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# Cycloheximide Stimulates Early Adenovirus Transcription if Early Gene Expression Is Allowed Before Treatment

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The inhibition of protein synthesis by cycloheximide markedly stimulated adenovirus 2 early transcription (assayed in vivo and in vitro in nuclei) when the drug was added 3 h after infection of HeLa cells. The stimulation was not uniform but ranged from three- to eightfold for region 1A to 10- to 30-fold for region 2 of the adenovirus genome. The increase was complete by about 60 min after treatment. The stimulation reversed rapidly after cycloheximide was removed. Treatment with cycloheximide either before infection with wild-type adenovirus type 5 or at 3 h after infection with *dl*312, a mutant which fails to express early gene products in HeLa cells, both resulted in levels of transcription at or substantially below the control (infection with wild-type adenovirus without treatment). Therefore we conclude that cycloheximide treatment and previous early gene expression interact to yield the maximum levels of transcription.

The adenovirus-specific mRNAs produced early after infection of HeLa cells are derived from the primary nuclear RNA transcripts of at least seven different transcription units (for a review, see Ziff [28]). Efficient early transcription of adenovirus sequences depends on normal function of the 1A transcription unit when cells are infected at a low multiplicity (MOI) (1, 9, 10). For example, a host-range mutant of adenovirus type 5 (Ad5), *dl*312, has a large deletion in early region 1A, and transcription of various early regions is greatly reduced in cells infected at a low MOI with *dl*312 (14). However, a high MOI with the *dl*312 mutant leads to transcription of all early transcription units (14) and finally to new virus production (22).

In addition to genetic analysis, a second experimental approach to determining the events that are necessary for adenovirus gene control has been the use of inhibitors of protein synthesis. Parsons and Green (18) found that cycloheximide (CH) added 1 h postinfection (p.i.) led to an increase in accumulation of labeled adenovirus-specific mRNA at later times, a finding that has been amply corroborated in many other studies (3, 5, 6, 27). Additional studies using variations in virus input and addition of various inhibitors of protein synthesis have been described (12, 18, 19), but no completely consistent picture of early virus mRNA control has emerged. When CH is added before infection at a high MOI, transcription of all early virus transcription units (except region 4) still apparently achieves a normal rate (16), but excess amounts of mRNA as detected by translation do not accumulate (19). Anisomycin has a similar effect when a high MOI is used (11, 12, 18), but the accumulation of individual mRNA species as judged by translation is not uniform. Nevins (14) found that even in cells infected with *dl*312, a mutant with a deletion in region 1a, protein synthesis inhibition causes a modest increase in transcription of early transcription units.

To clarify the established effect of CH on the accumulation of mRNA when it is added after infection, we examined the rate of viral transcription under several conditions. We found that when CH was added 3 h p.i., a marked stimulation of viral transcription from all transcription units was seen shortly afterwards. The increased transcription was demonstrated by transcription in isolated nuclei and by pulse-labels of cells in vivo. Increased amounts of virus-specific nuclear RNA could be detected by Northern blot analysis (24), presumably as a result of increased transcription. No strong effect on transcription was seen when CH was added before the virus, as was reported earlier (16). The lack of stimulation of transcription by inhibiting protein synthesis before infection is in accord with the results from other laboratories with other protein synthesis inhibitors (11, 18, 19). Thus, protein synthesis inhibition can cause a strong stimulation of viral transcription and RNA accumulation, but only if a period of uninhibited early infection is allowed before treatment.

## MATERIALS AND METHODS

The procedures for cell growth and purification of adenovirus were those described by Nevins (13), except that cells were maintained in 5% calf serum

instead of fetal calf serum. The MOI was 5,000 particles per cell (~500 PFU/cell) except for *dl*312, which was 1,000 particles per cell. CH treatment was with 35 μg/ml where indicated.

**In vivo labeling of nuclear RNA.** Pulse-labeling of nuclear RNA with [$^3$H]uridine (5 min) was done as previously described (13).

**In vitro transcription in isolated nuclei.** Nuclei were isolated by Dounce homogenization after swelling in reticulocyte standard buffer (10 mM NaCl, 10 mM Tris-hydrochloride [pH 7.4], 1.5 mM $MgCl_2$) with 0.5 mM dithiothreitol. After being washed in reaction buffer (5 mM $MgCl_2$, 1 mM $MnCl_2$, 10 mM Tris-hydrochloride [pH 8.0], 140 mM KCl, 14 mM β-mercaptoethanol, 10% glycerol), the nuclei were suspended in 1.5 ml of reaction buffer and 0.5 ml of reaction buffer containing also ATP, GTP, and CTP (4 mM each), and 0.25 to 0.5 mCi of [$^{32}$P]UTP (400 Ci/mmol; New England Nuclear Corp.) was added. Nuclei were warmed to 22°C and incubated for 5 to 20 min. EDTA was added to 10 mM to stop the reaction, the nuclei were spun out, and the nuclear RNA was extracted as previously described (13).

**Cell fractionation and RNA preparation.** Cell fractionation, RNA preparation, and polyuridylate-Sepharose column chromatography have been described by Nevins (13).

**Preparation of $^{32}$P-labeled RNA with polynucleotide kinase.** About 10 μg of polyadenylated [poly(A)$^+$] RNA was partially hydrolyzed as described (23) at 90°C for 30 min. Broken RNA was reacted with 150 μCi of [γ-$^{32}$P]ATP (1,000 to 3,000 Ci/mmol; New England Nuclear Corp.) and 10 U of T4 polynucleotide kinase (P-L Biochemicals). RNA of specific activity 1 × $10^7$ to 2 × $10^7$ cpm/μg was routinely obtained (23).

