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Attenuation in SV40 as a mechanism of transcription-termination by RNA polymerase B

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ABSTRACT

Nuclei which were isolated from SV40 infected cells with a hypotonic detergent-free buffer were used to establish in vitro conditions which lead to transcription-termination at the attenuation site of SV40. This system allowed us to identify regulatory elements involved in transcription-termination by RNA polymerase B transcribing SV40. Transcription-termination at the attenuation site was found to be ionic strength dependent. Efficient termination occurred at low (100 mM NaCl) but not at high (100 mM $(NH_4)_2 SO_4$ or 300 mM NaCl) ionic strength. When nuclei were prewashed with 300 mM NaCl, the efficiency of transcription-termination was low even when transcription was carried out at low ionic strength (100 mM NaCl). Efficient transcription-termination in the high salt prewashed nuclei was reconstituted by complementation with a high salt (300 mM NaCl) soluble factor extracted from nuclei of uninfected cells. In addition, the efficiency of transcription-termination was significantly reduced when ITP replaced GTP in the transcription reaction mixture. Our data indicate that a nuclear factor and RNA secondary structure are essential regulatory elements involved in transcription-termination by RNA polymerase B.

INTRODUCTION

In procaryotes, transcription-termination within as well as at the end of the operon plays an important role in regulating gene expression (for review see 1-3). Transcription-termination at the end of the operon prevents transcription of neighbouring genes, while transcription-termination within the operon, termed attenuation, causes premature termination of the transcripts and quantitatively regulates the level of gene expression (3). The sequences at the termination and attenuation site share common features. The site of termination is AT-rich while the DNA immediately preceding it is GC-rich and possesses dyad symmetry. The RNA transcript of this region can be folded into a GC-rich stem-and-loop structure followed by U-residues. Protein factors are also involved in the process of transcription-termination (for review see 1,2,4,5).

In eucaryotes, despite several attempts, transcription-termination sites

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of RNA polymerase B transcripts have not been clearly identified (6-8). Consequently, the regulatory elements involved in the process of transcription-termination could not be properly investigated, and they are almost unknown. The main reason for this failure is the immediate processing which occurs at the 3' end of most of the RNA polymerase B primary transcripts which complicates identification of the site of transcription-termination (9). This difficulty was partially overcome by the following approaches: (i) the use of mRNAs which lack polyA, such as histones mRNAs (7,8); (ii) the use of isolated nuclei in which the rates of transcription and processing in vitro are much slower than in vivo, but in which it is possible to obtain sufficiently high specific activity of RNA (6,9), and (iii) the use of the process of premature-termination at a defined attenuation site. Regarding the last point it is important to note that we have recently identified a site at which transcription of SV40 late RNA is attenuated in vitro (10,11). The DNA sequences where RNA synthesis is attenuated were found to be strikingly similar to those found at termination sites in procaryotes (12). However, in these studies the in vitro conditions which lead to a complete transcription-termination, in contrast to a long pause of the polymerase, have not been established.

In the present study we characterize a system of isolated nuclei in which an efficient transcription-termination at the SV40 attenuation site is occurring. This system allows us to start defining the regulatory elements involved in the mechanism of transcription-termination. The results reported here indicate the involvement of a nuclear factor and RNA secondary structure in the process of transcription-termination by RNA polymerase B.

MATERIALS AND METHODS

Preparation of transcription reaction mixture, in vitro transcription, purification of the viral RNA and its analysis by gel-electrophoresis

