MOLECULAR AND CELLULAR BIOLOGY, Sept. 1987, p. 3049–3056
0270-7306/87/093049-08$02.00/0

Vol. 7, No. 9

Adenovirus E1A Requires Synthesis of a Cellular Protein To Establish a Stable Transcription Complex in Injected *Xenopus laevis* Oocytes

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Received 6 March 1987/Accepted 20 May 1987

The *Escherichia coli*-expressed adenovirus E1A 13S mRNA product injected into *Xenopus* oocytes was active, as assessed by its ability to stimulate the transcription of an injected gene which is normally responsive to E1A in mammalian cells. In the presence of the protein synthesis inhibitors pactamycin or cycloheximide, E1A was correctly posttranslationally modified (phosphorylated) and transported to the nucleus; but it failed to stimulate the transcription of an injected gene containing the human heat shock protein 70 promoter. The basal (unstimulated) level of transcription of the gene was unaffected by these inhibitors. If oocytes were cultured in the presence of cycloheximide after E1A stimulated transcription, however, the high level of transcription was maintained for several hours without new protein synthesis. Results of competition studies with the same promoter (the heat shock protein 70 promoter) linked to two marked genes demonstrated that once the induction of transcription by E1A took place, the stimulated levels of transcription were maintained, even when they were challenged with excess competitor DNA. Results of these studies suggest that E1A requires the synthesis of a cellular protein to form a stable transcription complex.

The control of viral transcription in infected cells has served as a prototype for differential gene expression of eucaryotic cells in general. Viral systems have the advantage of not only having a highly ordered temporal sequence of gene transcription, but also, in several cases, the identity of the gene required for this temporal specificity is known as well. In adenovirus, for example, the E1A gene product is synthesized early following infection and is required for the efficient expression of other early adenovirus genes (2, 17, 38). Furthermore, E1A can stimulate the expression of certain cellular genes such as the heat shock protein 70 (HSP70) gene (18, 29, 41) and tubulin (35). In contrast, E1A can also inhibit gene expression by repressing enhancer activity (4, 37). The definition of the sequence elements of several of these genes which are either stimulated or repressed by E1A has shown that they bear little resemblance to one another (for a review, see reference 1). As a consequence of these diverse E1A-induced effects on unrelated DNA sequences and the observation that E1A does not bind DNA (9), it has been suggested that E1A is not the ultimate regulator of gene expression but rather that it might act in concert with cellular factors to exert unique effects on different substrates (1).

Two general approaches have been employed to identify such factors. The first has been to determine whether cellular proteins coimmunoprecipitate with E1A (13, 43). Although as many as nine cellular proteins are detected by this procedure, it is not known whether any bind DNA or affect transcription. An alternative approach has been to assess whether cellular proteins bind E1A-inducible promoters. Using gel shift and methylation protection assays, SivaRaman et al. (32) have shown that a HeLa cell nuclear protein interacts with a 17-nucleotide sequence in the adenovirus E2A promoter. However, the same level of binding activity was observed in the presence and absence of E1A. Kovesdi et al. (21, 22) have obtained similar results by using DNase I and exonuclease III protection assays, although these investigators found a second protein that bound to the E2A promoter. The binding activity of these cellular proteins, however, was greatly enhanced in the presence of E1A. Whether these E1A and DNA binding proteins are related, however, remains to be demonstrated.

As a system which is amenable to the manipulation of both DNA and protein, we employed microinjected *Xenopus* oocytes to determine whether cellular proteins are important for E1A-induced transcriptional stimulation. E1A protein, which is overexpressed in *Escherichia coli*, is functional in oocytes, as assessed by its ability to stimulate the transcription of injected genes which are normally E1A responsive in mammalian cells (8, 31). We found that continual oocyte protein synthesis is required for E1A-induced transcriptional stimulation, but not for basal (uninduced) levels of transcription. Once maximal levels of E1A-induced transcription were attained, however, they remained high even in the absence of protein synthesis or when challenged with competitor DNA. Results of these studies suggest that in injected *Xenopus* oocytes, a de novo-synthesized cellular protein is required for E1A-induced transcription.

MATERIALS AND METHODS

Plasmids and *E. coli*-expressed E1A. The promoters from the adenovirus type 5 E3 gene and the HSP70 gene were fused to the bacterial chloramphenicol acetyltransferase (CAT) gene as described elsewhere (E3/CAT, termed pKCAT23 in reference 16; pHSP70/CAT in reference 42). For the construction of HSP70/TK, a 220-base-pair (bp) *Hin*dIII-*Bam*HI fragment containing 70 bp of upstream and 150 bp of 5'-nontranslated human HSP70 sequences was removed from the plasmid HSPΔ70/CAT (kindly provided by B. Wu and R. Morimoto, Northwestern University,

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Evanston, Ill.) and cloned into the *Hin*dIII and *Bgl*II sites of the herpes simplex virus thymidine kinase (TK)-containing plasmid pAGO (6). This construct was fully inducible by E1A in mammalian cells. The overexpression of the E1A 13S mRNA product in *E. coli* and its subsequent purification have been described elsewhere (10).