**Agarose-formaldehyde gel electrophoresis and blotting of RNA.** RNA was separated according to size by electrophoresis through horizontal 1.5% agarose gels containing 2.2 M formaldehyde in a buffer containing 20 mM MOPS (morpholinepropanesulfonic acid, pH 7.0), 5 mM sodium acetate, and 1 mM EDTA for 18 h at 50 V. The RNA was pretreated with the above buffer in 50% formamide for 5 min at 60°C before being loaded. The RNA was blotted onto nitrocellulose paper as described by Thomas (24).

**Nick translation and hybridization for Northern blot analysis.** Nick translation was carried out by the method of Rigby et al. (21).

Baked filters were prehybridized for 30 min at 68°C in a buffer containing 5× SSC (1× SSC, 0.15 M NaCl plus 0.015 M sodium citrate), 10% dextran sulfate, 1× Denhardt solution, 0.1% sodium dodecyl sulfate, and 50 μg of salmon sperm DNA per ml. Hybridizations were carried out in the same buffer for 12 h at 68°C. After hybridization, filters were washed once in 2× SSC at 37°C for 30 min and three times in 0.5× SSC at 65°C and exposed to Cronex X-ray films for 1 to 3 days at −70°C.

**Hybridization of RNA to excess DNA.** Plasmid DNA was boiled for 5 min in 0.1 N NaOH and bound to 0.45-μm HA nitrocellulose filters (Millipore Corp.) as described previously (15). Hybridization of labeled RNA to at least a 20-fold excess of complementary DNA was performed in 2× TESS buffer (0.01 M TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] [pH 7.4], 0.3 M NaCl, 0.01 M EDTA, 0.2% sodium dodecyl sulfate) at 65°C for 40 h or in 30% formamide (Bethesda Research Laboratories)–0.1 M PIPES (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 7.0)–0.3 M NaCl–10 mM EDTA–0.2% sodium dodecyl sulfate at 45°C for 72 h. Carrier yeast RNA (100 μg/ml) and poly(A) (100 μg/ml) were added to the hybridization buffers. After hybridization, filters were washed extensively at 65°C in 2× TESS buffer and treated with pancreatic RNase in the same buffer minus sodium dodecyl sulfate. On occasion the supernatants from hybridization were rehybridized to ensure completeness.

## RESULTS

**In vivo and in vitro assays of transcription rate.** We used an in vivo and an in vitro assay to measure the viral transcription rate in Ad2-infected HeLa cells. In the in vivo assay, nuclear RNA was labeled by a brief pulse with [$^3$H]uridine (7, 15). In the in vitro assay, nuclei were isolated from infected cells, [$^{32}$P]UTP and the other unlabeled triphosphates were added, and the nuclei were warmed for a brief period to allow chain elongation and the consequent labeling of growing RNA chains (4, 25). In both assays nuclear RNA was extracted after the labeling, and excess DNA filter hybridization was carried out to specific cloned Ad2 DNAs representing various early regions.

In some experiments the percentage of total incorporated radioactivity specific to a given viral early region rather than the absolute amount of hybridized radioactivity is reported. Therefore it is important to describe the effects of CH treatment on total incorporation. Table 1 shows the total incorporation in vivo and in vitro as a function of the time of CH treatment before labeling. The total incorporation into nuclei in vitro remained about 75% of that in the control even when the nuclei were removed from the cells after 90 min of CH treatment. Thereafter CH caused a decline of total incorporation to about one half of control levels. In contrast, even a 30-min CH treatment caused a decline of

TABLE 1. Effect of CH on total incorporation in vivo and in vitro[a]

| CH added (min before labeling) | Ratio of total nuclear incorporation (cpm in treated cells/cpm in untreated cells) | |
|---|---|---|
| | In vitro | In vivo |
| 30 | 0.95 | 0.40 |
| 60 | 0.80 | 0.25 |
| 90 | 0.65 | 0.50 |
| 150 | 0.55 | 0.40 |
| 240 | 0.40 | 0.25 |

[a] Cultures of infected cells were divided at 3 h p.i. (first three lines) or 30 min p.i. (last two lines), and CH (35 μg/ml) was added to one half. At the indicated times after CH addition, nuclear RNA was labeled in vivo or in vitro as described in the text.

