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Pausing and premature termination of human RNA polymerase II during transcription of adenovirus *in vivo* and *in vitro*

(transcriptional pausing/transcriptional termination/5,6-dichloro-1-β-D-ribofuranosylbenzimidazole/transcription complex)

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ABSTRACT **The major late transcriptional unit of adenovirus type 2 has served as a model for studying transcription in eukaryotes. We report that pausing and premature termination are intrinsic to the transcription of this transcriptional unit by RNA polymerase II. *In vivo* and in isolated nuclei, transcription pauses at discrete sites proximal to the initiation site and can prematurely terminate at nucleotide 175 and possibly also at nucleotide 120. The prematurely terminated RNAs are not associated with the transcription complexes and accumulate in the cell nucleus *in vivo*, whereas paused RNAs remain associated with the transcription complexes and elongate into full-length transcripts. Pausing is also reproduced in the transcription complexes in a soluble system. 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole enhances pausing but not premature termination, and its action is reversible. The proposed premature termination site at nucleotide 175 in adenovirus type 2 bears sequence homology to the t_{R1} site in coliphage λ.**

In eukaryotes, the expression of both cellular and viral genes appears to be regulated primarily at the transcriptional level (1). In prokaryotes, transcription is regulated not only at the level of initiation but also by attenuation—the termination of transcription shortly following initiation (2). Little is known about the mechanism and regulation of transcription initiation in eukaryotes, and virtually nothing is understood about the events associated with elongation and termination.

Adenovirus type 2 (Ad-2)-infected HeLa cells contain small RNA species complementary to the promoter-proximal region of the major late transcription unit; these are represented in extramolarity compared to other regions of the unit at steady state *in vivo* (3) and in isolated nuclei (4). Synthesis of these RNAs persists in the presence of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), which apparently reduces the synthesis of productive transcripts (5). These results were interpreted to imply premature termination of transcription in adenovirus. Premature termination was also suggested to occur in Chinese hamster cells (6) and in globin-producing cells (7), where DRB had a similar effect. However, the critical experiments to ascertain the fate of these small transcripts have not been performed. Although promoter-proximal "attenuation" has been suggested to occur in simian virus 40 due to the appearance of a small RNA during transcription *in vitro* (8), the existence of this small RNA in simian virus 40-infected cells has not been demonstrated.

Although pausing during transcription has not been observed in the transcription of eukaryotic genes *in vivo*, it is a common occurrence in the transcription of bacterial and phage genes (9). Kadesch and Chamberlain (10) reported that polymerase II from calf thymus pauses during transcription of a T7 bacteriophage template *in vitro*. Coppola *et al.* (11) found that in a soluble system under limited substrate concentration, the human polymerase II pauses within 30 nucleotides (nt) following initiation in the Ad-2 major late transcription unit. Hatfield *et al.* (12) reported that the bacteriophage λ 4S termination site was used for pausing and premature termination during transcription *in vitro* of a plasmid containing Ad-2 major late promoter sequences. Thus, pausing can occur during transcription by eukaryotic polymerase II, at least in soluble systems.

We have used the adenovirus as a eukaryotic model for studying pausing and premature termination during transcription. We define transcriptional pausing as the slowing down of the polymerase at specific sites on the template. After pausing, transcription can continue. Premature termination is the halting of transcription at a specific site on the template prior to the normal 3′ termination event. The prematurely terminated RNAs are released from the template and are not capable of further elongation. In this report, we present evidence for pausing and premature termination *in vivo* and *in vitro* during transcription of the Ad-2 major late transcription unit by eukaryotic polymerase II. Transcriptional pausing, but not premature termination, is enhanced by DRB.

MATERIALS AND METHODS

Transcription *in Vivo*. HeLa cells were maintained in suspension culture and infected with Ad-2 as described (13). In some experiments DRB was added to a final concentration of 75 μM 1 hr prior to the beginning of labeling and maintained at the same concentration during labeling (14). Nuclear RNAs were extracted and separated on a 5–20% sucrose dimethyl sulfoxide gradient (13).

