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Premature Termination by Human RNA Polymerase II Occurs Temporally in the Adenovirus Major Late Transcriptional Unit

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We have recently demonstrated pausing and premature termination of transcription by eucaryotic RNA polymerase II at specific sites in the major late transcriptional unit of adenovirus type 2 in vivo and in vitro. In further developing this as a system for studying eucaryotic termination control, we found that prematurely terminated transcripts of 175 and 120 nucleotides also occur in adenovirus type 5-infected cells. In both cases, premature termination occurs temporally, being found only during late times of infection, not at early times before DNA replication or immediately after the onset of DNA replication when late gene expression has begun (intermediate times). To examine the phenomenon of premature termination further, a temperature-sensitive mutant virus, adenovirus type 5 *ts*107, was used to uncouple DNA replication and transcription. DNA replication is defective in this mutant at restrictive temperatures. We found that premature termination is inducible at intermediate times by shifting from a permissive temperature to a restrictive temperature, allowing continuous transcription in the absence of continuous DNA replication. No premature termination occurs when the temperature is shifted up at early times before DNA replication. Our data suggest that premature termination of transcription is dependent on both prior synthesis of new templates and cumulative late gene transcription but does not require continuous DNA replication.

Termination of transcription in eucaryotes is a process that has eluded description. In the last few years, it has become clear that in procaryotes, proper termination is important not only to confine transcription within relevant transcriptional units but also as a means of down regulating the level of mRNA synthesis after initiation. The latter case has been termed attenuation, since the termination sites are located immediately downstream from the initiation sites and before the structural genes in the operon. Studies of this type of premature termination have been very fruitful in understanding termination control in procaryotes (for reviews, see references 1 and 19). Many investigators have exploited adenovirus as a system for studying transcription in eucaryotes because its gene products have been extensively characterized. Based on the sequential onset of expression of different viral transcription units, the infectious cycle of the virus is customarily divided into early, intermediate, and late times. Replication of the viral template commences at the onset of the intermediate time and continues throughout the late time (for a review, see reference 7). Because cellular RNA polymerases transcribe all of the viral genes, adenovirus provides a well-defined system for studying the regulation of transcription in eucaryotes.

We have pursued our studies of premature termination in the major late transcriptional unit of adenovirus as a model of how transcriptional termination may occur and be regulated in eucaryotes. In our laboratory, we recently have shown that in HeLa cells infected with adenovirus type 2 (Ad2), transcription which has initiated at the major late promoter pauses at specific sites within the first 300 nucleotides after initiation and can terminate prematurely to yield two RNA species of 175 and 120 nucleotides (A. Maderious and S. Chen-Kiang, Proc. Natl. Acad. Sci. U.S.A., in press). Paused transcripts were distinguishable from prematurely terminated transcripts because they retained the potential to be elongated in pulse-chase experiments and were physically associated with the template. Based on these findings, we believe that prematurely terminated transcripts are products of aborted transcription, whereas paused transcripts represent transient intermediates which ultimately elongate to become mature RNA species. Although our system allows the study of both pausing and premature termination, this report is concerned only with premature termination.

Although our previous results demonstrated that premature termination occurs in vivo, additional evidence was needed to establish its biological significance. Here, we show that premature termination occurs during the late time of infection but not during the early or intermediate time. This is true both for Ad2-infected HeLa cells and for Ad5 *ts*107-infected KB cells under permissive conditions. The onset of DNA replication and the early-to-late switch of gene expression are thus not sufficient to explain the appearance of these prematurely terminated transcripts, because premature termination does not occur at intermediate times just after these events take place. To examine the mechanism of premature termination and its temporal expression, we dissociated DNA replication from the other events of adenovirus infection. To achieve this, we exploited the temperature-sensitive strain Ad5 *ts*107, an isolate of Ad5 *ts*125, which encodes a temperature-sensitive DNA binding protein necessary for viral DNA replication (3, 10).

Using temperature shift experiments, we found that prior DNA replication is required for premature termination to occur and that premature termination does not result simply from the accumulation of certain early gene products. Continuous DNA replication is not necessary, since premature termination could be observed late in infection after further DNA replication was halted by shifting to a restrictive temperature. Most important, we found that cumulative late gene transcription is a requirement for premature termina-

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tion, since it was inducible at intermediate times, but not at early times, by shifting from a permissive temperature to a restrictive temperature. The requirement of new template synthesis for late gene expression has been documented previously. On the basis of superinfection experiments, Thomas and Mathews have reported that the synthesis of new templates is a prerequisite for the expression of one late function, the synthesis of fiber polypeptide (17). Crossland and Raskas have recently presented evidence that template replication is also required for the expression of two intermediate genes, polypeptides IVa_2 and IV in Ad2 (6). The significance of premature termination in termination control of the adenovirus major late transcriptional unit is discussed further in this paper.

MATERIALS AND METHODS

Cells and viruses. HeLa and KB cells were maintained in suspension cultures in Joklik modified minimal essential medium supplemented with 5% fetal calf serum. Ad2 was studied in HeLa cells, whereas Ad5 *ts*107 (obtained from H. Young and H. Ginsberg) was studied in KB cells. The maintenance and infection of Ad2 were as described previously (5). Stocks of Ad5 *ts*107 were prepared by infecting KB cells at a concentration of 3×10^5 cells per ml with 10 PFU of virus per cell for 4 h at 32°C in the absence of serum. Thereafter, infected cells were maintained at half the initial concentration for 65 h at 32°C in medium containing 5% fetal calf serum. Infected cells were then concentrated to 4×10^7 cells per ml, and virus was released by six cycles of freezing and thawing in a dry ice-ethanol bath and a 32°C water bath, respectively. After sonication, Freon extraction, and purification by centrifugation through a CsCl gradient, the virus stock was stored in 20% glycerol with 0.02% (wt/vol) bovine serum albumin at −20°C. Infectivity of the viral preparation was quantitated by plaque assay.

In vivo transcription of Ad2. HeLa cells in mid-log phase of growth were concentrated to 10^7 cells per ml and infected with Ad2 in serum-free medium at room temperature for 30 min. Infected cells were then maintained at a concentration of 3×10^5 cells per ml at 37°C in medium with 5% fetal calf serum. During specific time periods postinfection (PI), HeLa cells infected with Ad2 were labeled in vivo with $[^{32}P]$phosphoric acid (2 mCi/10^8 cells) for 3 h at 37°C (4). During labeling, cells were maintained at a concentration of 2×10^6 to 4×10^6 cells per ml. A total of 2×10^9 cells infected at 100 PFU per cell were used for ^{32}P labeling at early times; 1×10^9 cells infected at 100 PFU per cell were used at intermediate times; and 1×10^8 cells infected at 20 PFU per cell were used at late times. Nuclear RNAs were extracted (13), fractionated on a 15 to 30% sucrose gradient in 0.05 M NETS (50 mM NaCl, 10 mM EDTA, 10 mM Tris-hydrochloride [pH 7.4], 0.2% sodium dodecyl sulfate), and centrifuged at 24,000 rpm for 14 h at 18°C in a Beckman SW41 rotor to separate low-molecular-weight RNAs from high-molecular-weight RNAs and DNAs.

