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Site of premature termination of late transcription of simian virus 40 DNA: Enhancement by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole

(isolated nuclei/simian virus 40 transcriptional control/attenuation/transcription termination/*in vitro* transcription)

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ABSTRACT **Sedimentation analysis of pulse-labeled RNA synthesized in nuclei isolated from simian virus 40-infected cells revealed an abundance of short cellular and viral RNAs. The relative amount of the short chains is increased in nuclei isolated from cells treated with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). The short viral RNAs were purified by hybridization to and elution from simian virus 40 DNA on filters, and their sizes were determined by gel electrophoresis. A major band of 93- to 95-nucleotide-long RNA was observed along with additional minor bands. Identical bands were revealed when the viral RNA was purified from nuclei of cells pretreated with DRB. The major band was identified as an aborted transcript of a RNA that initiated at the major initiation site (nucleotide 243). We have found that the DNA region where the RNA stops is A+T rich and is immediately preceded by a G+C-rich region that exhibits dyad symmetry, resembling the termination signal in prokaryotes. These observations show that RNA polymerase II responds to the same termination signal as the prokaryotic enzyme and suggest that a mechanism of attenuation regulates simian virus 40 late transcription.**

Transcription-termination sites in prokaryotes have several common features: G+C-rich sequences preceding the stop site, from which a stem-and-loop structure can be formed, and uridine residues in the terminus of the RNA transcripts (for review, see refs. 1–7). It has been suggested that the stem-and-loop structure causes retardation of polymerase movement through the termination region, whereas the uridine residues facilitate the release of the transcripts (8). This implies a general mechanism for transcription termination which is based in part on the physical chemistry of nucleic acid interactions. In eukaryotes, the mechanism of termination of RNA polymerase II transcription is not yet known, and the sequences that constitute terminators have not been determined.

In prokaryotes, transcription termination can occur within as well as at the end of the operon. Termination within the operon causes premature termination of the transcripts and quantitatively regulates the level of gene expression, by selectively reducing the transcription of distal portions of the operon. This mechanism has been termed "attenuation" (3). It is thought that regulation is achieved by altering the frequency with which transcription crosses the termination site.

It has been suggested that the attenuation mechanism also quantitatively regulates gene expression in eukaryotes (9–15). Quantitative transcriptional changes that occur during the cell cycle and cell differentiation have been attributed to this mechanism (16). Observations of aborted RNA in whole nuclear RNA (9, 10), in adenovirus type 2 (Ad-2) (11–13), and in simian virus 40 (SV40) (14, 15) provided experimental support for the existence of the attenuation mechanism in animal cells. Moreover, these studies have shown that the adenosine analog 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) enhances premature termination. It has not been determined, however, whether the premature termination occurs at unique sequences and whether DRB augments a normal mechanism of transcription termination.

The determination of the complete SV40 DNA sequences and the localization of the major and minor initiation sites of SV40 late transcription (17–22) have enabled us to define precisely the sequences where premature termination occurs, both without and with DRB treatment. We have noted that these sequences resemble transcription termination signals in prokaryotes. Our data suggest that an attenuation mechanism regulates SV40 late transcription.

MATERIALS AND METHODS

Growth of plaque-purified SV40 on BSC-1 monkey cells as well as concentration and purification of the virus from tissue culture lysates and preparation of SV40 DNA component I have been described (23). In these experiments, BSC-1 cells were infected with 50–100 plaque-forming units (pfu) of stock 777 SV40 per cell.

Nuclear fractions were prepared from infected cells with Nonidet P-40 as described (24), and RNA was extracted from nuclei with phenol/chloroform/isoamyl alcohol (23).

Purified nuclei capable of cell-free transcription were resuspended in 0.25 ml of 0.1 M $(NH_4)_2SO_4$/6 mM KCl/5 mM Hepes, pH 8.0/12.5% (vol/vol) glycerol containing ATP, CTP, and GTP at 0.4 mM each and 0.1 mCi of [α-^{32}P]UTP (100–400 Ci/mmol, Amersham; 1 Ci = 3.7×10^{10} becquerels). Incubation was carried out at 26°C. [^{32}P]RNA products were extracted as described (23).