Transcription in Isolated Nuclei. Nuclei were isolated from Ad-2-infected cells as described by Wolgemuth and Hsu (15). In some experiments DRB was added to infected cell cultures 1 hr prior to isolation but was absent during the isolation and labeling procedures. *In vitro* transcription was carried out essentially according to Derman *et al.* (16) at 10^8 nuclei per ml in a buffer containing 5 mM $MgCl_2$, 1 mM $MnCl_2$, 10 mM Tris·HCl (pH 8.0), 0.14 M KCl, 14 mM 2-mercaptoethanol, 1 mM *S*-adenosylmethionine, 1 mM (each) ATP, CTP, and GTP, and 10% glycerol at 31°C.

Isolation of Transcription Complexes. The isolation of transcription complexes from Ad-2-infected cells and RNA synthesis were carried out as detailed (14). When transcription complexes were isolated from [^{32}P]phosphoric acid-labeled cells, Nonidet P-40 was added to 0.1% (vol/vol) to release nuclei.

Selection and Analysis of RNAs. Labeled RNAs were hybridized in the presence of 50% formamide for 20 hr at 42°C to M13 single-strand DNA probes immobilized on nitrocellulose filters. Selected RNAs were eluted and analyzed by

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Abbreviations: Ad-2, adenovirus type 2; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; PI, postinfection; nt, nucleotide(s).

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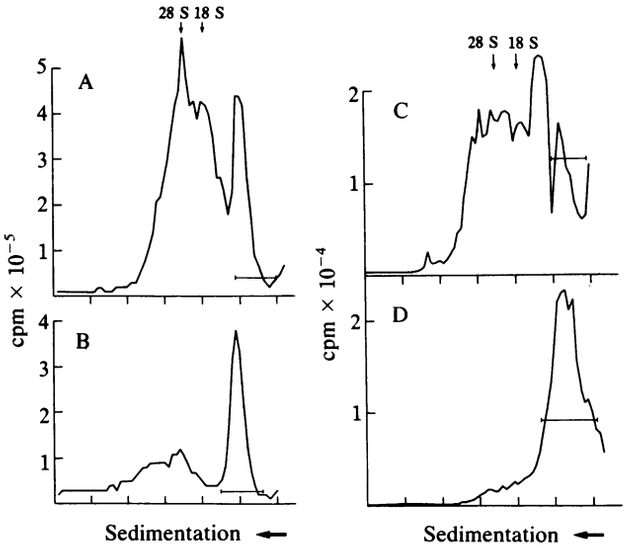

FIG. 1. Sedimentation profile of total nuclear RNAs late in Ad-2 infection and the effect of DRB *in vivo* and in isolated nuclei. HeLa cells were labeled *in vivo* from 16 to 19 hr PI with [^{32}P]phosphoric acid (8 mCi per 1.6 × 10^8 cells at 3 × 10^6 cells per ml; 1 Ci = 37 GBq) either in the absence (*A*) or in the presence (*B*) of 75 μM DRB. Nuclei from 2 × 10^8 untreated cells (*C*) or 4 × 10^7 cells treated DRB (*D*) were isolated at 18 hr PI and pulse labeled with [^{32}P]UTP for 3 min. Nuclear RNAs were extracted and separated on a 5–20% sucrose dimethyl sulfoxide gradient. The gradient was centrifuged for 42 hr at 40,500 rpm in an SW41 rotor at 22°C, and 60 fractions of each gradient were collected and assayed for Cerenkov counts. The small arrows designate the positions of 28S and 18S rRNAs. Fractions pooled for further analysis (Figs. 2 and 3) are indicated by bars (⟼).

electrophoresis on either 6.6% (Figs. 3 and 5) or 7% (Fig. 4) polyacrylamide gels containing 8 M urea/0.1% $NaDodSO_4$.

