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Secondary structure of the nascent 7S L RNA mediates efficient transcription by RNA polymerase III

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ABSTRACT

A structural motif at the 5′ end of human 7S L (srp) RNA that is recognized specifically by cellular proteins has been identified as an efficient activator of RNA polymerase (pol) III transcription in vivo and in vitro. Mutations affecting three double-stranded regions or a tetranucleotide bulge of this RNA motif result in strongly reduced expression rates. However, effective suppression is achieved by compensatory mutations restoring RNA sequence complementarity. This activation of transcription is also observed in the context of another pol III promoter and is position-dependent. The effects observed are reminiscent of the Tat-TAR trans-activation of the human immunodeficiency virus and attribute a novel function to the structure of cellular small stable RNA.

Keywords: pol III transcription; RNA binding proteins; RNA secondary structure; srp RNA; transcriptional activation

INTRODUCTION

The three eukaryotic transcription systems share common promoter elements and activator proteins. Transcription initiation is achieved by protein-protein interactions between factors specifically recognizing upstream activator elements and the general transcription factors bound to the proximal "core" promoter (Tjian & Maniatis, 1994). In general, pol III genes (genes transcribed by RNA polymerase III) fit to this model for transcription initiation. However, the finding of entirely gene-internal promoters in 5S RNA and tRNA genes (Sakonju et al., 1980; Hofstetter et al., 1981) seemed to reflect a special feature of the pol III transcription system. This notion was further substantiated by the discovery of exclusively gene-external pol III promoters of 7S K RNA (Murphy et al., 1987; Kleinert et al., 1988) and U6 RNA (Das et al., 1988) genes. Furthermore, split promoters located in part upstream as well as downstream of the transcription start site were found in EBER (Epstein-Barr-Virus) RNA (Howe & Shu, 1989) and human 7S L RNA (Ullu & Weiner, 1985; Bredow et al., 1990a) genes, the latter encoding the RNA constituent of the signal recognition particle (srp, Walter & Blobel, 1982; reviewed by Lütcke, 1995). Recently, it was demonstrated that 7S L RNA genes have changed promoter type during evolution (Heard et al., 1995). Thus, with respect to promoter structure, pol III genes reveal a remarkable flexibility that is reflected most impressively by the 7S L RNA genes.

The human 7S L RNA gene is composed of two domains: the Alu domain, which most likely gave rise to the highly repetitive Alu sequence element scattered throughout the human genome (Ullu & Tschudi, 1984) and the S domain, a 7S L RNA specific core sequence of 150 bp (Ullu et al., 1982). In addition to the gene-external promoter elements (Bredow et al., 1990b), the 5′ end of the human 7S L RNA gene contains two regions with limited homology to internal promoter elements of other pol III genes. The putative B-box around position +55 represents a 6 of 10 bp match to the consensus sequence (Galli et al., 1981; Ciliberto et al., 1983). In addition, two potential A-boxes, overlapping by 3 bp (+12 to +14), are located between positions +5 and +22. These sequences match to the corresponding A-box consensus (op. cit.) in 9 or 6 of 11 bp, respectively.

In this report, we describe a novel type of transcriptional activation of pol III genes in vivo and in vitro. In addition to the structural integrity of several promoter elements, efficient transcription of the human 7S L RNA gene also depends on the structure of the newly synthesized RNA. Evidence is presented that, during tran-

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scription, a structural motif of 7S L RNA is recognized specifically by cellular proteins. By several criteria, this type of regulation resembles the Tat–trans-activating response (TAR) trans-activation of human immunodeficiency virus (HIV-1) mRNA biosynthesis. Although the identity of the respective proteins remains to be clarified, it appears conceivable that 7S L RNA might represent a cellular target for some of those additional nonviral proteins required for Tat trans-activation of transcription from the viral promoter.

RESULTS

Maximal expression of the 7S L RNA gene in vivo requires the structural integrity of the RNA at its 5′ end

In our attempts to map more precisely the gene-internal part of the human 7S L promoter, we analyzed several different mutants. Earlier experiments had revealed that sequences downstream of the B-box are dispensable for full activity of the 7S L promoter in vivo and in vitro (G. Emde, unpubl.). In order to determine which of the two putative A-boxes might be functional, we introduced restriction sites at the 5′ end of the gene, allowing the insertion of synthetic oligonucleotides. To our surprise, we did not obtain any "neutral" mutation within the first 46 bp of the transcribed region. As proposed by Larsen and Zwieb (1991), the corresponding 7S L RNA sequences form a propeller-like secondary structure (Fig. 1A), with two hairpins being separated by a tetranucleotide bulge. All mutations introduced by us into that region resulted in severe loss of 7S L promoter activity in vivo. On average, expression rates obtained upon transfection into HepG2 cells were reduced to 20% of the wild-type (wt) activity (Fig. 1B). In addition, only basal level activity of about 5% was observed with a quadruple mutation (designated A-Box), which maps to position +11 to +14 at the first loop structure of the RNA. However, this further inactivation is likely to reflect the destruction of an A-box element, because both of these