BSC-1 cells were infected with SV40 (30-50 PFU/cell) as described (13). At 42-48 h post infection (p.i.) the cells were washed and collected in cold hypotonic buffer (50 mM Tris-HCl pH 7.9, 1.5 mM $MgCl_2$, 1 mM DTT) by centrifugation for 1 min at 1000g. Nuclei were isolated by resuspending $5x10^7$ cells in 10 ml of hypotonic buffer and pipeting up and down 10 times with a Pasteur pipette, followed by centrifugation for 2 min at 1000g. This step was repeated twice. High salt washed nuclei were prepared by resuspending the nuclei in 5 ml of 300 mM NaCl, 50 mM Tris-HCl pH 7.9, 1.5 mM $MgCl_2$, 1 mM DTT followed by centrifugation for 2 min at 1000g. The nuclei (approx-

imately $3x10^7$) were suspended in the transcription reaction mixture containing 5 mM KCl, 1.5 mM $MnCl_2$, 1 mM DTT, 12.5% glycerol and salt as indicated in the Fig. legends, 30 mM Hepes-NaOH pH 7.9, 400 µM each of ATP, GTP and CTP, 10 µM UTP, and 300-500 µCi of (α-^{32}P) UTP (400 mCi/mmole, Amersham, England), in a final volume of 0.6 ml. Transcription was carried out at 30°C for times as indicated in the Figs. legends, and stopped by the addition of 100 µg/ml RNase free DNase (14) for 1 min at 30°C followed by extraction with phenol-chloroform (15). The aqueous fraction was passed through G-25 Sephadex syringe column to remove free labeled nucleotides. After ethanol precipitation, the precipitate was resuspended in TKM (25 mM KCl, 2.5 mM $MgCl_2$, 50 mM Tris-HCl pH 6.7), 200 µg/ml of RNase free DNase were added, and incubation was carried out for 1 hr at 4°C. The DNase treatment was stopped by phenol-chloroform extraction and the RNA was precipitated with ethanol. SV40 specific RNA was isolated by hybridization to and elution from SV40 DNA on nitrocellulose filter. The hybridization was carried out in 70% formamide, 0.3 M NaCl, 10 mM Hepes-NaOH, pH 7.5, 1 mM EDTA and 0.1% SDS at 37°C for 40-48 h. At the end of the incubation time the filters were washed once with 0.5xSSC (SSC = 0.15 M NaCl /0.015 M Na citrate) and incubated in 35% formamide 0.01 M NaCl, 1 mM EDTA, 10 mM Hepes-NaOH pH 7.5 at 37°C for 30 min, and then washed 3 times with 0.5xSSC. The bound RNA was eluted by incubating the filter in 90% formamide 10 mM Hepes-NaOH pH 7.9 for 1 min at 90°C and subjected to electrophoresis on a 12% polyacrylamide gel (bis/acrylamide 1:29) in 7 M urea and TBE buffer (0.09 M Tris-HCl pH 8.3, 0.09 M boric acid, 2.5 mM EDTA).

Preparation of a nuclear extract containing a putative transcription-termination factor

Nuclei were prepated from BSC-1 cells using the hypotonic buffer procedure as described above. Approximately $2x10^8$ nuclei were resuspended in 8 ml of 300 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, 30 mM Hepes-NaOH pH 7.9 and 12.5% glycerol. After pipeting up and down 20 times with a Pasteur pipette, the nuclei were removed by centrifugation for 5 min at 1000g and the supernatant was collected. This step was repeated twice. The two supernatants were combined and concentrated by vacuum dialysis against 100 mM NaCl, 5 mM KCl, 1 mM DTT, 0.5 mM PMSF, 30 mM Hepes-NaOH, pH 7.9 and 12.5% glycerol using vacuum dialysis bags (Schleicher and Schull, Ultra-Thimbles UH). The concentrated supernatants were designated high salt soluble fraction ("HSF"). About 1 ml of "HSF" was prepared from approximately $2x10^8$ cells. The "HSF" was stored at -80°C.

RESULTS

The effect of ionic strength on the production of the attenuator RNA

We have recently shown that pulse-labeling with (α-^{32}P) UTP of SV40 transcriptional complexes (VTC), viral minichromosomes, nuclear matrices or isolated nuclei of SV40-infected cells leads to the production of 94-98 nt attenuator RNA (10,11,16-18). Fig. 1 shows the sequence, residue numbers and the two alternative conformations of the attenuator RNA. However, in these studies we have not established *in vitro* conditions which lead to a real and efficient transcription-termination as opposed to a pause of the RNA polymerase at the attenuation site. To this end nuclei were isolated from SV40-infected cells and incubated *in vitro* in the presence of various salt concentrations under "pulse" and "pulse-chase" conditions. It is assumed that if transcription-termination occurs at the attenuation site then the amount of the attenuator RNA synthesized during the "pulse" should remain constant following the "chase" periods.