Oocyte injections and CAT assays. Stage 6 *Xenopus laevis* oocytes (7) were manually defolliculated from excised ovarian fragments and cultured in Barth medium (26), which was supplemented with 10 μg of cycloheximide per ml or 10^{-6} M pactamycin, depending on the experiment. Plasmid DNA (the exact amounts are given in the figure legends) in 10 nl of 10 mM Tris hydrochloride (pH 8)–1 mM EDTA was injected into nuclei, and 10 ng of purified E1A in 10 nl of water was injected into the cytoplasms of oocytes. Injected oocytes were cultured for various times, as denoted in the figure legends. For CAT assays, injected oocytes were homogenized in 100 μl of 0.25 M Tris hydrochloride (pH 8), the yolk was removed by centrifugation at 12,000 × *g* for 10 min, and the supernatant was incubated with 5 μCi of [^{14}C]chloramphenicol (Amersham Corp., Arlington Heights, Ill.) and 1.65 μg of acetyl coenzyme A (16). Following incubation at 37°C for 1 h, the chloramphenicol was extracted with ethyl acetate, and the acetylated and nonacetylated forms of chloramphenicol were resolved by thin-layer chromatography and autoradiography. Quantitation of [^{14}C]chloramphenicol spots was determined by liquid scintillation spectrometry.

S1 nuclease mapping and analysis of in vivo-labeled nuclear RNA. S1 nuclease mapping and analysis of in vivo-labeled nuclear RNA was performed by the procedure described by Wu et al. (40). The probe for S1 nuclease mapping was prepared by 5′-end labeling at the HSP70 promoter-CAT *Bam*HI junction site (Fig. 1) and then by carrying out digestion within the HSP70 promoter at position −70. This yielded a 220-bp double-stranded probe which was radiolabeled on the coding strand. This probe contained HSP70 sequences only and, as such, could be used to map transcription from the complete gene or from the chimeric pHSP70/CAT gene. Correct initiation at the HSP70 cap site should yield an S1 nuclease-protected fragment of 150 bases.

Excess double-stranded probe was incubated with 3 μg of total RNA from injected oocytes in hybridization buffer (80% formamide, 0.4 M NaCl, 40 mM PIPES, [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 6.4], 2 mM EDTA) for 10 min at 90°C, followed by an overnight incubation at 54°C. A total of 300 μl of S1 nuclease digestion buffer (280 mM NaCl, 3 mM sodium acetate [pH 4.4], 4.5 mM $ZnCl_2$) and 300 U of S1 nuclease (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml was then added, and the mixture was incubated for 1 h at 37°C. The S1 nuclease-resistant DNA was recovered by ethanol precipitation and analyzed on a 6% acrylamide gel containing 50% urea. Control RNA was isolated from adenovirus type 5-infected HeLa cells at 18 h postinfection. Because E1A stimulates cellular HSP70 expression, S1 nuclease analysis of this RNA sample showed the elevated level of HSP70 mRNA in these cells.

For in vivo-labeled nuclear RNA analysis, DNA-injected oocytes were injected with about 2 × 10^5 cpm of [α-^{32}P]GTP (410 Ci/mmol; Amersham), and nuclei were isolated manually 45 min later. Nuclear RNA was extracted from 30 to 50 oocytes with phenol and chloroform, precipitated, and hybridized to 1 μg of linearized pHSP70/CAT DNA or the *Bgl*II-*Sma*I fragment of pHSP/TK, which was immobilized on nitrocellulose. Hybridization was carried out for about 15 h as described previously (36). The filters were washed, and the radioactivity was detected by autoradiography and quantitated by densitometry. Exposure times for autoradiographs ranged from 3 to 5 days.

Protein synthesis and immunoblotting. Relative rates of protein synthesis were determined by injecting oocytes with approximately 2 × 10^5 cpm of [^{35}S]methionine (1,200 Ci/mmol; New England Nuclear Corp., Boston, Mass.) and culturing them for 45 min. They were then homogenized in Barth medium and centrifuged to remove the yolk, and the supernatant was brought to 10% trichloroacetic acid. The precipitates were spotted onto paper disks; and then the disks were immersed in cold trichloroacetic acid for 10 min, boiled for 10 min, rinsed with cold absolute ethanol. The radioactivity on the dried disks was determined by liquid scintillation spectrometry.

For immunoblotting, E1A-injected oocytes were enucleated manually, and the nuclei were solubilized in sodium dodecyl sulfate (SDS) sample buffer (24). The cytoplasms were homogenized in 10 mM Tris hydrochloride (pH 7.5)–50 mM NaCl, and the yolk was pelleted by centrifugation at 12,000 × *g* for 5 min. The supernatant was brought to 4 volumes of absolute ethanol, and the protein was precipitated overnight at −20°C. The precipitated protein was collected by centrifugation at 12,000 × *g* for 10 min at 4°C, and the ethanol was evacuated. The precipitate was suspended in SDS sample buffer, and both nuclear and cytoplasmic fractions were resolved by SDS-10% polyacrylamide gel electrophoresis. The protein then was electroblotted onto nitrocellulose and reacted with rabbit E1A antiserum and peroxidase-conjugated goat anti-rabbit immunoglobulin G. The colorimetric peroxidase reaction then was carried out as described previously (19).