In vivo transcription of Ad5 *ts*107 under permissive conditions. KB cells in mid-log phase of growth were concentrated to 10^7 cells per ml and infected with *ts*107 at 10 PFU per cell in serum-free medium at room temperature for 30 min. Infected cells were then maintained at a concentration of 3×10^5 cells per ml at 32°C in medium supplemented with 5% fetal calf serum. During specific time periods PI, cells were labeled in vivo with $[^{32}P]$phosphoric acid (2 mCi/10^8 cells) for 3 h at 32°C (4). During labeling, cells were maintained at a concentration of 4×10^6 cells per ml. Nuclear and cytoplasmic RNAs were extracted separately (13, 16), and nuclear RNAs were fractionated as described above. Polyadenylic acid-containing $[poly(A)^+]$ RNAs were separated from nonpolyadenylic acid-containing $[poly(A)^-]$ RNAs on a polyuridylic acid-Sepharose affinity column by a modification of the method of Sawicki et al. (14) as previously described (5). A 1.4% formaldehyde-agarose gel was used for electrophoretic analysis of RNAs by a modification (5) of the method of Lebrach et al. (11).

In vivo transcription of Ad5 *ts*107 after temperature shifts. Infection and incubation of KB cells with Ad5 *ts*107 at 32°C were followed by temperature shifts to the nonpermissive temperature of 40.5°C at 12, 22, or 30 h PI for early, intermediate, and late times, respectively. Incubation was continued at 40.5°C for an additional 5 h, at which time infected cells were labeled in vivo with $[^{32}P]$phosphoric acid (1.2 mCi/10^8 cells) for another 3 h at 40.5°C. As a control, infected KB cells were incubated at 32°C for 35 h and labeled in vivo with 1.2 mCi of $[^{32}P]$phosphoric acid per 10^8 cells for 3 h at 32°C. RNA extraction and gel electrophoresis were performed as described above. Nuclear RNAs were fractionated on a 5 to 20% sucrose-dimethyl sulfoxide gradient instead of on a sucrose-NETS gradient (4) for 36 h at 40,000 rpm in a SW41 rotor.

DNA synthesis of Ad5 *ts*107 at permissive and nonpermissive temperatures. To measure total adenovirus-specific DNA in infected cells, KB cells were infected at 32°C with Ad5 *ts*107 as described above. At specific times, 10^7 cells were withdrawn from the culture, and either NaN_3 was immediately added to a final concentration of 20 mM to stop DNA synthesis or the cells were shifted to the nonpermissive temperature for 5 h before the addition of NaN_3. Total DNAs were extracted from the whole cells by the procedure

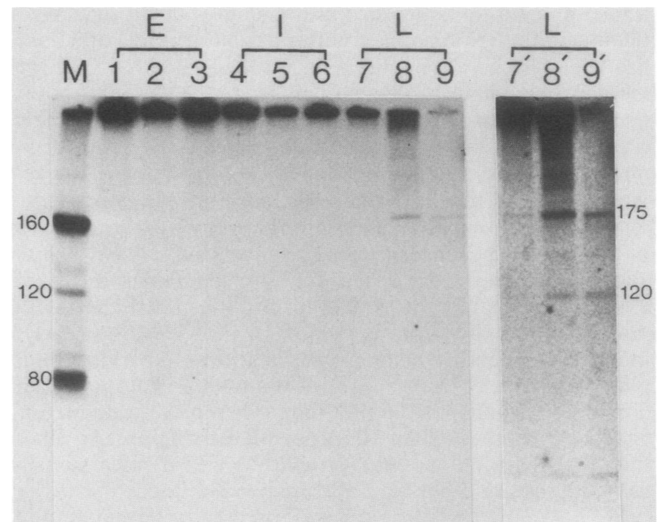

FIG. 1. Temporal expression of prematurely terminated RNAs from the Ad2 major late transcriptional unit. R14-selected nuclear RNA labeled in the early times at 3 to 6 h PI (E, lanes 1 to 3), in the intermediate times at 8 to 11 h PI (I, lanes 4 to 6), and in the late times at 16 to 19 h PI (L, lanes 7 to 9). Lanes 7′ to 9′ are a longer exposure of lanes 7 to 9. Similar longer exposures of lanes 1 to 6 did not reveal any further detail. RNAs from each label time period were fractionated into separate pools of RNAs larger than 1,000 nucleotides (lanes 1, 4, and 7), RNAs between 100 and 1,000 nucleotides (lanes 2, 5, and 8), and RNAs ca. 100 nucleotides (lanes 3, 6, and 9) and selected by R14 probe. Similar amounts (500 to 1,000 cpm) of selected RNAs were loaded in each lane. A 6.6% denaturing polyacrylamide gel was used for RNA analysis.

