JOURNAL OF VIROLOGY, June 1983, p. 737–748
0022-538X/83/060737-12$02.00/0

Vol. 46, No. 3

Identification of Adenovirus Genes That Require Template Replication for Expression

LYLE D. CROSSLAND† AND HESCHEL J. RASKAS*

Department of Pathology, Washington University School of Medicine, St. Louis, Missouri 63110

Received 15 November 1982/Accepted 1 March 1983

The relationship between adenovirus type 2 DNA replication and expression of intermediate stage viral genes was investigated. The 1.03-kilobase mRNA from early region 1b (E1b) and the mRNAs coding for proteins IX and IVa_2 were first detected between 6 and 8 h postinfection. Inhibition of viral DNA replication with hydroxyurea prevented expression of the IX and IVa_2 mRNAs, but not of the E1b mRNA. Pulse-labeling experiments demonstrated that the block of IX and IVa_2 expression in hydroxyurea-treated cells was at the level of transcription. By a series of superinfection experiments, it was determined that the viral and cellular factors present during the late stage of adenovirus infection are insufficient to activate IX gene expression. The viral DNA template must first replicate before IX transcription can begin.

Lytic infection by human adenoviruses is divided into four temporal stages of gene expression: pre-early, early, intermediate, and late (5, 19, 34). The assignment of genes to a particular stage has been based on the time of appearance of the gene product. mRNAs that appear between 6 and 8 h after infection have been assigned to the intermediate class (34, 38). Included in this class are the 0.52-kilobase (kb) mRNA from region E1a, the 1.03-kb mRNA from E1b, and the mRNAs for protein IVa_2 and protein IX (Fig. 1). Previous work has indicated that the appearance at intermediate times of the 0.52-kb and 1.03-kb mRNAs is due to increased cytoplasmic stability of mRNA species produced in the nucleus at early times (41). The IX gene, however, is regulated at the transcription level, and its promoter is not active until intermediate times (42). The mode of regulation of the IVa_2 gene has not been determined.

The promoter for the major late transcription unit (Fig. 1) is active at early times, although premature termination of the transcripts prevents formation of the mRNAs for late regions L2 through L5 (2, 29, 35). The onset of viral DNA replication has traditionally marked the beginning of the late stage of adenovirus infection (8). Thomas and Mathews (39) demonstrated that the gene products of late regions L2 through L5 are expressed only from a replicated DNA template; even though the major late promoter is active on parental genomes, replication and some coincident physical change in the template are required for expression of the downstream late regions. The relationship of intermediate gene expression to DNA replication is less certain. Although intermediate gene expression begins between 6 and 8 h after infection (about the same time that viral DNA replication begins), evidence of IX and IVa_2 gene expression has been found in cells infected in the presence of cytosine arabinoside, an inhibitor of DNA replication (24, 30). It is certain, however, that the expression of IX and IVa_2 mRNA is at its maximum level at late times after viral DNA replication has begun (24, 38, 42). Because all five early transcription units (28) and the major late transcription unit (2, 29, 35) are active by 3 h postinfection, while the IX transcription unit is inactive at this time (42), the IX promoter may be the last viral promoter to be activated. The exact times of activation of the other known viral promoters, IVa_2 and the alternative E2 promoter at map position 72, are not known.

The goal of this work was to determine the relationship between viral DNA replication and intermediate gene expression, focusing on the gene for protein IX. The first set of experiments was designed to determine how hydroxyurea (HU) blockade of DNA synthesis affects intermediate gene expression. HU was chosen because at 10 mM it blocks detectable DNA synthesis, does not break down during the course of the experiment (39), and does not significantly affect RNA synthesis. The second set of experiments examined the basis of the dependence of IX gene expression upon viral DNA replication.

† Present address: The Biological Laboratories, Harvard University, Cambridge, MA 02138.

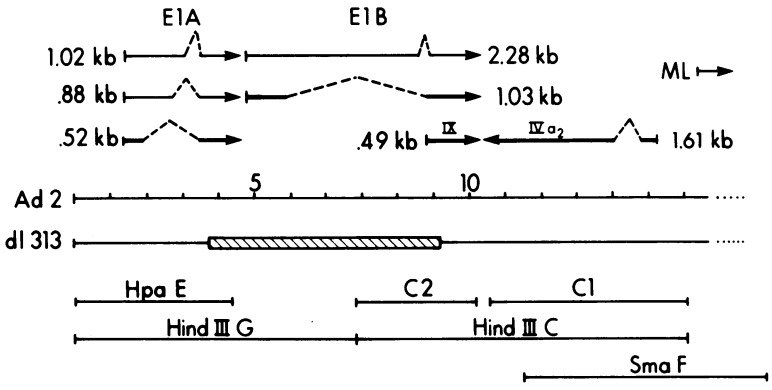

FIG. 1. Map positions of Ad2 left-end mRNAs (6, 9, 21) and DNA fragments cloned for use as probes. Spliced-out regions of mRNAs are indicated by dashed lines, and the approximate size in kb of each mRNA is given. ML is the major late promoter (13, 44). The hashed box indicates the region deleted in *dl*313 (18). All DNA fragments were inserted into pBR322, with the exception of *Sma* F which was inserted into M13 phage, and were used as recombinant DNAs. The M13-*Sma* F recombinant used released the left strand of *Sma* F in phage particles.

MATERIALS AND METHODS

Cells and virus. Cells were grown as monolayer cultures in Dulbecco modified Eagle medium (DME) containing 10% calf or horse serum (KC Biologicals) for KB or 293 cells, respectively. Cells were split 2 days before the experiments, to be approximately 70% confluent when infected. Virus stocks were prepared as described (37). *ts*125 virus was obtained from Jim Williams, *dl*313 was obtained from Tom Shenk, and Ad2$^+$ ND5 was obtained from Andrew Lewis.

Infection conditions. Cells for infection were grown in 150-mm dishes. The medium was aspirated off, and the monolayers were washed with serum-free DME. Virus was added in 5 ml of DME per plate, and absorption was continued for 1 h with gentle agitation every 15 min. The inoculum was removed, and 30 ml of DME containing 10% serum and, in some cases, 10 mM HU was added to each plate.

The superinfection protocol was identical to that for infection, except that a 45-min absorption was used. Great care was needed to avoid detaching the 293 cells when adding the superinfection inoculum and overlay medium.

Recombinant DNA probes. The left strand of the Ad2 *Sma*F fragment (map units 11.5–18.5) cloned in the single-stranded bacteriophage M13 was obtained from Mark Boguski. The *Hpa*E (map units 0–4.4), *Hin*dIII C (7.9–17.0), *Eco*RI C (89.7–100), *Hin*dIII H (72.8–79.9), and *Eco*RI F1 (72.8–75.9) fragments of adenovirus type 2 (Ad2) cloned in pBR322 were obtained from Beth Ladin, Elizabeth Slattery, Michael Tigges, D. Huang, and Richard Rosenthal, respectively. The *Hin*dIII C1 (11.5–17.0) and C2 (7.9–11.1) fragments were obtained by digesting the original *Hin*dIII C clone with *Sma*I, which cuts at 11.1 and 11.5. The linear DNA was then digested with a second enzyme that cuts the plasmid sequence near the insert. *Eco*RI digestion was used to excise the 11.5–17.0 region, and *Bam*I digestion was used to excise 7.9–11.1. The DNAs were then recircularized with T4 DNA ligase and used to transform *Escherichia coli* HB101.