RESULTS

In previous studies (8, 31), we have demonstrated that the *E. coli*-produced E1A 13S mRNA product stimulates expression of the adenovirus E3 promoter in gene-injected *X. laevis* oocytes. We have shown in this study that E1A also stimulates the expression of the HSP70 promoter in injected oocytes. The constructions of gene fusions between the adenovirus E3 and HSP70 promoters and the CAT gene as well as the HSP70 promoter and the herpesvirus TK gene are shown in Fig. 1. A total of 0.5 ng of pE3/CAT and pHSP70/CAT was injected into the nucleus (germinal vesicle) of each oocyte, and 10 ng of *E. coli*-expressed E1A was injected into the cytoplasm. Following 18 h of culture, oocyte homogenates were prepared and used for CAT assays (Fig. 2A). Results of these assays demonstrate that there was the same amount of CAT enzyme activity in oocytes injected with either pE3/CAT or pHSP70/CAT, as assessed by the degree of acetylation of [^{14}C]chloramphenicol. To control for an injection stress response of pHSP70/CAT, Fig. 2B shows CAT assays of gene-injected oocytes injected with E1A or bovine serum albumin. Although E1A stimulated CAT activity in these oocytes by about fourfold relative to that of controls (pHSP70/CAT), injected bovine serum albumin had no effect. Therefore, pHSP70/ACATCT does not respond to the injection of a nonspecific protein.

Several polypeptide variants arise from the E1A 13S mRNA in adenovirus-infected cells (12), as revealed by two-dimensional polyacrylamide gel electrophoresis. In one-dimensional SDS gels, there were two primary E1A proteins with apparent molecular weights of 43,000 and 45,000. The variants were due to posttranslational modifications, proba-

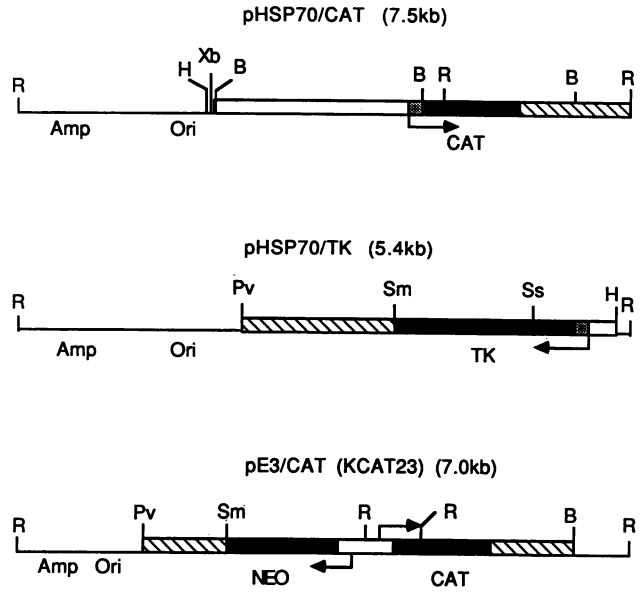

FIG. 1. Schematic diagram of the plasmids used in this study. pHSP70/CAT has been described by Wu et al. (40), who called it pHBCAT. It contains 2.4 kilobases (kb) of upstream (open area) and 150 bp of nontranslated (stippled area) human HSP70 sequences fused to the CAT coding sequence (solid area) and the necessary 3'-nontranslated control signals taken from the simian virus 40 early gene (striped area). The direction of transcription is denoted by the arrow. The plasmid pHSP70/TK contains 70 bp of upstream (open area) and 150 bp of 5'-nontranslated (stippled area) HSP70 sequences fused to the herpes simplex virus TK coding region (solid area) and 3'-nontranslated signals (hatched area). The 70-bp HSP70 upstream sequences are sufficient for maximum stimulation of this promoter by E1A (B. J. Wu, H. C. Hurst, N. C. Jones, and R. I. Morimoto, unpublished data). pE3/CAT has been described by Weeks and Jones (38), who called it pKCAT23. It contains a fragment of the adenovirus genome that incorporates the E2A and E3 early promoters (open area). The E3 promoter drives the CAT coding sequence, and the E2 promoter drives the neomycin phosphotransferase gene (NEO). The restriction sites are as follow: R, *Eco*RI; H, *Hin*dIII, Xb, *Xba*I; B, *Bam*HI; Pv, *Pvu*II; Sm, *Sma*I; Ss, *Sst*I.