RESULTS

Small Promoter-Proximal RNAs at Steady State *in Vivo*. Late in Ad-2 infection, HeLa cells were labeled with [^{32}P]phosphoric acid from 16-hr postinfection (PI) to 19 hr PI, and the nuclear RNAs were isolated and separated on a denaturing gradient. As reported by Fraser *et al.* (3), a bimodal size distribution of nuclear RNAs was seen in the Ad-2-infected cells. When DRB was present, only the production of small nuclear RNAs was maintained; RNAs larger than 1000 nt were greatly reduced (Fig. 1 *A* and *B*). The nuclear RNAs of up to 1000 nt were pooled and hybridized to restriction fragments of the Ad-2 genome blotted onto nitrocellulose. Fig. 2 reveals that, whereas the small RNAs from untreated cells represent transcription from every region of the genome, the DRB-treated cells synthesize small RNAs complementary only to the region proximal to the initiation site of the major late transcription unit at map unit 16.5—i.e., *Hin*dIII C, B; *Sma* I F, B; and *Kpn* I B, C.† These data are in agreement with previous conclusions that DRB reduces the synthesis of RNAs distal but not proximal to the transcription initiation site *in vivo* (3).

The small RNAs labeled *in vivo* were further analyzed through hybrid selection by phage M13 clones 404 and 390, the cloned rightward- and the leftward-reading strands, respectively, of a restriction fragment with boundaries 63 nt upstream and 495 nt downstream from the major late initiation site (bottom of Fig. 3). As expected, late in infection

†The Ad-2 genome is conventionally divided into 100 map units with each unit being about 366 base pairs. The major late transcription unit resides at map units 16.45–100.

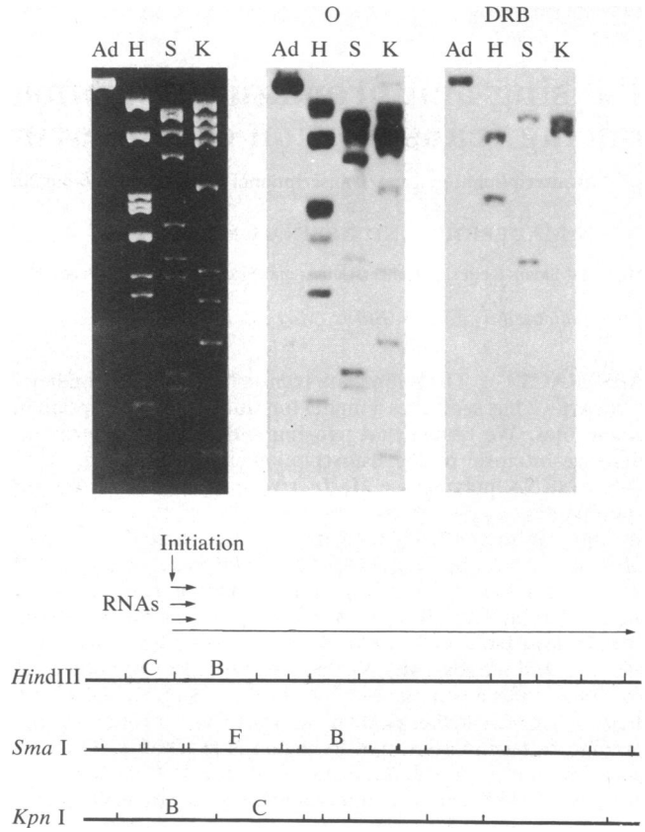

FIG. 2. Southern blot analysis of small nuclear RNAs synthesized *in vivo*. Ad-2 DNA was digested with restriction endonucleases *Hin*dIII (H), *Kpn* I (K), and *Sma* I (S) and chromatographed on a 1.2% agarose gel containing ethidium bromide. Duplicates of total Ad-2 and restriction digests were blotted onto nitrocellulose filters and hybridized to 30% of pooled [^{32}P]phosphoric acid-labeled small nuclear RNAs from control (O) and DRB-treated (DRB) cells as marked (⟼) in Fig. 1 *A* and *B*.

only RNAs complementary to the rightward-reading strand were synthesized (Fig. 3, compare lanes 2 and 5 to lanes 3 and 6). The major species in the control cells were 175 and 120 nt long (lane 2′), with a cluster of RNAs ≈160 nt in length. In the presence of DRB (lane 5′), the 175-nt species was not detectable and the 120-nt species was reduced, with the enhancement of the 160-nt cluster. Additional RNA species were seen in the presence of DRB—notably, the 150-nt, 185-nt, 210-nt, and three larger RNAs. A longer exposure of the same gel (lanes 2 and 5) revealed that these additional RNA species, albeit minor, were present in untreated cells. Likewise, a small amount of the 175-nt major species of the control cells was detected in DRB-treated cells.