Pulse-labeling RNA. To pulse-label cells, the medium was aspirated off and replaced with 8 ml per plate of DME with serum, containing 300 μCi of [^{3}H]uridine per ml and, where appropriate, 10 mM HU. After 5 or 10 min of incubation, the plates were placed on ice, the labeling solution was removed, and the cells were scraped into cold phosphate-buffered saline.

RNA isolation. Cytoplasmic, polyadenylic acid [poly(A)]-containing RNA was isolated by Nonidet P-40 leakage in isotonic buffer, extraction of the postnuclear supernatant with phenol-chloroform-isoamyl alcohol, and oligodeoxythymidylate-cellulose chromatography, all as described previously (25).

Total cell RNA was isolated by an adaption of the procedure of Sauerbrier and Brautigam (32). Harvested cells were pelleted from cold phosphate-buffered saline and taken up in 1 ml of lysis buffer (1% sodium dodecyl sulfate, 1 mM EDTA, 0.1 M NaCl, 0.1% diethyloxydiformate, and 10 mM Tris, pH 7.5). The mixture was vortexed vigorously, heated at 85°C for 30 s and cooled on ice. An 8-ml volume of 8.5 molal CsCl in lysis buffer containing 0.1% sodium dodecyl sulfate was added, and the solution was pipetted several times. The lysed cell mixture was layered over 2.5 ml of 9.5 molal CsCl in an SW41 tube and centrifuged at 26,000 rpm for 22 h at 8°C in an SW41 rotor. The supernatant was aspirated, and the RNA pellet was suspended in water and precipitated by the addition of sodium acetate to 0.2 M and 2.5 volumes of ethanol.

RNA blots. RNA samples for Northern analysis were incubated with 1 M glyoxal in 10 mM Na_2PO_4 (pH 6.8) at 50°C for 1 h (26) and then electrophoresed in 1.1 or 1.4% agarose gels. In all cases, RNA from an equivalent number of cells was loaded on each lane of a given gel. The RNAs were transferred to nitrocellulose as described by Thomas (40) and hybridized to nick-translated DNA probes.

RNA gradients. Pulse-labeled RNA for sucrose gradient fractionation was taken up in 250 μl of a solution containing 80% formamide, 2 mM EDTA, and 40 mM PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)] (pH 7.4). The mixture was heated at 68°C for 7.5 min

and layered on a preformed gradient of 5 to 20% sucrose in the same buffer solution. The gradients were centrifuged in an SW41 rotor at 30,000 rpm for 48 h at 4°C, and 350-μl fractions were collected from the bottom of the tube. The RNA in each fraction was precipitated with ethanol, taken up in buffer, and partially hydrolyzed with alkali as described by Nevins (27). Selected fractions were then hybridized to DNA bound to nitrocellulose filters as described previously (25).

DNA blots. To parepare DNA for Southern analysis, nuclei were isolated as described (16) and lysed by the addition of DNA extraction buffer (0.1 M NaCl, 10 mM EDTA, 0.5% sodium dodecyl sulfate, 50 mM Tris, pH 8.5). The mixture was digested with 1 mg of pronase B per ml overnight at 37°C, extracted with phenol-chloroform (1:1) three times, and precipitated with ethanol. The DNA was taken up in an appropriate buffer, digested with the restriction enzyme *Kpn*I, and electrophoresed on a 1% agarose gel. The DNA bands were transferred to nitrocellulose by the method of Southern (36) and hybridized to nick-translated Ad2 DNA.

RESULTS

Time course of intermediate gene expression. As a first step toward studying their regulation, the time of appearance of the intermediate mRNAs was examined. To determine the steady-state concentration of the mRNAs in the cytoplasm as infection progressed, poly(A)-containing cytoplasmic RNA was isolated from 2×10^7 KB cells infected for 4, 6, 8, or 10 h. The RNA was subjected to electrophoresis in an agarose gel and then transferred to nitrocellulose. The Ad2 *Hin*dIII C fragment (7.9 to 17.0 map units), cloned in pBR322, was nick-translated and hybridized to the RNA blot. The three intermediate mRNAs that hybridize to this probe, the 1.03-kb E1b mRNA and the IX and IVa_2 mRNAs, were first detected by this analysis between 6 and 8 h postinfection (Fig. 2, lane 3). As expected, the 2.28-kb early mRNA from E1b was detected at 4 h postinfection (Fig. 2, lane 1).

Intermediate gene expression in the absence of DNA replication. When DNA synthesis was blocked by the addition of 10 mM HU after virus absorption, the normally large quantity of IX mRNA present in 2×10^7 cells at 12 h postinfection was undetectable (Fig. 2, lanes 8 and 9). In contrast, the 2.28-kb and 1.03-kb E1b mRNAs were both present in HU-treated cells, although the 1.03-kb species was reduced in quantity. The *Hin*dIII C2 probe (7.9–11.1) used in this experiment does not hybridize IVa_2 mRNA. When the infection was continued for 15 h and the *Hin*dIII C probe (7.9–17.0) was used in the hybridization analysis, neither the IX nor the IVa_2 mRNA was detected in HU-blocked cells (Fig. 2, lanes 5 and 6). Similar experiments (not shown) suggest that the 0.52-kb E1a mRNA has the same time course and resistance to HU blockage as the 1.03-kb E1b mRNA. Although the time course of appearance of the four intermediate mRNAs was the same and the levels of the 0.52-kb E1a and 1.03-kb E1b mRNAs were reduced by HU, only the IX and IVa_2 mRNAs were dependent upon viral DNA replication for expression in the cytoplasm.

Activation of IX and IVa_2 expression is independent of template number. At 12 h postinfection, cells infected in the presence of HU contained fewer templates for viral transcription than control cells infected without drugs. To ensure that this difference in template number was not responsible for our inability to detect IX mRNA in HU-treated cells, infection conditions were determined that would place approximately the same number of templates in the nuclei of the control and HU-treated cells. Four plates of KB cells were infected at a multiplicity of infection (MOI) of 10, and at various times after infection nuclear DNA was isolated. A fifth plate was infected at an MOI of 500 in the presence of HU, and at 10 h postinfection its nuclear DNA was isolated. Equal amounts of these DNA samples were bound to nitrocellulose filters and hybridized to saturating amounts

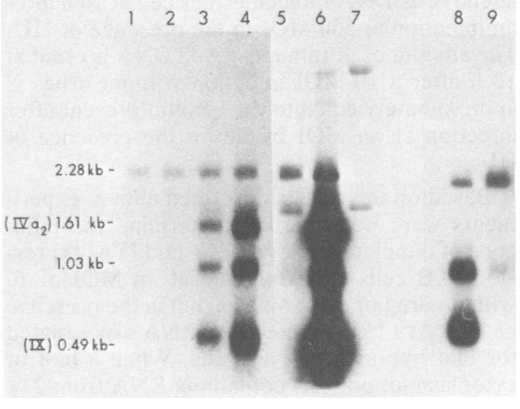

FIG. 2. Time course of intermediate mRNA expression. Cytoplasmic, poly(A)-containing RNA was isolated from cells infected for 4, 6, 8, or 10 h (lanes 1 through 4), for 12 h (lanes 8 and 9), or for 15 h (lanes 5 and 6). Lanes 5 and 9 are from cells infected in the presence of 10 mM HU. Lane 7 is a ^{14}C-labeled rRNA marker. The mRNAs were electrophoresed in an agarose gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled *Hin*dIII C (lanes 1 through 7) or *Hin*dIII C2 (lanes 8 and 9) DNA. The additional band comigrating with the 18S rRNA marker in lanes 3, 4, and 5 is occasionally seen with several Ad2 DNA probes. Presumably, it results from RNA aggregation due to rRNA contamination of poly(A)-containing RNA and subsequent overloading of this region of the gel.