For the preparation of restriction endonuclease fragments, SV40 DNA was cleaved with *Eco*RI, *Hpa* I, and *Bgl* I, and the fragments were separated by electrophoresis on 1.4% agarose gels. Transfer from the gel to nitrocellulose paper (Schleicher & Schuell BA85) was carried out by using the technique of Southern (25). Alternatively, for isolation of DNA fragments from the gel, the agarose in the corresponding bands was crushed and the DNA was eluted by shaking in 0.2 M NaCl/10 mM Tris·HCl, pH 7.4/5 mM EDTA. After 18 hr of incubation at room temperature, the suspension was filtered and extracted with phenol and the DNA was precipitated with ethanol. Recovery was 70–90%. The separated DNA fragments

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Abbreviations: SV40, simian virus 40; m.u., map unit (1 m.u. is the equivalent of 0.01 of the viral DNA length); Ad-2, adenovirus type 2; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; hnRNA, heterogeneous nuclear RNA; pfu, plaque-forming units.

were immobilized on nitrocellulose membrane filters and were used for hybridization as described (26).

For gel electrophoresis, RNA was dissolved in 3 μl of borate buffer (0.1 M Tris·HCl pH 8.3/0.1 M boric acid/2 mM EDTA) plus 7 μl of 10 M urea containing xylene cyanol marker. The sample was heated to 65°C for 5 min, cooled, and applied to a 10% polyacrylamide gel (bis/acrylamide, 1:19) in 7 M urea and borate buffer. Reservoir buffer was recycled and the electrophoresis was continued until the xylene cyanol was 20 cm from the origin (27).

RESULTS

Length Distributions of Mature and Nascent Cellular and Viral RNAs in Nuclei of SV40 Infected Cells. At 45 hr after infection, cells were labeled for 3 hr with [^{3}H]uridine. Nuclei were isolated and incubated *in vitro* for 1.5 and 5 min in the presence of [α-^{32}P]UTP, to allow elongation of preinitiated nascent RNA chains (26). The double-labeled RNAs were extracted and analyzed by velocity sedimentation through sucrose gradients and by blot hybridization to restriction fragments of SV40 DNA. Fig. 1 shows the profile of the *in vivo* [^{3}H]uridine- and *in vitro* [^{32}P]UMP-labeled RNAs. It is apparent that the *in vivo* labeled cellular and viral RNAs show typical size distributions of heterogeneous nuclear RNA (hnRNA) and viral RNA (23), respectively, indicating that the *in vitro* incubation did not cause any detectable RNA degradation. The profiles of the ^{32}P-labeled cellular and viral RNAs exhibit a prominent peak of short nascent chains sedimenting at about 5 S. Faster-sedimenting RNA components also were seen. A similar profile of nascent RNA was reported previously in HeLa cell nuclei (9, 10). The profile of the nascent viral RNA confirms our previous observations that promoter-proximal short RNA chains accumulate in a higher ratio than from any other region of the viral DNA (15). Indeed, on hybridization of the RNA to SV40 restriction fragments, the labeled viral RNA present in the 5 S peak hybridized predominantly to fragment e, that spans 0.67–0.76 map units (m.u.) and includes the initiation sites for late transcription (14, 21, 22, 29). Longer viral RNA transcripts hybridized to DNA fragments more distal to the initiation sites. Because there is almost no initiation of transcription by RNA polymerase B in the *in vitro* system (9, 26) and the rate of elongation is slow (26) we interpret these results as indicating that RNA polymerase pauses *in vivo* in a promoter-proximal region, leading to accumulation of short nascent chains. These chains are then elongated by a few nucleotides during the short *in vitro* incubation. Fig. 2 shows that accumulation of the short chains was not due merely to limited concentration of [α-^{32}P]UTP in the incubation mixture because [^{32}P]UMP incorporation was almost linear for the first 5 min. Moreover, similar results were obtained when the concentration of UTP in the reaction mixture was increased 10-fold.