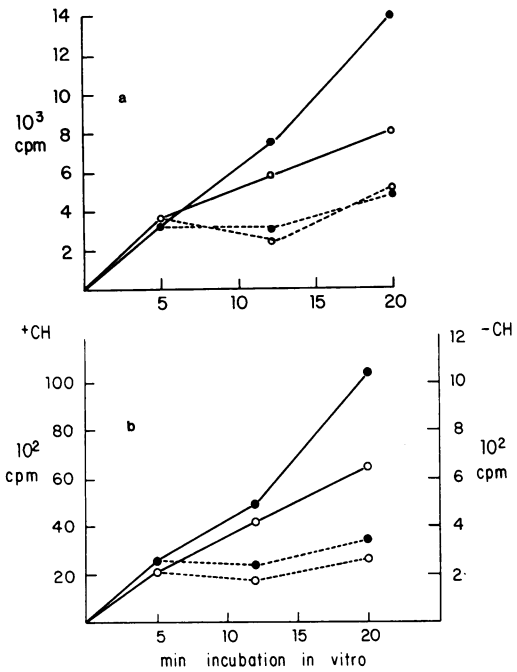


FIG. 1. Pulse-chase analysis of stability of in vitro-labeled RNA. A culture of Ad2-infected cells was split 3 h p.i. CH was added to one half, and the cells were harvested after 45 min and labeled for 5 min in vitro. The reaction mixtures were split, and a 50-fold excess of unlabeled UTP was added to half of each reaction mixture. Incubation was continued for 15 min. Samples were analyzed for total incorporation and E2A specific incorporation at 5, 10, 15, and 20 min. (a) Total incorporation; (b) E2A-specific incorporation. Symbols: ○, with CH; ●, without CH; ——, continuous label; -----, chase.

total in vivo incorporation of [$^3$H]uridine to about 30 to 40% of the control levels during a 5-min pulse. This probably reflects an inhibition of labeling of the acid-soluble pool within the 5-min [$^3$H]uridine pulse. Consistent with this idea, labeling by polymerases I and II was approximately equally inhibited, since about 30% of the total labeled RNA hybridized to cloned rDNA in both the control and CH-treated cells (data not shown).

Unlike the in vivo [$^3$H]uridine labeling assay, the in vitro assay of transcription is not dependent on equal rates of uptake and phosphorylation of the labeled precursor in treated and untreated cells since [$^{32}$P]UTP is added directly to the transcribing nuclei. The stability of the RNA labeled in vitro in nuclei from CH-treated or untreated cells was determined by adding unlabeled triphosphate after 5 min of incorporation. No loss in labeled RNA was observed in either case (Fig. 1).

From all of these considerations, it appears that comparing the percentage of in vitro-labeled RNA complementary to a particular viral transcription unit is a valid measure of the differential transcription rate of the adenovirus transcription units compared with all other transcription units in the cell, as well as being a valid measure of the rate of synthesis of adenovirus RNA in treated versus untreated cells. This is likely to be true for in vivo label as well since, as will be shown, the various changes in transcription patterns were quite similar whether in vitro or in vivo labeling was used.

**CH stimulation of early transcription.** Figure 2 shows a time course of the stimulation of viral transcription by CH. At 3 h p.i., an Ad2-infected culture of HeLa cells was divided. One half was treated, and the other served as a control. Transcription rates were assayed 40, 70, and 100 min after the addition of CH. Incorporation into RNA complementary to regions 1A, 1B, and E2A showed a large increase in treated samples compared with control samples. The stimulation was complete by less than 60 min. Table 2 shows a summary of the stimulation observed in a number of experiments for early regions 1A, 1B, and E2A and various times of incubation in CH. The results for regions other than 1A, 1B and E2A varied considerably between experiments, and therefore in most of the experiments described we analyzed only 1A, 1B and E2A

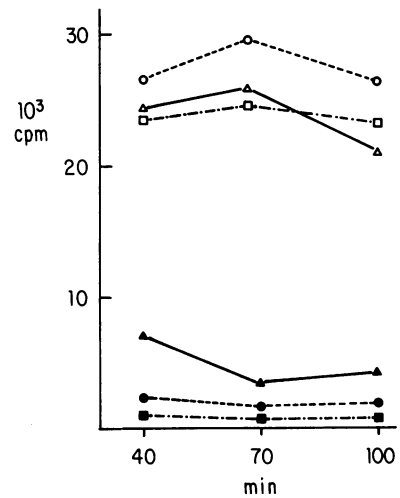


FIG. 2. In vitro transcription in isolated nuclei from regions 1A (△, ▲), 1B (○, ●) and E2A (□, ■). A culture of Ad2-infected cells was split at 3 h p.i., and CH (35 μg/ml) was added to one half. At 40, 70, 100 min, nuclei were isolated from the cultures and allowed to transcribe for 15 min. Labeled RNA was hybridized with filters containing 1A, 1B, and E2 probes in excess DNA. The hybridized counts per minute for 1B and E2A were divided by 1.5 and 5, respectively, to fit them on the same scale as those for 1A (actual counts). Open symbols, with CH; solid symbols, without CH.

TABLE 2. Stimulation of in vitro transcription from 1A, 1B, and E2 by addition of CH at 3 h p.i.[a]

| Expt | CH Treatment (min) | % Specific hybridization ratio (treated/control)[b] | | |
|---|---|---|---|---|
| | | E1A | E1B | E2 |
| 1 | 40 | 3.1 | 12.5 | 18.7 |
| | 70 | 7.0 | 20.8 | 26.0 |
| | 100 | 5.1 | 16.8 | 29.4 |
| 2 | 300 | 2.6 | 4.9 | 5.9 |
| | 90 | 4.6 | 6.5 | 12.0 |
| 3 | 30 | 3.3 | 5.8 | 7.1 |
| | 60 | 3.9 | 8.1 | 11.7 |
| 4[c] | 45 | 2.7 | 3.8 | 8.7 |
| 5 | 60 | 5.0 | 10.5 | 8.7 |

[a] Cultures were divided 3 h p.i., and CH was added to one half. At the indicated times after addition of the drug, nuclear RNA was labeled in isolated nuclei in vitro. RNA was hybridized in DNA excess to cloned Ad2 DNA as described in the text. The numbers are the ratio of the percentage of total incorporation specific to a given early region in treated compared with untreated cells. The actual counts per minute hybridized ranged from 500 to 15,000. The background of 25 cpm was subtracted.