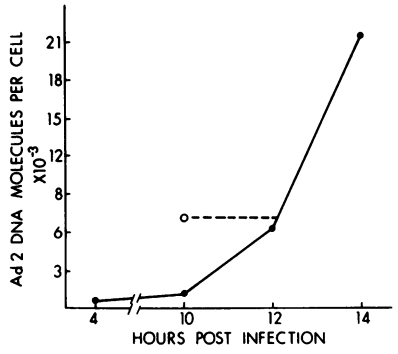

FIG. 3. Quantitation of Ad2 DNA in infected cells. The amount of Ad2 DNA per cell was determined by binding nuclear DNA from 3.3×10^4 infected KB cells to nitrocellulose and hybridizing to saturating amounts of ^{3}H-labeled Ad2 DNA. Under identical conditions, 3.9×10^{-3} μg of filter-bound Ad2 DNA (the equivalent of 9.4×10^7 Ad2 DNA molecules) hybridized 24,400 cpm. It was concluded that each 8.6 cpm hybridized to the experimental filters represented one Ad2 DNA molecule per cell. Symbols: ●, DNA from cells infected at an MOI of 10; ○, DNA from cells infected at an MOI of 500 in the presence of HU.

of ^{3}H-labeled Ad2 DNA (Fig. 3). The open circle in the center of Fig. 3 indicates the number of adenovirus DNA molecules per cell at 10 h after an infection at 500 MOI in the presence of HU. The amount of intranuclear Ad2 DNA present at 12 h after a 10 MOI infection without drugs is approximately equal to the amount present after infection at an MOI of 500 in the presence of HU.

Based on the results described above, experiments were performed to determine the influence of template number on IX and IVa_2 expression. KB cells were infected at an MOI of 10 with no drug or at an MOI of 500 in the presence of HU. At 12 h postinfection, RNA was isolated for blot hybridization analysis. When a blot of cytoplasmic, poly(A)-containing RNA from 2×10^7 cells was hybridized to the *Hin*dIII C probe (7.9–17.0), the IX and IVa_2 mRNAs were only detected in drug-free control cells, whereas the E1b 2.28-kb and 1.03-kb mRNAs were detected in control and HU-treated cells (Fig. 4, lanes 1 and 2) (the 1.03-kb band was too faint to reproduce in the photograph). When total cell RNA from 2×10^7 cells was hybridized to the *Hin*dIII fragment C2 (7.9–11.1) (Fig. 4, lanes 4 and 5) a similar result was observed, except that the level of the 1.03-kb mRNA was relatively greater in total cell RNA. Hybridization of the total cell RNA blots to the *Eco*RI C (89.7–100) (Fig. 4, lanes 7 and 8) probe demonstrated that the predominant early mRNAs from region E4 were present in equal or greater amounts in the HU-treated cells.

Newly synthesized IX and IVa_2 mRNA can only be detected when viral DNA replication occurs. Regulation of mRNA levels in the cytoplasm can occur at several levels (11). To determine whether the absence of IX and IVa_2 mRNA in HU-treated cells was because of lack of transcription of these genes, newly synthesized molecules were quantitated after a brief pulse label. Because the entire sequence of IX mRNA is shared with the E1b transcript, no DNA probe can specifically hybridize only IX mRNA. To quantitate IX transcription it was therefore necessary to size-fractionate IX mRNA and the E1b mRNAs before hybridization. KB cells were again infected at 10 MOI without drugs or 500 MOI in the presence of HU. At 12 h postinfection, when the number of viral DNA templates in the two sets of cells was equal, the cells were pulse-labeled with [^{3}H]uridine for 10 min. Total cell RNA was isolated and fractionated by sucrose gradient centrifugation. To locate the fractions containing mRNAs of interest, samples of fractions 9 through 25 were electrophoresed in an agarose gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled *Hin*dIII C DNA (7.9–17.0). Transcription activity was assayed by hybridization of gradient fractions to nitrocellulose-bound *Hin*dIII C2 DNA (7.9–11.1) to assay E1b and IX, or *Sma*F left-strand DNA (11.5–

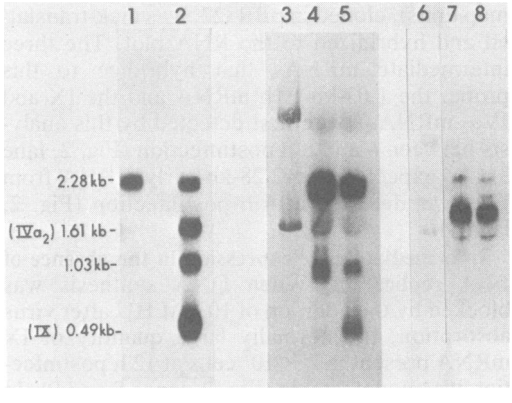

FIG. 4. Effect of HU on early and intermediate mRNA accumulation. KB cells were infected at an MOI of 500 in the presence of HU (lanes 1, 4, and 7) or at an MOI of 10 without drugs (lanes 2, 5, and 8). Twelve hours postinfection, cytoplasmic, poly(A)-containing RNA (lanes 1 and 2) or total cell RNA (lanes 4-8) was isolated, electrophoresed in an agarose gel, and transferred to nitrocellulose. The RNA blots were hybridized to ^{32}P-labeled *Hin*dIII C (lanes 1 and 2), *Hin*dIII C2 (lanes 3 through 5), or *Eco*RI E (lanes 7 and 8) DNA. Lanes 3 and 6 are ^{14}C-labeled rRNA markers.

18.2) to assay IVa_2 (Fig. 5). In both RNA gradients, a significant peak of [^{3}H]RNA, about 22S in size, hybridized to the *Hin*dIII C2 probe (7.9–11.1). This peak represents either the E1b precursor (2.36 kb) or the 2.28-kb message, or both. The absence of a peak corresponding to the 1.03-kb mRNA in either gradient suggests that 10 min is insufficient time for labeling and splicing of a significant amount of the precursor to occur. Transcription from the IX gene, represented by the 9S peak (see Fig. 5), and the IVa_2 gene, represented by the 17S peak, was not detectable after a 10-min pulse unless DNA replication was allowed. Similarly, after a 5-min [^{3}H]uridine pulse, transcription from the IX gene was not detected unless DNA replication was allowed (Fig. 6). Other pulse-labeling experiments (not shown) have demonstrated that IX mRNA continues to be transcribed if HU is added after viral DNA replication has begun. We conclude that, when viral DNA replication is blocked with HU, transcription of IX and IVa_2 is reduced at least 90% and is very likely absent.

Development of a superinfection protocol. Superinfection experiments were used to determine how IX transcription is related to viral DNA replication. Activation of the IX promoter might be dependent upon *trans*-acting, viral or cellular, regulatory factors that are produced only as the virus begins DNA replication. If this is the case, an unreplicated viral genome entering a previously infected, late-stage cell should begin transcribing the IX gene. Alternatively, the IX promoter might require activation by a *cis*-acting change in the template itself during replication. In this case, a superinfecting viral genome would first have to be replicated before it could act as a template for IX transcription, even if it had entered a late-stage cell. To distinguish between these possibilities, we performed a series of superinfection experiments utilizing adenovirus deletion and insertion mutants.