bly multiple phosphorylations, of the primary product. One or more of these phosphorylations induced a conformational change in the protein such that it had an altered mobility in SDS gels (N. C. Jones and J. D. Richter, manuscript in preparation). Thus, the 45,000-molecular-weight form is the product of the 43,000-molecular-weight form. These same two variants were detected in E1A-injected *Xenopus* oocytes, indicating that these cells have the capability for at least this posttranslational modification. Interestingly, the modification took place in the cytoplasm of injected oocytes, and it is the modified 45,000-molecular-weight form that is the predominant one in the nucleus (30, 31). We determined whether continual protein synthesis in injected oocytes is required for this E1A modification or nuclear translocation. Oocytes were injected with 10 ng of *E. coli*-expressed E1A and cultured for 15 h in the absence or presence of cycloheximide, and the nuclei and cytoplasms were separated manually. The presence of modified E1A (i.e., the 45,000-molecular-weight form) in each of these cellular fractions then was assessed by immunoblotting (Fig. 3). Greater than 95% of the E1A which was injected into the cytoplasm was modified and translocated to the nucleus in oocytes cultured

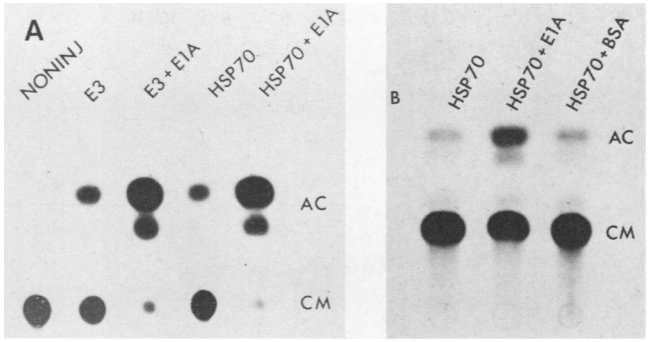

FIG. 2. CAT enzyme activities from injected oocytes. (A) Thirty oocytes were injected with 0.5 ng of pE3/CAT (E3) or pHSP70/CAT (HSP70) only or with each of these plasmids plus 10 ng of *E. coli*-expressed E1A (E3 + E1A and HSP70 + E1A, respectively). The DNA injections were nuclear, and the E1A injections were cytoplasmic. The oocytes were cultured for 18 h, homogenized, and incubated with [^{14}C]chloramphenicol and acetyl coenzyme A. The chloramphenicol was then extracted, and the acetylated (AC) and nonacetylated (CM) forms were resolved by thin-layer chromatography and detected by autoradiography. NONINJ refers to noninjected oocytes. (B) Ten oocytes were injected with 0.5 ng of pHSP70/CAT alone (nuclear) or together with 10 ng of E1A or 10 ng of bovine serum albumin (BSA) (cytoplasmic). The oocytes were cultured for 15 h, and CAT assays were performed as described above.

in the absence or presence of cycloheximide. Thus, continuous protein synthesis is not required for the posttranslational modification or nuclear localization of E1A or for its remarkable stability (see below). We must emphasize that we are assessing only that phosphorylation event(s) which induces the conformational change in E1A. It is possible, although unlikely, that some phosphorylations which do not affect E1A mobility in one-dimensional SDS gels are affected by protein synthesis inhibitors.

E1A requires continuous protein synthesis to stimulate transcription. We used injected oocytes to determine whether *trans* activation by E1A requires continuous protein synthesis. Oocyte nuclei were injected with pHSP70/CAT, and cytoplasms were injected with the *E. coli*-expressed E1A. Oocytes then were cultured in the absence or presence of the protein synthesis inhibitors pactamycin or cycloheximide, and the RNA was extracted and CAT transcripts were analyzed by S1 nuclease mapping (Fig. 4). Three results

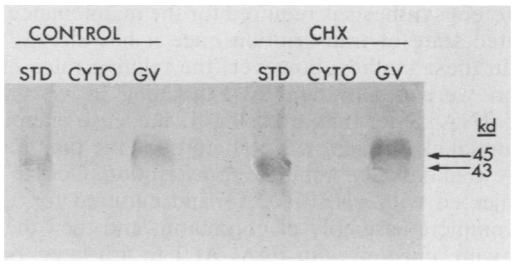

FIG. 3. Subcellular distribution of injected E1A. Five oocyte cytoplasms were injected with 10 ng of E1A and cultured in the absence (CONTROL) or presence (CHX) of cycloheximide for 15 h. Nuclei then were separated manually from cytoplasms, and the presence of E1A in each cellular compartment was assessed by an immunoblot. STD refers to a 50-ng standard of noninjected *E. coli*-expressed E1A, CYTO refers to cytoplasm, and GV refers to germinal vesicles (nuclei). The apparent molecular sizes (in kilodaltons [kd]) of the two forms of E1A are denoted and were derived by comparison with known molecular weight standards.