Fig. 2 shows the results of these experiments in which the incubation mixture contained salt of either high (100 mM $(NH_4)_2$ SO_4) or low (100 mM NaCl) ionic strength. In the presence of 100 mM $(NH_4)_2$ SO_4 following a 5 min pulse with 0.3 µM (α-^{32}P) UTP a major intense band of 94 nt is revealed

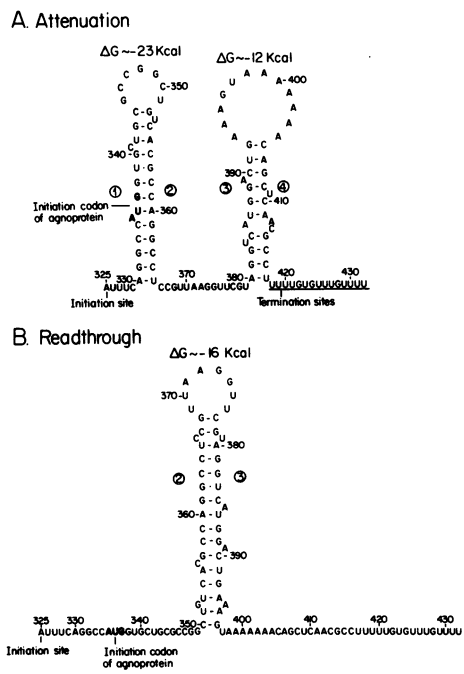

Fig. 1. Schema of alternative conformations in the attenuator RNA
(A) Attenuation conformation showing typical pausing (1+2) (16) and termination signals (3+4) (10,11).
(B) Readthrough conformation. For details see Hay et al. (10). The ΔG were calculated as described by Tinoco et al. (37). The residue number is from Tooze (38).

(Fig. 2A,P). However when the "pulse" was followed by a 10 min "chase" the majority of the radioactivity in the 94 nt band disappeared and appeared at the upper part of the gel (Fig. 2A, C_1). Only a small fraction (~10%) of the 94 nt RNA was not chased. These results indicate that during the pulse the majority of the RNA polymerase molecules paused but did not terminate transcription at the attenuation site. A major band of 94 nt was also produced following a 5 min "pulse" in the presence of 100 mM NaCl (Fig.2B,P). However, in contrast to incubation at high ionic strength, "chase" for 10 or even 30 min under the present conditions had almost no effect on the intensity of the 94 nt band (Fig. 2B, C_1 and C_2). These results indicate a real and efficient transcription-termination rather than pausing of the RNA polymerase molecules at the attenuation site at low ionic strength incubation.

The results of Fig. 3 provide additional evidence for a real transcription-termination at the attenuation site under conditions of low ionic strength. In this experiment isolated nuclei were incubated in the presence of 10 μM (α-^{32}P) UTP for 20 min. It is evident that the 94 nt attenuator RNA was produced when the incubation mixture contained 100 mM NaCl (Fig. 3A) but not when it contained 100 mM $(NH_4)_2$ SO_4 (Fig. 3B). Note the production of a ~64 nt band in Fig. 3B. In a separate study we have characterized it

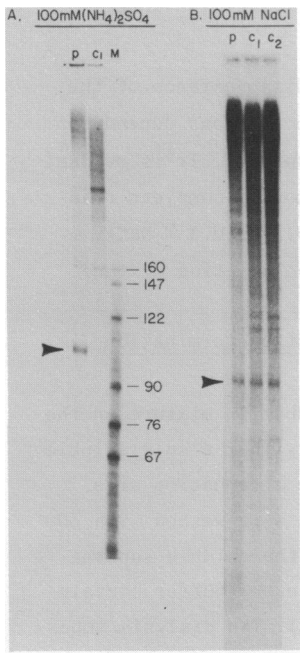

Fig. 2. Size analyses of viral RNAs produced in isolated nuclei incubated in the presence of salt of high (A. 100 mM $(NH_4)_2$ SO_4) and low (B. 100 mM NaCl) ionic strength under "pulse" and "pulse-chase" conditions