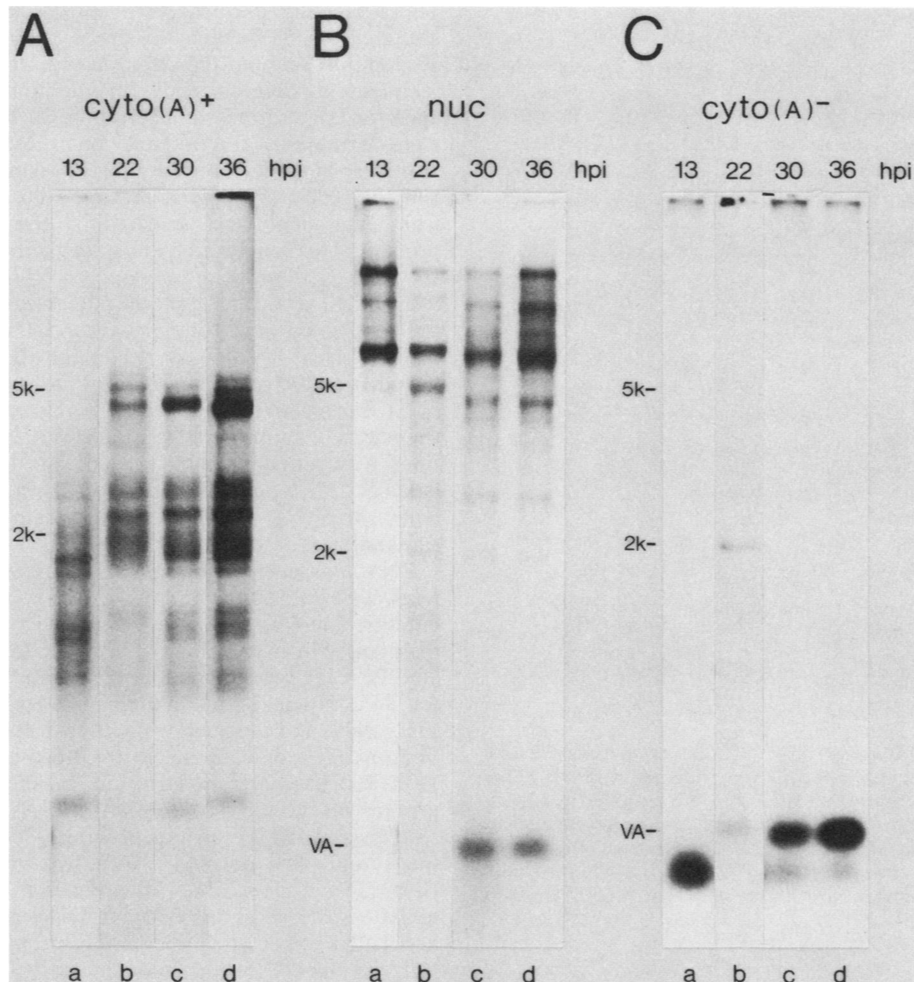

FIG. 2. Profiles of RNA synthesis at 32°C in Ad5 *ts*107-infected KB cells at various times after infection. Infected KB cells were labeled with [^{32}P]phosphoric acid, and RNAs were extracted as described in the text. (A) Cytoplasmic poly(A)$^+$ RNAs at 13 (lane a), 22 (lane b), 30 (lane c), and 36 (lane d) h PI. (B) Sucrose gradient-fractionated nuclear RNAs at 13 (lane a), 22 (lane b), 30 (lane c), and 36 (lane d) h PI. (C) Cytoplasmic poly(A)$^-$ RNAs at 13 (lane a), 22 (lane b), 30 (lane c), and 36 (lane d) h PI. The lengths of the 28S and 18S ribosomal RNAs and the VA RNA are marked in the margins. RNAs from ca. 10^6 cells were used for each lane. A 1.4% formaldehyde-agarose gel was used for RNA analysis.

of Carter and Ginsberg (3). After ethanol precipitation, DNAs were digested by the restriction enzyme *Kpn*I, electrophoresed on a 1.2% agarose gel, blotted onto nitrocellulose filters, and hybridized with nick-translated Ad2 DNA (5).

To follow the kinetics of the cessation of DNA synthesis in Ad5 *ts*107 after the temperature shift to 40.5°C, 10^7 infected cells were pulse-labeled with 500 μCi of [^{3}H]thymidine for 20 min in 50 ml of medium at various times after the temperature shift. At the end of the pulse period, NaN_3 was added to 20 mM to stop DNA synthesis. Total DNAs were extracted, sheared by sonication (3), and hybridized in 2× SSC (0.3 M NaCl, 30 mM sodium citrate [pH 7.0]) at 65°C for 40 h to Ad2 DNA immobilized on nitrocellulose filters. The filters were washed, then dried, and counted in a toluene-based scintillation fluid.

RNA selection and analysis. Specific nuclear RNA species were selected by hybridization to phage M13-cloned Ad2 DNA fragments immobilized on nitrocellulose filters, followed by elution (11). The R14 probe is derived from the r-strand of the *Bal*I-*Hin*dIII restriction fragment (map position, 14.68 to 17.06) of Ad2 and complements transcripts containing sequences in the first 197 nucleotides of the Ad2 major late transcriptional unit. This probe was a kind gift from Jim Lewis. The 390 probe was derived from the l-strand of the *Hpa*II restriction fragment (map position, 16.35 to 17.87) of Ad2. This probe was a generous gift from Richard Roberts. Low-molecular-weight, M13 probe-selected nuclear RNA species were separated by electrophoresis on denaturing 6.6% polyacrylamide gels containing 8 M urea and 0.1% sodium dodecyl sulfate.

RESULTS

Temporal expression of premature termination in Ad2. To determine the biological significance of premature termination, we first examined how premature termination was correlated with the temporal expression of the Ad2 major late transcriptional unit. Early in infection, before viral DNA replication, the promoter of the major late transcriptional unit is active at a level similar to those of early transcriptional units, but only the 5′ portion of the transcriptional unit is expressed. The termination site of transcription is unknown,

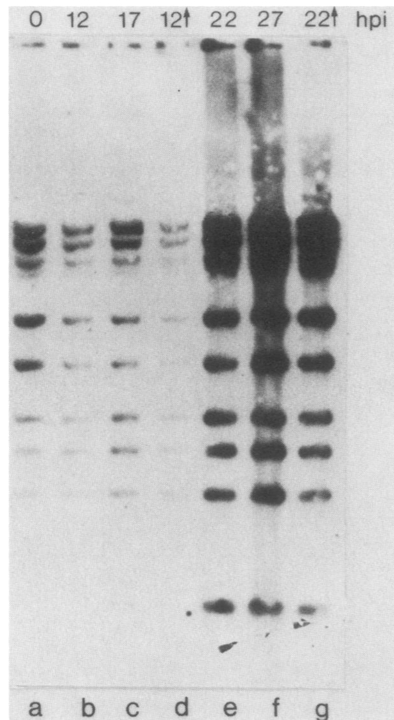

FIG. 3. Analysis of Ad5 *ts*107 DNA synthesis at permissive and nonpermissive temperatures. KB cells were infected with *ts*107 and maintained at 32°C; portions of the culture were removed at 12 and 22 h PI for incubation for 5 h at 40.5°C. Each lane represents DNA from 10^6 cells. Ad5 DNA at 0 (lane a), 12 (lane b), and 17 (lane c), h PI after a temperature shift to 40.5°C and 12 (lane d), 22 (lane e), and 27 (lane f) h PI after a temperature shift to 40.5°C at 22 h PI (lane g).

but only one mRNA of this L1 family is processed and translated at this time (2, 12, 15). Concurrent with the onset of viral DNA replication, the entire major late transcriptional unit (L1 to L5) becomes active with at least 19 mRNAs being generated by differential RNA processing.

There were several testable hypotheses concerning the nature of premature termination. First, if premature termination is merely a reflection of transcriptional inefficiency, one would predict the presence of prematurely terminated RNAs throughout the infectious cycle. Second, if premature termination is related to a general tendency early in infection for transcription to terminate in the 5′ region of the transcriptional unit, the only region of this unit expressed before DNA replication, one would expect prematurely terminated RNAs to be more plentiful early in infection. Third, if the start of premature termination coincides with the full expression of the transcriptional unit, one would predict that prematurely terminated RNAs should first become detectable at intermediate times, when the late gene expression commences. Finally, if premature termination occurs only late in infection, premature termination may require more than the synthesis of new templates and the onset of expression of the entire transcriptional unit. In other words, the continued production of RNAs from the entire transcriptional unit would also be necessary.