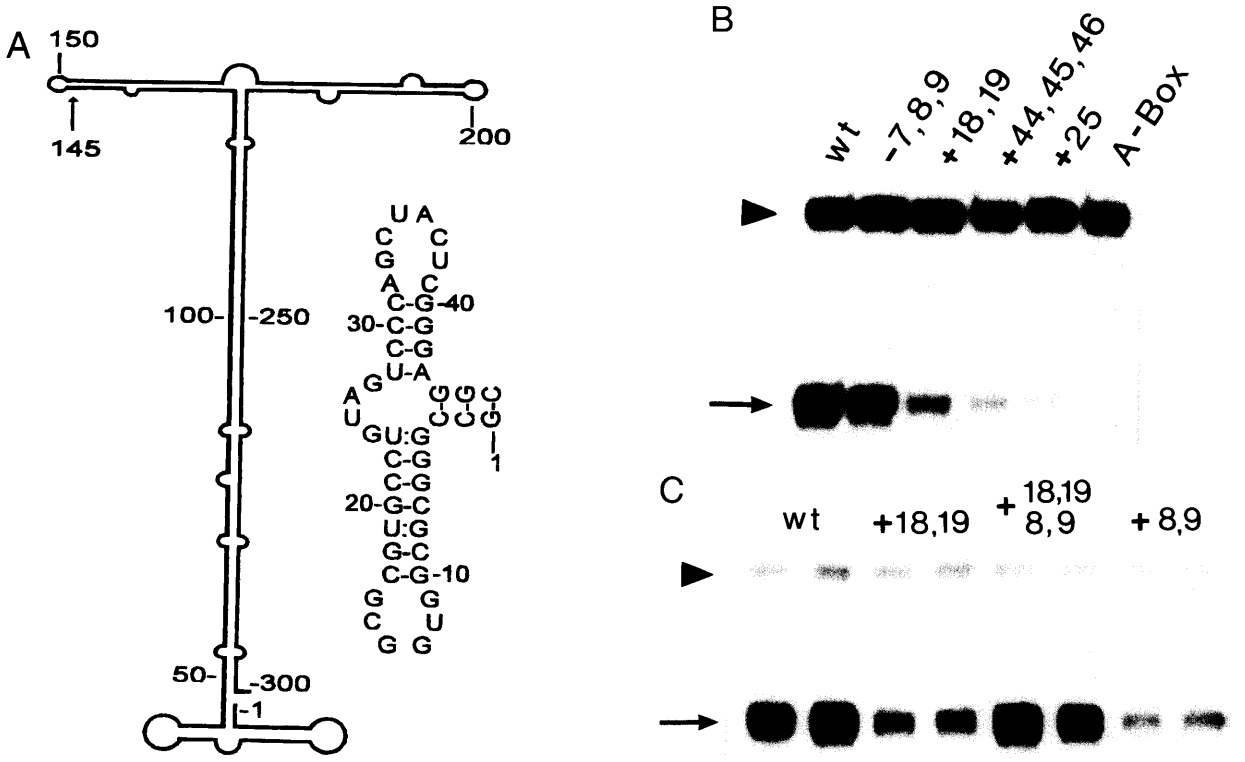

FIGURE 1. The 5′ end secondary structure of 7S L RNA and its influence on expression in vivo. **A:** Secondary structure of human 7S L RNA as proposed by Larsen and Zwieb (1991). The insert shows the sequence of the 5′ end propeller-motif. **B:** Expression rates of transfected 7S L RNA mutant genes. Hep G2-cells were transfected with 7S L RNA wt or mutant constructs and a 7S K reference-gene (see Materials and Methods). After 36 h, total RNA was isolated and analyzed for S1-nuclease protection of labeled DNA probes. Reference and 7S L RNA-specific bands are indicated throughout by arrowheads and arrows, respectively. Mutations are described by position numbers relative to the transcription start-site (+1). A-Box is a +12 to +15 mutant affecting either one of the two potential A-boxes. **C:** Effect of suppressor mutations on the expression of 7S L RNA mutant genes. The +18/+19 mutations in B were compensated by opposite strand mutations at positions +8/+9, restoring complementarity of the second stem of the propeller-motif. As a control, a construct containing the +8/+9 mutations alone was included. Analysis of constructs was as in B; however, all four constructs were tested in duplicates.

potential homologies share this sequence. In contrast, mutations introduced upstream of the transcription start site between −5 and −20 (see −7, 8, 9) had no effect on promoter activity.

With the exception of one construct (A-Box; +11 to +14), all of the several mutants we had analyzed did not affect conserved nucleotides of the two putative A-Box elements (consensus: 5′-GGCNNARYGG-3′) that are located between positions +5 to +14 and +13 to +22, respectively. On the other hand, all these internal mutations caused the described repression of 7S L RNA transcription of about 80% or more. From there it appeared that some other principle than just promoter disruption might contribute to the observed inhibition of 7S L RNA expression. Therefore, we analyzed whether or not the secondary structure of the newly synthesized RNA was involved. For this, the +18/+19 mutations of the first stem-loop structure were compensated by additional mutations in the opposite strand (+8/+9), allowing the restoration of an RNA double strand. As is evident from the results in Figure 1C, these compensatory mutations were able to suppress efficiently the inhibitory effects observed with either of the two one-sided mutations (+18/+19 or +8/+9) alone. In addition, neither double mutation alone can revert the effects of a noncompensatory mutation (for example +8,9 with +25; data not shown). This excludes the possibility that one of the stem mutations acts as some kind of an omnipotent suppressor. With respect to the role of the two potential A-box elements, it should be noted that the +18/+19 mutations of the second homology (between positions +13 and +22) led to an even improved match with the consensus sequence (5′-GGCNNARYGG-3′), whereas the +8/+9 mutations affected two variable positions of the first homology located between positions +5 and +14. Therefore, because both of the one-sided mutations by themselves reduced transcription strongly, the full activity observed in case of the compensatory mutations can only be the result of a restoration of the authentic 5′-structure of 7S L RNA.

Efficient transcription from the 7S L promoter in vitro also depends on the 7S L RNA propeller motif

For being able to detect transcripts originating from the transfected gene, constructs were used with a VA I RNA reporter sequence fused to the 7S L RNA gene at position +145 (see arrow in Fig. 1A). Aside from the 5′ end propeller structure, these constructs do not allow formation of the extended double-stranded structure of the 7S L wt RNA. On the other hand, such a mutant RNA cannot be incorporated into the appropriate ribonucleoprotein complex, i.e., the srp. Therefore, it appeared conceivable that an altered structure at the very 5′ end of this mutant RNA might introduce a marked change in RNA stability. In particular, expression rates of transiently transfected genes might be affected significantly by an increased susceptibility to degradation of the newly synthesized RNA. To exclude this possibility, in vitro transcription reactions in HeLa cell nuclear extracts were performed with the mutant genes described. The results shown in Figure 2 demonstrate that in vitro efficient transcription of the human 7S L RNA gene depends on the integrity of the secondary structure at the 5′ end. Again, upstream mutations had no effect and nucleotide exchanges within the first loop (A-Box) resulted in basal level transcription of about 5%. As before, however, compensatory mutations allowing for double-strand formation of the RNA were able to restore wt transcription rates. Furthermore, the comparison of the wt (+145) construct with the full-length 7S L RNA gene (pUC8-L) showed that our reporter construct was transcribed as efficiently in vitro as the authentic gene. This indicates that the structure of the 3′ half of 7S L RNA does not contribute to transcriptional activation of the gene.

The 7S L RNA propeller motif is functional in the context of a heterologous promoter

By several criteria, the promoter of the human 7S L RNA gene appears to be somehow exceptional among all pol III promoters studied so far. Therefore, we

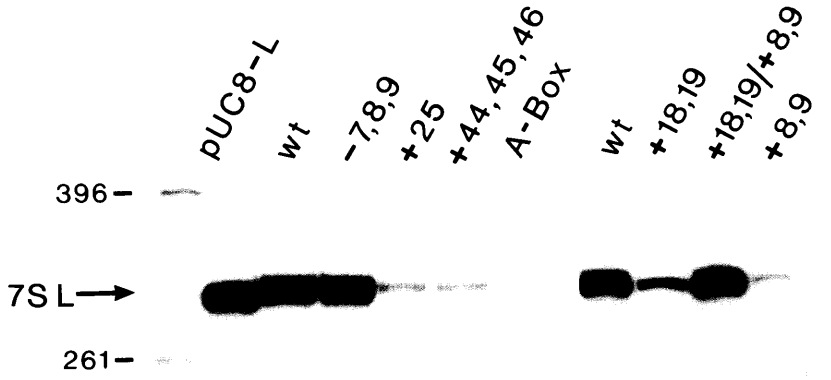

FIGURE 2. In vitro transcription of 7S L mutant genes in nuclear extracts. Five-hundred nanograms of plasmid DNA were transcribed in 10 μL of HeLa cell nuclear extract and RNA analyzed as described under Materials and Methods. Wild-type and mutant constructs were as in Figure 1. pUC8-L represents the authentic full-length human 7S L RNA gene. The size of DNA marker fragments is indicated.