(P) nuclei were incubated in a transcription reaction mixture containing 100 μCi (0.3 μM) of (α-^{32}P) UTP for 5 min ("pulse") as described in Materials and Methods. (C_1), nuclei were incubated as in (P) but following the "pulse" 20 μM of unlabeled UTP were added and transcription continued for 10 min ("chase"). (C_2), as in (C_1) but transcription in the presence of unlabeled UTP continued for 30 min. The labeled RNAs produced were purified and analyzed by gel electrophoresis. The arrows point to the position of the 94 nt attenuator RNA. M - size markers obtained by end labeling of the Hpa II restriction fragments of pBR322 DNA.

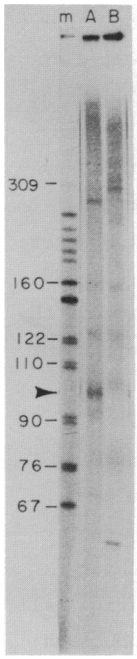

Fig. 3. Size analyses of viral RNAs produced in isolated nuclei, incubated in the presence of salt of high and low ionic strengths for a long duration

Nuclei were incubated in a transcription reaction mixture containing 10 μM (α-^{32}P) UTP for 20 min in the presence of salts of low (100 mM NaCl) in A or high (100 mM $(NH_4)_2$ SO_4) in B, ionic strengths. The labeled RNAs produced were purified and analyzed by gel-electrophoresis. The arrow points to the position of the 94 nt attenuator RNA. m - size markers as in Fig. 2.

as the SAS RNA (19) (manuscript in preparation). The production of the SAS RNA indicates that our isolated nuclei system can faithfully synthesize viral RNA.

Fig. 4 shows that readthrough which leads to the disappearance of the 94 nt band during the "chase" or prolonged in vitro incubations depends on the ionic strength and is not specific for ammonium sulfate. It is evident that the 94 nt band also disappeared and there was almost a complete readthrough when the incubation mixture contained either 0.2 or 0.3 M NaCl. In the following experiments the standard transcription reaction mixture contained 100 mM NaCl and 10 μM UTP.

Possible involvement of a cellular factor in transcription-termination at the attenuation site

The effect of high salt concentrations in the incubation mixture on the production of the attenuator RNA raised the possibility that a salt soluble factor is involved in transcription-termination at the attenuation site. To verify this possibility, nuclei of uninfected cells were washed with 300 mM NaCl, precipitated by low speed centrifugation and the soluble supernatant fraction was concentrated by vacuum dialysis against a buffer containing 100 mM NaCl (for details see Materials and Methods). Two dialysis tubes

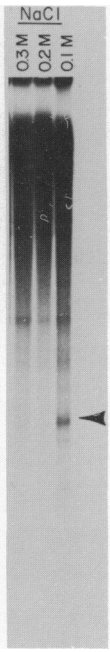

Fig. 4. Size analyses of viral RNAs produced in isolated nuclei in the presence of various salt concentrations

Nuclei were incubated in a transcription reaction mixture containing 10 μM (α-^{32}P) UTP for 15 min in the presence of various salt concentrations, as indicated. The labeled RNAs produced were purified and analyzed by gel-electrophoresis. The arrow points to the position of the 94 nt attenuator RNA.