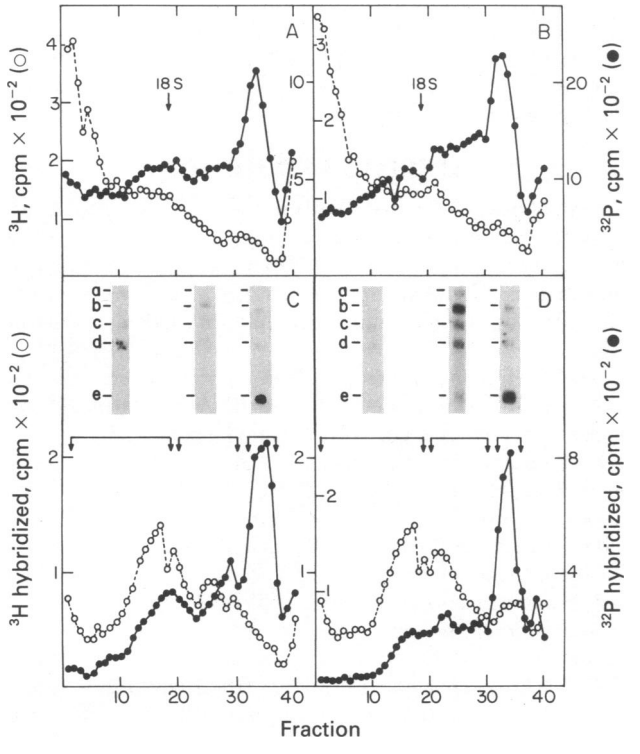

FIG. 1. Analysis of viral RNA by sedimentation through a sucrose gradient and blot hybridization. At 48 hr after infection, cells were labeled with [^{3}H]uridine for 3 hr. Nuclei were prepared and labeled *in vitro* with 100 μCi of [α-^{32}P]UTP for 1.5 min (*A*) and 5 min (*B*). The RNA was extracted, denatured in formamide, and sedimented through 15–30% sucrose gradients in NaDodSO$_4$ buffer (28) (25,000 rpm for 22 hr in the Spinco SW 27.1 rotor at 21°C). The position of 18 S was determined from the sedimentation of ^{32}P-labeled rRNA in a parallel tube. Aliquots (20 μl) of each fraction were precipitated with trichloroacetic acid and assayed. Aliquots (150 μl) of each fraction were hybridized to nitrocellulose filters bearing SV40 DNA (1 μg) for 1.5 min (*C*) or 5 min (*D*). (*Insets*) Results of hybridization of the pooled fractions, as indicated, with filter blots containing the five *Eco*RI, *Hpa* I, and *Bgl* I restriction fragments as in Fig. 4*A*.

Promoter-Proximal Discrete RNA. In order to examine the length of ^{32}P-labeled short RNA chains, isolated nuclei of SV40 infected cells were labeled *in vitro* in the presence of [α-^{32}P]UTP for 3, 8, and 13 min. ^{32}P-Labeled RNAs were extracted, viral RNAs were purified by hybridization to and elution from SV40 DNA on filters, and the ^{32}P-labeled viral RNAs were subjected to gel electrophoresis (27). One major band in a position corresponding to the length of 93–95 nucleotides could be recognized in the three RNA preparations (Fig. 3). Longer exposure of the gel to x-ray film permitted detection of additional minor bands of short RNA. Long RNA molecules remained at the origin of the gel. It also appears that, with the increase in labeling time, longer RNA molecules were synthesized in addition to the major band. This suggests the existence of at least two viral RNA species, one terminating 93–95 nucleotides downstream from the initiation site and a second one elongating beyond this site. The ratio between the two species of molecules depended on the salt concentration

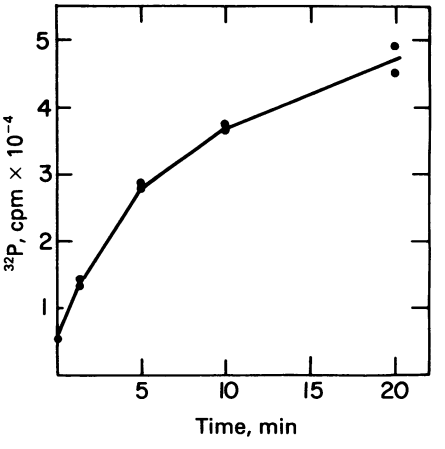

FIG. 2. Kinetics of [α-^{32}P]UMP incorporation in isolated nuclei. At 48 hr after infection, nuclei were isolated and incubated *in vitro* at 26°C in a standard incubation mixture containing [α-^{32}P]UTP. At the indicated times, samples of equal volumes were removed, precipitated with trichloroacetic acid, and assayed.

