***In vivo* photocrosslinking reveals that transcription factor binding to the mammalian ATF recognition sequence is required for E1A-induced transactivation in injected *Xenopus laevis* oocytes**

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ABSTRACT

The adenovirus E1A 13S mRNA product transactivates genes injected into *Xenopus laevis* oocytes that are normally E1A-inducible in mammalian cells. However, E1A-stimulated transcription, but not basal (uninduced) transcription, was inhibited if oocytes were incubated in the presence of protein synthesis inhibitors. This suggests that a cellular protein(s) is required for E1A-induced transactivation, but that it is dispensable for basal transcription. In order to identify such a protein from *Xenopus* oocytes that interacts with the adenovirus E3 promoter, gel shift assays, a new *in vivo* photocrosslinking assay, and immunoselection of biotinylated oligonucleotides were employed. A protein of molecular size 75 kd, which bound to the mammalian ATF recognition sequence *in vivo*, was found to be essential for E1A-induced transactivation. Although cycloheximide treatment of oocytes inhibited factor binding, E1A exerted no effect on factor binding. These data suggest that E1A modulates the activity of an oocyte transcription factor, either directly or indirectly, but not its ability to bind DNA.

INTRODUCTION

Defined protein products of viruses that transactivate viral genes have proven to be exceptionally useful paradigms for the study of differential gene transcription in eukaryotic cells. The 289 amino acid product of the adenovirus E1A 13S mRNA, for example, induces the transcription of all the other early viral genes, whose products in turn are required for a productive infection by the virus (1). E1A also activates the transcription of some cellular genes such as HSP70 (2,3) and B-tubulin (4). Antithetically, E1A can repress the transcription of some genes by interfering with enhancer activity (5,6,7). DNA sequence and mutational analysis of several gene promoters that are induced by E1A have not revealed conserved elements that could indicate a specific site of E1A interaction (8,9, reviewed in ref. 1). These observations, coupled with experiments that indicate that E1A is not a sequence-specific DNA binding protein (10), suggest that diverse cellular factors may mediate the regulatory activity of E1A.

One approach for the identification of such factors has been to use gel mobility shift assays and enzymatic or chemical 'footprinting' techniques to examine the cellular proteins that bind to E1A inducible promoters, and to assess whether their binding activities are enhanced by E1A. At least two HeLa cell factors bind the adenovirus E2A promoter (11), and the binding activity of one, termed EIIF, is enhanced when protein extracts are prepared from adenovirus infected cells (11,12). The adenovirus E3 promoter is bound by four cellular factors (13,14), although the binding of none of these appears to be regulated by E1A (14). Three factors interact with the adenovirus E4 promoter (15). One of these, E4F1, is required for E1A-induced transcription *in vitro* (i.e., in extracts prepared from 293 cells, which constitutively express the E1A gene), but its binding activity is unaffected

by E1A (15). A close inspection of the DNA sequences that are bound by factors in these three adenovirus promoters revealed a common sequence, (A/T)CGTCA, which resides 50–70 nucleotides upstream of the start of transcription. This sequence is required for E1A-induced transactivation and is bound by a factor termed ATF (16).

In addition to adenovirus promoters, an ATF-like sequence is present in several promoters that are activated by cAMP (reviewed in ref. 17), where they have been designated cAMP response elements (CRE). This observation would suggest that cAMP and E1A can induce transcription of at least some promoters via a common DNA element. In this regard, it has recently been shown that cAMP can induce the adenovirus E2A and E3 promoters and requires the ATF sequence (17).

We have examined E1A-induced transactivation in injected *Xenopus* oocytes (18–21). Injected *E. coli*-expressed E1A stimulates the transcription of injected genes that are under the control of promoters that are normally E1A-inducible. However, E1A-stimulated transcription, but not basal transcription, is repressed if oocytes are incubated in the presence of protein synthesis inhibitors (21), which suggests that the induction of transcription by E1A requires factors that are not necessary for basal transcription. To identify this factor, we have developed an *in vivo* photocrosslinking procedure using oligonucleotides that contain the nucleotide analog BrdU. Our results demonstrate that in injected *Xenopus* oocytes, the binding of a factor to the ATF recognition sequence is required for E1A-induced transactivation.