wanted to know whether the activation exerted by the newly transcribed RNA could also be observed within the context of a different pol III promoter. Such an analysis required an exclusively gene-external pol III promoter—as present, for example, in 7S K and U6 RNA genes (Carbon et al. 1987; Murphy et al., 1987; Das et al., 1988; Kleinert et al., 1988). For this experiment, a −1/+1 hybrid gene (K/L, Fig. 3A) was cloned, with the 5′ flanking human 7S K promoter (Krüger & Benecke, 1987) being fused to the transcribed region of the human 7S L RNA gene. As shown in Figure 3B, in vivo expression rates obtained upon transfection of this chimeric 7S K/7S L RNA gene construct into HepG2 cells again demonstrated the significance for transcription of the 7S L RNA secondary structure. In the context of this heterologous promoter, too, mutation of the double-stranded RNA regions resulted in severe inhibition of transcription (Fig. 3B). The ability to extend the conclusions drawn previously to another motif of the propeller structure, in this case the compensatory effect of opposite strand mutations, was demonstrated by affecting the second stem-loop structure. For this, a

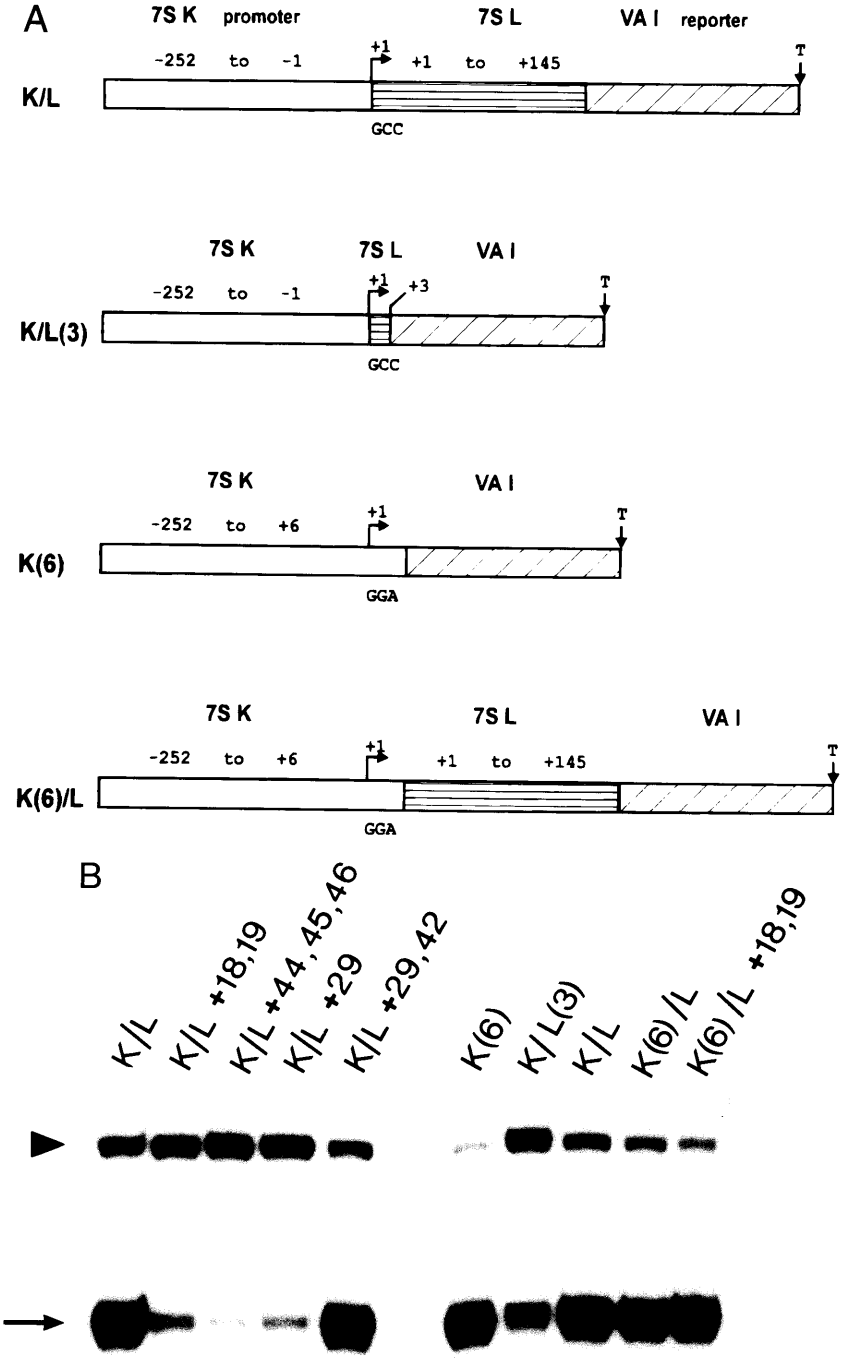

FIGURE 3. Effect of 7S L RNA sequence mutations in the context of the human 7S K RNA promoter. **A:** Schematic presentation of 7S K promoter constructs used in B and C. Chimeric genes were constructed by fusion of the 7S K promoter to the 7S L coding sequence. The boxes representing 7S K, 7S L, and VA I RNA gene sequences are not drawn to scale and numbers above the boxes refer to the respective nucleotide positions, relative to the transcription start site (+1). T, termination signal. Letters below the boxes depict the first three bases of the initiator element. **B:** Expression rates of 7S K/7S L −1/+1 fusion constructs. Mutations introduced into the 7S L RNA sequence were as before, except that, in this case, compensatory suppressor mutations were analyzed that affected the second stem-loop structure of the propeller (+29 versus +29/+42). Expression rates obtained with K/L constructs (arrow) and the 7S K reference (arrow-head) were determined as in Figure 1. **C:** Displacement of 7S L RNA structural mutations downstream of the 7S K promoter. Wild-type or mutant 7S L RNA sequences were fused to position +6 of the 7S K RNA gene [K(6)/L and K(6)/L +18,19]. To demonstrate the effect of different initiator elements on transcription from the 7S K promoter, two 7S K/VA I reporter fusion constructs are included [K(6) and K/L(3)]. K(6) represents a fusion of the 7S K RNA promoter (down to +6) with the adenoviral VA I cassette, used as reporter sequence throughout. In contrast, K/L(3) contains the 7S K promoter (to −1) fused to the first 3 bp (+1 to +3) of the 7S L RNA sequence and then again followed by the VA I cassette. Therefore, these two constructs differ only by the initiator element and provide evidence that the 7S L RNA sequence indeed represents a poor initiator element for the 7S K promoter. Analysis of expression rates in vivo was as above.