[b] In all experiments the amount of hybridized RNA was more than tem times the background. The cloned segment of E1A was about 500 bases lone and of E2 was 4,000 bases long, of which ~1,500 was complementary to mRNA and 3,500 to nuclear RNA.

[c] In experiment 4, transcription from regions E3, E4, and the L1 region of the late transcription unit were also assayed and found to be stimulated 7.8-, 15.1-, and 2.5-fold, respectively.

transcription. However, all viral transcription units were stimulated by CH treatment for 45 min or longer in all experiments by a substantial factor, provided that the CH was added at 3 h p.i. In one experiment (Table 2, experiment 4), transcription rates from all known early regions were measured.

Pulse-chase experiments in vitro were performed to ensure that the apparently greater transcription rate from viral early regions in nuclei from CH-treated cells was not due to degradation of viral RNA in the control nuclei. Both the total incorporated radioactivity and the radioactivity incorporated into virus-specific RNA were stable for at least 15 min in both treated and control cells (Fig. 1).

Since the effect of CH on protein synthesis is reversible, the effect on viral transcription rate might be expected to be reversible as well. In fact, within 30 min after CH was removed, stimulation of the E2 transcription rate was reversed (Table 3). The effect of CH on the rate of accumulation of newly synthesized 1A mRNA was also reversed rapidly (data not shown).

**Correlation of in vitro and in vivo measurements.** To determine whether the increased transcription observed in vitro was also occurring in vivo, and to examine whether the increased transcription resulted in increased cytoplasmic mRNA, we performed the following experiment. CH was added to a culture of Ad2-infected cells at 3 h p.i. At various times thereafter, transcription rates from 1A, 1B, and E2A were measured both in vitro as described above and in vivo by 5-min pulse-labels with [$^3$H]uridine. In addition, samples of unlabeled cytoplasmic RNA were extracted, and the poly(A)-containing RNA was selected by polyuridylate-Sepharose chromatography; this was alkali broken and end-labeled with [$^{32}$P]ATP by polynucleotide kinase. Both nuclear and cytoplasmic RNA samples were then assayed by hybridization to 1A-, 1B-, and E2A-specific cloned DNA fragments in excess DNA. The results of the transcription rate measurements were expressed as the percentage of total incorporation hybridizing to a particular early region.

There was substantial stimulation of viral transcription with a similar time of onset both in vivo and in vitro (Fig. 3), and the results of the two measurements agreed within a factor of two. The abundance in the cytoplasm of mRNA from the same early regions increased rapidly when CH was added 3 h p.i. and the increase in the transcription rate was about the same as the cytoplasmic increase (Fig. 3). Therefore, under these conditions most of the cytoplasmic increase in mRNA could be accounted for by the increased transcription rate caused by CH.

**Northern blot analysis of nuclear and cytoplasmic RNAs.** To determine whether the increased transcription that we observed in vivo and in vitro resulted in normal RNA species in the nucleus and cytoplasm, we carried out Northern blot analysis of nuclear and cytoplasmic RNAs. CH was added to an infected culture at 3 h p.i.,

TABLE 3. Stimulation of E2 transcription by CH added at 3 h p.i. is reversible

| Duration of CH treatment (h) (time p.i. [h]) | Nuclei prepared (time p.i. [h]) | Ratio of E-2 specific labeled RNA (cpm in treated cells/cpm in control)[a] |
|---|---|---|
| 0.5 (3–3.5) | 3.5 | 3.1 |
| 1 (3–4) | 4 | 5.2 |
| 2 (3–5) | 5 | 13.8 |
| 1 (3–4)[b] | 5 | 1.5 |

[a] Label in the hybrid exceeded 5,000 cpm in all cases.

[b] CH was removed after 1 h by washing the cells, and the cells were incubated for another hour without CH.