In all of the superinfection experiments, *dl*313 was used as the preinfecting virus. *dl*313 is an Ad5 deletion mutant lacking part of E1, including the IX promoter (18). The Ad5-transformed human cell line 293 contains integrated Ad5 DNA from region E1 (1, 17); *dl*313 can lytically infect 293 cells because the missing E1 functions are provided by the host cell. However, because the viral IX promoter is deleted and the integrated IX gene cannot be activated by infection, no IX mRNA or protein is produced during the infection (37).

It was essential that *dl*313-infected cells be in the late stage of infection and thus be producing any factors that might be necessary for IX transcription before superinfection. To determine when the late stage begins, *dl*313-infected

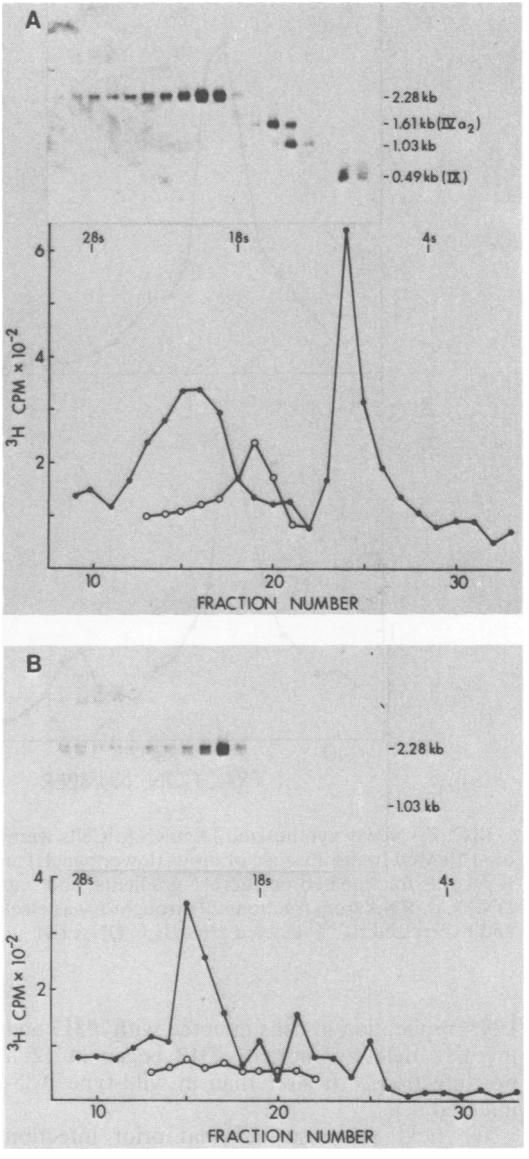

FIG. 5. Newly synthesized IX and IVa_2 mRNA. Cells infected at an MOI of 10 without drugs (A) or at an MOI of 500 in the presence of HU (B) were pulse-labeled for 10 min with [^{3}H]uridine. Total cell RNA was isolated and fractionated on sucrose gradients, and fractions were hybridized to nitrocellulose-bound *Hin*dIII C2 DNA (●) or *Sma*F left-strand DNA (○). The locations of the 28S, 18S, and 4S RNA markers in the gradient are indicated. The insets show agarose gels of fractions 9 through 25, transferred to nitrocellulose and hybridized to ^{32}P-labeled *Hin*dIII C DNA.

293 cells were pulse-labeled with [^{3}H]thymidine at various times after infection. DNA, isolated from cells from each time point, was hybridized to nitrocellulose-bound Ad5 DNA (Fig. 7). Viral

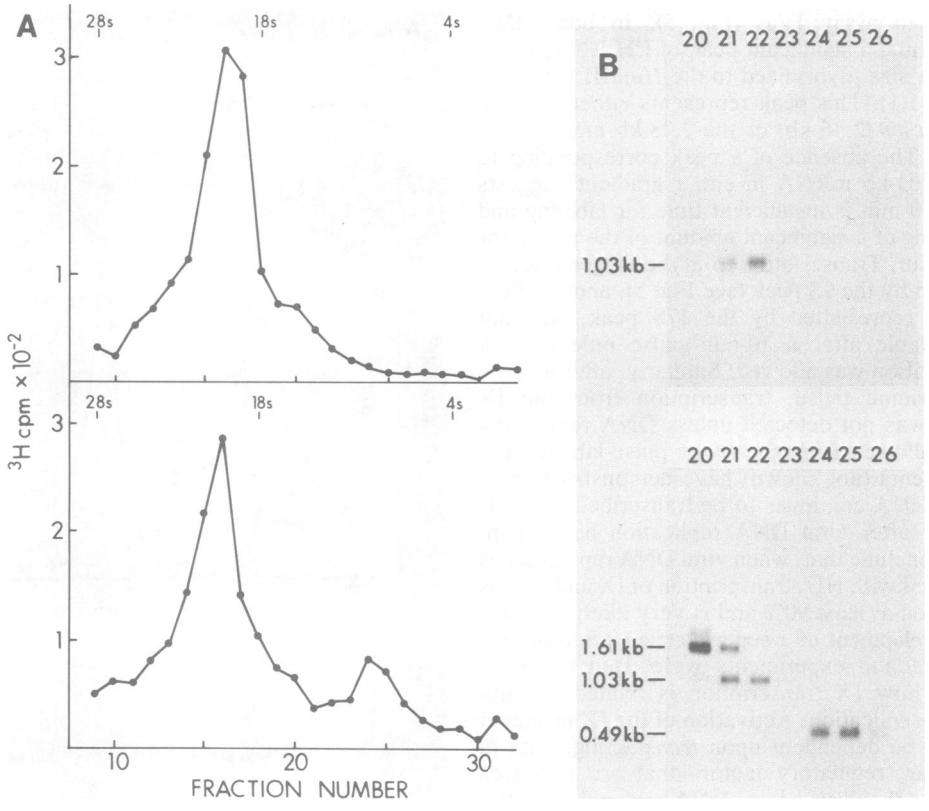

FIG. 6. Newly synthesized IX mRNA. Cells were infected at 500 MOI in the presence of HU (upper panels) or at 10 MOI in the absence of drugs (lower panels) and were pulse-labeled for 5 min with [^{3}H]uridine. Total cell RNA was fractionated on sucrose gradients, and fractions were hybridized to nitrocellulose-bound *Hin*dIII C2 DNA (A). RNA from fractions 20 through 26 was electrophoresed in an agarose gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled *Hin*dIII C DNA (B).

DNA replication in cells infected with *dl*313 and the E1a deletion mutant *dl*312 began at 12 h postinfection, 6 h later than in wild-type Ad5-infected cells.

We next demonstrated that prior infection with *dl*313 can provide functions needed for a superinfection to proceed. For this experiment we utilized *dl*313 and, as a superinfecting virus, the temperature-sensitive Ad5 mutant *ts*125. *ts*125 codes for a temperature-sensitive DNA-binding protein and cannot replicate viral DNA at 40°C (12, 43). Mock-infected and *dl*313-infected 293 cells were shifted from 37 to 40°C at 13 h postinfection and superinfected with *ts*125. Southern blot analysis (36) of the viral DNA in these cells revealed that mock-infected, *ts*125-superinfected cells contained only about as much *ts*125 DNA after 12 h at 40°C as a similar preparation blocked with HU (Fig. 8, lanes b and e). Little viral DNA replication had occurred, and the bands observed were presumably due mostly to parental *ts*125 DNA. *dl*313-infected, *ts*125-superinfected cells contain DNA from both viruses. To observe the replication of only the superinfecting virus by Southern blot hybridization, a marker specific for *ts*125 DNA was required. Because of the deletion in *dl*313, the *Hin*dIII G fragment present in Ad5 (and *ts*125) is missing. Prior infection with *dl*313 allowed significant production of *ts*125 DNA in 12 h at 40°C, as evidenced by the density of band G in lane a versus lane b of Fig. 8. A similar result was obtained when *ts*125 was allowed 24 h to replicate (Fig. 8, lanes c and d). These results prove that the superinfecting *ts*125 DNA enters the nucleus, where an early protein coded by *dl*313 can complement its missing function.