MATERIALS AND METHODS

Plasmids and Oligodeoxynucleotides

E3CAT (pKCAT23), containing the adenovirus E3 promoter and the chloramphenicol acetyltransferase (CAT) gene, has been described (22). Other plasmids contain three tandemly linked wild type or mutant ATF binding sites, which were derived from the adenovirus E4 promoter (sequences are shown below). These sequences were fused to the E3 TATA region that was immediately 5' of the CAT gene. Complementary oligodeoxynucleotides containing the adenovirus E3 TATA, ATF, API1, and NF1 binding sites (cf. Figure 1) were synthesized and annealed at 50°C for 60 min in 0.125 M KCl containing 0.5 mg/ml of each oligonucleotide. The annealed oligomers were then end-labeled with ³²P-ATP using T4 DNA kinase to a specific activity of about 2000 cpm/ng. The synthesis of a double stranded oligonucleotide containing BrdU was carried out as follows. A long oligonucleotide of the sequence (5') AGCTTGCGGGCGGCTTTCGTC-ACAGGGTGCGGTCGC, which spans the E3 ATF promoter sequence (cf. Figure 1), was annealed to a complementary extension oligonucleotide of the sequence (5') GCGACCGCAC as described above except that the concentration of the long oligonucleotide was 0.185 µg/µl and that of the extension oligonucleotide was 0.05 µg/µl. The oligonucleotides were then diluted to 50 ng per µl and 1 µl was incubated with 1 mM each of dGTP, dATP, 5-bromo-2'-deoxyuridine 5'-triphosphate (BrdU, Pharmacia), 50 µCi ³²P dCTP (3000 Ci/mmol, Amersham), 0.1 M Tris-HCl, pH 8.3, 0.14 M KCl, 10 mM MgCl₂, 28 mM β-mercaptoethanol, and 40 U MuLV reverse transcriptase (Bethesda Research Laboratories) for 45 min at 37°C. Five mM each of dCTP and dTTP was added to the reaction mixture and the incubation was continued for another 20 min. The mixture was then centrifuged through a 1 ml Sephadex G-50 column and the eluate was extracted with phenol and chloroform and precipitated with ethanol. The specific activity of BrdU-containing ³²P-labeled oligonucleotides was about 10,000 cpm/ng.

Additional ^{32}P -labeled BrdU-containing oligomers that contained three randomly linked ATF sites from the E4 promoter were synthesized. For this synthesis, the long oligomer was (5') TCGACTTAACCGTTACGTCATTTTATTAACCGTTACGTCATTTTATTAACCGTTACGTCATTTGCATG and the extension oligomer was (5') CATGCAAATG. Two mutant derivatives of the E4 ATF sequences were synthesized and used for ^{32}P and BrdU labeling. The long oligomer for mutant 1 was (5') TCGACTTAACCGTTACTCCATTTTATTAACCGTTACTCCATTTTATTAACCGTTACTCCATTTGCATG and the extension oligomer was the same as that used above. The long oligomer for mutant 2 was (5') TCGACTTAACCGTTACGCCATTTTATTAACCGTTACGCCATTTTGCATG and the extension oligomer was the same as that used above.

In some cases, oligonucleotide synthesis reactions included biotinylated dATP. A long oligonucleotide identical to that used in the BrdU-containing reaction for the ATF E3 promoter was synthesized except that it contained the Eco R1 recognition sequence GAATTC at its 5' and 3' ends. An extension oligonucleotide of the sequence (5') GAATTCCTCGC was synthesized and both oligonucleotides were annealed and used for a reverse transcription reaction as described previously except that BrdU and ^{32}P dCTP were omitted but dCTP, dGTP, dATP, and dTTP (1 mM each) were included. The oligonucleotide was then centrifuged through a Sephadex column, precipitated with ethanol, and digested with Eco R1. Following phenol/chloroform extraction and ethanol precipitation, the protruding ends were filled in with biotin-7-dATP (Bethesda Research Laboratories) and dTTP with the Klenow fragment of DNA polymerase I. The oligonucleotide was finally separated from unincorporated nucleotide as described above.