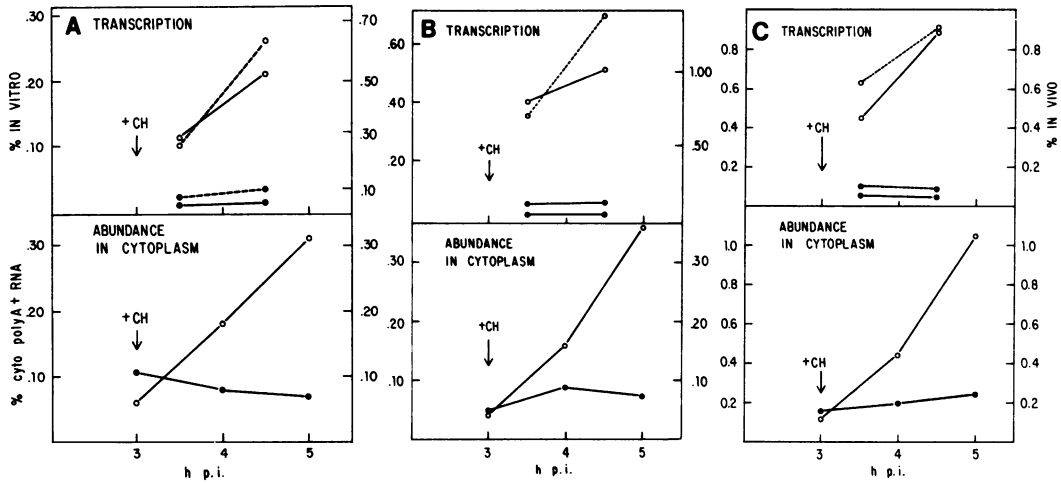


FIG. 3. Rapid stimulation by CH of early Ad2 transcription measured in vivo (——) and in vitro (-----) and its effect on cytoplasmic abundance. Samples from cultures treated with CH as described in the legend to Fig. 2 were labeled for 5 min in vivo with [$^3$H]uridine or in vitro as described in the legend to Fig. 1 at 30 and 90 min after CH addition. Cytoplasmic poly(A)$^+$ mRNA was isolated 0, 60, and 120 min after CH addition, alkali broken, and end labeled as described in the text. All labeled RNA samples were hybridized with 1A, 1B, and E2 filters. (A) 1A-specific hybridization; (B) 1B-specific hybridization; (C) E2-specific hybridization. ○, with CH; ●, without CH. All measurements are expressed as the percentage of total incorporation. For the top half of each panel, scales for in vitro and in vivo transcription are to the left and right, respectively.

and nuclear and cytoplasmic RNAs were extracted from the treated and control cultures at various times. The denatured RNA was subjected to electrophoresis through a formaldehyde-agarose gel, blotted on nitrocellulose, and hybridized with nick-translated $^{32}$P-labeled 1B or E2A DNA (Fig. 4). There was a rapid increase in both nuclear and cytoplasmic RNAs from these regions in the presence of CH. All species observed in the CH-treated cells were also present in the control cells, indicating that the increased transcription in CH gave rise to normal precursor and mRNA species that were present in greater abundance after a brief CH treatment.

**Effect of CH addition at 0.5 h p.i.** Next, the effect of CH on viral transcription was determind when CH was added just after, instead of 3 h after, infection. The level of viral proteins present in the cells should be markedly reduced by having CH present during most of early infection; thus, the stimulation of transcription by CH might be reduced if viral proteins are necessary for the stimulation to occur. On the other hand, if the stimulation is independent of the synthesis of viral proteins, then the longer incubation in CH might be expected to give the same or a greater degree of stimulation than the brief (30 to 90 min) treatments discussed above.

Cells were infected, and 0.5 h after the addition of virus CH was added to half of the culture. At various times thereafter transcription rates and the cytoplasmic abundance of 1A, 1B, E2A, and E3 RNAs were measured as described above (Fig. 5). The degree of stimulation of the transcription rate appeared to increase as infection proceeded. After 1 h of CH treatment, transcription rates from the various regions assayed were similar in control and CH-treated cells. In addition to the changing rates of transcription in the control described previously (16), progressively greater stimulation of transcription by CH occurred as the experiment proceeded. The main conclusion from this experiment is that the CH stimulation of viral transcription (Fig. 2, 3, and 4) is not caused by an equal CH treatment during the earlier stages of infection. The stimulation observed when CH was added at 0.5 h p.i. only developed after 2 to 4 h of treatment and never reached the levels observed when the drug was added at 3 h p.i. For example, CH added for 60 min at 3 h p.i. resulted in about a 10-fold stimulation of E2A transcription (Table 1). A 3-h CH treatment from 0.5 to 3.5 h, however, resulted in no transcriptional stimulation at all.

This result suggested that the stimulation of viral transcription that occurred when CH was added at 3 h p.i. was dependent on accumulation of the viral proteins that are normally responsible for regulating early transcription. Nevins et al. (16) found little or no stimulation of early transcription by CH when it was added before the virus, a result we repeated in this study (Table 4 and Fig. 6). Therefore the 0.5 h of uninhibited infection in this experiment must

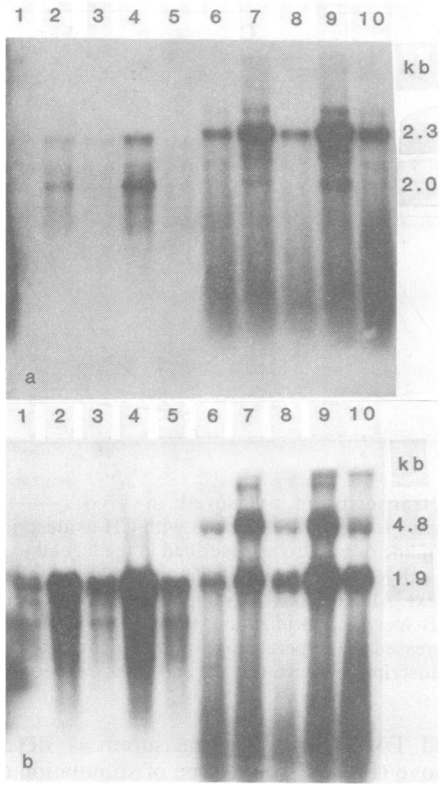


FIG. 4. Northern blot analysis of nuclear and cytoplasmic RNAs after CH addition at 3 h p.i. Nuclear and cytoplasmic RNAs were extracted from cultures at 3, 4, and 5 h p.i.; at 3 h the culture was divided, and CH was added to one portion. Total nuclear and cytoplasmic RNAs from $2 \times 10^6$ cells were subjected to electrophoresis on a 1.5% agarose gel containing formaldehyde as a denaturant. RNA was transferred to nitrocellulose paper and hybridized with a nick-translated 1B (a) or E2A (b) probe. Lanes: 1 through 5, cytoplasmic RNA; 6 through 10, nuclear RNA; 1 and 6, and 3 h p.i. (time of addition of CH); 2 and 7, 4 h p.i., CH added; 3 and 8: 4 h p.i., no CH; 4 and 9: 5 h p.i., CH added; 5 and 10: 5 h p.i., CH. kb, Kilobases.

have allowed enough synthesis of early proteins to give some degree of CH stimulation.