Only replicated templates can express IX mRNA. The superinfection protocol was utilized to assay IX expression from unreplicated and replicated templates in a late environment. In addition to IX, the superinfecting virus should code for an early RNA (one that is expressed prior to viral DNA replication) that can be

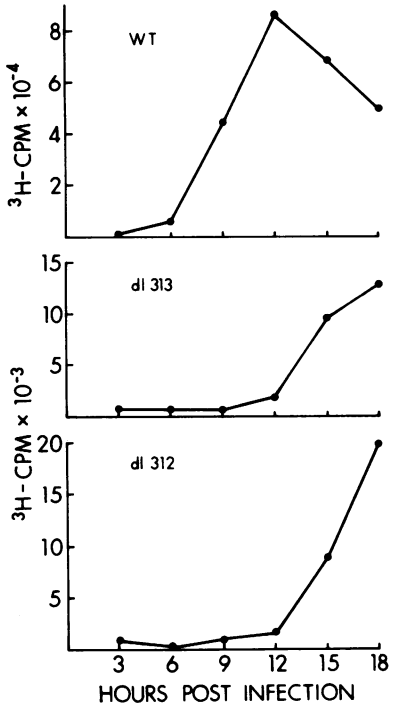

FIG. 7. Viral DNA replication in deletion mutant-infected cells. Infected cells were pulse-labeled with [^{3}H]thymidine for 1 h at the times indicated. Their DNA was isolated and hybridized to nitrocellulose-bound Ad5 DNA, and the quantity of hybridizable [^{3}H]DNA was determined. WT, Wild-type virus.

detected against a background of *dl*313 mRNAs. Ad2$^+$ND5 is a nondefective derivative of Ad2 carrying a 1.3-kb insertion of simian virus 40 (SV40) DNA in early region 3 (E3) (15, 20, 23). Early in Ad2$^+$ND5 infection, mRNAs of 1.5 and 2.3 kb were detected by RNA blot hybridization with SV40 DNA as a probe (Fig. 9, lane a). The 2.3-kb mRNA hybridized to *Hin*dIII H DNA (72.8–79.9) (Fig. 9, lane e) but not to *Eco*RI F1 DNA (72.8–75.9) (lane d). This suggests that the 2.3-kb mRNA originates at the E3 promoter at 76 map units. The structure of the 1.5-kb mRNA is unknown. Late in AD2$^+$ND5 infection, mRNAs of 3.1, 3.5, 4.0, and 6.8 kb hybridized to SV40 DNA (Fig. 9, lane b). These species also hybridized to the *Eco*RI F1 DNA (72.8–75.9) (lane c), suggesting that they result from fusion of late region L4 mRNA bodies with SV40 sequences.

The superinfection protocol consists of infection of 293 cells with *dl*313, followed by superinfection with AD2$^+$ND5 in the presence of HU. This procedure allows observation of the ability of the unreplicated, superinfecting template to transcribe the IX gene while in a late environment. Through analysis of the Ad2-SV40 hybrid mRNAs, one can independently confirm that the superinfecting template is transcriptionally active for an early promoter. Ad2$^+$ND5 replication was monitored in *dl*313-infected, Ad2$^+$ND5-superinfected cells by [^{3}H]thymidine pulse-labeling and hybridization of cellular DNA to SV40 DNA. Replication of the superinfecting viral DNA was just detectable at 5 to 7 h post-superinfection (data not shown). It was expected that a superinfection allowed to proceed for 5 to 7 h before the addition of HU would produce Ad2$^+$ND5-coded mRNAs whose expression was dependent upon template replication.

To perform the superinfection experiment, four plates of 293 cells were infected with *dl*313,

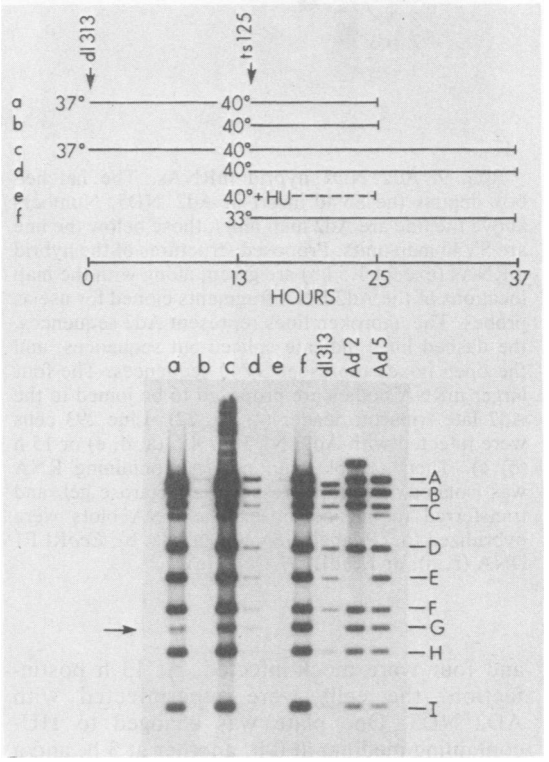

FIG. 8. *dl*313 preinfection can complement defective early function of superinfecting *ts*125. Line 293 cells were preinfected with *dl*313 (a and c) or mock-preinfected (b and d). After 13 h at 37°C, the cells were shifted to 40°C, superinfected with *ts*125, and incubated for 12 h (a and b) or 24 h (c and d) more. For (e), cells were infected with *ts*125 at 40°C in the presence of HU and incubated for 12 h. For (f), cells were infected with *ts*125 at 33°C and incubated for 24 h. DNA from each sample was digested with *Kpn*I, electrophoresed in a 1% agarose gel, and transferred to nitrocellulose. *Kpn*I-digested *dl*313, Ad2, and Ad5 DNAs were included as markers. The DNA blot was hybridized to ^{32}P-labeled Ad5 DNA.