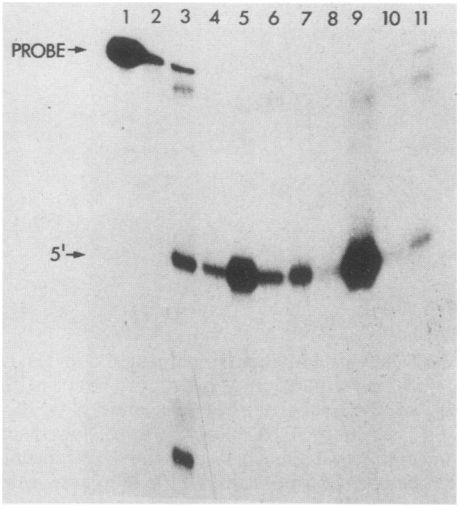

FIG. 4. S1 nuclease mapping analysis of CAT mRNA from gene-injected oocytes. Approximately 30 oocytes were injected with 0.5 ng of pHSP70/CAT or with the same DNA plus 10 ng of E1A and cultured for about 15 h. Total RNA then was extracted, and CAT mRNA levels were assessed by S1 nuclease analysis. The bands denoted as 5′ are 150 bp in length. Lane 1, probe only; lane 2, probe plus tRNA; lane 3, adenovirus-infected HeLa cells; lanes 4 and 8, pHSP70/CAT- injected oocytes cultured in control medium; lanes 5 and 9, pHSP70/CAT and E1A cultured in control medium; lanes 6 and 10, pHSP70/CAT-injected oocytes cultured in the presence of pactamycin and cycloheximide, respectively; lanes 7 and 11, pHSP70/CAT- and E1A-injected oocytes cultured in the presence of pactamycin and cycloheximide, respectively. Oocytes from two different females were used for these experiments (lanes 4 to 7 are from female 1 and lanes 8 to 11 are from female 2).

were obtained from this experiment. First, CAT mRNA from gene-injected oocytes had the correct 5′ end when compared with endogenous HSP70 mRNA from adenovirus-infected HeLa cells. Second, E1A stimulated pHSP70/CAT transcription by 15- to 25-fold (compare lanes 3 versus 4 and 8 versus 9) but failed to stimulate transcription in injected oocytes cultured in the presence of pactamycin or cycloheximide (compare lanes 5 versus 6 and 10 versus 11). Third, the protein synthesis inhibitors did not affect basal levels of transcription (for example, compare lanes 4, 6, and 7).

The experiments shown in Fig. 4 suggest that a de novo-synthesized cellular protein is involved in the stimulation of transcription by E1A. We next determined whether continuous protein synthesis is required for the maintenance of the stimulated state of transcription once it has already taken place. In these studies, however, the relative rates of transcription were determined by examining in vivo-labeled nuclear RNA rather than by using S1 nuclease mapping.

The initial experiment was to determine the time required for E1A to maximally stimulate transcription. Oocyte nuclei were injected with pHSP70/CAT and cultured for 12 h to allow complete assembly of chromatin, and then the cytoplasms were injected with E1A. At 1 to 5 h later, oocytes were injected with [^{32}P]GTP and incubated for 45 min. The nuclei were then isolated manually, and the RNA was extracted and hybridized to excess CAT DNA which was immobilized on nitrocellulose. The maximal stimulation of transcription by E1A was reached by about 4 h postinjection (Fig. 5).

Based on these results, oocytes were injected with pHSP70/CAT, cultured for 12 h, injected with E1A, and cultured for an additional 5 h. They then were incubated in the presence of cycloheximide for 2 to 12 h, and analysis of labeled nuclear RNA was performed. Although cycloheximide reduced oocyte protein synthesis to less than 10% of control levels within 2 h, relative CAT transcription remained high for up to 8 h and then declined to control levels by 12 h (Fig. 5). These data suggest that although continual protein synthesis is required for the initial E1A-induced transcriptional stimulation, once maximal transcription is attained, protein synthesis is not required for a prolonged period of time.

We determined whether the induction of transcription by E1A is reversible. Oocytes were injected with pHSP70/CAT and E1A and incubated for 5 h in control medium and then cultured for 12 h in the presence of cycloheximide. By following this regimen, E1A-induced transcription returned to basal levels (see above). The oocytes then were again cultured in control medium, and the labeled nuclear RNA was analyzed at several intervals. Oocyte protein synthesis resumed within 2 h after the cells were removed from cycloheximide (Fig. 5). Futhermore, E1A again was able to induce transcription, as assessed by the enhanced level of nascent CAT transcripts detected by hybridization. The concentration of cycloheximide used in these experiments (10 μg/ml) was found to be the minimum amount which could inhibit oocyte protein synthesis of 90% but still be washed out of oocytes when they were subsequently cultured in control medium. However, the level of protein synthesis in oocytes cultured initially in cycloheximide and then in control medium never exceeded 80% of the level of protein synthesis in oocytes never exposed to the drug.

How E1A *trans* activates a gene is not known, and therefore, it is not clear whether activation of the HSP70 promoter and the early adenovirus promoters is accomplished by the same mechanism. This is likely to be the case, however, because it has been shown that in HeLa cells HSP70 induction by E1A is 13S mRNA specific (41); the E1A 12S mRNA product fails to stimulate HSP70. This also is the case for the early adenovirus promoters. In addition to the HSP70/CAT studies described above, we also examined the requirements for E1A induction of E3/CAT in oocytes. In experiments similar to those described for HSP70/CAT, E1A induction of E3/CAT was inhibited by cycloheximide, although basal transcription was unaffected (data not shown). Therefore, the requirements for HSP70 and E3 induction by E1A appear to be the same.