To test these hypotheses, HeLa cells were infected with Ad2 and maintained in suspension culture at 37°C. At 3 h PI (early time), 8 h PI (intermediate time), or 16 h PI (late time), cells were labeled in vivo with [^{32}P]phosphoric acid for 3 h. These labeling periods were chosen in light of the Ad2 infectious cycle, which has been extensively studied (7). Taking into account the differences in the number of active templates at different times in infection, the ratio of cells used for infection was chosen to be 20:10:1. Nuclear RNAs were extracted, fractionated on sucrose gradients, and pooled into three fractions: larger than 1,000 nucleotides, 100 to 1,000 nucleotides, and ca. 100 nucleotides. RNAs from each pool were selected by the phage M13-cloned probe R14 and analyzed by polyacrylamide gel electrophoresis. The R14 probe complements RNAs with sequences transcribed from the first 197 nucleotides of the major late transcriptional unit. Similar amounts (500 to 1,000 cpm) of these selected RNAs were loaded onto each lane. Premature termination did not occur at early times (Fig. 1, lanes 1 to 3) or at intermediate times (Fig. 1, lanes 4 to 6); however, prematurely terminated transcripts of 175 and 120 nucleotides were unequivocally present late in infection (Fig. 1, lanes 7 to 9). No bands were revealed in longer exposures of lanes 1 to 6 (Fig. 1). Thus, in Ad2, premature termination of transcription is temporally regulated.

RNA transcription in Ad5 *ts*107 under permissive conditions. To establish the biology of Ad5 *ts*107 under permissive conditions, KB cells were infected with the virus and maintained in suspension culture at 32°C. After 13, 22, 30, or 36 h PI, cells were labeled in vivo with [^{32}P]phosphoric acid for 3 h. Nuclear and cytoplasmic RNAs were extracted separately, and the steady-state RNA species were resolved by formaldehyde-agarose electrophoresis. Cells labeled after 13 h PI were early in infection, as indicated both by the profiles of cytoplasmic poly(A)$^+$ RNAs and nuclear RNAs synthesized and by the lack of viral-associated (VA) RNA in the cytoplasmic poly(A)$^-$ RNA fraction (Fig. 2, lanes a). Cells labeled after 22 h PI were at an intermediate time in infection, just after the onset of DNA replication (3; Fig. 3 and 4). As expected, the cytoplasmic poly(A)$^+$ and nuclear RNA profiles changed from an early to a late pattern of synthesis. Also, VA RNA began to be synthesized (Fig. 2, lanes b). RNAs from cells labeled after 30 and 36 h PI represented times late in infection; this was demonstrated most clearly by the increasing levels of VA RNA synthesized (Fig. 2B and C, lanes c and d). Thus, we determined that under permissive conditions at 32°C, 13 h PI represented an early time point; 22 h PI represented an intermediate time point; and 30 and 36 h PI represented late time points with respect to RNA synthesis in Ad5 *ts*107-infected KB cells.

DNA synthesis of *ts*107 under permissive and nonpermissive conditions. Having ascertained that Ad5 *ts*107 under permissive conditions behaved as a wild-type virus in its transcriptional patterns through the infectious cycle (Fig. 2), we performed direct measurements of viral DNA synthesis to verify the times of infection and to ascertain the stringency of the mutation in *ts*107. By Southern blot analysis, it was apparent that at 12 h PI under permissive conditions, there was no increase in total viral DNA (Fig. 3, cf. lanes a and b). Although there might have been a slight increase in viral DNA at 17 h PI at 32°C, the culture which was shifted to 40.5°C at 12 h PI and maintained under nonpermissive conditions for an additional 5 h did not exhibit any additional viral DNA synthesis (Fig. 3, cf. lanes b and d). The halting of DNA synthesis by shifting to a nonpermissive temperature was confirmed at another time of infection. At 22 h PI under permissive condition, viral DNA synthesis had begun (Fig. 3e), but further incubation for 5 h after shifting to the nonpermissive temperature (Fig. 3g) again did not result in additional viral DNA synthesis. The control culture which

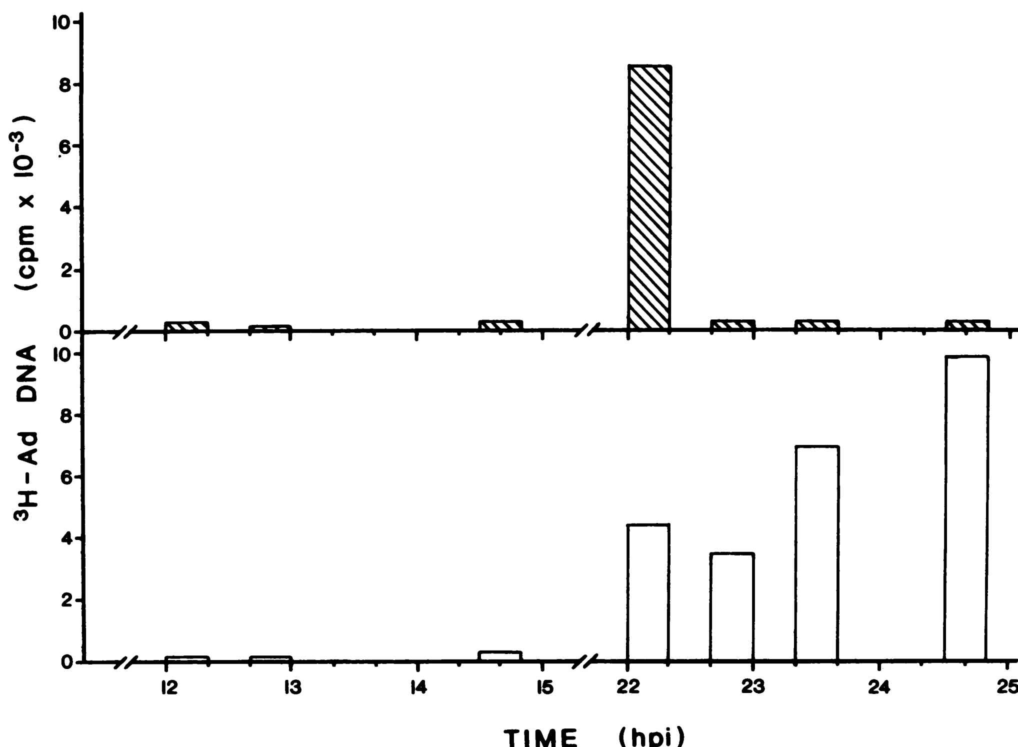

FIG. 4. Effect of temperature shift on DNA synthesis in Ad5 *ts*107. Infection of KB cells with Ad5 *ts*107, temperature shift, DNA extraction, and hybridization to Ad2 DNA immobilized on nitrocellulose filters were carried out as described in the text. The plain histogram represents cells maintained under permissive conditions, and the hatched histogram represents cells after a temperature shift to 40.5°C at 12 or 22 h PI.

was maintained at 32°C for 27 h, however, did continue to synthesize viral DNA (Fig. 3f).