It is apparent that some RNA species are increased by DRB treatment but not the two major species seen in its absence. Because these results represent a steady-state situation, the major RNAs could represent either prematurely terminated RNAs or processing products that DRB stabilizes differentially. Alternatively, all of the RNAs could be paused RNAs with DRB arresting transcription at certain pause sites differentially. A third possibility is that in the control cells, the 175-nt species, which represents ≈75% of the promoter-proximal small RNA population, and the 120-nt species are prematurely terminated RNAs that accumulated during steady-state labeling, whereas the minor species are RNAs at pause sites. DRB could function to arrest elongation of transcription at the existing pause sites, preventing the accumulation of prematurely terminated RNAs as well as the synthesis of complete transcripts. The reduction of large RNAs (Figs. 1 and 2) and the 175-nt and 120-nt RNAs (Fig.

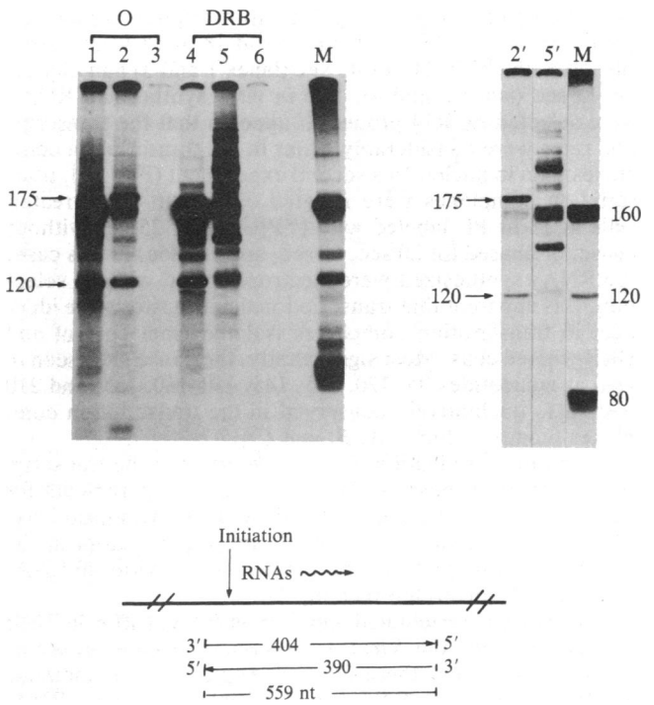

FIG. 3. Accumulation of Ad-2 promoter-proximal nuclear RNAs *in vivo*. [^{32}P]Phosphoric acid-labeled small nuclear RNAs from control (O) and DRB-treated (DRB) cells (40% of pooled small RNAs in Fig. 1 *A* and *B*, respectively) were hybridized to probes 404 and 390, eluted, and analyzed by polyacrylamide gel. Lanes 1–3, pooled small RNAs from control cells without selection, 404-selected, and 390-selected; lanes 4–6, pooled small RNAs from DRB-treated cells without selection, 404-selected, and 390-selected; lanes 2' and 5', lighter exposures of lanes 2 and 5; lane M, RNA size markers (shown in nt).

