation sites within the SV40-like promoter, but for simplicity, only the three major starts are indicated in the diagram at the bottom. The expansion of the upper portion of the gel is from a separate experiment and is shown to clarify the position of the start sites at the top of the gel. The bands labeled ALU show the location of the start site expected from polymerase III transcription of the *Alu* sequence. The bands labeled SV mark the point where the probe and vector sequences diverge (at the *Bam*HI site shown) and represent transcription originating to the right of this point.

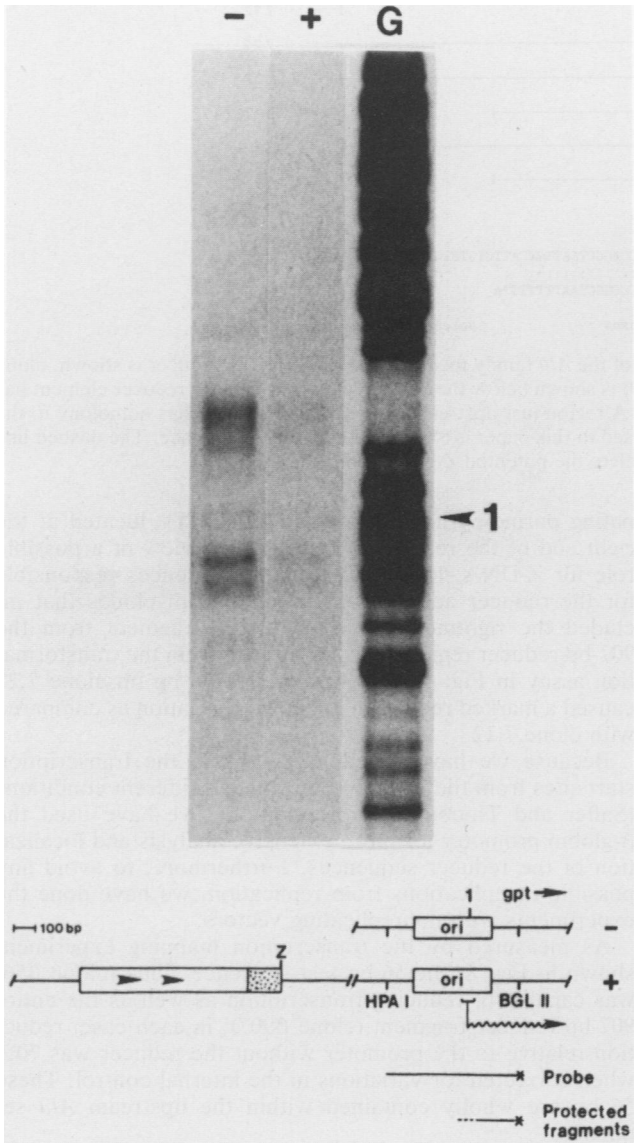

FIG. 5. Effect of the reducer sequence on the SV40 early promoter. Vector pSV2Agpt, with (+) or without (−) the reducer, was transfected into COS1 cells and total RNA was isolated 48 h later. Equal amounts of RNA were hybridized to a probe, shown at the bottom, which was end labeled within vector sequences and which covered the SV40 early promoter. S1 nuclease-resistant hybrids were analyzed as described above. Nucleotide 1 within the viral origin is indicated. Lane G is a G-sequencing reaction of the probe.