Carter and Ginsberg (3) have reported that in Ad5 *ts*125, a temperature-sensitive mutant defective in the single-strand DNA binding protein, viral DNA synthesis ceases within 1 h after the temperature shift. The thermolability of DNA binding protein for DNA synthesis has since been reproduced in vitro (9, 18). For the purpose of following the kinetics of DNA replication in *ts*107, an isolate of *ts*125, we measured the incorporation of [^{3}H]thymidine into newly synthesized viral DNA, using the pulse-labeling experimental protocol designed by Carter and Ginsberg (3). There was no viral DNA synthesis in three selected 20-min pulse-labeling periods after 12 h PI, under either permissive or nonpermissive conditions (Fig. 4). After 22 h PI under permissive conditions, [^{3}H]thymidine was incorporated into newly synthesized viral DNA in amounts increasing with time. However, viral DNA synthesis was reduced to an undetectable level within 40 min after a shift to nonpermissive conditions. These results corroborate the initial characterization of the lesion of this mutant in vivo (3).

Induction of premature termination of transcription in Ad5 *ts*107. Temperature shifts from 32 to 40.5°C at 12, 22, or 30 h PI were followed by continued incubation at the restrictive temperature for 5 h and [^{32}P]phosphoric acid labeling in vivo for 3 h (Fig. 5B). Cells subjected to a temperature shift at 12 h PI remained early in infection for at least 8 h, as indicated by the lack of viral DNA synthesis (Fig. 3 and 4), the profile of cytoplasmic poly(A)$^+$ RNAs, the abundant host ribosomal RNAs reaching the cytoplasm, and the absence of VA RNA (Fig. 5A, lanes a, e, and i). Selection of the cytoplasmic poly(A)$^-$ RNAs for ribosomal RNAs, using the 28S rRNA-specific probe I-19, was included to verify the identities of the 5- and 2-kilobase bands and to assess the stringency of this method of RNA selection (Fig. 5A, cf. lanes a to d and e to h). Cells after the temperature shift at 22 h PI remained at intermediate times of infection, as demonstrated by the late cytoplasmic poly(A)$^+$ RNA profile, the appearance of VA RNA, and the slight decrease in rRNA (Fig. 5A, lanes b, f, and j). Cells after the temperature shift at 30 h PI remained late in infection, as shown by the increased VA RNA synthesized and the markedly diminished host rRNA (Fig. 5A, lanes c and g). The cells after the temperature shift at 30 h PI and the no-shift control cells were labeled at the same time; however, a comparison of the levels of rRNA and VA RNA revealed again that the temperature shift had the effect of halting the progression of adenovirus lytic infection (Fig. 5A, cf. lanes c and d). This is in agreement with our Southern blot analysis of DNA synthesis in *ts*107 and the finding by Carter and Ginsberg that DNA replication is quickly arrested at restrictive temperatures in *ts*125, and Ad5 strain with the identical DNA binding protein defect (3).

Having characterized the gross transcriptional and DNA synthesis patterns of Ad5 *ts*107 throughout the infectious cycle under permissive conditions and after temperature shifts, we turned our attention to premature termination. Under permissive conditions, prematurely terminated transcripts isolated from small nuclear RNAs, using the phage M13-cloned probe R14, were detectable only late in infection after 30 and 36 h PI (Fig. 6A, lanes c and d). The approximate lengths of the prematurely terminated transcripts were 120 and 175 nucleotides as in Ad2. No small RNAs were selected by the leftward-reading probe 390 which served as a