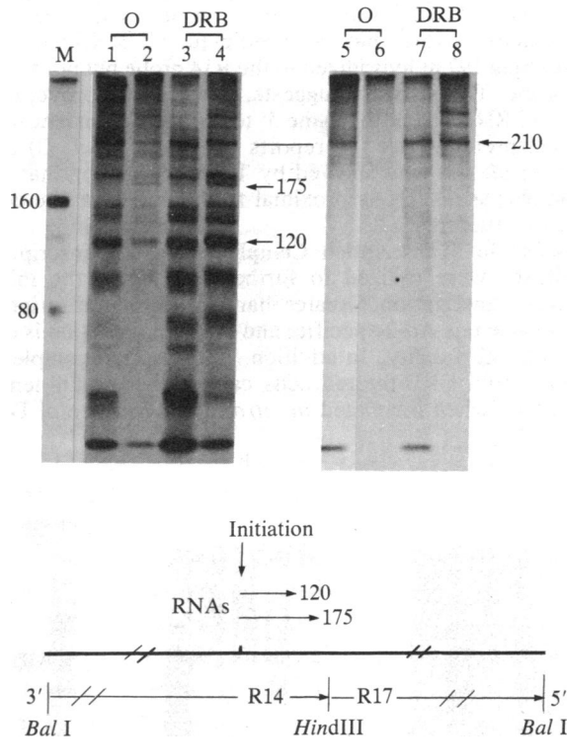

FIG. 4. Paused and prematurely terminated RNAs during pulse-chase in isolated nuclei. At 18 hr PI, nuclei were isolated from 1×10^9 control cells (O) and 2×10^8 DRB-treated cells (DRB) and labeled with [^{32}P]UTP at 0.25 mCi per 10^8 nuclei for 3 min. At the end of the pulse-labeling period, half of the nuclei were subjected to chase with unlabeled UTP for another 6 min. RNAs were extracted and probe R14- and R17-specific RNAs were selected and analyzed by polyacrylamide gel. Lanes 1–4, R14-selected RNAs. Lane 1, control nuclei, pulse-labeled; lane 2, control nuclei, pulse-chased; lane 3, DRB-treated nuclei, pulse-labeled; lane 4, DRB-treated nuclei, pulse-chased. Lanes 5–8, R17 probe-selected RNAs. Lane 5, control nuclei, pulse-labeled; lane 6, control nuclei, pulse-chased; lane 7, DRB-treated nuclei, pulse-labeled; lane 8, DRB-treated nuclei, pulse-chased. Lane M, RNA size markers (shown in nt).

3) in the presence of DRB (as well as further evidence presented below) support the third model.

Transcription in Isolated Nuclei. To examine the nature of these RNAs at the transcriptional level, we performed pulse-labeling and pulse-chase experiments using nuclei from Ad-2-infected cells isolated at 18 hr PI. All incubations were performed in the absence of DRB regardless of DRB pretreatment. Since DRB enhances the synthesis of the Ad-2 initiation site-proximal RNAs *in vivo*, only one-fifth as many cells were used when DRB was added. As shown in Fig. 1 *C* and *D*, the size distribution of RNAs pulse-labeled with [^{32}P]UTP in nuclei isolated from the control and DRB-treated cells is similar to that of the nuclear RNAs at steady state *in vivo*. This implies that the small RNAs found *in vivo* result from transcription and not from subsequent RNA metabolism or degradation (or both).

The production of these small RNAs was further analyzed by a 3-min pulse-labeling of isolated nuclei followed by a 6-min chase. The small RNAs generated during transcription were hybridized to two sets of phage M13 clones. R14 and L14 are the rightward and leftward reading strands of a restriction fragment encompassing map units 14.7–17.0. This includes the first 197 nt of the major late transcription unit. R17 and L17 are immediately downstream at positions 17.0–21.0. No RNAs complementary to L14 or L17 were identified (unpublished data). The RNAs selected by the R14 and R17 probes were eluted and analyzed by denaturing polyacrylamide gel electrophoresis (Fig. 4).

These results indicate that shortly after initiation, RNA polymerase II appears to pause at distinct sites (lane 1) as pulse-labeling marks the 3' end of the growing RNA chains initiated *in vivo*. The majority of these sites are qualitatively maintained during an *in vitro* chase, when the UTP concentration is not limiting (lane 2). Pretreatment of cells with DRB prior to isolation of nuclei enhances the extent of pausing by ≈10-fold (since only one-fifth cell equivalent was used in lanes 3 and 4) but does not alter the sites of pausing. The major *in vivo* promoter-proximal RNA of 175 nt is synthesized during pulse-labeling (lanes 1 and 3) and accumulates during chase (lanes 2 and 4) in nuclei isolated from both control and DRB-pretreated cells. These results are consistent with transcriptional termination at nt 175. They also demonstrate the reversibility of the effect of DRB.