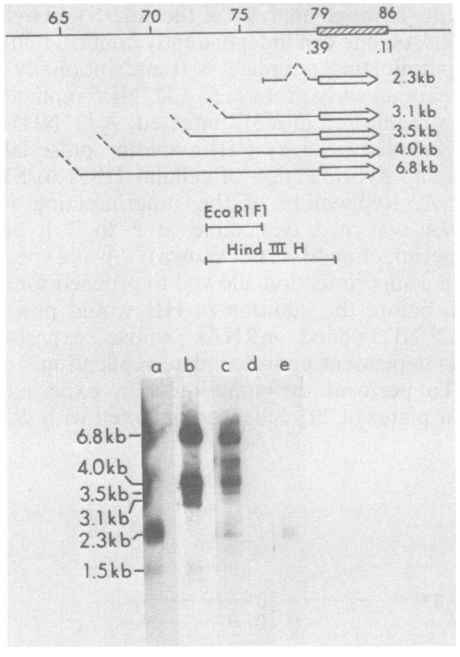

FIG. 9. Ad2$^+$ND5 hybrid mRNAs. The hatched box depicts the SV40 insert in Ad2$^+$ND5. Numbers above the line are Ad2 map units; those below the line are SV40 map units. Proposed structures of the hybrid mRNAs (except 1.5 kb) are given, along with the map locations of the Ad2 DNA fragments cloned for use as probes. The unbroken lines represent Ad2 sequences, the dashed lines indicate spliced-out sequences, and the open boxes represent SV40 sequences. The four larger mRNA bodies are proposed to be joined to the Ad2 late tripartite leader (4, 10, 22). Line 293 cells were infected with Ad2$^+$ND5 for 4 h (a, d, e) or 15 h (b, c). Their cytoplasmic, poly(A)-containing RNA was isolated, electrophoresed in an agarose gel, and transferred to nitrocellulose. The RNA blots were hybridized to ^{32}P-labeled SV40 DNA (a, b), *Eco*RI F1 DNA (c, d), or *Hin*dIII H DNA (e).

and four were mock-infected. At 13 h postinfection, the cells were superinfected with AD2$^+$ND5. One plate was changed to HU-containing medium at 0 h, another at 3 h, and a third at 6 h post-superinfection; all were harvested at 9 h. Cytoplasmic, poly(A)-containing RNA was isolated from each plate, electrophoresed in an agarose gel, and transferred to nitrocellulose. Duplicate RNA blots were hybridized to nick-translated *Hin*dIII C (7.9–17.0) DNA or SV40 DNA (Fig. 10 and 11). The 0.49-kb IX mRNA was not detected by the *Hin*dIII C probe when DNA replication was blocked immediately after superinfection (Fig. 10, lane a) or when it was blocked 3 h post-superinfection (lane b). Only when more than 3 h was allowed for the superinfecting virus to enter and replicate was IX expression detected (Fig. 10, lanes c and d). The 0.66-kb mRNA hybridizing the *Hin*dIII C probe is transcribed from *dl*313 and is a fusion product of E1a and E1b sequences (37).

Hybridization with the SV40 probe detected low levels of the 2.3- and 1.5-kb early hybrid mRNAs from the superinfecting virus, even when DNA replication was blocked immediately after superinfection (Fig. 10, lane a). A low level of the early hybrid mRNAs is to be expected after superinfection into late-stage cells, as E3 promoter activity declines late in infection (28). The 3.1-, 3.5-, 4.0-, and 6.8-kb late hybrid mRNAs were detected only when more than 3 h was allowed for the superinfecting virus to enter and replicate (Fig. 10, lanes c and d).

The results presented above demonstrated that IX mRNA was expressed only in cells in which the superinfecting viral DNA had replicat-

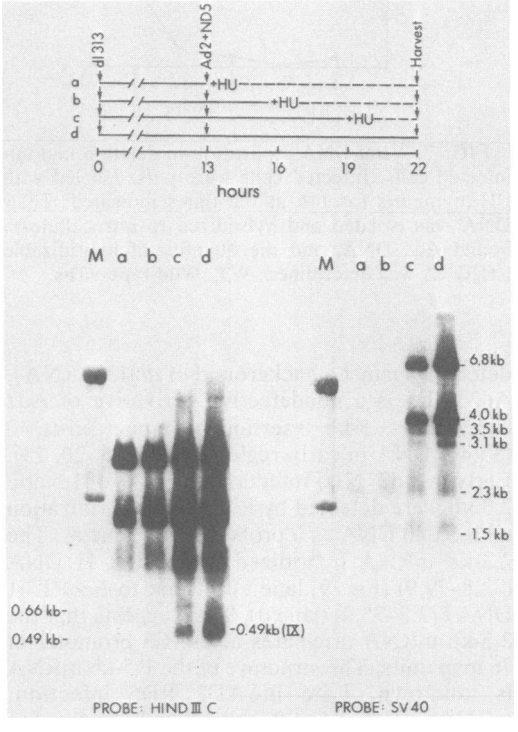

FIG. 10. Expression of IX mRNA requires replication. Line 293 cells were infected with *dl*313 (a through d) and 13 h later were superinfected with Ad2$^+$ND5. The cells were changed to medium containing 10 mM HU at the times indicated (dashed lines indicate the presence of HU) and were harvested 9 h after superinfection. Cytoplasmic, poly(A)-containing RNA was isolated, electrophoresed in an agarose gel, and transferred to nitrocellulose. The RNA blots were hybridized to ^{32}P-labeled *Hin*dIII C DNA or SV40 DNA as indicated. Autoradiogram of the hybridized blot. M denotes ^{14}C-labeled rRNA markers.

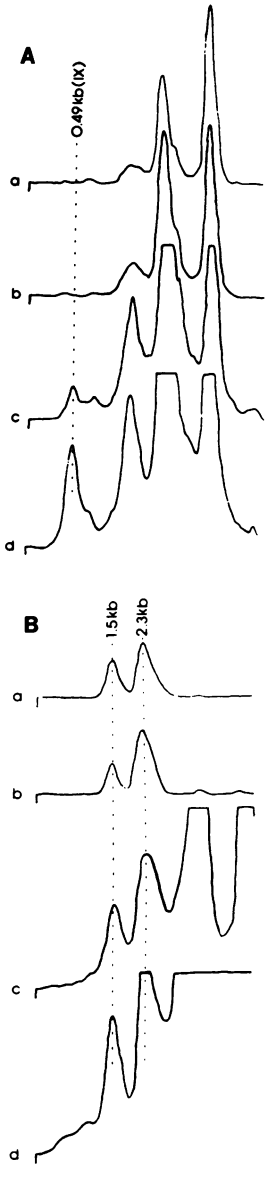

FIG. 11. Densitometer scans of lanes a through d of Fig. 10, hybridized to *Hin*dIII C DNA (A) and SV40 DNA (B).

ed. Due to replication, those cells also contained a larger number of potential templates for IX transcription. To demonstrate that the replication-dependent regulation of IX transcription is independent of template number, the amount of superinfecting virus was varied 10-fold. *dl*313-infected 293 cells were superinfected with $Ad2^{+}ND5$ at 10 or 100 MOI. Half of each set of cells was incubated in the presence of HU, and half was incubated without drugs. Nine hours post-superinfection, the poly(A)-containing RNA was isolated from 10^7 cells for analysis as before. Hybridization with *Hin*dIII C probe (7.9–17.0) revealed IX mRNA from the 10-MOI and 100-MOI, no-drug superinfections (Fig. 12, lanes a and b), but no IX mRNA from the 10-MOI and 100-MOI plus HU superinfections (lanes a and c). Despite a 10-fold greater input of superinfecting virus, expression of IX mRNA could not be detected in the absence of DNA replication.

DISCUSSION

This paper describes experiments designed to test the effect of template replication upon the expression of Ad2 intermediate genes. All four intermediate mRNAs assayed appeared at 6 to 8 h postinfection, a time course of expression in agreement with previous work (30, 38).