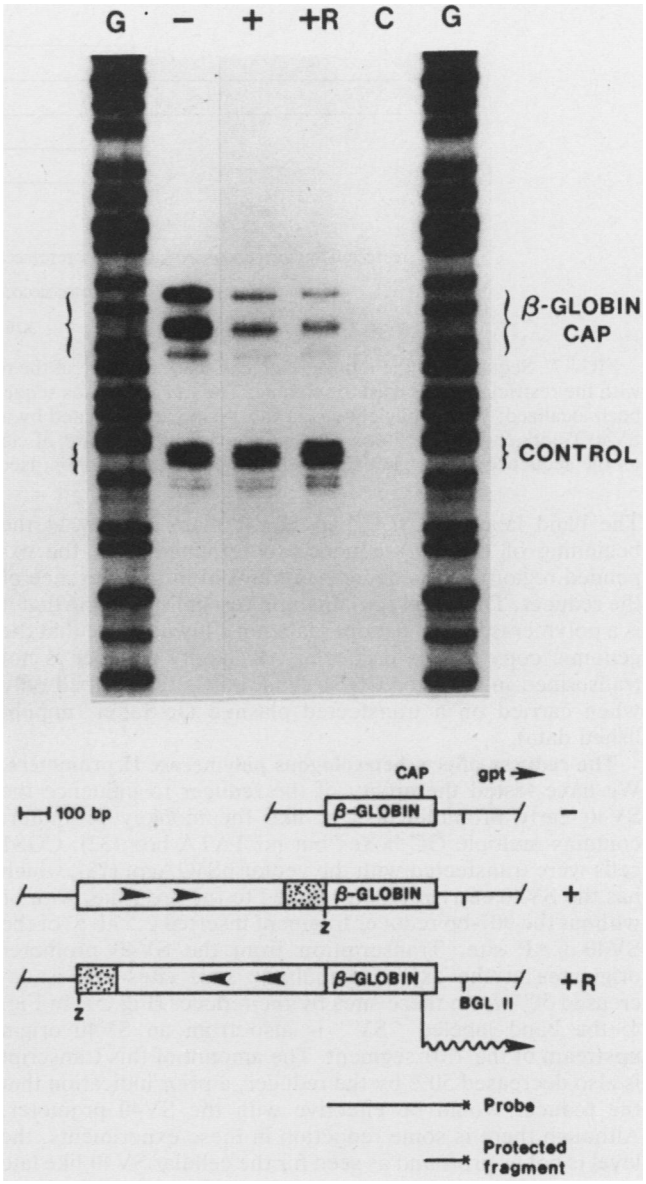

FIG. 6. Effect of reducer sequence on the human β-globin promoter. The human β-globin promoter was inserted into vector pAngpt.LR in the absence (−) or presence of the reducer element in its natural (+) or reversed (+R) orientations. The plasmids were made to contain an SV40 viral origin in place of the enhancer element and were transfected into COS1 cells along with a cotransfected control plasmid, pSV2Agpt. Total RNA was prepared and S1 mapped as above, using a probe which was end labeled within vector *gpt* sequences and which covered the entire β-globin promoter sequence. The position of transcripts arising from the β-globin CAP site is indicated. RNAs from the cotransfected control are detectable with the same probe and will give rise to a band at the point of divergence between the SV40 promoter-*gpt* construct and the β-globin promoter-*gpt* construct (labeled CONTROL). Lane C is an S1 assay, using no RNA, and lane G is a G-sequencing reaction of the probe.

increase transcript yield from the naturally weak early direction promoter, the vector also contained an SV40 origin, and transfection was into COS1 cells which provide the T antigen required for replication.

S1 mapping of plasmid-specific transcripts is shown in Fig. 4. The expected multiple early direction start sites were all reduced in intensity fivefold due to the presence of the reducer, but were not totally eliminated. The additional start sites from the *Alu* sequence and the SV40 origin found near the top of the gel will be discussed below. We conclude from these experiments that the reducer decreases transcription from the 7.02 promoter in the early, as well as the late, direction.

The pUC8-7.01 construction used in Fig. 4 was designed to permit the detection of transcripts originating from the *Alu* sequence. This particular *Alu* family member is transcribed by polymerase III in vitro, with termination of the polymerase III transcript occurring in the vector sequences beyond the region covered by the S1 probe (unpublished results).

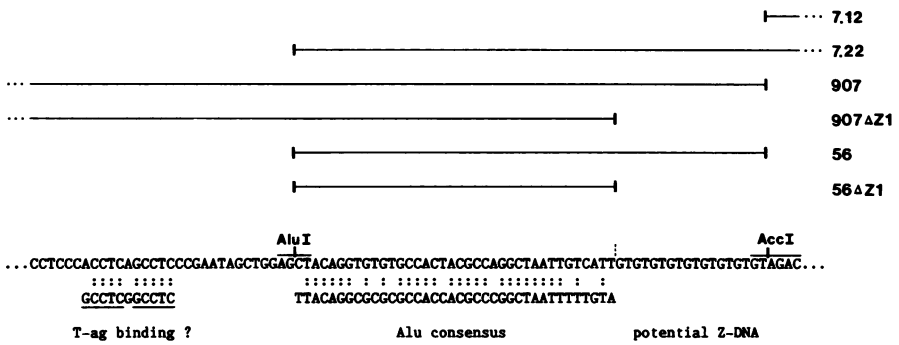

FIG. 7. Sequence of the reducer element. The sequence of the region of the *Alu* family member that contains the reducer is shown, along with the restriction sites used for cloning. The *Alu* consensus sequence (9) is shown below the 38-bp region in which the reducer element has been localized; similar nucleotides in this region are indicated by a ":." A region just upstream of the reducer, which has homology to the SV40 T-antigen-binding site, is also indicated. The endpoints of clones used in this paper are depicted above the sequence. The dashed line on the sequence shows the exact endpoint of the Bal deletion used to delete the potential Z-DNA segment.