Gel Shift Assays

Nuclei (germinal vesicles or GVs) from control (untreated) oocytes, from oocytes incubated for 6 hr in the presence of 10 $\mu\text{g}/\text{ml}$ cycloheximide, or from oocytes injected (into the cytoplasm) with 1 or 10 ng of *E. coli*-expressed E1A 13 S mRNA product (18) and incubated for 8 hr, were isolated manually with watchmakers forceps. Approximately 50 GVs were homogenized in 25 mM Hepes- NaOH, pH 7.5, 1 mM EDTA, 5 mM dithiothreitol, 0.15 M NaCl, and 10% glycerol, centrifuged for 5 min at 12,000 $\times$ g, and stored at -80°C until use. Zero or 5 GV equivalents were incubated in the buffer described above together with about 0.2 ng of ^{32}P -labeled oligonucleotide, 0.2 μg double stranded poly (dI).poly (dC) (Pharmacia), and 2 or 5 ng of competitor oligonucleotide for 45 min at 25°C . The mixture was then applied to a 10% polyacrylamide minigel in $0.5\times$ Tris-borate-EDTA (23) and exposed to X-ray film after electrophoresis. To perform the *in vitro* BrdU photocross-linking, a gel shift assay similar to that described above was performed with various amounts of nuclear protein (i.e., GV equivalents). Following electrophoresis, the gel was placed on a UV light box (302 nm light) and irradiated at 4°C for 45 min. The gel was then wrapped in Saran wrap, exposed to X-ray film, and the shifted band, which was detected by autoradiography, was excised with a razor blade. This gel fragment was incubated in 10% SDS and 1 mM B-mercaptoethanol for 1 hr and then placed in a well of a SDS 10% polyacrylamide gel. Following electrophoresis of this gel, the photocrosslinked protein was detected by autoradiography.

Oocyte Injections

Oocyte nuclei were injected with 10 nl volumes containing various amounts of oligonucleotides. Following incubation (usually 3–4 hr), GVs were isolated manually and

Figure 1. Structural features of the adenovirus E3 promoter. The sequences and their positions relative to the start of transcription (+1) that are protected from DNase I digestion following reconstitution with HeLa cell nuclear extracts are shown (14). The tentative identification of the factors that bind these sequences is also denoted.

Figure 2. Gel shift assays with oligonucleotides derived from the E3 promoter. Double stranded oligodeoxynucleotides corresponding to the sequences shown in Figure 1 were synthesized, end-labeled with ³²P-dATP, and reconstituted with oocyte nuclear extracts (GVs). The extracts were prepared from control oocytes (none) or from oocytes incubated in cycloheximide (CHX) or injected with 1 or 10 μg of *E. coli*-expressed E1A. The mixture was then resolved by electrophoresis through 10% polyacrylamide and autoradiographed. For the ATF gel shift experiment in the right panel, a ³²P-labeled ATF oligomer was mixed with 10 or 25 fold excess of an oligomer containing the same sequence (WT) or an oligomer with a point mutation (G→C) prior to the addition of the nuclear extract (5 GV equivalents).

Figure 3. *In vitro* photocrosslinking. A ^{32}P -labeled BrdU containing oligonucleotide with the same ATF sequence as that shown in Figure 1 was used in a gel shift assay with various amounts of oocyte GV extract. Following electrophoresis, the gel was irradiated with ultraviolet light and autoradiographed (left panel). The shifted band in the 3 GV lane was excised with a razor blade and placed in the well of a 10% polyacrylamide SDS gel for electrophoresis and autoradiography (right panel).

placed, in groups of 40–80, in a well of a 96 well microtiter plate. The GVs were irradiated, from the top, with an inverted UV light box (302 nm light) for 45 min at 4 °C. Following irradiation, the GVs were dissolved in SDS gel loading buffer and subjected to SDS gel electrophoresis and autoradiography. In one case, intact irradiated GVs were incubated with 5 $\mu\text{g}/\text{ml}$ DNase I for 0–20 min. In another instance, irradiated GVs were treated with 1 $\mu\text{g}/\text{ml}$ V8 protease (Boehringer Mannheim Biochemicals) prior to electrophoresis.

Some oocyte nuclei were injected with 0.1 ng of plasmid DNA and the cytoplasm with 10 ng *E. coli*-expressed E1A 13S mRNA product (18). Following 15–18 hr of incubation, the oocytes were homogenized and protein extracts used for CAT assays as described previously (19). Total nucleic acid from other oocytes injected with ^{32}P -labeled oligonucleotide was extracted by phenol/chloroform and resolved by electrophoresis in a 12% polyacrylamide gel followed by autoradiography.