**Effect of CH on *dl*312 transcription.** To analyze the effect of CH in the presence and absence of viral proteins, we performed the following experiment. Cells were infected with wild-type (WT) Ad5 and with *dl*312, a deletion mutant that lacks a functional 1A gene and which (at low or moderate MOIs) undergoes much less transcription of regions 1B, 2, 3, or 4 during early infection than does WT Ad5 (14, 22). In addition, one culture infected with WT virus was treated with CH beginning 5 min before virus addition. The *dl*312 infection provided a means of assaying the stimulatory effect of CH added at 3 h p.i. in the absence of putative viral regulatory proteins. The infections were at equivalent MOIs, as determined by assay of the particle-to-cell input ratio. The purified virus was assayed for particles by UV absorption and plaque formation on 293 cells, which are permissive for both viruses. The MOI used, 100 PFU/cell (1,000 particles per cell) was chosen to give sufficient transcription in the *dl*312-infected cells while still preserving the much lower rate of transcription of E1B and region 2 in *dl*312-infected cells than in WT Ad5. Although this MOI allows the transcription of some viral RNA (14, 22), the levels are still far below the levels in a WT infection (Fig. 6), and we assume that the levels of viral protein are correspondingly low. The level of 1A gene products is of course zero in a *dl*312 infection at any MOI.

At 3 h p.i., WT- and *dl*312-infected cultures were divided, and CH was added to half of each culture. At 4 h p.i., in vitro transcription was measured for 1A, 1B, E2A, and E4 in all cultures, including the one to which CH was added 5 min before the addition of virus (Table 4). Transcription rates from viral templates in *dl*312-infected cells were drastically reduced compared with rates in WT Ad5-infected cells. A 60-min CH treatment increased viral transcription of E1B and E2A in *dl*312-infected cells, but the levels were still a small fraction of those in WT Ad5-infected cells (1%). The same length of CH treatment in WT Ad5-infected cells gave a dramatic stimulation of transcription, as already described. The viral transcription rates in *dl*312-infected cells treated with CH were only about 0.1% of the rates in WT Ad5-infected cells given an identical CH treatment. Also, when CH was added to WT Ad5-infected cells just before infection rather than 3 h after, the transcription rates for 1A, 1B, and E4 were the same as control levels and considerably below the levels in cells to which CH was added at 3 h p.i. E2 transcription was actually depressed by CH addition before infection. These results for CH addition before infection confirm those of Persson et al. (20), who also found no stimulation of transcription at 5 h p.i. by anisomycin addition to infected cells 30 min before infection. These authors also reported inhibition of region 2 transcription, as did we (Table 4). Thus, the very high levels of transcription seen in normal infection with CH added at 3 h p.i. were not seen when the levels of viral proteins in the cells at the time of treatment were kept low either by drug treatment or infection with the deletion mutant.

Northern blot analysis of nuclear RNA in this experiment (Fig. 6) showed that the abundance of 1A and 1B RNAs in the nuclear RNA followed the pattern of transcription. Normal-sized 1B nuclear RNA was also found in CH-treated *dl*312 cells (Fig. 6). The abundance of this spe-