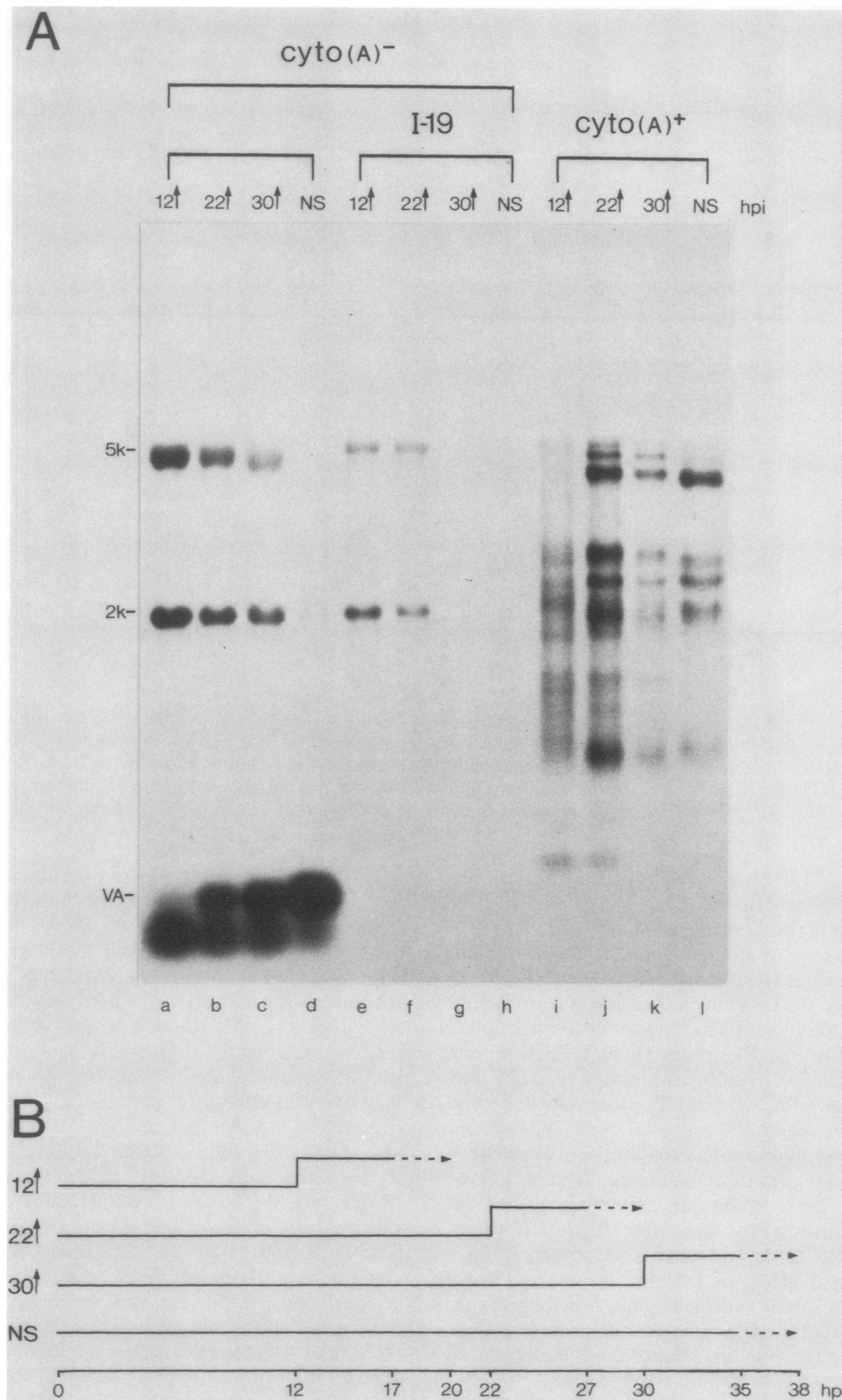

FIG. 5. Profiles of RNA synthesis in Ad5 *ts*107-infected KB cells after a temperature shift up at different times. KB cells were maintained at a permissive temperature until 12, 22, or 30 h PI, at which time the incubating temperature was shifted to 40.5°C for 5 h. The cells were then labeled in vivo for 3 h with [^{32}P]phosphoric acid, and RNAs were extracted as described in the legend to Fig. 2. (A) Cytoplasmic poly(A)$^-$ RNAs labeled after a temperature shift at 12 (lane a), 22 (lane b), or 30 (lane c) h PI and after 35 h PI at 32°C (control; no shift [NS]) (lane d). I-19-selected cytoplasmic poly(A)$^-$ RNAs labeled after shifts at 12 (lane e), 22 (lane f), or 30 (lane g) h PI and at 35 h PI at 32°C (control) (lane h). The I-19 probe is specific for 28S ribosomal RNA and also hybridizes to 18S rRNA. A comparison of lanes a to d and e to h reveals that this method of RNA selection is quite stringent but also shows that the hybridization efficiencies can be variable under these conditions. Cytoplasmic poly(A)$^+$ RNAs labeled after a shift at 12 (lane i), 22 (lane j), or 30 (lane k) h PI and after 35 h PI at 32°C (lane l). A 1.4% formaldehyde-agarose gel was used for RNA analysis. Cell equivalents (lanes): a, 5×10^6; b, 3.3×10^6; c, 1×10^7; d, 1.7×10^7; e, 1.5×10^7; f, 1×10^7; g, 5×10^6, h, 5×10^6; i, 1×10^7; j, 1×10^7; k, 2×10^7; and l, 3.3×10^7 cells. (B) Strategy of the temperature shift experiment. NS represents nonshifted control cells.

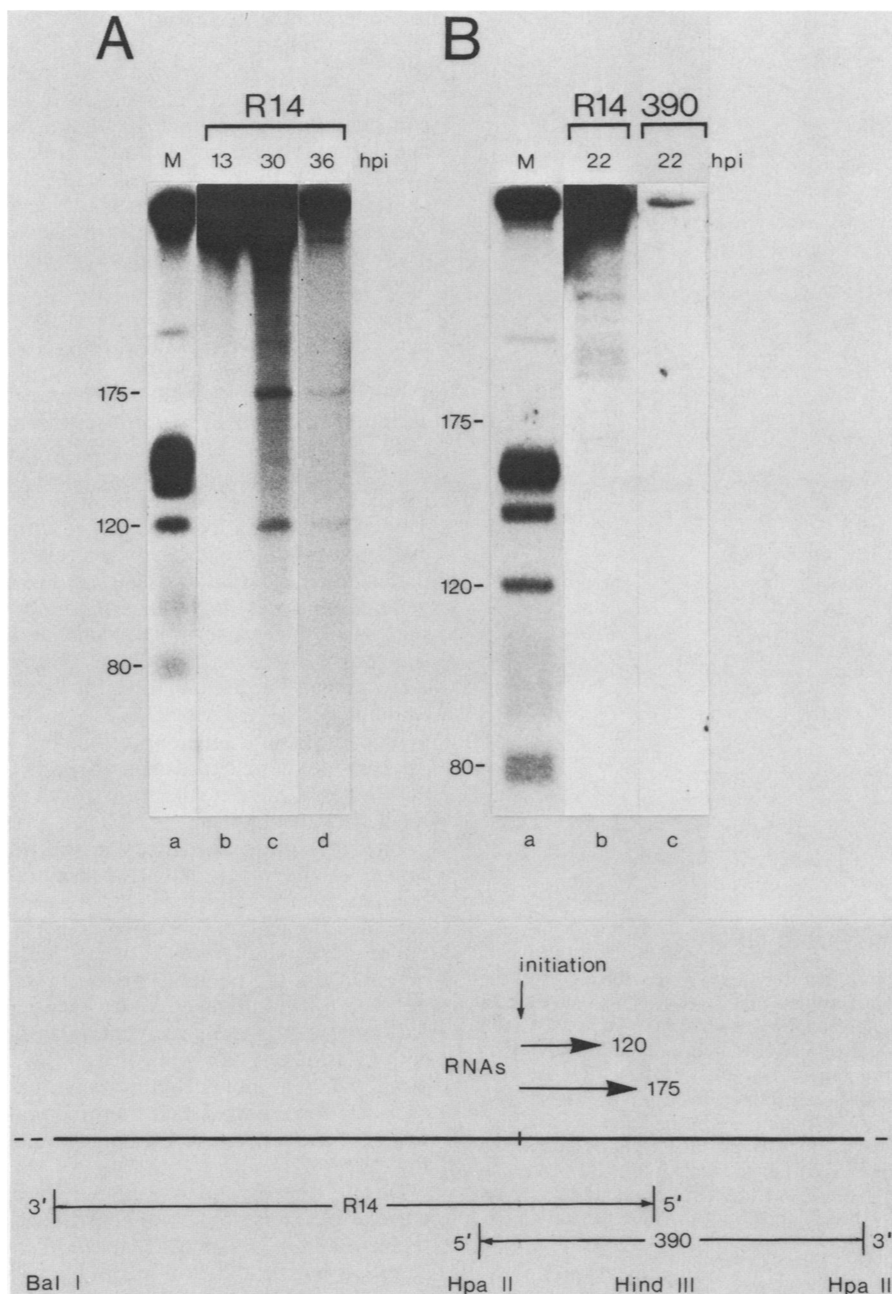

FIG. 6. Temporal expression of prematurely terminated RNAs from the Ad5 *ts*107 major late transcriptional unit. KB cells infected with Ad5 *ts*107 at 32°C were labeled in vivo at indicated times as described in the text. The labeled small nuclear RNAs were selected by probes R14 or 390, and ca. 2,000 cpm of the selected RNAs were loaded in each lane of a 6.6% denaturing polyacrylamide gel. The hybridization efficiencies for selecting RNAs without breakage varied from 5 to 15%. (A) Cytoplasmic poly(A)$^-$ RNAs used as RNA size markers (lane a); R14-selected nuclear RNA labeled at 13 (lane b), 30 (lane c), and 36 (lane d) h PI. (B) Cytoplasmic poly(A)$^-$ RNA marker (lane a), R14-selected nuclear RNA labeled at 22 h PI (lane b), and 390-selected nuclear RNA labeled at 22 h PI (lane c). The relationships of the R14 and 390 probes to the major late transcription unit is shown at the bottom of the figure.