Immunoselection of Biotinylated Oligonucleotides

Oocytes were incubated in Barth's medium (23) containing 0.5 mCi/ml ^{35}S -methionine for about 15 hours and then injected (into GVs) with biotinylated oligonucleotide. Following another 4 hr of incubation (in medium containing ^{35}S -methionine), GVs were isolated manually, disrupted in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton-X-100, 0.5% Na deoxycholate, and 0.05% SDS), and incubated for 2 hr at 25 °C with rabbit IgG anti-biotin antibody (Enzo Biochemicals). Protein A Sepharose CL-4B (Sigma) was then added and the mixture incubated another 2 hr with constant agitation. The Sepharose beads were then briefly centrifuged to pellet, the supernatant removed, and the pellet washed 5 times with 1 ml of RIPA buffer. The beads were finally boiled in SDS gel loading buffer and the proteins resolved by SDS polyacrylamide gel electrophoresis and autoradiography.

RESULTS

We have demonstrated previously that E1A protein injected into *Xenopus* oocytes transactivates injected genes that contain the adenovirus E3 or human HSP70 promoters, which are normally E1A-inducible in mammalian cells (21). E1A failed to transactivate these promoters, however, if oocytes were incubated in the presence of protein synthesis inhibitors (21). This suggested that a cellular protein(s), which presumably was not stored in large amounts in oocytes and whose steady state level was maintained by continual synthesis, mediated E1A-induced transactivation. In our initial attempts to identify such a protein, we used oocyte nuclear extracts for DNase I and methylation protection footprinting experiments. Unfortunately, the results were not conclusive, due perhaps to the limited number of germinal vesicles (oocyte nuclei) that could be isolated manually. We therefore have used an alternative approach, which is based on the footprint pattern of the E3 promoter by HeLa cell nuclear factors. Using both DNase I and methylation protection footprinting, Hurst and Jones (14) identified four E3 promoter sequences that were bound by HeLa cell factors (Figure 1). These sequences contained the consensus TATA, ATF, AP1, and NF1 binding sites (see ref. 14, for review). Suspecting that oocyte factors might bind the same regions of the E3 promoter as do HeLa cell factors, we synthesized double stranded oligonucleotides that contained the sequences mentioned above (Figure 1). The oligonucleotides were then used in a series of gel mobility shift assays with nuclear extracts from untreated oocytes, and from oocytes that had been injected with E1A or incubated in medium containing cycloheximide. Figure 2 shows that although oocytes contain factors that bind to oligomers containing TATA, ATF, AP1, and NF1 sequences, the binding activities of these factors was unaffected by E1A. Similarly, cycloheximide treatment of oocytes did not affect the binding of factors to the TATA, AP1, or NF1 oligomers. However, a factor(s) that interacted with the ATF oligomer was nearly undetectable when oocytes were incubated with cycloheximide. We should note that specific oligonucleotide competition experiments would be required to confirm the identity of any of the factors reported here. In addition, the oligomers might interact with more than one factor; this could apply particularly to the TATA oligomer since factor interaction with this sequence is weak in the absence of other factors that stabilize the complex.

To ensure that the binding of an oocyte factor(s) to the ATF oligomer was specific, a competition experiment was performed. ³²P-labeled wild type ATF oligomer was mixed with different amounts of unlabeled wild type ATF oligomer or an oligomer containing a point mutation (a G→T transversion corresponding to nucleotide -56 of the E3 promoter) that has been shown to have a low affinity for HeLa cell ATF protein (14). A nuclear extract from oocytes was then added and a gel shift assay was performed (Figure 2, right panel). Although the wild type ATF sequence was an effective competitor for factor binding, the mutant sequence did not compete. These data therefore show that oocytes contain a newly synthesized protein that either interacts with a sequence containing an ATF binding site or that modifies a DNA-binding protein to increase its binding activity.

In Vitro Photocrosslinking

The results described above do not reveal the number or sizes of factors (proteins) that bind to the ATF oligomer *in vitro*. Consequently, we have synthesized a radiolabeled ATF oligonucleotide that contains the photoactivatable protein crosslinking analog BrdU (24) and have used it in a gel shift assay with an oocyte nuclear extract (Figure 3, left panel).

Figure 4. Stability of injected ATF oligonucleotide. The ^{32}P -labeled ATF oligonucleotide used for *in vitro* photocrosslinking was injected into oocyte nuclei and the oocytes cultured for 1 to 7 hours. Total nucleic acid was then extracted and resolved by electrophoresis through polyacrylamide followed by autoradiography (left panel). A similar experiment was also conducted with the same oligomer that was mixed with 10 fold excess nonspecific double stranded oligomer prior to injection (right panel).