+29-nt exchange was compensated by a complementary opposite strand mutation at position +42. The recovery of transcription in the context of this promoter also must be attributed again to a restoration of RNA structure, because the +8/+9 and +42 mutations alone had revealed the same inhibition observed with the other single-side mutations before (data not shown).

Aside from the initiator sequence (unpubl.), the human 7S K promoter is virtually independent of any gene-internal elements. Therefore, such a construct enabled us to ask whether, in addition to the structure of the RNA, the position of this propeller-motif is also important for transcription activation. This question was addressed by placing the 7S L RNA sequence 6 bp downstream of the transcription start site. Figure 3C shows in vivo expression rates of chimeric 7S K/7S L constructs in which the 7S L RNA sequence (+1) was fused to the 7S K promoter at position +6 (to preserve the 7S K initiator element) of the 7S K RNA gene [K(6)/L]. As is evident from the comparison of the wt propeller construct [K(6)/L] with a corresponding construct containing the +18/+19 mutation analyzed previously [K(6)/L +18,19], no negative effect can be attributed to the propeller mutation any more. Furthermore, these unaltered expression rates, which are observed in spite of that propeller mutation, demonstrate unambiguously that the reduced 7S L RNA expression rates observed previously in response to the propeller mutations definitely did not reflect decreased RNA stability. This notion is further supported by the fact that transcripts from both the K(6)/L and the K(6)/L +18,19 constructs contain unpaired 5′ ends, respectively. Therefore, these RNA molecules should be even more readily susceptible to ribonuclease degradation, if compared, for example, with the K/L transcripts.

In this analysis (Fig. 3C), the basic constructs K(6) and K/L(3) were included to demonstrate that the 7S K promoter is more active in the presence of its own initiator element (GGA) than with the GCC sequence provided by the 7S L RNA gene (see Fig. 3A). Furthermore, the comparison of K(6) and K/L(3) strongly argues against a dominant negative effect on the 7S K promoter of the mutated propeller element, as might have been concluded from the results shown in Figure 3B alone. These two constructs just differ by the initiator element, which, in case of the 7S L initiator, leads to an 80–90% inactivation of the K promoter. This inactivation has to be taken into account for all K/L hybrid constructs of Figure 3B. Therefore, it appears that, in case of those K/L constructs, the positive effect of the intact RNA propeller motif is balanced by the negative effect on 7S K promoter activity of the 7S L initiator element.

In our hands, in vitro transcription reactions in HeLa cell nuclear extracts are not hampered by remarkable ribonuclease activity. Yet, to be sure that the propeller-dependent modulation of 7S L RNA transcription indeed reflects control of synthesis rather than structural protection against degradation, we performed in vitro transcription reactions to analyze RNA stability. First, the kinetics of 7S L wt and mutant RNAs were compared in a short-interval time course of synthesis. As is evident from Figure 4A, transcription from both templates follows very similar kinetics, but with the mutant RNA showing clearly reduced rates of synthesis again. In case of a preferential degradation of mutant transcripts, with increasing times of synthesis, one would expect to reach a plateau or even to observe a decrease in the amount of this RNA. It should be noted that the described reduction of mutant RNA synthesis is clearly observed from the very beginning. This result supports directly the conclusion of a transcriptional activation by the intact propeller. Secondly, pulse-chase experiments were performed with in vitro-transcribed RNA (Fig. 4B). For this, after 30 min of standard transcription reaction (time 0 in Fig. 4B), a 1,000-fold molar excess of unlabeled UTP was added and the reaction continued for the times indicated. As verified by PhosphorImager quantification, no significant turnover was observed with both RNAs. Therefore, this analysis also rules out the possibility that the different levels of 7S L wt and mutant RNAs are due to differential stabilities of the respective RNA products.

Furthermore, to analyze the effect of exogenous RNA on the in vitro transcription reaction, RNA synthesis was performed in the presence of 7S L competitor RNA, obtained with the bacteriophage T7 RNA polymerase system in vitro. In these experiments, 7S L wt RNA or the +25 (bulge) mutant template were transcribed in nuclear extracts in the absence or presence of 1 μg wt or mutant RNA. The results in Figure 5 again reveal drastically reduced in vitro transcription efficiencies of the +25 mutant versus the wt propeller construct. However, preincubation of the nuclear extract with the two 7S L competitor RNAs resulted in different responses. In the presence of wt competitor, transcription of the 7S L wt RNA template was reduced to 60%. In contrast, only very slight reduction (5%) was observed upon preincubation of the extract with exogenous 7S L mutant RNA [in this case, mutant RNA was transcribed from a T7 template with three point mutations converting the 7S L RNA bulge-sequence (at positions +24 to +26) from G-U-A to U-G-C]. A different picture emerged if the +25 (bulge) mutant construct was transcribed in the presence of exogenous competitor RNA. Now, no difference was observed in the presence of wt or mutant RNA, as compared to the reaction without competitor. In the case where disruption of the propeller might facilitate degradation of the mutant RNA, one would expect, however, that the additional presence of easily accessible substrate RNA would allow the detection of even increased levels of newly synthesized 7S L transcripts. One might argue that the