control (Fig. 6B, lane c). Although no premature termination in the major late transcriptional unit was found at early (Fig. 6A, lane b) or intermediate (Fig. 6B, lane b) times, transcriptional pausing was evident after 22 h PI (Fig. 6B, lane b). This demonstration follows our previous findings (Maderious and Chen-Kiang, in press) that pausing and premature termination are distinct. The presence of paused RNAs at 22 h PI also serves as an internal control indicating that the absence of prematurely terminated RNAs at this time of infection was not due to the lack of RNA synthesis specific to the initiation site-proximal region. Consistent with these observations are results of Northern blot analyses in which nuclear RNAs from Ad2-infected cells were probed with either R14 or another phage M13 probe specific for the Ad2 L1 family; extramolar synthesis of RNAs from the promoter-proximal region was seen late in infection but not early (K. Wu and S. Chen-Kiang, unpublished data).

In a temperature-shift experiment (Fig. 7), no premature

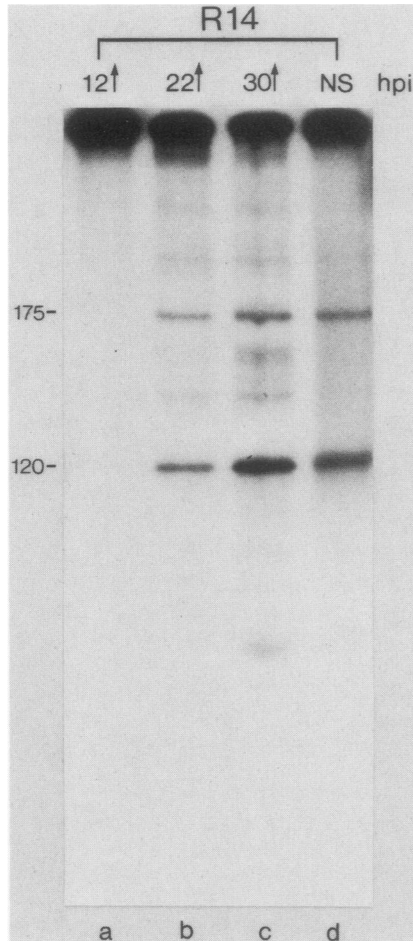

FIG. 7. Expression of prematurely terminated RNAs from the Ad5 *ts*107 major late transcriptional unit after various temperature shift-up times. KB cells were infected with Ad5 *ts*107 at 32°C and labeled with [^{32}P]phosphoric acid after temperature shifts at various times as described in the text. The small nuclear RNAs were selected by probe R14 and electrophoresed on a 6.6% denaturing polyacrylamide gel. R14-selected nuclear RNA labeled after shifting the temperature to 40.5°C at 12 (lane a), 22 (lane b), and 30 (lane c) h PI, and R14-selected nuclear RNA labeled at 35 h PI at 32°C, i.e., no temperature shift (lane d). The RNA transcripts are marked according to their approximate lengths in nucleotides. Approximately 1,000 cpm were loaded in each lane.

termination was detected after the temperature shift at 12 h PI, indicating that premature termination as well as late gene expression was dependent on prior DNA replication and not on the accumulation of early gene products, which continued to be synthesized at the elevated temperature (Fig. 7a). Interestingly, premature termination was found after the temperature shift at 22 h PI, unlike after 22 h PI under permissive conditions (cf. Fig. 7b and Fig. 6B, lane b). The cells shifted to the restrictive temperature at 22 h PI remained at the intermediate time of infection judging from the replication and transcription patterns, even though they had been infected for a total of 30 h. Since DNA replication was arrested at restrictive temperatures, the proportion of new templates to old templates should have been maintained after the temperature shift and should have been no greater than that at the corresponding time under permissive conditions. Therefore, the most likely explanation to account for the induction of premature termination was the cumulative late gene transcription during the 8 h after the temperature shift. As expected, premature termination was observed after the temperature shift at 30 h PI and in the no-shift control cells (Fig. 7c and d). Again, the prematurely terminated transcripts were 120 and 175 nucleotides in length in all cases. Taken together, these results also demonstrate that continuous DNA replication is not necessary for premature termination to occur, since restrictive conditions which prevent DNA synthesis did not prevent the appearance of these transcripts.

DISCUSSION

Our results indicate that premature termination of transcription of the major late transcriptional unit occurs temporally in both Ad2- and Ad5-infected human cells. The 120- and 175-nucleotide transcripts are present in nuclei late in infection but not in the early or intermediate times. Since premature termination is not found throughout the infectious cycle, it cannot be explained as a reflection of transcriptional inefficiency. In addition, this phenomenon is not related to a general tendency for transcription to terminate in the 5′ portion of the transcriptional unit during production of L1 transcripts, because premature termination is not seen at early times. The finding that the occurrence of premature termination is not coincidental with the onset of DNA synthesis and late gene expression but is some time after the onset of late gene expression suggests that premature termination might have a role in or result from a transcriptional regulatory mechanism.

The nucleotide sequences in the initiation site-proximal region of the major late transcriptional unit where these premature terminations occur are nearly identical in Ad5 and Ad2 (see the legend to Fig. 8). We have previously pointed out sequence similarities between the premature termination region of the 175-nucleotide species in Ad2 and the termination site t_{R1} in coliphage λ and between an upstream site in Ad2 and the N protein recognition site nutR in λ (Maderious and Chen-Kiang, in press). As the nutR site in λ, the predicted stem-loop structure centered at position 59 of this transcription unit in Ad2 is thermodynamically unstable with $\Delta G = -3.2$ kcal. These sequence homologies are depicted in Fig. 8.