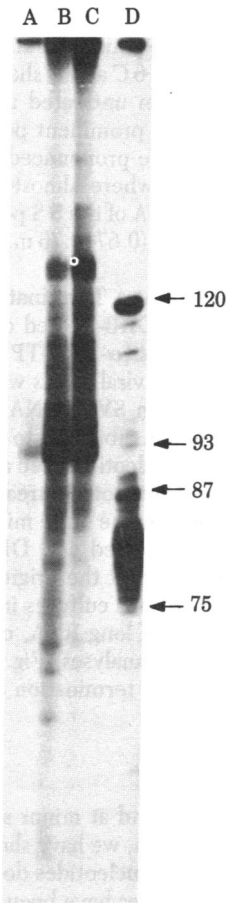

FIG. 3. Size analysis of viral RNA by gel electrophoresis. Isolated nuclei were labeled *in vitro* with [α-^{32}P]UTP for 3 min (lane A), 8 min (lane B), and 13 min (lane C). RNA was extracted and the viral RNA was selected by hybridization to and elution from SV40 DNA on filters. The ^{32}P-labeled viral RNAs were subjected to electrophoresis on a 10% polyacrylamide gel in 7 M urea for 22 hr at 180 V/20 cm. Lane D shows length markers of *Escherichia coli* RNA (a generous gift of M. David).

and temperature of the incubation mixture. Low salt concentration and low temperature enhanced premature termination; high salt concentration and high temperature allowed read-through. Furthermore, the presence of substances that stabilize secondary structure (e.g., spermine) in the reaction mixture caused complete termination but the presence of substances that destabilize secondary structure (e.g., proflavine) allowed read-through (unpublished data). These results suggest that the secondary structure of the RNA is involved in the process of premature termination.

In order to map the RNA present in the major band on the physical map of SV40 DNA, the RNA was eluted from the gel and hybridized to two sets of restriction fragments. The RNA hybridized to fragment e (0.67–0.76 m.u.) in one set (Fig. 4*A*) and to fragment d (0.73–0.82 m.u.) in the second set (Fig. 4*B*). These data indicate that the RNA in the major band has originated from a region of the genome spanning 0.73 to 0.76 m.u. Moreover, the labeled RNA hybridized to the "late" strand of the fragment spanning 0.67 to 0.76 m.u. (data not shown), indicating that the correct template was used (23). It has been shown previously that the major initiation site for late transcription resides in this region of the genome at nucleotide 243 (0.73 m.u.) (29). We suggest that transcription of the RNA in the major band has been initiated at the major initiation site and terminated 93–95 nucleotides downstream. Therefore, the aborted RNA spans from nucleotide 243 to nucleotides 335–339. It is unlikely that the short RNA has originated from a processing event that occurred *in vivo* during transcription because the 3′ end of the RNA is still attached to the template.

The possibility that RNA processing is occurring *in vitro* 93–95 nucleotides downstream from the major initiation site also seems unlikely because there is no accumulation of short RNA having their 5′ end at nucleotides 335–339 (unpublished

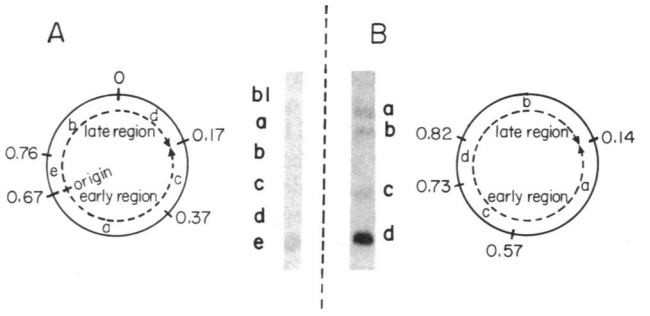

FIG. 4. Hybridization of the major RNA band (93–95 nucleotides) to restriction fragments of SV40 DNA. The RNA in the major band in the polyacrylamide gel as shown in Fig. 3 (93–95 nucleotides) was eluted from the gel by electrophoresis and hybridized to two sets of restriction fragments. (*A*) Five fragments were obtained by digestion of SV40 DNA with *Eco*RI, *Hpa* I, and *Bgl* I. (*B*) Four fragments were obtained by digestion of SV40 DNA with *Taq* I, *Hpa* II, *Hae* II, and *Bam*HI.

data). The map location of the aborted RNA has been confirmed by the following observations. RNA extracted from viral transcriptional complexes (26) of the deletion mutant dl 1626 in which the entire region between the major initiation site (nucleotide 243) and the suggested termination site (nucleotides 335–339) is deleted (30) failed to give the major band. The major band was also missing in dl 802 and dl 808 that delete nucleotides 262–341 and 223–383, respectively (31). Moreover, in dl 1811 (32), in which the major initiation site is at nucleotide 208 and 40 nucleotides are deleted between this site and the suggested termination site (nucleotides 335–339), the major band was found to be 88–92 nucleotides long (unpublished data).