We have compared the steady-state levels of the IX and IVa_2 mRNAs in cells replicating viral DNA and cells blocked in DNA synthesis. IX or IVa_2 mRNA was detectable by RNA blot hybridization only when viral DNA replication was allowed. Densitometer scans of the autoradiographs from these experiments were used to quantitate the reduction in IX and IVa_2 mRNA levels. In three experiments in which both sets of cells were infected at the same MOI, HU treatment reduced the steady-state level of IX

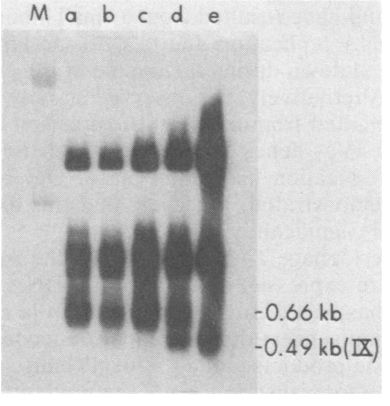

FIG. 12. Expression of IX mRNA is independent of MOI. Line 293 cells were infected with *dl*313 (MOI, 20; lanes a through d) or mock-infected (e) and 13 h later were superinfected with $Ad2^{+}ND5$ as follows: lane a, MOI of 10 plus HU; lane b, MOI of 10 without HU; lane c, MOI of 100 plus HU; lanes d and e, MOI of 100 without HU. Nine hours after superinfection, cytoplasmic, poly(A)-containing RNA was isolated, electrophoresed in an agarose gel, and transferred to nitrocellulose. Each lane represents the material from 10^7 cells. The RNA blot was hybridized to ^{32}P-labeled *Hin*dIII C DNA.

mRNA an average of 800-fold and that of IVa_2 mRNA an average of 350-fold. Under infection conditions that resulted in equal numbers of viral templates in both sets of cells, HU treatment reduced the steady-state level of IX mRNA 500-fold and that of IVa_2 mRNA 50-fold. These figures represent minimum values, as the films were probably exposed beyond the linear response range in the no-drug lanes in an effort to reveal faint bands in the HU-treated lanes.

We have also compared the rate of transcription of IX and IVa_2 mRNAs in cells replicating viral DNA and cells blocked in DNA synthesis. When equal numbers of viral templates were present in both sets of cells, newly synthesized IX and IVa_2 mRNA could only be detected when viral DNA replication was allowed. Because of the high backgrounds inherent in this pulse-labeling experiment, we can say with certainty only that the rate of transcription of IX and IVa_2 mRNAs is reduced 10-fold in the absence of viral DNA replication. The accumulated evidence suggests, however, that no IX or IVa_2 mRNA is produced when viral DNA replication is blocked.

Other investigators have observed some IX protein (30) or IVa_2 mRNA (24) in cells infected in the presence of the DNA synthesis inhibitor cytosine arabinoside, although the quantity of these products present was not directly compared to that present in control cells. Cytosine arabinoside has been reported to break down during prolonged incubation (39). The IX and IVa_2 gene activity observed by these investigators could have resulted from a small amount of viral DNA replication due to cytosine arabinoside breakdown during the course of the experiment. Alternatively, the observed activity could have resulted from inefficient expression of the IX and IVa_2 genes in cells in which no viral DNA replication had taken place. Our results have demonstrated, however, that this expression is insignificantly low.

Bacteriophage T4 late genes, like the Ad2 IX gene, are expressed only after viral DNA replication has begun. Investigations of T4 late-gene regulation suggest that both phage-encoded soluble gene products and a physical change in the template for transcription are required for phage late-gene expression (31). The superinfection experiments presented in this paper demonstrate that the Ad2 IX promoter cannot be activated on an infecting, parental template. Diffusible, *trans*-acting factors produced late in infection with *dl*313 are not sufficient to activate the IX gene on a parental template. The possible objection that *dl*313 does not produce the needed regulatory factors because of mutation is dismissed by the observation that *dl*312, which was derived from the same parent virus and thus should differ only in region E1 (18), produces IX mRNA and protein when infecting 293 cells. Besides IX, the only E1 mRNA missing from *dl*313-infected 293 cells is the 0.52-kb mRNA from E1a (37), which is also absent from *dl*312-infected 293 cells.

Activation of the IX gene promoter is probably dependent upon a change in the physical structure of the template during replication. An alteration in the distribution or composition of nucleosomes (33), exposing the IX promoter to the action of RNA polymerases, could be responsible for this change. Another possibility is that soluble regulatory factors necessary for IX promoter activity cannot bind to the parental DNA because of the nucleosome structure. During or after replication, accessibility to the binding site of these regulatory factors on the template is afforded by the perturbation of the deoxyribonucleoprotein during replication. This model of IX regulation resembles the stable, active and inactive transcription complexes observed by Bogenhagen et al. in the *Xenopus* 5S RNA gene (7). A third possibility is that the replicated genomes enter a specific nuclear compartment, and only in that compartment are the factors necessary for IX transcription available. Further work will be needed to determine which, if any, of these models is correct.

Although IX and late regions L2 through L5 are activated by the same event, viral DNA replication, IX protein is observed earlier during infection than proteins encoded by L2 through L5 (14, 30). This time difference may result from more rapid transcription, processing, transport, and translation of the small, unspliced IX message (3). Also, the different mechanisms of regulation—promoter activation for the IX gene, versus antitermination and poly(A) site selection for L2 through L5—may be responsible for some of the time difference. The observation that fiber protein from L5 is not seen until 2 h after the appearance of hexon protein protein from L3 (30) indicates that late-gene activation is complex and may involve factors or events other than DNA replication.

Several characteristics of the IX and IVa_2 genes differentiate them from the 0.52- and 1.03-kb intermediate genes from E1: (i) IX and IVa_2 are the only Ad2 transcription units to produce a single transcript and polypeptide, whereas the other intermediates are members of families of mRNA species originating at the same promoter; (ii) IX and IVa_2, and possibly the E2 promoter at map position 72, are the last Ad2 promoters to be activated during lytic infection, whereas the other intermediates derive from promoters active at early times; (iii) IX and IVa_2 are regulated by promoter activation, whereas the other intermediates are regulated by mRNA stability.

ACKNOWLEDGMENTS

We thank the colleagues who kindly provided recombinant DNAs for this study. We also thank Ellen Johnson and Scott Hauser for technical assistance, and Betsy Klein for typing the manuscript.

This work was supported by research grants from the American Cancer Society (MV-31H) and the National Cancer Institute (CA-16007). Cell culture media were prepared in a Cancer Center facility funded by the National Cancer Institute (CA-16217). This study was also supported by Brown Williamson Tobacco Corp., Philip Morris, Inc., R. J. Reynolds Tobacco Co., and the United States Tobacco Co.