Despite these sequence homologies and their functional correlations, it is unlikely that termination of transcription is regulated analogously in eucaryotes and procaryotes. Transcription and translation are not physically coupled in eucaryotes, and RNA must undergo complex nuclear processing before entering the cytoplasm. In phage λ, premature termination at t_{R1} occurs during the early times of lytic infection. Transcription antiterminates later after the appearance of an early phage gene product, N protein. This process allows the expression of late phage genes. In the adenovirus major late transcriptional unit, the temporal expression of premature termination is opposite that in λ; that is, premature termination does not occur at the early times but only when the late genes become fully expressed. This presents a paradox. In searching the adenovirus genome for these λ-like sequences, we have also noticed sequences similar to those at the λ nutR site and the λ t_{R1} site beginning at 324 and 374 nucleotides, respectively, downstream from the polyadenylation site of the L1 family, which is the only portion of the adenovirus major late transcriptional unit expressed in the early times of infection (2, 12, 15). This suggests the intriguing possibility of a scheme involving differential utili-

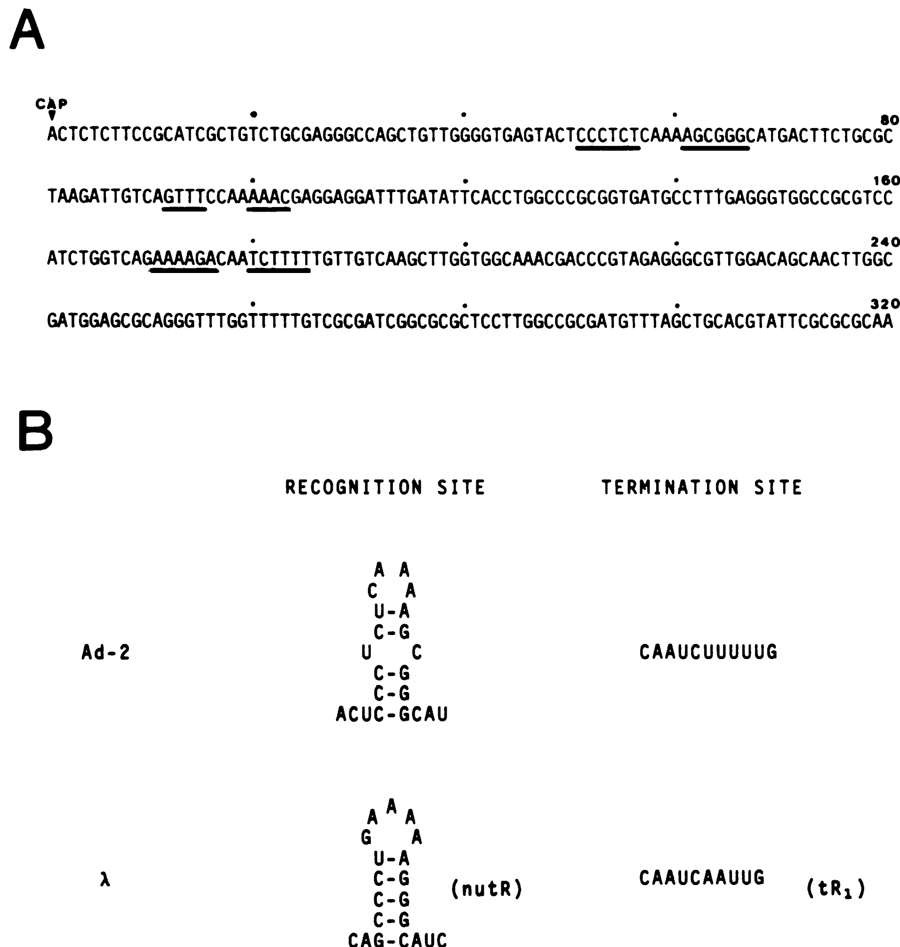

FIG. 8. Initiation site-proximal region of the major late transcriptional unit in Ad2. (A) The first 320 nucleotides of this transcriptional unit in Ad2. Three sequences which contain dyadic symmetry are underlined. The first one, centered at position 59, shares sequence homology with the nutR site in λ; the second is centered at position 98; and the third is centered at position 179. The sequence of Ad5 at this region is identical to that of Ad2 except for a G at position 57 and an A at position 147. (B) A comparison of the RNA sequences in Ad2 and λ which exhibit homology. On the left are possible hairpin structures centered at position 59 in Ad2 and the nutR site in λ. On the right are the sequences in the premature termination region of the 175-nucleotide RNA species in adenovirus and that at the termination site t_{R1} in λ. This sequence resembles those present at the 3′ termini of *Drosophila* and yeast transcripts, 5′-CAA$^{U}_{G}$CUUUG-3′, as described by Henikoff et al. (8). Note that the adenovirus sequences in the premature termination regions of the 175- and 120-nucleotide RNA species are not similar.

zation of termination sites during the early-to-late switch of gene expression.

The mechanism by which the expression of the major late transcriptional unit qualitatively changes in the transition from early times of infection to the late times could involve an alteration in transcriptional termination. Only the 5′ portion (L1) of this transcriptional unit is expressed at early times, whereas the entire transcriptional unit (L1 to L5) is active at intermediate and late times (2, 12, 15). We propose as a working model that the presumed termination site(s) 3′ to the polyadenylic acid addition site of L1 would be used at early times. At intermediate and late times, with the advent of newly replicated DNA templates which are in some way modified compared to the old templates, this site(s) is no longer the only one(s) utilized, so that the entire transcriptional unit could be transcribed. In fact, our results demonstrate also that new templates are needed before the late pattern of transcription appears; preventing adenovirus DNA replication by shifting to a restrictive temperature 12 h PI precludes the appearance of RNAs typical of late time (Fig. 5A, lanes a, e, and i). These results are in agreement with those of Thomas and Mathews (17) and Crossland and Raskas (6).

In addition, we propose that the initiation site-proximal premature termination sites would be utilized only on newly synthesized templates. Although premature termination normally occurs at late times, we have shown that it is inducible at intermediate times shortly after the onset of viral DNA replication by shifting to a restrictive temperature which halts further template synthesis. The most likely explanation for the delayed appearance of premature termination after the onset of DNA replication under permissive conditions is that there is a dependence on cumulative late gene transcription. Thus, premature termination may be involved in a feedback modulation of late gene expression, although the precise relationship of these two events requires further elucidation.

Along with initiation of transcription, termination has

been established as an important component in the regulation of transcription in procaryotic genes. In contrast, transcriptional termination in eucaryotes has remained poorly understood; the mechanism of this process is bound to be more complex. The coupling of transcription and translation is an important factor in the system of termination control present in procaryotes. These processes are not coupled in eucaryotes due to the nuclear-cytoplasmic compartmentalization. Eucaryotic systems must therefore either ignore the relationship between the transcription of a gene and its ultimate product (an unlikely event) or generate new regulatory mechanisms to circumvent this problem. Our findings that premature termination occurs at specific times in the adenovirus infectious cycle and is associated with the appearance of both new templates and cumulative late gene transcription suggest that termination control may be important as one of the mechanisms for regulating gene expression in eucaryotes. In addition, our ability to manipulate transcription in vivo and in vitro in the adenovirus major late transcriptional unit makes this an attractive model system for studying transcriptional termination in eucaryotes.

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