Transcription Termination Signal. We examined the aborted RNA for potential secondary structures that might relate to the transcription termination event. We found that the DNA region 93–95 nucleotides downstream from the major initiation site is

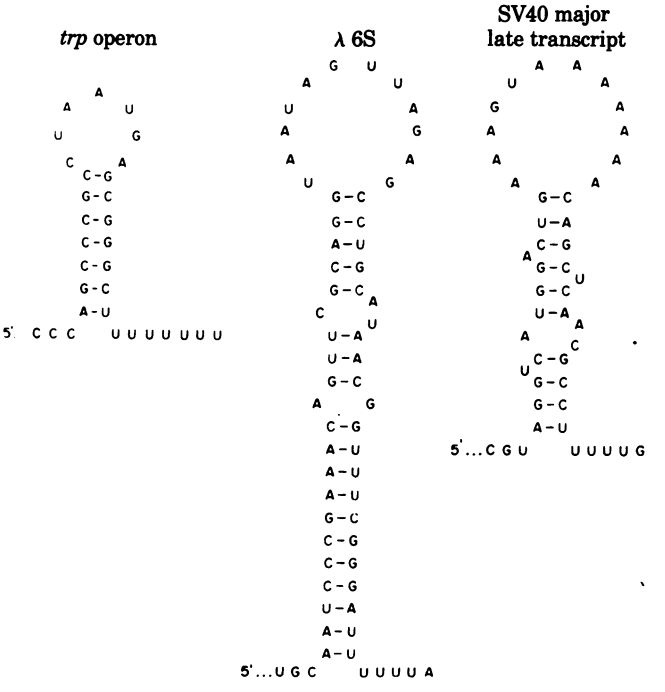

FIG. 5. The 3′ end of λ 6S transcript of the *trp* attenuator and of the 95-nucleotide aborted RNA. The 3′ ends of the three transcripts are shown in their potential stem-and-loop configuration. Termination can occur at one of the uridine residues (1–7). The secondary structure of λ 6S transcript and of the *trp* attenuator are from ref. 4.

A+T rich and is immediately preceded by a G+C-rich region that exhibits dyad symmetry. The potential of these sequences to signal transcription termination has been discussed (1–7). In Fig. 5, the secondary structure of the 3′ end of the aborted RNA, the attenuation region of 6S RNA of bacteriophage λ, and the tryptophan attenuator of *E. coli* are compared. A striking similarity is apparent. We conclude that premature termination in SV40 occurs at sequences previously found to signal transcription termination in prokaryotes.

Effect of DRB on Premature Termination of Cellular and Viral RNAs. It has been reported (15) that, when SV40-infected cells are pretreated with DRB and then labeled *in vivo* or *in vitro*, they synthesize short labeled viral RNAs that hybridize almost exclusively with a DNA fragment spanning 0.67 to 0.76 m.u. In the present study, we compared the length of the viral RNAs synthesized in isolated nuclei prepared from untreated and DRB-treated SV40-infected cultures in order to obtain some insight into the mode of action of this drug.

Nuclei isolated from untreated and DRB-treated cultures were incubated *in vitro* in the presence of [α-^{32}P]UTP for 10 min. [^{32}P]RNAs were extracted and purified. One portion of each preparation was sedimented through a sucrose gradient and a second portion was hybridized to restriction fragments prepared as shown in Fig. 4*A*. The profiles of [^{32}P]RNA from both untreated and DRB-treated cultures had a prominent peak at about 5 S (Fig. 6 *A* and *B*). Hybridization to restriction fragments showed that the majority of the viral RNA molecules in these preparations are complementary to fragment e (0.67–0.76 m.u.). It is also evident that DRB enhanced the accumulation of the short RNA and the hybridization to fragment e. Because the viral RNA comprises only a small fraction of the total RNA (2–5%) (23) this result indicates an accumulation of short nascent cellular RNA and its enhanced accumulation in DRB-treated cells. Similar observations were reported previously for RNA synthesized in nuclei of HeLa cells (9, 10). Fig. 6 *C* and *D* shows the profiles of the viral RNAs extracted from untreated and DRB-treated cultures, respectively. Again, a prominent peak at about 5 S is recognizable; this peak is more pronounced in RNA prepared from DRB-treated cultures, where almost no RNA longer than 5 S is apparent. The [^{32}P]RNA of the 5 S peak hybridized almost exclusively with fragment e (0.67–0.76 m.u.) (*Insets* in Fig. 6 *C* and *D*).