LITERATURE CITED

1. **Aiello, L. R., R. Guilfoyle, K. Huebner, and R. Weinmann.** 1979. Adenovirus 5 DNA sequences present and RNA sequences transcribed in transformed human embryonic kidney cells (HEK-Ad5 or 293). Virology **94**:460–469.
2. **Akusjarvi, G., and H. Persson.** 1981. Controls of RNA splicing and termination in the major late adenovirus transcription unit. Nature (London) **292**:420–426.
3. **Alestrom, P., G. Akusjarvi, M. Perricaudet, M. B. Mathews, D. F. Klessig, and U. Pettersson.** 1980. The gene for polypeptide IX of adenovirus type 2 and its unspliced messenger RNA. Cell **19**:671–681.
4. **Berget, S. M., C. Moore, and P. A. Sharp.** 1977. Spliced segments at the 5' terminus of adenovirus 2 late mRNA. Proc. Natl. Acad. Sci. U.S.A. **74**:3171–3175.
5. **Berk, A. J., F. Lee, T. Harrison, J. Williams, and P. Sharp.** 1979. Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. Cell **17**:935–944.
6. **Berk, A. J., and P. A. Sharp.** 1978. Structure of the adenovirus 2 early mRNAs. Cell **14**:695–711.
7. **Bogenhagen, D. F., W. M. Wormington, and D. D. Brown.** 1982. Stable transcription complexes of Xenopus 5S RNA genes: a means to maintain the differential state. Cell **28**:413–421.
8. **Carter, T. H., and H. S. Ginsberg.** 1976. Viral transcription in KB cells infected by temperature-sensitive "early" mutants of adenovirus type 5. J. Virol. **18**:156–166.
9. **Chow, L. T., T. R. Broker, and J. B. Lewis.** 1979. Complex splicing patterns of RNAs from the early regions of adenovirus 2. J. Mol. Biol. **134**:265–303.
10. **Chow, L. T., R. E. Gelinas, T. R. Broker, and R. J. Roberts.** 1977. An amazing sequence arrangement at the 5' ends of adenovirus 2 messenger RNA. Cell **12**:1–8.
11. **Darnell, J. E.** 1982. Variety in the level of gene control in eukaryotic cells. Nature (London) **297**:365–371.
12. **Ensinger, M. J., and H. S. Ginsberg.** 1972. Selection and preliminary characterization of temperature-sensitive mutants of type 5 adenovirus. J. Virol. **10**:328–339.
13. **Evans, R., J. Weber, E. Ziff, and J. E. Darnell.** 1979. Premature termination during adenovirus transcription. Nature (London) **278**:367–370.
14. **Everitt, E., and L. Philipson.** 1974. Structural proteins of adenovirus. XI. Purification of three low molecular weight virion proteins of adenovirus type 2 and their synthesis during productive infection. Virology **62**:253–269.
15. **Flint, S. J.** 1977. The topography and transcription of the adenovirus genome. Cell **10**:153–166.
16. **Goldenberg, C. J., and H.. J. Raskas.** 1979. Splicing patterns of nuclear precursors to the mRNA for adenovirus 2 DNA binding protein. Cell **16**:131–138.
17. **Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36**:59–72.
18. **Jones, N., and T. Shenk.** 1979. Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cell **17**:683–689.
19. **Jones, N., and T. Shenk.** 1979. An adenovirus type 5 early gene function regulates expression of other early viral genes. Proc. Natl. Acad. Sci. U.S.A. **76**:3665–3669.
20. **Kelly, T. J., and A. M. Lewis.** 1973. Use of nondefective adenovirus-simian virus 40 hybrids for mapping the simian virus 40 genome. J. Virol. **12**:643–652.
21. **Kitchingman, G. R., S.-P. Lai, and H. Westphal.** 1977. Loop structures in hybrids of early RNA and the separated strands of adenovirus DNA. Proc. Natl. Acad. Sci. U.S.A. **74**:4392–4395.
22. **Klessig, D. F.** 1977. Two adenovirus mRNAs have a common 5' terminal leader sequence encoded at least 10 kb upstream from their main coding regions. Cell **12**:9–21.
23. **Lebowitz, P., T. J. Kelly, D. Nathans, T. N. H. Lee, and A. M. Lewis.** 1974. A colinear map relating the simian virus 40 (SV40) DNA segments of six adenovirus-SV40 hybrids to the DNA fragments produced by restriction endonuclease cleavage of SV40. Proc. Natl. Acad. Sci. U.S.A. **71**:441–445.
24. **Lewis, J. B., H. Esche, J. E. Smart, B. W. Stillman, M. L. Harter, and M. B. Mathews.** 1980. Organization and expression of the left third of the genome of adenovirus. Cold Spring Harbor Symp. Quant. Biol. **44**:493–508.
25. **McGrogan, M., and H. J. Raskas.** 1977. Species identification and genome mapping of cytoplasmic adenovirus type 2 RNAs synthesized late in infection. J. Virol. **23**:240–249.
26. **McMaster, G. K., and G. C. Carmichael.** 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. U.S.A. **74**:4835–4838.
27. **Nevins, J. R.** 1980. Definition and mapping of adenovirus 2 nuclear transcription. Methods Enzymol. **65**:768–785.
28. **Nevins, J. R., H. S. Ginsberg, J.-M. Blanchard, M. C. Wilson, and J. E. Darnell.** 1979. Regulation of the primary expression of the early adenovirus transcription units. J. Virol. **32**:727–733.
29. **Nevins, J. R., and M. C. Wilson.** 1981. Regulation of adenovirus 2 gene expression at the level of transcriptional termination and RNA processing. Nature (London) **290**:113–118.
30. **Persson, H., U. Pettersson, and M. B. Mathews.** 1978. Synthesis of a structural adenovirus polypeptide in the absence of viral DNA replication. Virology **90**:67–79.
31. **Rabussay, D. P., and E. P. Geiduschek.** 1977. Regulation of gene action in the development of lytic bacteriophages, p. 1–196. In H. Fraenke-Conrat and R. Wagner (ed.), Comprehensive virology, vol. 8. Plenum Publishing Corp., New York.
32. **Sauerbrier, and A. R. Brautigam.** 1970. A simple method for isolating RNA from bacteria. Biochim. Biophys. Acta **199**:36–40.
33. **Sergeant, A., M. A. Tigges, and H. J. Raskas.** 1979. Nucleosome-like structural subunits of intranuclear parental adenovirus type 2 DNA. J. Virol. **29**:888–898.
34. **Sharp, P. A., J. Manley, A. Fire, and M. Gefter.** 1980. Regulation of adenovirus mRNA synthesis. Ann. N. Y. Acad. Sci. **354**:1–15.
35. **Shaw, A. R., and E. B. Ziff.** 1980. Transcripts from the adenovirus-2 major late promoter yield a single early family of 3' coterminal mRNAs and five late families. Cell **22**:905–916.
36. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
37. **Spector, D. J., D. N. Halbert, and H. J. Raskas.** 1980. Regulation of integrated adenovirus sequences during adenovirus infection of transformed cells. J. Virol. **36**:860–871.
38. **Spector, D. J., M. McGrogan, and H. J. Raskas.** 1978. Regulation of the appearance of cytoplasmic RNAs from region 1 of the adenovirus 2 genome. J. Mol. Biol. **126**:395–414.
39. **Thomas, G. P., and M. B. Mathews.** 1980. DNA replication and the early to late transition in adenovirus infection. Cell **22**:523–533.
40. **Thomas, P. S.** 1980. Hybridization of denatured RNA and

small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. 77:5201–5205.

41. **van der Vliet, P. C., A. J. Levine, M. J. Ensinger, and H. S. Ginsberg.** 1975. Thermolabile DNA binding proteins from cells infected with a temperature-sensitive mutant of adenovirus defective in viral DNA synthesis. J. Virol. 15:348–354.
42. **Wilson, M. C., and J. E. Darnell.** 1981. Control of messenger RNA concentration by differential cytoplasmic half-life. Adenovirus messenger RNAs from transcription units 1A and 1B. J. Mol. Biol. 148:231–251.
43. **Wilson, M. C., N. W. Fraser, and J. E. Darnell.** 1979. Mapping of RNA initiation sites by high doses of uv irradiation: evidence for three independent promoters within the left 11% of the Ad2 genome. Virology 94:175–184.
44. **Ziff, E. B., and R. M. Evans.** 1978. Coincidence of the promoter and capped 5′ terminus of RNA from the adenovirus 2 major late transcription unit. Cell 15:1463–1475.