E1A induces the formation of stable transcription complexes. Results of the experiments presented in Fig. 5 suggest that once formed, the E1A-induced transcription complex is stable for several hours in the absence of protein synthesis. The stability of transcription complexes, however, can be determined directly by DNA completion assays. Before these assays could be performed, it was necessary to determine the level of pHSP70/CAT which is rate limiting for maximal stimulation by a constant amount of E1A. Therefore, oocytes were injected with a constant 10 ng of E1A and progressively greater amounts of pHSP70/CAT; relative rates of transcription then were determined by in vivo labeling of nuclear RNA. It is demonstrated in Fig. 6 that 10 ng of E1A maximally stimulated the transcription of 0.1 ng of pHSP70/CAT DNA.

Based on these results, oocytes were injected with an amount of pHSP70/CAT, to ensure that this DNA substrate was rate limiting for transcription (0.5 ng), plus 10 ng of E1A; cultured for 5 h; and subsequently injected with 0.01 to 50 ng pHSP70/TK or up to a 70-fold molar excess of this competitor DNA. The relative rates of transcription of these plas-

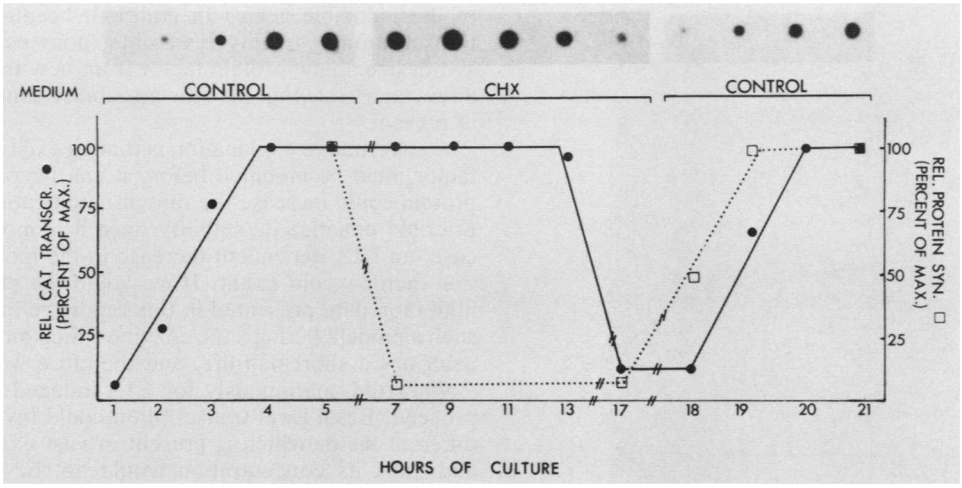

FIG. 5. Analysis of in vivo-labeled nuclear RNA from injected oocytes. Oocytes were injected with 0.5 ng of pHSP70/CAT, cultured for 12 h, injected with 10 ng of E1A, and cultured for various periods in the absence or presence of cycloheximide (CHX; cf. text for times of exposure to this drug). They were then injected with [^{32}P]GTP, and the in vivo-labeled nuclear RNA was extracted and hybridized to excess CAT DNA immobilized on nitrocellulose. Dot blot hybridizations were quantitated by densitometry (relative CAT transcription [REL. CAT TRANSCR.]), and relative protein synthesis (REL PROTEIN SYN.) rates were determined by trichloroacetic acid insolubility of injected [^{35}S]methionine. It should be noted that this figure is a composite of results from three separate experiments (hours of culture, 1 to 5, 5 to 17, and 17 to 21). Therefore, the values for transcription and protein synthesis are relative for each individual experiment and not necessarily between experiments.

mids were then determined by in vivo nuclear RNA labeling. pHSP70/CAT transcription continued unabated in the presence of up to a 14-fold molar excess of pHSP70/TK and only began to diminish when the competitor was present in 70-fold molar excess (Fig. 7A). Conversely, pHSP70/TK was transcribed at a constant basal level and increased significantly only when it was present at a 70-fold molar excess. Thus, once E1A-induced transcription complexes are formed, they are stable and interact very little with other DNA templates.

In a final experiment, we determined whether the E1A-induced transcription complex, once formed and subsequently dismantled, has the ability to reform on any appropriate DNA template. Oocytes were injected with pHSP70/CAT and E1A, cultured for 5 h, injected with 0.01 to 10 ng of pHSP70/TK, and then cultured in cycloheximide for another 12 h. This regimen allowed for the maximal stimulation of HSP70 by E1A and its subsequent return to basal levels of transcription. Oocytes then were cultured in control medium, which once again allowed for the induced level of transcription. Transcription of both CAT and TK genes was then assessed by in vivo nuclear RNA labeling (Fig. 7B). In this experiment, the amount of transcription from each plasmid varied as a function of the TK/CAT ratio. As the concentration of pHSP70/TK increased relative to that of pHSP70/CAT, there were progressively more TK transcripts and fewer CAT transcripts. pHSP70/CAT transcription declined by about 50% when it and pHSP70/TK were present in approximately equal molar concentrations. These results should be contrasted with those presented in Fig. 7A, in which it was shown that pHSP70/TK was unable to effectively compete with E1A-induced pHSP70/CAT transcription complexes once those complexes were formed. Our interpretation of these data is that E1A-induced transcription complexes, once dismantled, can reform on any suitable DNA template.