DRB Enhances Premature Termination at a Termination Signal. Nuclei isolated from untreated and DRB-treated cultures were labeled *in vitro* in the presence of [α-^{32}P]UTP for 3, 8, and 13 min. [^{32}P]RNAs were extracted, viral RNAs were purified by hybridization to and elution from SV40 DNA on filters, and the ^{32}P-labeled viral RNAs were subjected to gel electrophoresis. The major band of 93–95 nucleotides (see also Fig. 3) was present in RNA preparations from both untreated and DRB-treated cultures (Fig. 7). Moreover, the same minor bands are recognizable in the RNA of untreated and DRB-treated cultures. The absence of radioactivity at the origin of the gels in the RNA samples from DRB-treated cultures indicates that the drug inhibited the synthesis of long RNA, confirming the data obtained by sedimentation analyses (Fig. 6). We conclude that DRB enhances premature termination at a physiological termination site.

DISCUSSION

SV40 late transcription initiates at a major and at minor sites (15, 21, 22, 39). In the present communication, we have shown that late mRNA chains can terminate 93–95 nucleotides downstream at unique sequences. Similar sequences have been implicated previously in transcription termination in prokaryotes (1–7) and of 5 S RNA in eukaryotes (40). Thus, the eukaryotic RNA polymerases II and III and the analogous prokaryotic enzyme respond to similar sequences that constitute terminators. This conclusion implies that transcription termination is based,

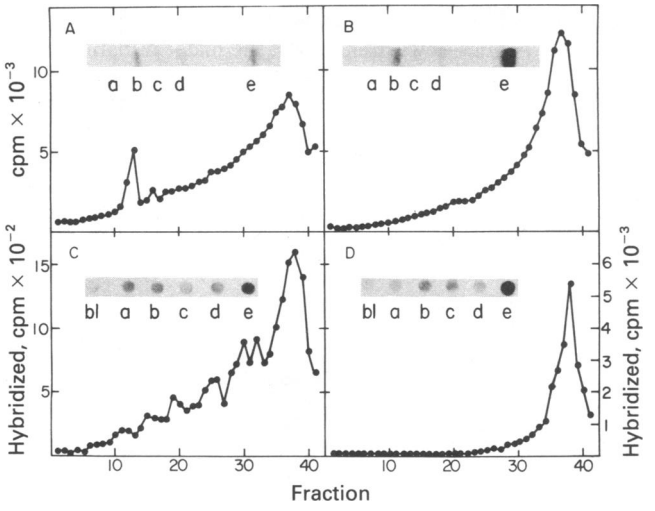

FIG. 6. Sucrose gradient analysis and blot hybridization of RNA extracted from untreated and DRB-treated cultures. At 48 hr after infection, cultures were treated with DRB (25 μg/ml) for 45 min. Control infected cultures received no DRB. Nuclei were prepared and incubated at 26°C in the presence of 50 μCi of [α-^{32}P]UTP for 10 min. Labeled RNAs were extracted, denatured, and centrifuged through 15–30% sucrose gradients in NaDodSO$_4$ buffer (25,000 rpm for 22 hr in the Spinco SW 27.1 rotor at 21°C). Samples (20 μl) of each fraction were precipitated with trichloroacetic acid. (*A*) RNA from untreated cultures. (*B*) RNA from DRB-treated cultures. Aliquots (150 μl) of each fraction were hybridized with SV40 DNA bound to nitrocellulose filters. (*C*) Viral RNA from untreated cultures. (*D*) Viral RNA from DRB-treated cultures. *Insets* in *A* and *B* are the results of hybridization of total RNA to blots containing the five *Eco*RI, *Hpa* I, and *Bgl* I restriction fragments (see Fig. 4*A*). *Insets* in *C* and *D* are the hybridization of pooled fractions (33–38) with similar DNA fragments.