were used, one with a M.W. cut-off of 10k and the second with a M.W. cut-off of 75k. We designated these high salt soluble fractions "10 kd HSF" and "75 kd HSF", respectively. In order to determine whether these fractions contain a transcription-termination factor, nuclei of SV40-infected cells were washed with 300 mM NaCl and divided into four groups. The first contained the high salt prewashed nuclei (Fig. 5a), the second contained the same as the first but in addition the "10 kd HSF" (Fig. 5b), the third contained the same as the first but in addition the "75 kd HSF" (Fig. 5c) and the fourth contained the same as the third but the "75 kd HSF" was heated at 80°C for 10 min before adding it to the high salt prewashed nuclei (Fig. 5d). Following preincubation of the nuclei with "HSF" for 5 min at 20°C, the various components of the standard transcription reaction mixture were added and transcription was allowed to proceed for 15 min. Fig. 5a shows that washing the nuclei with high salt (300 mM NaCl) before incubating the nuclei at low salt (100 mM NaCl) abolished the production of the attenuator RNA (see Figs. 2, 3 and 4). Fig. 5 (b and c) show that transcription-termination at the attenuation site was restored upon addition of the two "HSF". The results of Fig. 5d show that the activity of the "HSF" was inactivated by heating.

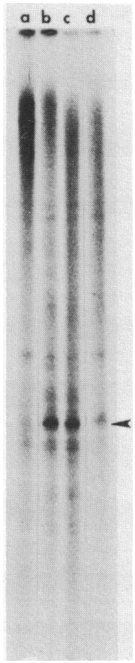

Fig. 5. Size analyses of viral RNAs produced in isolated nuclei with and without the presence of "HSF"

Isolated nuclei were washed with 300 mM NaCl and preincubated for 5 min either without "HSF" (a) with "10 kd HSF" (b) or with "75 kd HSF" (c). (For details see Materials and Methods and the text). (d) is the same as (c) but the "75 kd HSF" was heated at 80°C for 10 min before addition to the strandard transcription reaction mixture. "HSF" extracted from $1x10^8$ nuclei was added to $3x10^7$ nuclei. Incubation was then carried out for 15 min at standard reaction mixture. The labeled RNAs produced were purified and analyzed by gel-electrophoresis. The arrow points to the position of the 94 nt attenuator RNA.

The interpretation of the above results is that a nuclear factor or a multimer of it having a M.W. higher than 75k and which is heat labile is involved in transcription-termination by RNA polymerase B. It is worth noting that Leer et al. (20) have found a high salt soluble factor which is involved in transcription-termination of ribosomal RNA (i.e. a polymerase A transcript), and Stunnenberg et al. (8) have suggested that a high salt soluble factor is involved in transcription-termination of histone mRNA (i.e. a polymerase B transcript).

The secondary structure of the attenuator RNA is involved in transcription-termination

The resemblance of the possible secondary structure of the attenuator RNA (see Fig. 1) to the termination signal of the procaryotic polymerase (12) and the observation that the intercalating drug, proflavine, abolishes attenuation (10) led us to suggest that the secondary structure of the attenuator RNA is involved in pausing (16) and in transcription-termination (10-12, 16-18,21) of the RNA polymerase B which transcribes SV40. In the following experiment we have examined this suggestion more directly, by replacing GTP with its analog ITP in the standard transcription reaction mixture. In bacteria and bacteriophages, ITP was shown to abolish transcription-termination

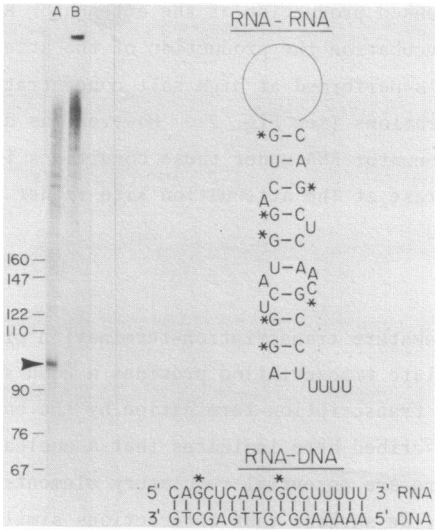

Fig. 6. Size analyses of viral RNAs produced in isolated nuclei incubated in the presence of GTP or ITP in the transcription reaction mixture