The band labeled "ALU" in Fig. 4 maps exactly at the beginning of the *Alu* sequence (determined from the expended region) and is decreased fourfold in the presence of the reducer. This band is α-amanitin resistant, proving that it is a polymerase III transcript (data not shown). Note that the genomic copy of this particular *Alu* family member is not transcribed in CV-1 or COS1 cells, but is transcribed only when carried on a transfected plasmid (J. Saffer, unpublished data).

The reducer affects heterologous polymerase II promoters. We have tested the ability of the reducer to influence the SV40 early promoter which, like the monkey promoter, contains multiple GC boxes but no TATA box (32). COS1 cells were transfected with the vector pSV2Agpt (18), which has the SV40 early promoter linked to the *gpt* gene, with or without the 907-bp reducer fragment inserted 2.5 kb 5′ of the SV40 CAP site. Transcription from the SV40 promoter originates at the expected multiple start sites and is decreased 50% from these sites by the reducer (Fig. 5). In Fig. 4, the band labeled "SV" is also from an SV40 origin upstream of the 7.01 segment. The amount of this transcript is also decreased 50% by the reducer, a prior indication that the reducer would be effective with the SV40 promoter. Although there is some reduction in these experiments, the level is not as profound as seen for the cellular SV40-like late promoter. This difference may be due to features of the SV40 promoter that are not found in the monkey promoter, such as the ability to replicate, or due to some loss of efficiency over the increased distance.

To examine the effect of the reducer element on a typical TATA box-containing promoter, a 400-bp fragment containing the human β-globin promoter was inserted into pAngpt.LR. The reducer was then cloned 380 bp from the CAP site (see Fig. 6, bottom). Transfection into COS cells included a cotransfected control plasmid, pSV2Agpt, whose transcripts were detected by the same S1 mapping probe. Quantitation of transcription efficiencies in this and subsequent experiments was expressed relative to the internal control. Transcripts initiating at the human β-globin promoter CAP site were decreased 70% when the 907-bp reducer was present and 80% when it was in reversed orientation (Fig. 6). These experiments show the reducer can affect very different promoter types and further support the position and orientation independence of this effect.

The DNA sequences responsible for reducer activity are wholly located within an *Alu* sequence. A notable feature of the DNA segment containing the reducer is 20 bp of alternating purine-pyrimidine including $(dG\text{-}dT)_9$ located at the right end of the restriction fragment. In view of a possible role for Z-DNA, localization of the sequences responsible for the reducer activity was initiated with clones that included the rightmost 56-bp *Alu*I-*Acc*I fragment from the 907-bp reducer region (Fig. 7). As seen from the transformation assay in Fig. 1, inclusion of that 56 bp on clone 7.22 caused a marked reduction in colony formation as compared with clone 7.12.

Because we have found variations in the transcription start sites from the 7.02 promoter under different conditions (Saffer and Thurston, in preparation), we have used the β-globin promoter for more extensive analysis and localization of the reducer sequences. Furthermore, to avoid any possible complications from replication, we have done the experiments with nonreplicating vectors.

As measured by the transcription mapping experiment shown in Fig. 8, the 56-bp test sequence alone (clone β56) was capable of reducing transcription as well as the entire 907-bp reducer fragment (clone β907); in each case, reduction relative to the promoter without the reducer was 70% when corrected for variations in the internal control. These 56 bp are wholly contained within the upstream *Alu* sequence.