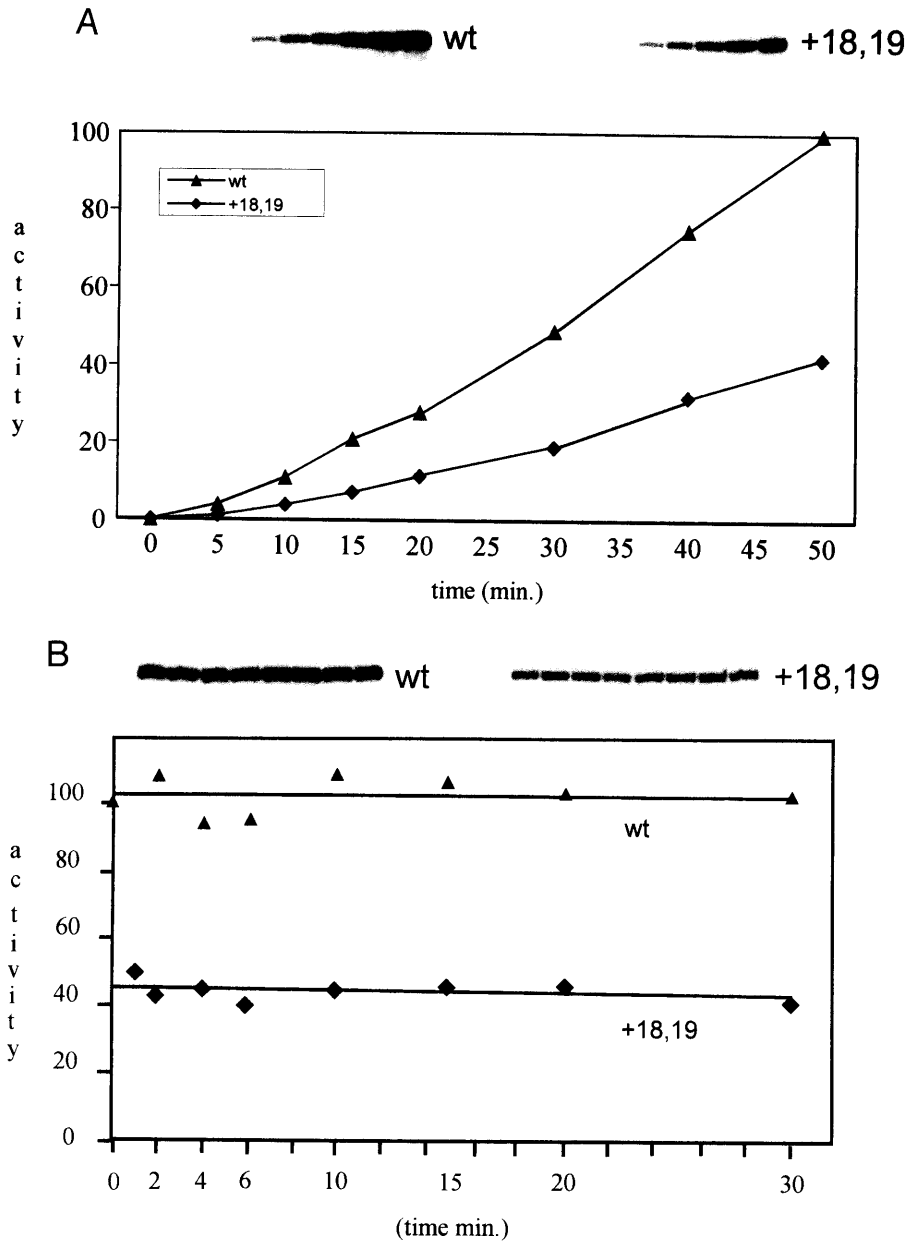

FIGURE 4. Stability of in vitro-transcribed wt and mutant RNA. **A:** 7S L wt or mutant (+18,19) RNA was synthesized in vitro (as in Fig. 2). At the times indicated, the reaction was terminated by addition of 0.6% SDS and 0.5 mg/mL proteinase K. After incubation for 30 min at 37 °C and phenol extraction, RNAs were analyzed as described before. The inserts above the diagram show the bands of the autoradiogram obtained for the wt or mutant RNA at the time points 5, 10, 15, 20, 30, 40, and 50 min, respectively. The diagram depicts the activity of bands as obtained by PhosphorImager quantification (Fujix BAS 1000; TINA 2.09). **B:** Pulse-chase analysis of in vitro synthesized wt or mutant RNA. Standard in vitro transcription reactions were performed for 30 min. At this point (time 0; wt = 100%), a 1,000-fold molar excess of unlabeled UTP was added to the reaction mixture and incubation continued for the times indicated. Termination of the reaction and processing of RNA was as in A. The autoradiogram inserts on top (chase for 2, 4, 6, 10, 15, 20, and 30 min, respectively) and the diagram below were obtained as before.

overall transcription activity of the +25 mutant construct simply was too low to exclude a differential effect of wt and mutant competitor RNA. The differential responses of wt 7S L transcription toward both competitors, however, clearly support the notion that the wt propeller structure, but not the mutant RNA sequence, is able to bind a cellular protein required for efficient transcription of the human 7S L RNA gene. It should be noted that competition of wt transcription by the exogenous propeller RNA did not go down to the transcription level observed with the +25 mutant, although competitor RNA was present in large excess. Therefore, in addition to the *cis*-effect of the nascent RNA, the 7S L RNA propeller motif may also act in *trans*, albeit much less efficiently. This result argues against a simple model of sequestering a limiting factor. Rather, it appears that efficient activation via the propeller motif requires a close association of this RNA structure with the initiation and/or elongation complex during transcription of the gene. Together, these results demonstrate that the dependence of efficient 7S L RNA synthesis on the integrity of the 5′ secondary structure reflects transcriptional activation of the gene.

Cellular proteins specifically recognize the 7S L RNA propeller motif

Finally, we wanted to see whether a cellular protein can be detected that binds specifically to the 7S L RNA propeller structure. For this experiment, labeled 7S L RNA (truncated at position +51) was synthesized by T7 RNA polymerase in vitro and incubated with in-

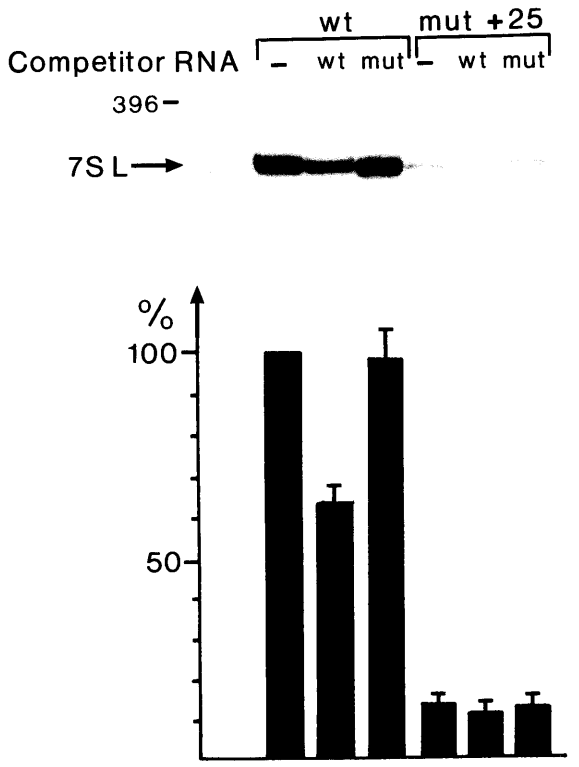

FIGURE 5. In vitro transcription of 7S L constructs in the presence of wt or mutant 7S L competitor RNA. In vitro transcription reactions were as in Figure 2, however, nuclear extracts were preincubated with 1 μg of competitor RNA. Templates were the 7S L wt RNA construct or the +25 bulge mutant. In the case of the mutated competitor RNA, 3 nt (+24/+25/+26) were exchanged. Furthermore, both competitor RNAs consisted only of the first 145 nt of the 7S L RNA sequence, i.e., lacked the VA I reporter element. The diagram below shows the mean values of the PhosphorImager quantification of five independent experiments, with the standard deviations indicated.

creasing amounts of S100 extract proteins. Figure 6A reveals that, with the wt propeller RNA, S100 extract proteins formed four different complexes (I–IV), with the weak complex II being observed in varying amounts among different extract preparations. The RNA mobility shift experiments in Figure 6B were performed just to demonstrate that identical patterns as with S100 were obtained with nuclear extracts. As outlined in the Discussion (see below), it appears that formation of these RNP complexes is not due to the binding of the srp proteins p9/p14.