Figure 5. Photocrosslinking in injected *Xenopus* oocytes. Left Panel. One hundred oocytes were injected with about 0.2 ng ^{32}P -labeled BrdU-containing ATF oligomer and cultured for 3 hr. The nuclei were isolated and 50 were irradiated with 302 nm light for 45 min (+UV) while another 50 were not irradiated (-UV). The nuclei were then disrupted in SDS gel loading buffer and analyzed by SDS gel electrophoresis and autoradiography. Right Panel. 0.2 ng of ^{32}P -labeled BrdU-containing ATF oligomer was injected into oocyte nuclei alone, or first mixed with either 1 and 5 ng of the same oligomer that was unlabeled (WT), or with 1 and 5 ng of an oligomer containing a point mutation (mutant) prior to injection. Following 4 hr of culture, the nuclei were isolated and the photocrosslinked proteins were analyzed as described above. Thirty oocytes were injected with the ^{32}P and BrdU-labeled ATF oligomer; the nuclei were then isolated, irradiated, disrupted, and incubated with V8 protease. The photocrosslinked proteins were then analyzed as described above.

Figure 6. Isolation of an oocyte factor that binds to the ATF recognition sequence with biotinylated oligonucleotide. 180 oocytes were incubated overnight in medium containing ^{35}S -methionine; 90 of these oocytes were then injected with a biotin-labeled ATF oligomer and cultured for an additional 4 hr. The nuclei from injected (+DNA) and noninjected (-DNA) oocytes were then isolated, disrupted in RIPA buffer, and mixed with anti-biotin antibody and protein A Sepharose. Following incubation, the beads were washed and boiled in SDS gel loading buffer (the selected material). Nonselected refers to material that did not bind to the Sepharose beads. One and 5 refer to the volume, in μl (out of a 50 μl reaction) of the nonselected material that was resolved by electrophoresis.

Figure 7. Effects of cycloheximide and E1A on factor interaction with the ATF oligomer. Left Panel. Thirty-five oocytes were incubated for 5 hr in medium containing cycloheximide (CHX) and were then injected with ^{32}P -labeled BrdU-containing ATF oligomer. Thirty-five additional oocytes were injected with 10 ng E1A, cultured for 15 hr and were then injected with the oligomer (E1A). The oligomer was also injected into a third group of 35 untreated oocytes (CONT). Following oligomer injection, all oocytes were cultured for 4 hr and the photocrosslinked proteins were detected as described in Figure 5. Right Panel. Eighty oocytes were incubated for 5 hr in cycloheximide (CHX); one-half of this group was then incubated for an additional 5 hr in control medium (CHX washout). The ^{32}P -labeled BrdU-containing ATF oligomer was then injected into these oocytes, as well as 40 untreated oocytes (CONT), and the photocrosslinked proteins were resolved as described in Figure 5.

Following electrophoresis, the gel was irradiated; the shifted band was detected by autoradiography, excised, and the photocrosslinked protein(s) resolved on a second polyacrylamide gel that contained SDS. The autoradiograph in Figure 3 (right panel) shows that a protein of molecular mass 75 kd was detected by this procedure, suggesting that it was responsible for the retarded mobility of the ATF oligomer.

In Vivo Photocrosslinking

Although the results described above demonstrate that an oocyte protein binds the ATF oligomer upon reconstitution *in vitro*, the extent to which this occurs *in vivo* is unknown. As a consequence, we have assessed whether the 75 kd protein also photocrosslinks to the ATF oligomer in injected *Xenopus* oocytes. To perform such experiments, however, it was important to first assess the stability of the ³²P-labeled BrdU-containing ATF oligomer injected into oocyte nuclei. Figure 4 (left panel) shows that the half-life of this oligonucleotide was less than one hr, which would probably make *in vivo* photocrosslinking unfeasible. However, when the oligonucleotide was mixed with a 10 fold excess of unlabeled nonspecific oligonucleotide (i.e., a double stranded 40-mer with no putative ATF binding sites), its half-life was extended to about 4 hr. Therefore, in all subsequent injection experiments, specific, labeled oligonucleotides were mixed with a 10 fold excess of nonspecific oligomer to prolong the half-life of the specific oligomer of interest.