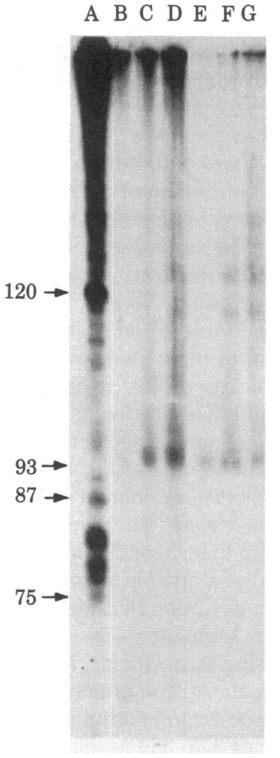

FIG. 7. Size analyses, by gel electrophoresis, of viral RNA of untreated and DRB-treated cultures. Cells were treated with DRB (25 μg/ml) for 45 min before preparation of the nuclei. Labeling *in vitro* was carried out with 50 μCi of [α-^{32}P]UTP for 3 min (lanes B and E), 8 min (lanes C and F), and 13 min (lanes D and G). RNAs were extracted and viral RNAs were selected and subjected to electrophoresis on 10% polyacrylamide gels as in Fig. 3. Lanes: B, C, and D, RNAs of untreated cells; E, F, and G, RNAs of DRB-treated cells; A, length markers of *E. coli* RNA.

at least in part, on the physical chemistry of nucleic acid interactions. However, we have not yet determined whether the aborted RNA in SV40 results from true termination as a part of an attenuation control mechanism or from a pausing process (41–43). Experiments aimed at identifying the aborted RNA *in vivo* have indicated, so far, that it does not accumulate in the viral infected cells (unpublished data). It is worth noting that, in prokaryotes, direct evidence for the synthesis of an attenuator RNA *in vivo* has been provided only for the *trp* operon (33) and most recently for the *his* operon (34).

There are several reasons that lead us to believe that a true premature termination event exists in SV40, including the following. (*i*) There is a striking similarity between the synthesis of the aborted RNA in SV40 and the attenuation mechanism in prokaryotes; thus, the termination signal lies within the leader region of the major 16S mRNA (29, 35) as in the case of the attenuator RNAs of amino acid operons in bacteria (3) and of 6S RNA of bacteriophage λ (4). In addition, the leader of the viral 16S mRNA (36) and the prokaryotic leaders encode for a small protein (4, 7). (*ii*) At the end of the termination signal, there is a sequence of uridine residues that are thought to facilitate the release of the transcript (8). (*iii*) Under controlled *in vitro* conditions, viral RNA 93–95 nucleotides long can be released from the template (unpublished data). (*iv*) Discrete aborted RNAs have been observed in Ad-2-infected cells that most likely represent premature termination products (11). By analogy to the attenuation mechanism in prokaryotes, it is possible that a number of additional features are involved in the process of premature termination in SV40, such as the presence of G+C-rich sequences distal to the termination site that can form secondary structures (29, 37, 38), protein that accentuates the termination event [a likely candidate is the "agnoprotein" (36)], and protein that functions as an antiterminator [a likely candidate is one of the early gene products (29)]. Moreover, the specific tertiary structure of the SV40 minichromosome exhibiting a "gap" in the region encompassing the initiation and attenuation sites could also play a role in the attenuation mechanism (44–46).

In regard to the mode of action of DRB, the present results strongly support the idea that DRB enhances a normal mechanism of transcription termination. This could be achieved by interaction of the drug with the RNA polymerase molecule itself (13, 15) or with a component that plays a positive or a negative role in the attenuation mechanism or by stabilizing the conformation of the secondary structure of the RNA that signals transcription termination.

As far as the physiological significance of the attenuation mechanism in SV40 is concerned, it is speculated that it plays roles in determining the ratio between the production of viral proteins and DNA and presumably also in the early/late switch of viral RNA synthesis.

Finally, in addition to SV40, it has been suggested that attenuation regulates gene expression in Ad-2 (11–13), vesicular stomatitis virus (43), retroviruses (47), and whole cells (9, 10). It appears, therefore, that attenuation is a general control mechanism in animal cells.

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