The site at nt 120 is apparently one of several strong pause sites for transcription (lanes 1–4). Since the 120-nt RNA accumulates at steady state to a much greater extent than do other paused RNAs *in vivo*, this site may also serve as a weak termination site. Indeed, like the prematurely terminated 175-nt RNA and unlike other paused RNAs, the amount of the 120-nt RNA at steady state *in vivo* is not enhanced by DRB (Fig. 3, lane 5'). Integration and comparison of peak sizes obtained by densitometer scanning of the autoradiograph seen in Fig. 4 showed that the 120-nt RNA species is conserved, relative to other paused RNA species in the same lane, during chase in isolated nuclei (data not shown). The overall loss of label during chase in control isolated nuclei is due to loss of ≈30% of the sample (lane 2).

Only RNAs pausing at sites further than 197 nt (the common boundary of R14 and R17 probes) from the initiation site

were expected to hybridize to both probes. The results in Fig. 4 demonstrate that, as predicted, all RNA species shorter than 197 nt hybridized to the R14 probe but not to the R17 probe. This strongly suggests, but does not prove, that the small RNAs have the same 5′ terminus. Consistent with this interpretation are the reports by Fraser *et al.* (3) and Evans *et al.* (4), who showed by T1 fingerprinting that the extramolar small RNAs proximal to the initiation site contain cap structures.

Pausing in Transcription Complexes. Ad-2 transcription complexes were utilized to further investigate the initial stages of transcription. Greater than 85% of the transcription in this system is Ad-2-specific, and polyadenylylation is carried out with fidelity. In addition, transcription complexes isolated from DRB-treated cells can synthesize full-length transcripts when incubated *in vitro* in the absence of DRB (14). In one experiment (Fig. 5*A*), transcription complexes from control and DRB-treated cells at 18 hr PI were pulse-labeled with [^{32}P]UTP for 45 sec (lanes 1 and 3) and chased for 75 sec (lanes 2 and 4). The *in vitro* synthesized RNAs were selected by R14 probes. It appears that the transcription rates were considerably faster in the transcription complexes than in nuclei. In a second experiment (Fig. 5*B*), transcription complexes were isolated only from DRB-treated cells at 18 hr PI, labeled with [^{32}P]UTP for 25 sec without chase, or chased for 20 sec, 65 sec, and 155 sec. In this case, the RNAs synthesized were electrophoresed without selection. It is apparent that transcriptional pause sites were identical in transcription complexes isolated from control and DRB-treated cells. Most significantly, the pause sites seen *in vivo* at nucleotides 95, 120, 135, 145, 150–160, 185, and 210 were also qualitatively conserved in the transcription complexes (compare Fig. 5 *A*, *B*, and *C*).

Premature termination was not observed in the transcription complexes, possibly because certain requirements for factors and conditions are not met by the system used here. The importance of ionic strength and other factors in the generation of prematurely terminated simian virus 40 RNAs has been demonstrated recently (8).

Prematurely Terminated and Paused RNAs Differ in Their Association with the Viral Templates. The data presented above suggest that there are two populations of initiation site-proximal small RNAs in Ad-2-infected cells—RNAs temporarily arrested at pause sites and RNAs prematurely terminated. The paused RNAs have the potential to be elongated, as shown in transcription complexes (Fig. 5). They are also expected to be associated with the viral templates, whereas prematurely terminated RNAs should be free. To test this hypothesis, Ad-2-infected cells, with or without DRB treatment, were labeled with [^{32}P]phosphoric acid from 16 to 19 hr PI. Labeled transcription complexes were isolat-