A - a standard reaction mixture. B - ITP (400 μM) replaced GTP in the standard transcription reaction mixture. Transcription was allowed to proceed for 15 min. The labeled RNAs produced were purified and analyzed by gel-electrophoresis. The arrow points to the position of the 94 nt attenuator RNA. Possible RNA-RNA and RNA-DNA interactions at the 3' end of the attenuator RNA (see Fig. 1) are also represented. The (*) indicates a G-residue which was replaced by an I-residue during transcription in the presence of ITP. The position of the size markers, as in Fig. 2, are shown on the left side of the autoradiogram.

by destabilizing RNA stem-and-loop structure (22-25).

As shown in Fig. 6 there was a significant increase in readthrough transcripts when ITP replaced GTP in the standard reaction mixture. Incorporation of ITP into RNA can reduce the stability of either RNA-DNA or RNA-RNA hybrids. In the case of SV40 attenuator RNA, it is evident that incorporation of ITP has a much higher effect on the stability of the RNA-RNA as compared to the RNA-DNA hybrid.

From the illustration of the base-pairing in Fig. 6 it is evident that in the RNA-DNA hybrid the incorporation of ITP can destabilize only 2 of the 8 G-C bonds immediately preceding the attenuation site. In the RNA-RNA duplex the incorporation of ITP can destabilize all the G-C bonds, thus eliminating the entire RNA secondary structure. This may suggest that it is the RNA stem-and-loop structure which is essential for transcription-termination.

High ionic strength can also stabilize RNA secondary structure and

therefore lead to an increased production of the attenuator RNA. Indeed, during a short in vitro incubation the production of the attenuator RNA is enhanced when incubation is performed at high salt concentrations as compared to low salt concentrations (see Fig. 2). However, as discussed above, the production of the attenuator RNA under these conditions is due to pausing of the RNA polymerase at the attenuation site rather than to transcription-termination.

DISCUSSION

The existence of a premature transcription-termination process, i.e. attenuation, during SV40 late transcription provides a good system for studying the mechanism of transcription-termination by the eucaryotic RNA polymerase B. The work described here indicates that a nuclear factor and the RNA secondary structure are essential regulatory elements involved in transcription-termination. We suggest that interactions similar to those involved in procaryotic transcription-termination (5) operate in eucaryotes. We further suggest that, similar to the mechanism of transcription-termination in procaryotes, the intramolecular hairpin structure in the RNA transcript (resulting from dyad symmetry in the template) impedes the progress of the RNA polymerase and leads it to pause at the start of the U-residues which follow it. Whether the RNA polymerase pauses or terminates transcription probably depends on a delicate balance between the rate of formation of the RNA hairpin and the stability of the rU-dA hybrid region (5). This balance can be affected by the in vitro conditions. Indeed, we have observed in the present studies that a combination of low UTP concentration and high ionic strength enhances the pause of the RNA polymerase at the terminator. Transcription-termination is however inefficient. The most efficient transcription-termination was found to occur with a combination of limited UTP and low ionic strength. We therefore speculate that high ionic strength in the transcription reaction mixture has two effects: (i) it stabilizes the RNA hairpin structure which enhances pausing of the RNA polymerase and (ii) it removes a nuclear factor which is involved in transcription-termination and therefore reduces the efficiency of the termination process. At low ionic strength transcription-termination is efficient due to the formation of the RNA hairpin structure, the exceptionally unstable rU-dA interaction, and the presence of a termination factor. The termination factor apparently brings the enzyme to a complete stop, and leads to the release of the RNA transcript from the template. The increased termination observed

in the present study, at low UTP concentration, is presumably due to the slower elongation rate which increases the duration of the pause. As a result there could be a better chance for the termination factor to interact with the RNA polymerase and to lead it to terminate transcription. That the concentration of the ribonucleotides may influence the extent of termination has also been observed in procaryotes (26).