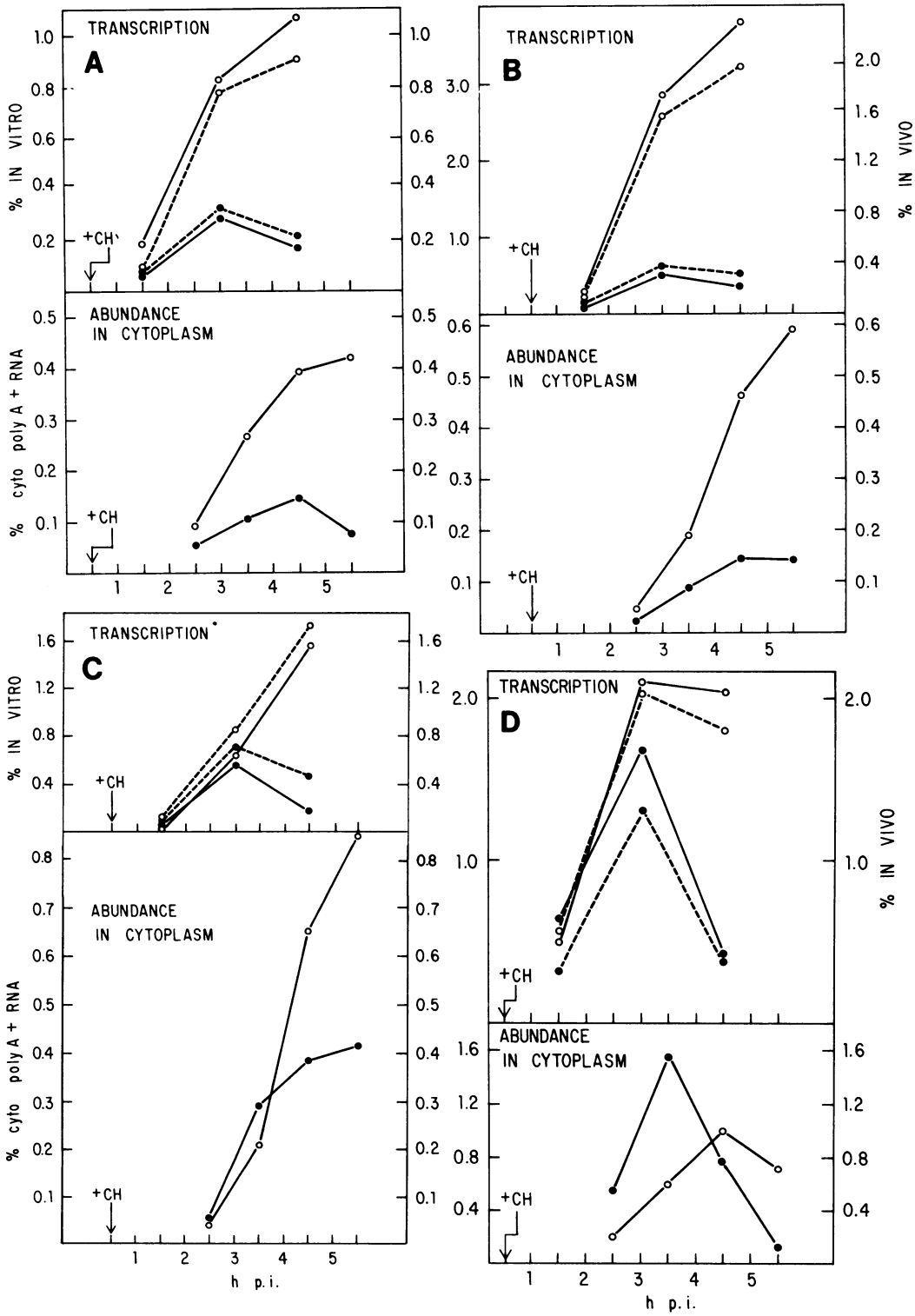


FIG. 5. Effect of CH addition at 0.5 h p.i. A culture of infected cells was split 0.5 h p.i., and CH was added to one half. Transcription rates were measured in vivo and in vitro at 1.5, 3, and 4.5 h p.i., and the cytoplasmic abundance of specific RNAs was measured at 1.5, 2.5, 3.5, and 4.5 h p.i. All measurements and symbols are as described in the legend to Fig. 3. (A) 1A-specific hybridization; (B) 1B-specific hybridization; (C) E2-specific hybridization; (D) E3-specific hybridization.

TABLE 4. Effect of CH at 3 h after *dl*312 infection[a]

| Region | % of input radioactivity hybridized[b] | | | | |
|---|---|---|---|---|---|
| | *dl*312-infected cells | | WT Ad5-infected cells | | |
| | No CH | CH present from 3 to 4 h p.i. | No CH | CH present from 3 to 4 h p.i. | CH present from 0 to 4 h p.i. |
| 1A | ND[c] | ND | 0.014 | 0.070 | 0.020 |
| 1B | 0.0002 | 0.0012 | 0.028 | 0.21 | 0.034 |
| E2 | 0.0007 | 0.0060 | 0.120 | 1.0 | 0.046 |
| E4 | 0.0003 | 0.0004 | 0.008 | 0.031 | 0.004 |

[a] HeLa cells were infected with *dl*312 or WT Ad5 at 1,000 particles per cell, and cultures were divided in half. CH was added after infection to one half of each sample and at the time of the infection to the other half of the WT Ad5-infected cells. At 4 h p.i., nuclear RNA was labeled in isolated nuclei.

[b] The actual counts per minute ranged from 35 to 700 above the background of 15 for the *dl*312 samples and from 223 to 63,000 above the background of 77 in the WT Ad5 samples.

[c] ND, Not done.

cies increased with CH treatment in parallel with the transcription rate measurements. (The same band is just visible in lane 1 in the original autoradiograph.)

## DISCUSSION

These experiments showed that early Ad2 transcription was strongly stimulated by the addition of CH after the establishment of early infection. This transcription resulted in the same size precursor and mRNA species, as detected by Northern blot analysis. Although we cannot rule out small differences in the sizes or splice sites of these RNAs, it seems likely that CH caused a simple increase in the activity of the viral transcription units that were already active. (In this connection, we found that migration of the cap 1 and cap 2 structures of 1A mRNA from CH-treated cells had the same mobilities on DEAE paper as did 1A caps 1 and 2 from control infected cells, which argues for the use of the same nucleotide in 1A initiation [unpublished data].) When CH was added before the virus, no stimulation of transcription was observed, in accord with previous reports (16, 20). One point about previously described results (26) which was raised but not settled by the present experiments is the possible effect of CH on increasing mRNA half-life when it is added before infection. We previously measured the rate of accumulation of [$^3$H]uridine in Ad2 mRNA in control and CH-pretreated infected cells and concluded that CH induced an increased half-life. However, in the present experiments we did not detect any substantial increase in steady-state levels of mRNA when CH was added before infection (data not shown). In the experiments in which CH was added p.i., the increase in steady-state levels was matched by a proportional increase in transcription rates (Fig. 3 and 5). Therefore, additional experiments are in order to reinvestigate the role of CH on mRNA half-life. However, it is the clearly demonstrated effect of CH on transcription when added after a period of normal infection on which we wish to concentrate in this paper.