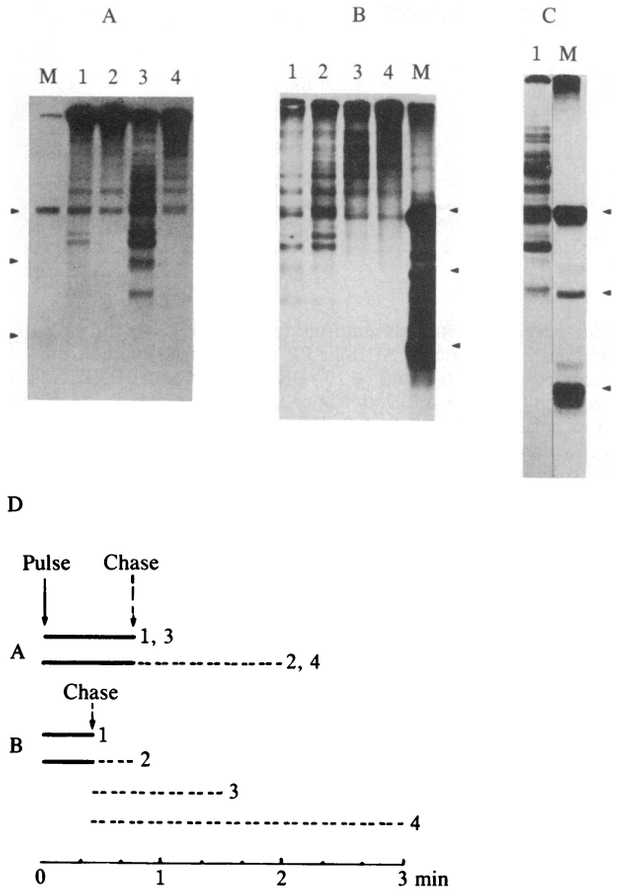

FIG. 5. Kinetics of pulse-chase during *in vitro* transcription of transcription complexes. Transcription complexes were isolated from Ad-2-infected cells at 18 hr PI and pulse-labeled *in vitro* with [^{32}P]UTP. The strategy for the pulse-chase experiments in *A* and *B* is depicted in *D*. In *A*, transcription complexes were isolated from 2 × 10^7 cells untreated or treated with DRB. The isolated transcription complexes were divided into two aliquots and each was pulse-labeled with 20 μCi of [^{32}P]UTP for 45 sec. One aliquot was then followed by a 75-sec chase with unlabeled UTP while the other was not. RNAs were extracted and selected by M13 probe R14: control pulse-labeled (lane 1) or pulse-chased (lane 2) or DRB-pretreated and pulse-labeled (lane 3) or pulse-chased (lane 4). In *B*, transcription complexes were isolated from 2 × 10^7 cells treated with DRB and divided into four aliquots, each pulse-labeled with 10 μCi of [^{32}P]UTP for 25 sec followed by chase. RNAs were not selected from the 25-sec pulse, no chase (lane 1), 20-sec chase (lane 2), 65-sec chase (lane 3), or 155-sec chase (lane 4). Lane 1 in *C* represents clone 404-selected RNAs from DRB-treated cells labeled *in vivo* (see lane 5′ in Fig. 3). Lane M, RNA size markers. The arrowheads indicate 160, 120, and 80 nt.

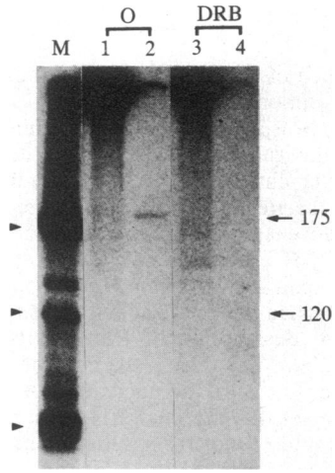

FIG. 6. Prematurely terminated RNAs are not associated with the DNA templates. At 16–19 hr PI, 2 × 10^8 HeLa cells either with or without DRB treatment were labeled with [^{32}P]phosphoric acid at 80 μCi/ml. Nuclei were isolated from the labeled cells by the Nonidet P-40 method. Transcription complexes and RNAs were then isolated (14), mixed with [^{3}H]thymidine-labeled transcription complexes from a separate experiment for marker purposes, and separated in a 10-ml 10–30% sucrose $(NH_4)_2SO_4$ gradient containing 0.025% Sarkosyl. The gradients were centrifuged at 34,600 rpm for 15 hr in an SW41 rotor, and 30 fractions were collected. RNAs were extracted from both the [^{3}H]thymidine-labeled transcription complexes (bound) and soluble fractions (free) and then hybridized to R14 clones. A 6.6% polyacrylamide gel was used for RNA analysis. Lanes 1 and 2, template-bound and free RNAs from untreated cells; lanes 3 and 4, template-bound and free RNAs from DRB-treated cells; lane M, RNA size markers with 160, 120, and 80 nt indicated (arrowheads).