To test the requirement of the potential Z-DNA segment for reducer activity, this region was deleted from clone β56 (resulting in clone β56ΔZ1 containing only 38 bp of the reducer fragment) and from β907 (resulting in clone β907ΔZ1). The truncated reducer element was still able to reduce expression by 75% in both clones (Fig. 8), thereby showing that the potential Z-DNA segment is not essential for reducer activity. It is still possible that the Z-DNA region plays a role in vivo, perhaps by regulating access of factors to this region or affecting nuclear localization, but in these expression vector tests the Z-DNA region had only fortuitously led us to the involved sequences.

We have tested the ability of directly repeated 38-bp reducer segments to reduce transcription. We find that two tandem 38-bp regions function the same as one (data not shown). However, we have also found that direct repeats of the 907-bp fragment containing the reducer do not serve to reduce transcription. Relative to the internal control, transcription from the β-globin promoter in the presence of the tandem 907-bp fragments (clone $\beta 907^2$) was 95% of that from the promoter alone (clone β) as determined by densitometry (Fig. 8). This unexpected behavior of the tandem 907-bp fragment was not due to competition for limiting factor since

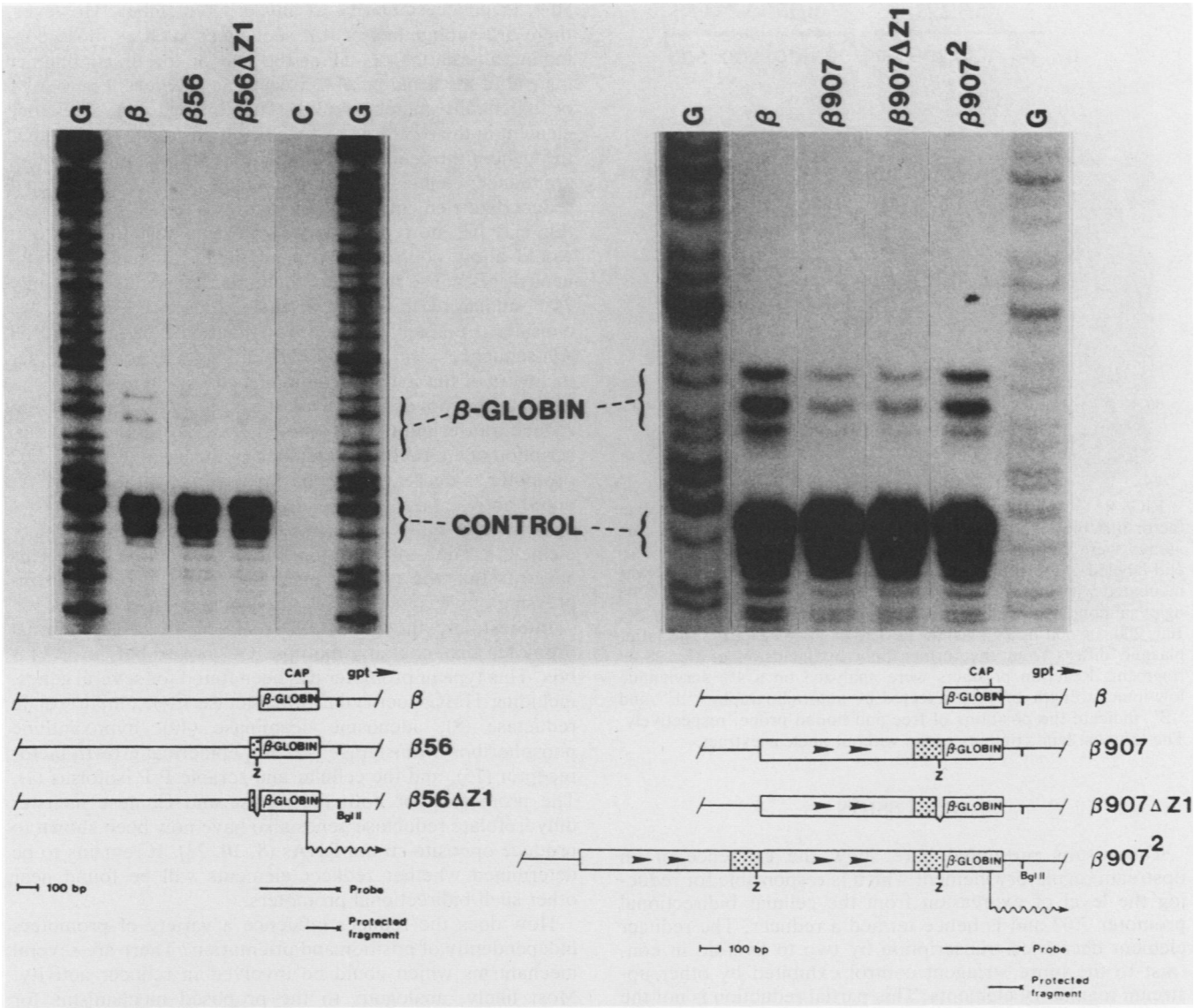

FIG. 8. Localization of the DNA sequences responsible for reducer activity. Clones containing the human β-globin promoter with the portions of the reducer fragment indicated were constructed. The endpoints of the clones are indicated in Fig. 7. Clone β907² was made to contain two tandem reducer fragments. Plasmids were transfected into COS1 cells along with a cotransfected control of pSV2Agpt. S1 mapping was carried out, and the same S1 probe can detect both the experimental and control transcripts. Lane C is a control reaction using no RNA, and lane G is a G-sequencing reaction of the probe.

tandem 38-bp fragments did not show the same behavior. Note that the similarity in the reducer activity of one or two copies of the 38-bp region and the cancelling effect of two copies of the 907-bp fragment are in contrast to the additive effect of multiple copies of the SV40 enhancer (19).