Oocytes injected with the oligomers described in Figure 4 were incubated for 3 hr; the germinal vesicles were then isolated manually, irradiated with 302 nm light, and the resulting photocrosslinked proteins resolved by SDS gel electrophoresis and autoradiography. Figure 5 (left panel) shows that a single protein, of molecular mass 75 kd, photocrosslinked to the ATF oligonucleotide. No proteins were detected, however, when UV irradiation was omitted.

Several additional experiments were performed to assess the specificity of the 75 kd protein that photocrosslinked to the ATF oligomer following injection. The detection of this protein was sensitive to treatment with V8 protease (Figure 5, right panel) and DNase I (data not shown), demonstrating that the 75 kd band detected by autoradiography was a protein that was photocrosslinked to radioactive DNA. In addition, a limit DNase I digestion does not increase the mobility of the photocrosslinked protein in SDS-polyacrylamide gels. Thus, the oligomer would not appear to significantly affect the deduced molecular mass of the crosslinked protein.

The wild type and mutant oligonucleotides used for the *in vitro* competition experiments described in Figure 1 were used for similar experiments in injected oocytes. The ³²P-labeled, BrdU-containing ATF oligonucleotide was mixed with 5 and 25 fold excess wild type and mutant oligomers and injected into oocyte nuclei. The germinal vesicles were subsequently isolated, irradiated, and the photocrosslinked protein was detected by SDS polyacrylamide gel electrophoresis and autoradiography (Figure 5, right panel). The wild type oligomer, but not the oligomer containing the point mutation, was an efficacious competitor with the radiolabeled BrdU-containing oligonucleotide for protein binding. Therefore, the binding of protein to an oligonucleotide containing an ATF sequence occurs *in vivo* as well as *in vitro*. We also note here that two proteins of similar molecular mass (75 and 77 kd) appear to bind the ATF oligomer. These could be two different proteins or unmodified and modified forms of the same protein (see Discussion).

Immunoselection of Biotinylated Oligonucleotide

We have confirmed the data obtained with photocrosslinking by a procedure that does not require UV irradiation. An ATF oligonucleotide with the same sequence as that shown

Figure 8. Transcription of injected plasmids that contain wild type and mutant ATF sequences. Oocytes were injected with a plasmid that contains three tandemly linked wild type ATF sequences linked to the CAT gene. Oocytes were also injected with similar plasmids that contained point mutations in the ATF sequences (mutants M1 and M2). One-half of each group of these oocytes was also injected with 10 ng E1A. Following an 18 hr incubation, the oocytes were homogenized and used for CAT assays. Each assay represents 35 oocytes. The percent conversion from the nonacetylated chloramphenicol (CM) to the acetylated form (AC) was determined by liquid scintillation spectrometry.

Figure 9. Competition between mutant and wild type ATF oligomers. Oocytes were injected with and 0.2 ng ³²P-labeled BrdU-containing ATF oligomer, or this same oligomer plus either 0.4 or 2 ng unlabeled wild type oligomer (ATF), 0.4 or 2 ng mutant 1 oligomer (M1), or 0.4 or 2 ng mutant 2 oligomer (M2). The oocytes were cultured for 3 hr and the photocrosslinked proteins were detected as described in Figure 5. Each lane represents 40 injected nuclei.

in Figure 1 was synthesized except that Eco RI recognition sequences were added to both ends. Following second strand synthesis, the oligomer was digested with Eco RI and the overhanging ends filled in with biotin-dATP. The oligomer was then injected into oocytes that had been incubated overnight in the presence of ^{35}S -methionine. Following another 4 hours of incubation, the oligonucleotide (and associated proteins) were immunoselected with anti-biotin antibody and protein A Sepharose, and the resulting radioactive material resolved by SDS gel electrophoresis and autoradiography (Figure 6). The nonselected proteins from control (–DNA) and DNA injected oocytes were identical. Two proteins, of sizes 75 and 77 kd, were immunoselected from DNA injected oocytes, but were absent from oocytes that were not injected with DNA. These data, together with the results from the photocrosslinking experiments, clearly show that *Xenopus* oocytes contain two protein species that specifically bind the mammalian ATF recognition sequence.

Cycloheximide, but not E1A, Affects Factor Binding In Vivo

The gel shift assays presented in Figure 1 showed that extracts prepared from oocytes incubated in cycloheximide exhibited significantly reduced factor binding to the ATF recognition sequence. We have used photocrosslinking of oligomers injected into oocytes to determine whether this also occurs *in vivo*. The ^{32}P -labeled BrdU-containing ATF oligomer was injected into oocytes that were either incubated in cycloheximide or injected with E1A; the photocrosslinked proteins were then detected as described previously. The autoradiograph in Figure 7 (left panel) demonstrates that the level of factor binding decreased (by about 3 fold) when oocytes were incubated in cycloheximide, but was unaffected when oocytes were injected with E1A.