Because these RNA mobility-shift analyses revealed quite a number of complexes, we determined their specificity by using mutant RNA molecules. For this, mutations were compared that affected different sections of the 7S L RNA propeller structure. The first mutation (+25) changed the sequence of the bulge. The second construct contained three nucleotide exchanges at positions +18, +19, and +29, respectively. These three point mutations affected two duplex regions of the propeller, whereas the third construct (+18,19) destabilized only one of the two hairpin structures. The analysis of these mutants in comparison with the wt sequence is shown in Figure 7A. The pattern obtained with the first mutant (+25) indicates that complex IV is sensitive to mutations affecting the bulge. Furthermore, this mutation also affected the formation of complex III, yet not as intensively as complex IV. In case of the bulge mutant, we observed, reproducibly, a significantly increased formation of complex I. However, a different pattern was obtained with mutations affecting the stem-loop structures of the propeller motif (see +18,19,29 and +18,19). Whereas complex IV was absent again, complex I was also suppressed now. Together, these results indicate that the formation of different complexes, such as I and IV, depends on the integrity of different structural elements of the propeller-motif. In addition to the analysis of these mutant transcripts, mobility shift analyses were also performed with RNA containing the compensatory mutations analyzed for transcriptional activation previously. The results in Figure 7B show that restoration of the double-stranded structure of the RNA by these compensatory mutations also allowed the formation of the wt pattern of shifted complexes again. Essentially, this result allows us to establish a correlation between the effects of the mutations on transcript level and on factor binding.

Finally, competition experiments were performed with labeled wt RNA and unlabeled competitor sequences. As is shown in Figure 8A, all four complexes were sensitive to an excess of unlabeled wt propeller RNA. In contrast, the results in Figure 8B reveal that two complexes (III and IV) were resistant toward competition by mutant (+25) RNA. Whereas complex III remained unaffected throughout, it appeared that formation of complex IV gradually decreased in the presence of higher concentrations of this competitor RNA (up to 100-fold molar excess). This response, however, is clearly different from that observed with the same excess of wt competitor RNA before (compare Fig. 8A). Therefore, we conclude that, with cellular proteins, the 7S L RNA propeller-motif is able to form at least two specific complexes (III and IV) of slightly different stability. The formation of these complexes appears to be bulge sequence-dependent. However, our present data do not exclude the possibility that a third specific complex (I) exists that might depend only on the structural integrity of the two hairpins. Formation of this complex was even increased by the bulge mutation (Fig. 7A). Consequently, an excess of +25 mutant RNA competed effectively against complex I (Fig. 8B).

DISCUSSION

Pol III genes have been found to be controlled by four different types of promoters (reviewed by Willis, 1993). Among those, the split promoter of the human 7S L

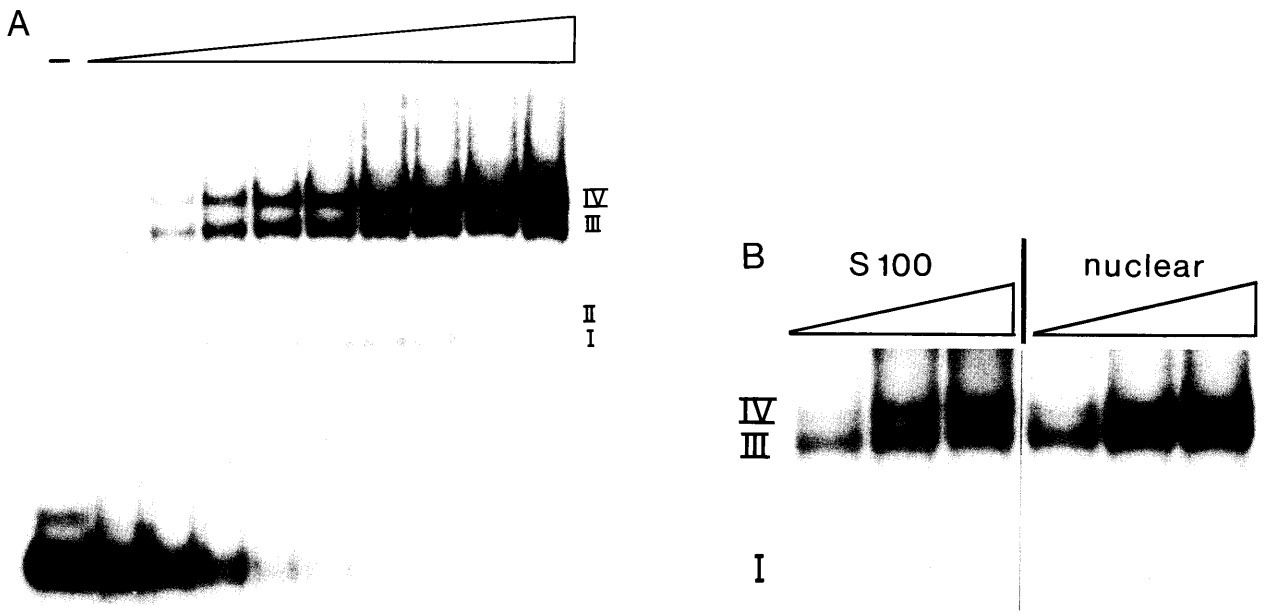

FIGURE 6. Electrophoretic mobility-shift analysis of 7S L RNA complexes with cellular proteins. **A:** HeLa cell S100 extract proteins were incubated with 6 ng of labeled 7S L RNA (truncated at position +51) and analyzed in native 6% polyacrylamide gels. Increasing protein concentrations were: 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, and 22.5 μg, respectively, with "–" representing a minus-protein control. The complexes observed are numbered I–IV. **B:** Same as A, however, labeled wt 7S L RNA (+51) was incubated with 5, 7.5, or 10 μg of protein obtained from HeLa S100 (left) or nuclear (right) extract, respectively.

RNA gene (and of EBER RNA genes) is least understood, at present. The results of this work demonstrate that the promoter-dependent control of 7S L RNA synthesis is superimposed by another principle of transcriptional activation. This is supported by the finding that efficient transcription of this gene also depends on the structural integrity of the 5′ end of the RNA itself. In that aspect, the 7S L RNA gene clearly is different from other pol III genes. Only in the case of the ribosomal 5S RNA genes is a contribution to transcriptional regulation by the RNA product well established. In that system, an excess of the mature RNA binds the gene-specific transcription factor TFIIIA, which, consequently, is not available any more for initiation complex formation at the internal control region (Pelham & Brown, 1980). However, with such an analogy in mind, one would have to expect an even increased transcription of mutant 7S L genes, because their RNA product should not be able to sequester an essential initiation factor. Yet, this definitely is not the case. In addition, the competition experiments in Figure 5 indicate that the down-regulation by a large excess of exogenous 7S L RNA is much less efficient than the inhibition of transcription observed with propeller-mutants. Together, these data argue strongly against a simple model of sequestering an essential transcription factor. It rather appears that a close association of the nascent 7S L RNA with the transcription machinery is required.