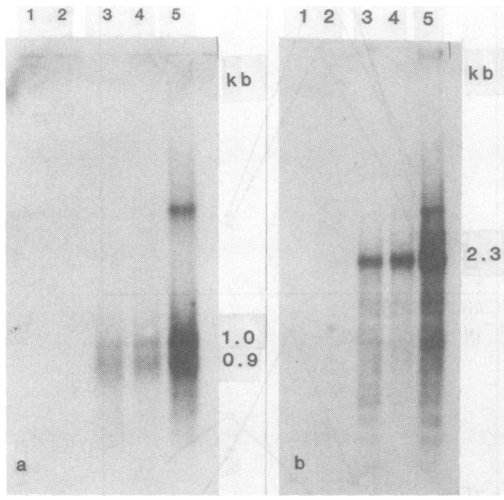


FIG. 6. Levels of nuclear 1A and 1B RNAs in *dl*312-infected cells compared with WT Ad5 with and without CH at 0 or 3 h. p.i. Nuclear RNA was extracted from cultures (see Table 4). Poly(A)$^+$ RNA from $10^7$ cells was selected and separated by a 1.2% agarose gel and hybridization to 1A (a) and 1B (b) probes were as described in the legend to Fig. 4. Lanes: 1 and 2, *dl*312-infected cells; 3, 4, and 5, WT Ad5-infected cells; 1 and 3, no CH treatment; 2 and 5; CH added at 3 h p.i., 4, CH added before virus. kb, Kilobases.

The presence of some product of the viral 1A region brings about increased viral transcription (14) (Table 4). Thus, stimulation of transcription by the 1A product(s) and by CH interacted to produce the maximum level of early Ad2-specific transcription (Table 4 and Fig. 6).

There are two possible interpretations of this result, and in the absence of additional genetic and biochemical data we are unable to choose

between them. One possibility is that, as suggested by Nevins (14), there is a cellular repressor of Ad2 transcription, which is destroyed or otherwise inactivated by 1A gene products, and which is sufficiently short lived that continuous treatment with protein synthesis inhibitors from the beginning of infection leads to substantial viral transcription. If such a repressor exists, and if both 1A products and protein synthesis inhibitors act to remove it, then it is possible that the combination of 1A products and protein synthesis inhibition at 3 h remove the inhibitor much more effectively than do either 1A products or protein synthesis inhibition alone. This would occur if, for example, 1A products decrease the functional half-life of the repressor and CH stops its synthesis. In this case, major stimulation of transcription would be expected by adding CH after early infection is established and 1A products are plentiful, and lesser stimulation (or none) if CH is added before or shortly after the virus. In addition, it would be expected that the stimulation in CH would be reversible, since the cell factor could be resynthesized quickly after CH was removed. All these expectations were met by the data obtained.

Persson et al. (19) showed that in 293 cells, which express 1A products constitutively, blocking protein synthesis before infection causes a large stimulation of E4 transcription only; other early regions are not stimulated. This suggests that whereas E4 may be directly stimulated by inhibiting protein synthesis in the presence of E1A products, the CH stimulation of other early regions (1A, 1B, E2, E3, and L1) which we demonstrated may depend on the previous expression of other early genes besides 1A. The E4 stimulation in 293 cells recalls the experiments of Nevins and Winkler (17) and Nevins et al. (16), who showed that region 4 was inhibited by a product of region 2. Inhibition of protein synthesis in the presence of 1A protein (as would be true in 293 cells) should, in light of these early results, stimulate region L1, and it does.

A second way to account for our results is to suppose that the rate of early transcription is a balance of stimulation and repression and that the repressive element(s) is less stable than the stimulatory element(s). The actual level of transcription at any given time during early infection would depend on the balance of these independent effects. It is known that early regions 2, 3, and 4 all increase transcriptional activity early in infection and later fall off, sometimes quite sharply (Fig. 5) (16). The failure to observe the maximum levels of transcription in cells incubated in protein synthesis inhibitor from the beginning of infection would result from the much lower amounts of activator protein(s) present in these cells. Because neither the known activation of transcription nor the postulated repression of transcription would occur with no protein synthesis, the resulting levels of transcription would be hard to predict in advance. Both of the above models assume the existence of repressors of Ad2 transcription which are short lived. The first assumes the repressor to be a cellular product. The second makes no assumptions about the origin of the repressor. Both models could obviously be expanded by assuming the existence of multiple activators and repressors for various different early regions.

Both of these models could fully account for the data presented here. Obviously, further experimentation of a different kind is required to fully work out the cause of the response to CH that we have described.

Fire et al. (8) showed that at least some extracts made from Ad2-infected cells late in infection have different in vitro transcription activities than do extracts from early-infected or mock-infected cells when early versus late transcription units are assayed. Although they found no difference between mock- and early-infected cell extracts, it would be interesting to know whether a difference would be seen between early-infected extracts and extracts from cells which are given CH after the establishment of early infection. If the transcription ability of the extracts from CH-treated cells were increased on early Ad2 regions, this would argue for a *trans*-acting repressor that is inactivated by CH treatment.

In summary, we showed that when CH was added after early infection had started early transcription was strongly stimulated, and this stimulation required the previous synthesis of some viral protein(s). Understanding the mechanism of this stimulation should increase our understanding of the regulation of Ad2 early transcription.

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