A nuclear factor binds specifically to the reducer element. As with other regulatory sequences, it is likely that reducer activity is mediated by a protein factor which binds to the 56ΔZ1 38-bp region. We have used this fragment in gel shift assays to determine whether CV-1 cell nuclei have a factor that binds specifically to this region. A retarded band was found when CV-1 nuclear extracts were incubated with the 38-bp probe (Fig. 9). Plasmid pβ.LR did not inhibit retardation of the probe, but pβ56ΔZ1.LR, which differs from pβ.LR only by the 38-bp reducer fragment, did compete for factor binding. Although these data confirm the presence of a factor that binds specifically to the reducer element, it remains to be directly demonstrated that the factor is responsible for mediating reducer activity.

As expected, the assay results in Fig. 9 show competition for factor binding by a reducer-containing plasmid. However, competition was not consistently seen in our assays. Under many conditions, the gel shift assays were complicated by what appears to be an aggregation phenomenon: formation of more slowly moving bands which may be multimers, and a lack of competition by reducer-containing sequences at the concentrations used above (data not shown). It is noteworthy that Perelygina et al. have previously identified an *Alu*-specific binding protein, ABP, which also shows this behavior (31). The binding site for ABP was not localized, but ABP may be the same factor as seen in our assays.

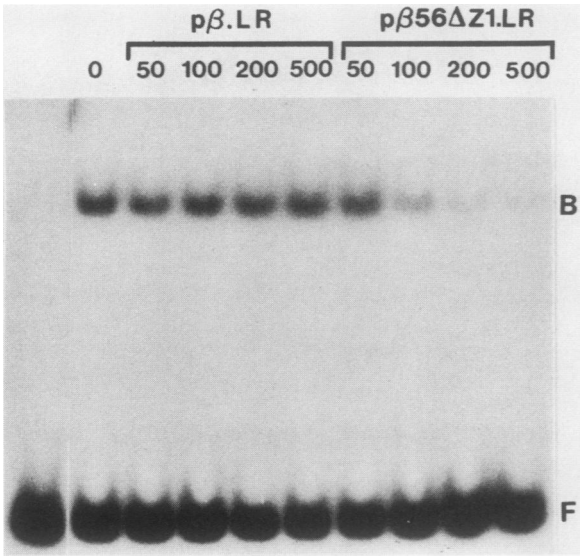

FIG. 9. Gel shift assay demonstrating the presence of a nuclear factor that binds to the reducer. Gel electrophoresis DNA-binding assays were carried out by the method of Singh et al. (39). An end-labeled 38-bp probe (0.5 ng), shown as 56ΔZ1 in Fig. 7, was incubated with 4 μg of CV-1 nuclear extract in the presence of 200 ng of salmon DNA. Binding was performed in the presence of 0, 50, 100, 200, or 500 ng of plasmid pβ.LR or pβ56ΔZ1.LR. The latter plasmid differs from the former only by inclusion of the 38-bp fragment. Reaction products were analyzed on a 4% acrylamide low-ionic-strength gel and detected by autoradiography. "F" and "B" indicate the positions of free and bound probe, respectively. The leftmost lane contains probe without nuclear extract.