Trans-acting region of 7S L RNA functionally resembles TAR

In certain aspects, the type of transcriptional activation described here is reminiscent of the Tat-TAR interaction involved in regulation of mRNA synthesis of the human immunodeficiency virus type 1 (Cullen, 1990; Karn & Graeble, 1992). Like the HIV-1 TAR element, the propeller motif of the human 7S L RNA is located at the 5′ end of the transcription unit and overlaps with the initiator element. No sequence homology is observed, but both structures include a hexanucleotide loop stabilized by a 4-bp stem. This hairpin is separated from a second duplex structure by a bulge. In contrast to the trinucleotide bulge of the TAR sequence, the two stem-loop elements of the 7S L RNA are separated by four unpaired nucleotides. However, in both cases, destabilization of the duplex structures and/or mutations of the bulge sequence are deleterious, yet without abolishing transcription completely.

Tat protein strongly stimulates transcription from the HIV-1 long terminal repeat (LTR) through recognition of the TAR element of the nascent RNA. Genetic and biochemical analyses revealed that trans-activation by Tat requires additional cellular factors (Madore & Cullen, 1993; Wu-Baer et al., 1995; Zhou & Sharp, 1995, 1996). Two different modes have been suggested for trans-activation by the Tat protein. In reactions in vitro, Tat primarily stimulates the formation of more-

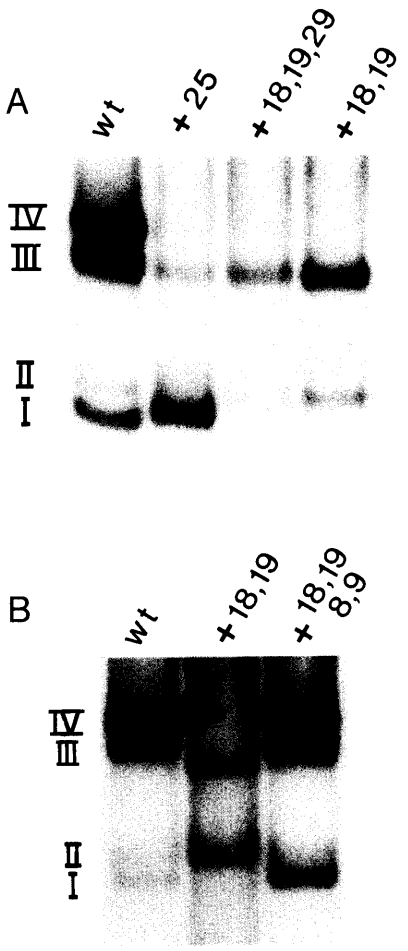

FIGURE 7. RNA mobility-shift analyses with different mutant 7S L RNAs. **A:** Labeled wt or mutant 7S L RNA (+51) was incubated with 10 μg of S100 protein and complexes analyzed as above. Positions of mutated nucleotides are indicated again by numbers. **B:** Same as A, however, in this experiment, the effect of compensatory mutations (+8,9/+18,19) was analyzed in comparison with wt and mutant (+18,19) RNAs.

processive elongation complexes (Marciniak & Sharp, 1991; Kato et al., 1992; Rittner et al., 1995). In addition, short RNA molecules that account for the majority of HIV transcripts have been observed in the absence of Tat (Kao et al., 1987; Muesing et al., 1987; Laspia et al., 1989; Ratnasabapathy et al., 1990; Feinberg et al., 1991). On the other hand, Tat has been reported to interact in vivo with DNA sequence-specific transcription factors, such as TBP (Kashanchi et al., 1994; Veschambre et al., 1995) and SP1 (Kamine & Chinnadurai, 1992; Jeang et al., 1993). Therefore, it was concluded that Tat stimulates both the initiation and elongation of transcription.

At present, it is not known whether the structure of the nascent 7S L RNA affects transcription at the level of initiation or elongation or both. It is quite conceivable that the described structural motif of the 7S L RNA interacts directly with factors of the transcription machinery or with RNA polymerase III itself. One possibility is that the propeller stimulates the formation of more-processive elongation complexes. On the other hand, at least in the context of the 7S K promoter, the 7S L RNA propeller motif is nonfunctional when displaced downstream of the transcription start site. This finding might point to an involvement in initiation complex formation. Furthermore, so far, we were unable to detect short RNA molecules transcribed from 7S L mutant templates, which would be indicative for stalled elongation complexes or abortive transcription. However, these results do not exclude the possibility of regulating 7S L RNA synthesis via processivity of the pol III complex.

Specific complexes of 7S L RNA with cellular proteins

The RNA mobility-shift experiments presented here indicate that at least two cellular proteins specifically recognize the propeller-like structure of 7S L RNA. Biogenesis of the signal recognition particle involves binding of proteins p9 and p14 to this sequence ele-

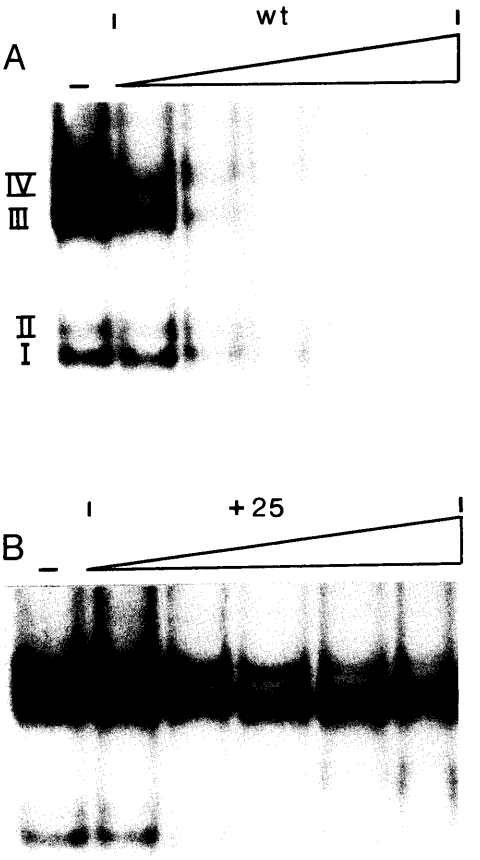

FIGURE 8. Competition assay for specificity of shifted wt RNA complexes. **A:** Labeled wt 7S L RNA (+51) was analyzed as in Figure 6 either without ("−") or with increasing concentrations of wt competitor RNA. The molar excess of unlabeled competitor RNA was 2-, 10-, 25-, 50-, and 100-fold, respectively. Shifted complexes again are numbered I–IV. **B:** Same as A, however, unlabeled bulge mutant (+25) RNA was used for competition.