Previous experiments demonstrated that the cycloheximide inhibition of E1A-induced transactivation was reversible since oocytes switched from cycloheximide-containing to cycloheximide-lacking medium were capable of supporting E1A-stimulated transcription (21). To determine whether factor binding to DNA also was reversible, oocytes were first cultured in medium containing cycloheximide and then switched to control medium; the ^{32}P -labeled BrdU-containing oligomer was then injected and photocrosslinking performed. Figure 7 (right panel) shows that cycloheximide again reduced factor binding, in this case about 7 fold. Following washout of cycloheximide, factor binding returned to control levels. Thus, E1A-induced transactivation paralleled factor binding in injected oocytes.

Factor Binding to the ATF Recognition Sequence is Required for E1A-Induced Transactivation

The data described thus far suggest a strong correlation between the ability of a cellular factor to bind the ATF recognition sequence and E1A-induced transactivation. To carry this further, we have constructed CAT genes that contain 3 tandemly linked wild type (in this case, from the adenovirus E4 promoter, which conforms more closely to the ATF consensus binding site than does the E3 ATF site) or mutant ATF sites in their promoters and have assessed whether they are induced by E1A. Oocytes were injected with DNA (nuclear) and *E. coli*-expressed E1A (cytoplasmic) and cultured for 18 hr, after which they were homogenized and used for CAT assays (Figure 8). E1A injection resulted in a 3 fold stimulation of transcription from the promoter containing a wild type ATF binding site. A promoter containing a TC substitution for GT in the ATF recognition sequence (M1) was not induced by E1A, but was still capable of supporting basal level transcription. However, another promoter carrying a T→C transition in the ATF binding sequence (M2) was induced by about 4 fold by E1A.

Oligomers with the wild type and mutant sequences described above were used in a

series of competition experiments similar to those shown in Figure 5. A ^{32}P -labeled BrdU-containing oligonucleotide spanning the E4 ATF binding site was injected into oocytes with or without competitor oligomers, followed by photocrosslinking and SDS gel electrophoresis (Figure 9). When injected in 10 fold excess, unlabeled wild type ATF oligomer was an effective competitor with the labeled oligomer for protein binding. Mutant 1, which was not transactivated by E1A (Figure 8), did not compete when similar amounts were injected. Mutant 2, which was stimulated by E1A, was also an effective competitor for factor binding. These data therefore demonstrate that factor binding to the ATF recognition sequence is required for E1A-induced transactivation in injected *Xenopus* oocytes.

DISCUSSION

Our previous studies have demonstrated that E1A-induced transactivation required ongoing protein synthesis in injected *Xenopus* oocytes (21), which suggested that a cellular protein(s) mediated this E1A activity. Initial attempts to identify such a protein by footprinting the adenovirus E3 promoter proved unsuccessful, possibly due to the relatively few oocyte nuclei that could be isolated manually. Since the HeLa cell factor binding sites on the E3 promoter had been mapped (14), we anticipated that double stranded oligonucleotides corresponding to these sequences could be used in gel shift assays with oocyte nuclear extracts since such experiments require much less protein. These assays, using either double stranded poly(dI).poly(dC) (Figure 2) or salmon sperm DNA as nonspecific competitors (data not shown), demonstrated that oocytes contain factors that bind to the TATA, ATF, AP1, and NF1 sites. In no instance was binding affected by E1A injection, but in the case of the ATF oligomer, binding was decreased by treatment with cycloheximide. We therefore have further examined the factor(s) that bind the ATF oligomer, and have employed a photocrosslinking procedure in which DNA/protein interactions may be examined *in vivo*.