DISCUSSION

The results presented here show the existence of an upstream regulatory element which is responsible for reducing the level of expression from the cellular bidirectional promoter 7.02 and is hence termed a reducer. The reducer element decreases transcription by two to fivefold in contrast to the more stringent control exhibited by other upstream regulatory elements. This partial reduction is not the consequence of limiting reducer-binding factor since the level of reduction is unaffected by competing sequences cotransfected with the test plasmid (data not shown). Obviously, cells must be able to control genes by more than all-or-nothing mechanisms, and the reducer element appears well suited for the fine regulation of gene expression.

The reducer shares several characteristics with enhancer sequences: it functions independently of orientation, it functions over distances of at least 2.5 kb, and it influences different promoters. Thus, the reducer element is similar to the yeast *HMR* silencer (5), the murine c-*myc* dehancer (33), the human T-cell lymphotrophic virus type I NRE (35), the rat insulin gene upstream negative element (20), and the rat α-fetoprotein upstream negative element (24), all of which also have enhancerlike properties.

However, the reducer has some important differences from these negative regulatory elements. The reducer element is wholly contained in a member of the *Alu* family of interspersed repeated sequences. *Alu* sequences are a family of dimeric elements consisting of direct repeats of an approximately 130-bp sequence. The second monomer contains a 31-bp insert, and the end of each monomer is characterized by an A-rich sequence. Each *Alu* family member has about 80% sequence similarity to any other member. However, there are subfamilies of *Alu* sequences such as those containing alternating dG-dT at the end of the first monomer instead of the usual poly(A) tract which represent perhaps 1 of 100 family members (37). The finding of a regulatory element in the *Alu* family which occurs as much as 10^6 times throughout the genome raises many questions. For example, are reducer elements found near a majority of genes? Even if reducerlike elements are found in only a few *Alu* sequences, this may be one type of conserved regulatory element that would allow control of whole families of genes simultaneously. Because the 38 bp containing the reducer are only 74% similar to the *Alu* consensus sequence (Fig. 7), we consider it unlikely that reducer function is common to all *Alu* sequences. But reducer elements may be found in other members of the Z-DNA-containing *Alu* subfamily.

A second novel feature of the reducer is its ability to reduce transcription from a polymerase III promoter. Transcription of an *Alu* family member on the late side of the 7.02 promoter is decreased by the presence of the reducer. As noted above, this late-side *Alu* is not transcribed in CV-1 cells in vivo, but is transcribed in CV-1 cells if the reducer element is not included on the transfected clone. This suggests that the reducer element may be involved in repressing transcription of the *Alu* sequence in vivo.

Interestingly, the reducer is associated with a bidirectional promoter that contains multiple GC boxes but no TATA box. This type of promoter has been found for several genes, including HMG coenzyme A reductase (34), dihydrofolate reductase (8), adenosine deaminase (40), hypoxanthine phosphoribosyltransferase (22, 30), epidermal growth factor receptor (15), and the cellular and scrapie PrP isoforms (1). The promoters for both the mouse and Chinese hamster dihydrofolate reductase genes also have now been shown to produce opposite-strand RNAs (8, 10, 23). It remains to be determined whether reducer elements will be found near other such bidirectional promoters.

How does the reducer influence a variety of promoters independently of position and orientation? There are several mechanisms which could be involved in reducer activity. Most likely, analogous to the proposed mechanisms for enhancers, the reducer is a binding site for a negative regulatory factor which interacts with the promoter through a looping mechanism. While much needs to be done to prove a looping mechanism, the data are consistent with interaction of DNA-bound factors over large distances. To be promoter nonspecific, however, the reducer-binding negative factor would likely interact with a positive factor common to both polymerase II and polymerase III transcription units. There are similarities between the promoters for the different polymerases as suggested by a functional TATA box upstream of the 7SK RNA gene (27) and an octamer motif implicated in both polymerase II and III transcription (7).

The 38 bp responsible for reducer activity are located at the beginning of the 3′ monomer of an *Alu* family member and, interestingly, are adjacent to a region similar to the SV40 T-antigen-binding site found in many *Alu* sequences (17). This proximal relationship of the T-antigen-binding site to the reducer element suggests the possibility that T antigen may directly influence reducer activity. Demonstration of such a mechanism would be important for understanding the T-antigen-induced alteration of cellular gene expression.

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