ment. Because large amounts of free srp proteins have been found associated with the cytoplasmic fraction of HeLa cells (Andreazzoli & Gerbi, 1991; Strub et al., 1991), one might speculate that these proteins were involved. However, preliminary evidence suggests that the specific complexes observed with our truncated RNA were not due to recruitment of p9/p14 proteins. First, binding of these two srp proteins includes the 3′ end of 7S L RNA (Andreazzoli & Gerbi, 1991), which is absent in the RNA shifted. Secondly, indirect labeling of proteins bound to (+51) RNA and analyzed after UV crosslinking and RNase A digestion revealed two complexes with an apparent molecular mass of about 100 and 125 kDa, respectively (data not shown). In addition, the inhibitory effects described here were also observed with the (+25) bulge-mutant, a position that is not included in the p9/p14 binding site (Andreazzoli & Gerbi, 1991). Nuclear extracts have been found to contain only very small amounts of free p9/p14. Yet, supplementation of nuclear extracts with recombinant p9/p14 proteins had no stimulatory effect on in vitro transcription reactions. Finally, antibodies directed against human p9 protein (kindly provided by Dr. R. Maraia, NIH) had no effect, neither on in vitro transcription, nor on the shift of complexes (data not shown). Therefore, we conclude that the as yet unidentified regulatory proteins specifically recognizing the 5′ end of human 7S L RNA are not identical to these srp proteins.

Pol III transcription and viral infection

Increased levels of pol III transcription in response to regulatory proteins of several DNA viruses are well established (Gaynor et al., 1985; Hoeffler & Roeder, 1985; Jang & Latchman, 1989). This effect has been attributed to increased concentration or activity of the cellular pol III transcription factor TFIIIC. More recently, the HIV Tat protein has also been found to increase pol III transcription (Jang et al., 1992). Although this increase again was attributed to the activity of TFIIIC, it is interesting to note that, in these experiments, transcription of human repeated Alu sequences was analyzed. Within the region of interest, which is highly conserved among various Alu sequences, these Alu elements are closely related to the 7S L RNA gene studied here (Ullu et al. 1982; Ullu & Tschudi, 1984). Therefore, our findings may also contribute to the understanding of the evolution and biological significance of transcribed Alu sequences. In this context, it is interesting to note that this 5′ propeller structure of 7S L RNA was also found to be extremely conserved among various archaebacteriae (Kato, 1990).

Irrespective of whether or not the mechanisms and factors involved in transcriptional activation via the nascent 7S L RNA and the HIV TAR element are related, the results described here add a new facet to cellular gene expression. So far, the remarkable phenomenon of RNA sequence-mediated regulation of transcription has only been observed in the primate immunodeficiency viruses and in related nonprimate lentiviruses. Now it appears that this is a more general phenomenon that applies to cellular genes as well. One has to expect that those nonviral auxiliary factors required for Tat trans-activation must also possess some physiological function. It is quite conceivable that, for these proteins, the type of activation of 7S L RNA synthesis described here provides an analogous cellular function. In addition, one might expect that 7S L RNA will not remain the only small stable RNA species having adopted this principle of transcriptional activation.

MATERIALS AND METHODS

Templates

The basic clone referred to as wt 7S L RNA construct represents the full-length 7S L promoter (−178 to +145) fused to an adenoviral VA I cassette as reporter sequence (see arrow in Fig. 1A). This cassette contains the VA I RNA sequence from position +71 to +153, supplemented with the 7S K terminator region (*Hin*d III at +269 to *Eco*R I, 25-bp downstream of the 3′ end). With 308 nt, the length of transcripts derived from this template is close to the 299 nt of the authentic human 7S L RNA. The fusion of the VA cassette to the +145 site of 7S L RNA included a 9-bp linker-sequence containing an *Xba* I restriction site. For S1-nuclease protection analyses, this site was filled (Klenow enzyme) in a 3′ end-labeling reaction. Propeller mutations were obtained by introducing synthetic double-stranded oligonucleotides (synthesized in a Pharmacia GeneAssembler) between the "native" *Ava* I restriction site at position +41 and an artificial *Kpn* I site introduced in the 5′ flanking sequence around position −10. This site had been verified to represent a "neutral" mutation. For competition experiments and RNA mobility-shift analyses, transcripts synthesized in vitro were obtained by use of bacteriophage T7 RNA polymerase (as outlined by Melton et al., 1984), with 7S L RNA templates truncated at position +51 or +145, respectively.

Mutations introduced were at positions −9 to −7 (TCT → GTA); +8,9 (GC → CT); +12 to +15 (A-Box; TGGC → GTTA); +18,19 (GT → AG); +25 (T → C); +24,25,26 (GTA → CAT); +29 (C → G); +42 (G → C); and +44,45,46 (GGC → CCG), respectively.

Transfections

Hepatoma HepG2 cells cultivated under standard conditions were transfected for 36 h with plasmid DNA via calcium phosphate co-precipitation. For reasons of normalization, a reference plasmid (10 μg) was co-transfected together with the construct (15 μg) to be analyzed. The reference construct consisted of the human 7S K promoter fused to the procaryotic pAT153 vector sequence described previously (construct II in Kleinert & Benecke, 1988). Extraction of cellular RNA, hybridization to 3′ end-labeled probes, S1-nuclease digestion,

and analysis of protected fragments in 6% polyacrylamide gels containing 8 M urea was as described in Sambrook et al. (1989). Exposure of the dried gels to X-ray films for 6 h was in the presence of a Cronex intensifier screen.

In vitro transcription reactions

Nuclear extracts (8–10 mg/mL protein) were prepared from HeLa cells as described in detail by Dignam et al. (1983). Five-hundred nanograms of plasmid DNA were transcribed in 10 μL of nuclear extract in the presence of 70 mM KCl, 16 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, and 1 μg/mL of α-amanitin. Reactions in a final volume of 50 μL were started by addition of nucleotides (0.5 mM each of ATP, GTP, CTP, and 10 μCi of [α^{32}-P]UTP) and incubated for 1 h at 30 °C. An excess of 1 μg of unlabeled T7 RNA polymerase transcripts as competitor RNAs was preincubated with extract proteins for 15 min at 37 °C, prior to addition of template. Analysis of phenol-extracted RNA in denaturing gels was as described above.

RNA binding assays

RNA mobility-shift analyses were performed with HeLa cell S100 extract proteins (20 mg/mL protein; Weil et al., 1979) or nuclear extracts (see above) in the presence of 150 mM KCl, 20 mM HEPES-KOH, pH 7.9, 4 mM $MgCl_2$, 1 μg of tRNA and 6% (v/v) glycerol. Routinely, 7.5 μg of extract protein, together with 6 ng of labeled RNA, were kept for 10 min on ice, followed by incubation for another 10 min at 37 °C. Complexes were analyzed in the presence of 0.25 × TEB [1×: 89 mM Tris, 89 mM boric acid, 2 mM EDTA] in native 6% polyacrylamide gels. In competition experiments, unlabeled RNA was added together with the labeled probes at the concentrations indicated in the legends to the figures. Electrophoresis was for 180 min at 150 V and 10 mA. Exposure of dried gels to the film was for 2 h at −70 °C, using an intensifier screen.

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