Photocrosslinking of a Factor to an ATF Oligomer

In vitro photocrosslinking of a BrdU-containing ATF oligomer in an oocyte nuclear extract revealed the presence of a 75 kd protein. A protein of identical size was observed when oocytes were injected with the same oligomer and photocrosslinking was performed *in vivo*. Since the size of this protein differs from that of HeLa cell ATF, (45 kd, 14), yeast ATF (66 kd, 25) and rat brain CRE binding protein (CREB), (43 kd, 26), it was possible that the photocrosslinking was altering the electrophoretic mobility of the protein, or that the procedure was nonspecific. We have addressed these possibilities in two ways. First, a biotin-labeled ATF oligomer was injected into oocytes in which proteins had been metabolically labeled, which was followed by anti-biotin antibody immunoselection and electrophoresis and autoradiography. Since this procedure does not involve covalent bonding, all radiolabeled DNA-bound proteins should be resolved without bias due to the DNA. Two proteins, of 75 and 77 kd, were identified. Although these species may be distinct polypeptides or unmodified and modified forms of the same polypeptide (by phosphorylation, for example), it is nevertheless clear that the photocrosslinked 75 kd protein was neither a dimer nor a protein whose electrophoretic migration was significantly altered by the covalently attached DNA. Second, we have assessed the specificity of photocrosslinking by double oligomer competition experiments. An oligomer containing a G-T transversion in the ATF binding site was an ineffective competitor with the wild type ATF oligomer for protein binding in HeLa cell (14) or oocyte extracts (Figure 2), or in injected oocytes (Figure 5). These aggregate data therefore strongly suggest that

oligonucleotide photocrosslinking in injected oocytes accurately reflects specific DNA/protein interactions.

E1A-Induced Transactivation Requires Factor Binding to the ATF Site

The data presented in Figure 8 show that one of two plasmids containing mutated ATF recognition sequences is transactivated by E1A (M2). Although this in itself does not demonstrate the requirement of factor binding for E1A stimulated transcription of the E3 promoter, this experiment should be viewed with the results of the *in vivo* oligonucleotide competition studies, which show that only the oligomer containing the ATF sequence that was transactivated by E1A competes for factor binding (Figure 9). Taken together, these data present a compelling argument that factor binding to the ATF site is required for E1A-induced transactivation *in vivo*, and are in agreement with the studies of Lee et al. (16). However, Garcia *et al.* (13) have demonstrated that although deletion of nucleotides -52 to -82 of the E3 promoter lowers basal expression, the induction of transcription by E1A is relatively unaffected. It is also important to note that not all promoters that are activated by E1A do so via the ATF sequence. For example, the E1A-enhanced transcription of the adenovirus E1B promoter may not involve ATF (27). It is therefore likely that E1A-induced transactivation requires different factors for different promoters. Moreover, multiple factors are probably required for E1A-induced transactivation of any given promoter, such as the TATA box-binding factor (28).

Studies with mammalian cells have shown that mutations in DNA sequences, including the ATF recognition sequence, that lower E1A-induced transcription also lower basal-level transcription (25,29,30). Yet, in injected oocytes, factor binding to the ATF site appears to be dispensible for basal transcription. The explanation for this discrepancy is not readily apparent, for if oocytes contain additional proteins that bind mutated ATF sequences, they are not identified by photocrosslinking. However, transcription of injected DNAs can be promiscuous; it is possible that in the absence of E1A-induced transactivation, other normally cryptic sites are used as promoter elements.

Comments on In Vivo Photocrosslinking

We note here several points concerning the crosslinking procedure that should be considered. First, when synthesizing the ³²P dCTP-labeled BrdU-containing oligonucleotide, it is important to chase the reverse transcription reaction with radioinert dCTP and to remove as much unincorporated nucleotide as possible. We have found that ³²P-dCTP can form nonspecific adducts with proteins to give what would appear to be photocrosslinked proteins. Second, photocrosslinking experiments conducted with oocytes from different females show a high degree of variability with respect to quantity of crosslinked protein. We also noted previously the ability of oocytes from different females to transcribe injected DNAs varied considerably (19). Whether the variability in these two parameters is linked is unknown, but it is clear that oocytes from several different females should be used for crosslinking experiments. Finally, not all injected oligomers are efficiently photocrosslinked. We have synthesized a BrdU-containing oligonucleotide that spans the adenovirus E3 AP1 site (Figure 1), but have been unable to detect photocrosslinking of this oligomer. With any oligomer, it is difficult to estimate the efficiency of crosslinking. For example, Figures 5, 7, and 9 show that most of the injected oligomer is not cross-linked to protein and migrates with the bromphenol blue dye front. This apparent inefficiency could be due to the large amount of oligomer injected relative to the amount of specific protein (i.e., ATF) present in oocytes. In addition, although germinal vesicles are visibly transparent, UV light is almost certainly rapidly attenuated, which would significantly lower cross-linking efficiency.

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