ed and separated by gradient centrifugation. The ^{32}P-labeled RNAs associated with viral templates and those not associated with templates were distinguished by their positions in the gradient by using as references [^{3}H]thymidine-labeled transcription complexes. The two RNA populations were selected by probe R14 and analyzed. As predicted, the prematurely terminated RNAs of 175 nt and 120 nt were present only in the soluble fraction from the control cells (Fig. 6, lane 2). No prematurely terminated RNAs were observed in either the template-associated or free fraction from DRB-treated cells (Fig. 6, lanes 3 and 4). Pause sites (Fig. 3, lane 5′) were more pronounced in the template-associated fraction in DRB-treated cells than in untreated cells (Fig. 6, lanes 1 and 3). These results are in agreement with the *in vivo* and *in vitro* data that prematurely terminated RNAs differ from paused RNAs and that *in vivo* DRB enhances pausing but not premature termination. The presence of promoter-proximal RNAs associated with DRB-treated viral templates also confirms the idea that pausing is intrinsic to transcription *in vivo* and is not an aberration of isolated nuclei or transcription complexes.

DISCUSSION

We have presented evidence that human RNA polymerase II pauses during transcription of the Ad-2 major late transcription unit *in vivo* and that the promoter-proximal pause sites are recapitulated in isolated nuclei and in transcription complexes. The pause sites are discrete, but examination of the nucleotide sequences involved reveals no apparent sequence homology. Although all of these sites reside in the first intron of the major late transcription unit, pausing could also occur at other sites closer to the initiation site (11), since the acrylamide gel system used in this study does not allow resolution of RNAs smaller than ≈50 nt. At least one of these sites, the pause site at nt 120, may also serve as a site for premature termination—reminiscent of transcriptional pausing in prokaryotes (18). The ratio of pausing to premature termination at this site is not known. Since little is known about the transition from initiation to elongation during transcription in eukaryotes, one might postulate that at these pause sites, elongation complexes undergo modifications such that subsequent transcription and processing will be carried out accurately.

It has been suggested that premature termination during transcription of the major late transcription unit occurs at multiple sites and is enhanced by the presence of DRB (3, 4). Our data indicate that premature termination occurs only at two sites, 175 nt and most likely also at 120 nt downstream from the initiation site, and that DRB enhances only pausing but not premature termination. DRB was initially observed by Tamm to reduce the synthesis of nuclear RNAs larger than ≈700 nt in HeLa cells (19). Our observations indicate that *in vivo* DRB does not impede initiation of transcription but increases pausing at specific natural sites and, secondarily, affects further loading of polymerase II. The DRB action is reversible because transcription elongation rates were indistinguishable in nuclei or transcription complexes isolated from treated and untreated cells.

We show here that, unlike paused RNAs, prematurely terminated RNAs are not associated with templates. They accumulate in the nucleus but remain undetected in the cytoplasm (unpublished data). Unlike the sequences at pause sites, the sequence at the major premature termination site at nt 175, 5′ C-A-A-U-C-U-U-U-U-U-G 3′, shares sequence homology with the phage λ premature termination site t_{R1}, 5′ C-A-A-U-C-A-A-U-U-G 3′. We also found a sequence upstream to nt 175, centered at position 59, similar to the antitermination protein N-recognition site (nutR site) in λ. It is tempting to postulate that these sequences serve a common purpose in transcriptional termination, however complicated the mechanism and regulation may be in eukaryotes. Indeed, Henikoff *et al.* (17) have found that a 5′ C-A-A-G/U-C-U-U-U-G 3′ sequence is present at the 3′ termini of *Drosophila* and yeast transcripts. In Ad-2, the sequence of the possible premature termination site at nt 120 does not share homology with the site at nt 175. This is not without precedence in prokaryotes (20).

We have recently obtained evidence that premature termination occurs temporally in both Ad-2 and adenovirus type 5. Further, premature termination can be induced by means of cumulative transcription in the absence of continuous DNA replication, suggesting the intriguing possibility of its role in biological regulation (21).

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