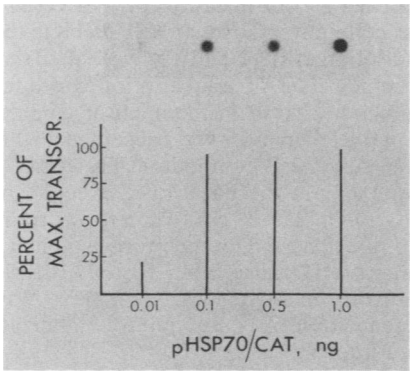

FIG. 6. Rate-limiting amount of injected pHSP70/CAT maximally stimulated by 10 ng of E1A. Oocytes were injected simultaneously with various amounts of pHSP70/CAT and a constant 10 ng of E1A. They were cultured for 5 h, and analysis of in vivo-labeled nuclear RNA was performed. MAX. TRANSCR., Maximum transcription.

DISCUSSION

The results presented in this report demonstrate that the E1A-induced stimulation of transcription observed in injected *Xenopus* oocytes requires continuous protein synthesis. This implies that either the injected E1A induces the transcription of a new gene, the protein product of which is important for E1A *trans* activation, or that oocytes constitutively synthesize a protein that is required for E1A stimulation and that has a half-life of less than 10 h. The results also indicate that while the inhibition of a protein synthesis completely blocks E1A-stimulated transcription, uninduced or basal-level transcription is unaffected.

One possible explanation for these results is that the role of E1A in *trans* activation is to induce the synthesis of a limiting transcription factor that interacts with E1A-inducible promoters. This factor, in turn, would be required

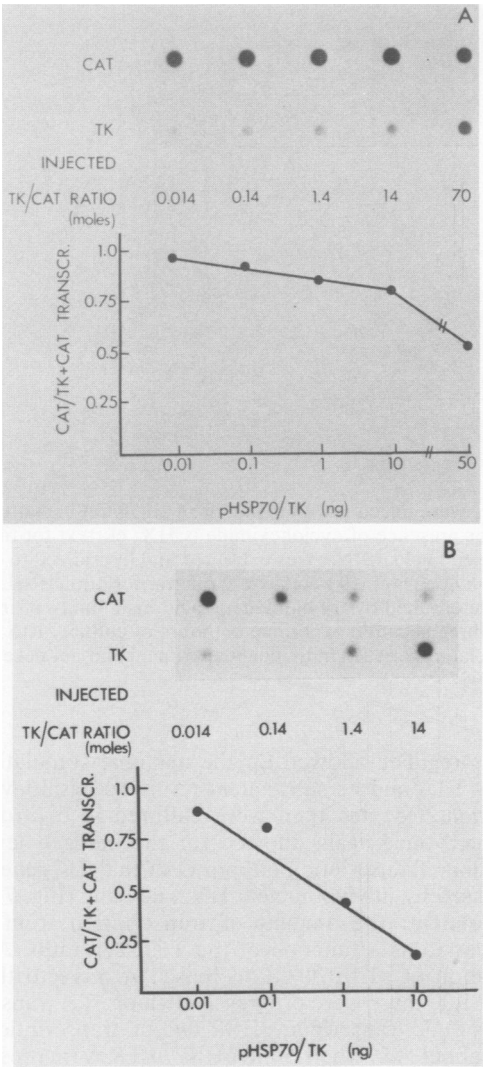

FIG. 7. Competition between pHSP70/CAT and pHSP70/TK for E1A-induced transcription (TRANSCR.) complexes. (A) Oocytes were injected with 0.5 ng of pHSP70/CAT and 10 ng of E1A, cultured for 5 h, and then injected with various amounts of pHSP70/TK. Analysis of in vivo-labeled nuclear RNA for CAT and TK were performed at several subsequent time periods. (B) Oocytes were injected with the same materials as described for panel A, but following pHSP70/TK injection, they were cultured for 12 h in the presence of cycloheximide. The oocytes were subsequently cultured for 5 h in control medium, and CAT and TK transcripts were detected by analysis of in vivo-labeled nuclear RNA.

for efficient transcription; in the absence of this factor, the promoter could still function, but only inefficiently, and thus would give a basal level of activity. This proposal is consistent with the findings of Kovesdi et al. (21, 22), who demonstrated that a HeLa cell nuclear protein binds to the E1A-inducible adenovirus E2 promoter; the apparent level of this protein is significantly increased on the infection of cells with wild-type adenovirus, but not with the E1A mutant *dl*312. To be consistent with this model, however, one would have to envision that E1A could stimulate the transcription of an endogenous gene to such an extent that its protein product, in turn, could enhance the transcription of a substantial fraction of the large number of injected genes. (Maximal stimulation was obtained with about 2×10^7 injected copies of the inducible gene.) In addition, because E1A-induced transcription is readily reversible, oocytes would have to synthesize a new protein factor from new transcripts in just a few hours (cf. Fig. 5). This possibility cannot be ruled out at present.