Support for the conclusion that the eucaryotic polymerase B can respond to the procaryotic terminator is provided by the observation that in a cell free eucaryotic system, the bacteriophage lambda 4S RNA terminator caused human polymerase B to pause on the template and to partially terminate transcription of transcripts initiated at the adenovirus 2 major late promoters (27). The observation that only partial termination occurred and that the polymerase failed to respond to the lambda t_{int} and t_{R1} terminators may indicate the lack of a termination factor in the in vitro system and/or the involvement of a specific sequence in the termination process.

Similar terminators were shown to occur at the 3' end of other polymerase B transcripts, such as in the minute virus of mice (MVM), an autonomous parvovirus (28), and at the 3' end of human U_1 RNA (29). It is worth noting that a termination site has been identified for the mouse β-major globin gene that is transcribed by RNA polymerase B. This site is located about 1000 bases downstream from the poly(A) site, but the nature of the terminator is still unknown (6). The mitochondrial polymerase appears to respond to a similar termination signal at the 3' end of the 16S rRNA (30); likewise, polymerase C responds to a similar terminator at the 3' end of the VA RNA in Ad (31). Moreover, based on the DNA sequence of several viruses we have suggested that there is a potential of forming terminators at promoter proximal regions (12). Terminators which contain only the hairpin but not the run of Us or only the run of Us were also reported. Birchmeier et al. (7) have shown that the 3' terminus of histone mRNA may be defined by dyad symmetry 30 to 40 bases downstream from the protein stop codon. These sequences are necessary for generating the 3' terminus, although downstream sequences are also involved. AT-rich dyad symmetries with no U-residues following them were also found at regions involved in transcription-termination of various yeast genes (32). This terminator resembles the rho dependent terminator in bacteria. The run of the Us was shown as an essential element for RNA polymerase C transcription-termination (33). Based on these observations we suggest that there are several mechanisms for transcription-termination in eucaryotes. The SV40 attenuator resembles the

rho independent terminator of procaryotes. Nevertheless, the finding that a cellular factor is involved in transcription-termination at the SV40 attenuator is not surprising, because it is possible that in procaryotes the rho factor actually is needed to recognize these signals in the bacterium and it enhances termination in vitro (1). It is interesting to note that in order to see the effects of the rho factor and the cellular factor in vitro, it is necessary to use conditions of relatively low ionic strength.

It has been previously shown that a cellular factor with an estimated M.W. of 50-100k is involved in transcription-termination of rRNA in Tetrahymena-a polymerase A transcript (20). Similarly, a cellular factor with estimated M.W. of 200-250k was needed to generate the 3' end of histone mRNA, a polymerase B transcript (8). Both termination factors were extracted from nuclear fractions with high salt buffers. Similar to the rho factor these eucaryotic transcription-termination factor(s) could be active in multimeric form (1). Our factor as well as the others are still crude, and further purification and characterization are needed to determine whether there are more than one eucaryotic transcription-termination factors. In this regard it is interesting to note that, based on idiosyncrasies in termination efficiencies of histone gene transcription in xenopous oocyte and HeLa cells, Bendig and Hentschel have suggested the presence of termination factors which are relatively species or even tissue specific (34).

We would like to mention the possibility that the SV40 attenuator is functioning also as a terminator when the RNA polymerase reaches it again during transcription of the circular genome. A dual function of the SV40 terminator is supported by the observation that, following injection of SV40 DNA into xenopous oocytes, RNA species corresponding in lengths to the 94-98 nt attenuator RNA (J. Mertz, personal communication) and to a genome length transcript accumulate (35).

Finally, we have recently presented a model in which attenuation and modulation of mRNA secondary structure in a feedback control mechanism regulate SV40 gene expression (10,21). In this model we have suggested that the agnoprotein (36) stabilizes the attenuation conformation (see Fig. 1) and enhances transcription-termination. We now have experimental evidence for this speculation (Hay and Aloni, manuscript in preparation). It should therefore be remembered that while attenuation provides a model to study the mechanism of transcription-termination, it is controlled by more specific regulatory factors.

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