An alternative explanation is that an existing transcription factor must be modified before it can become active. E1A protein could increase the rate of modification of this factor or could enhance its stability once it is modified; in either case, an E1A-dependent increase in the modified transcription factor would result. How would the protein synthesis inhibition data presented in this report be incorporated into such a model? Perhaps the enzyme which modifies the factor itself has a short half-life, and therefore would have to be synthesized continuously for E1A-induced transcription to proceed. Basal level transcription could involve an entirely different factor which is present in vast excess in oocytes, and thus, its concentration would not be greatly affected over the time course of the experiments described here. In fact, the oocyte is known to store large quantities of transcription factors for use during early embryogenesis (for a review, see reference 33). Alternatively, basal and stimulated transcription might employ the same factor; but while stimulated transcription requires a modified factor, basal transcription could proceed in the absence of the modification. Irrespective of the modification, the factor would interact with the same promoter elements. Such a model would be consistent with the results of a number of studies in which it has been shown that mutations made in E1A-inducible promoters that lower the level of induced transcription also reduce basal transcription (14–16, 20, 25, 27). Furthermore, nuclear factors that interact with promoter elements in the E2A, E3, and E4 adenovirus genes have been identified; and in most cases they are present at similar levels in uninfected and adenovirus-infected cells (32; F. Lee and M. Green, submitted for publication; H. C. Hurst and N. C. Jones, submitted for publication). Distinguishing between any of these models, however, requires the isolation and characterization of cellular factors that are essential for E1A activity.

E1A induces stable transcription complex formation. We performed a series of double DNA injection experiments to assess whether the transcription complex induced by E1A is stable once it has formed. Rate-limiting amounts of one DNA template (pHSP70/CAT) and E1A were injected and allowed to reach maximal levels of stimulated transcription. This was followed by an injection of a severalfold molar excess of the competitor DNA (pHSP70/TK); the relative rates of transcription of CAT and TK were then determined. In this case, enhanced CAT transcription was affected little by a 14-fold molar excess of the competitor, and declined by only 50% when the competitor was present in a 70-fold molar excess. Conversely, the competitor TK gene was transcribed continuously at the basal rate, except when it was present in a 70-fold molar excess, when it was transcribed at the stimulated rate. These data therefore establish that E1A, like the polymerase III transcription factor TFIIIA (3, 5, 39), induces the formation of a stable complex. Whether the continual presence of E1A is required for stabilization of the complex is not known.

When HeLa cells are infected with a high multiplicity of an adenovirus mutant defective for E1A (*dl*312), transcripts from the other early genes are detectable, but their appearance is greatly delayed (27). This observation suggested to Gaynor and Berk (11) that E1A might catalyze the rate of transcription of these early genes but that it was not abso-

lutely required for their expression. As a consequence, they first infected HeLa cells with *dl*312 and then, after allowing sufficient time for early region transcription to occur, superinfected HeLa cells with a virus which had a wild-type E1A gene but a genetically marked E3 gene. Their results showed that for several hours following superinfection, only the *dl*312 transcript was detected. Gaynor and Berk (11) inferred from these data that a stable transcription complex was formed on *dl*312 E3 chromatin, a process which is presumably catalyzed by E1A. Our data with E1A and gene-injected oocytes are entirely consistent with those of Gaynor and Berk (11).

To observe the effects of E1A over prolonged periods of culture, our experiments required that this protein remain stable in injected oocytes. In fact, the *E. coli*-expressed E1A has a half-life of at least 12 to 18 h (31). This extraordinary stability of E1A is in marked contrast to the stability of E1A synthesized in adenovirus-infected cells, in which it has a half-life of about 30 min (34). We presently do not know the cause of this stability of *E. coli*-expressed E1A, although it is also quite stable in injected mammalian cells (23). We are presently investigating this phenomenon further.

Injected *Xenopus* oocytes have proven to be a valuable system for examining E1A activity. One of the most important set of experiments yet to be performed is the identification of cellular proteins which are required for E1A *trans* activation. In addition, it will be interesting to assess whether these proteins have unique functions during development.

ACKNOWLEDGMENTS

We thank J. Greenberg (Yale University, New Haven, Conn.) for the pactamycin and B. Wu and R. Morimoto (Northwestern University) for a plasmid containing the heat shock promoter.

This study was supported by Public Health Service grants CA 40189 and GM 34554 from the National Institutes of Health (to J.D.R.). J.D.R. is a Cancer Research Scholar of the American Cancer Society, Massachusetts Division.

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