Loss of a Phosphorylated Form of Transcription Factor CREB/ATF in Poliovirus-Infected Cells

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Host cell RNA synthesis is inhibited by poliovirus infection. We have studied the mechanism of poliovirus-induced inhibition of RNA polymerase II-mediated transcription by using the adenovirus early region 3 (E3) promoter. In vitro transcription from the E3 promoter was severely inhibited in extracts prepared from poliovirus-infected HeLa cells. Four regions in the E3 promoter have been shown to serve as binding sites for cellular transcription factors. These regions contain binding sites for transcription factors NF-1 (site IV), AP-1 (site III), CREB/ATF (site II), and the TATA factor (site I). Binding to these four regions was not significantly altered by poliovirus infection as assayed by DNase I footprinting analysis; furthermore, gel retardation assays failed to reveal dramatic differences in the total amount of CREB/ATF-, AP-1-, and NF-1-binding activity present in mock- or poliovirus-infected cell extracts. Gel retardation assays, however, did reveal significant qualitative differences in the DNA-protein complexes formed with a CREB/ATF-binding site in extracts prepared from poliovirus-infected cells as compared to mock-infected cell extracts. Radioimmunoprecipitation reactions performed with antiserum against CREB/ATF revealed a severe reduction in a phosphorylated form of the protein present in poliovirus-infected cell extracts. However, in vitro kinase reactions demonstrated that mock- and poliovirus-infected cell extracts contained similar levels of CREB/ATF. Expression from the E3 promoter was shown to be activated by CREB/ATF in vivo; this induction was dependent upon the phosphorylation of CREB/ATF. Thus, we propose that poliovirus infection inhibits transcription from the E3 promoter, at least in part, through the dephosphorylation of CREB/ATF.

Infection of susceptible cells with members of the picornavirus family results in the rapid inhibition of host cell RNA synthesis (see reference 24 for a review). Infection of HeLa cells with poliovirus, for example, causes a severe decrease in transcription catalyzed by RNA polymerases (pol) I, II, and III. It has been shown that the inhibition of transcription observed in vivo can be recapitulated in vitro by using extracts prepared from either mock- or poliovirus-infected cells (6, 10, 26, 40). For each of the three polymerase systems, in vitro analysis has revealed that the inhibition of transcription by poliovirus infection is a consequence of inactivation of specific transcription factors (10, 26, 40). We previously showed that pol II-mediated transcription from the relatively simple adenovirus major late promoter was reduced in extracts prepared from poliovirus-infected cells relative to extracts prepared from mock-infected cells; virus-infected cell extracts were found to be deficient in an activity which copurified with TFIID, the TATA-binding transcription factor (26). Studies from other laboratories have revealed that additional proteins required for efficient pol II transcription, including pol II itself, are modified during the course of poliovirus infection, although the nature of these modifications remains unclear (28, 38).

The promoters of the adenovirus early-expressed genes have been the subject of intense investigation, and their analysis has provided insight into the proteins and mechanisms which regulate pol II-mediated transcription. Studies of the adenovirus E3 promoter have revealed four regions which serve as binding sites for cellular transcription factors (12, 22, 27). These regions contain binding sites for the transcription factors NF-1 (site IV), AP-1 (site III), CREB/

ATF (site II), and the TATA-binding factor, TFIID (site I). Recent studies performed with site-directed mutants in each of the four binding sites revealed the importance of sites II and III for efficient basal and E1A-induced levels of transcription in vivo (27). Mutations in sites I and IV had little effect on either basal or E1A-induced transcriptional levels in vivo. Analysis of the mutant E3 promoters in vitro, however, showed that an intact TATA element was required in addition to binding sites II and III for efficient basal-level transcription. Mutations in site I, II, or III reduced transcription in vitro approximately 10-fold; a double mutation in sites II and III virtually eliminated E3 promoter activity in vitro. Thus, transcription factors AP-1, CREB/ATF, and TFIID appear to be important for expression of the E3 promoter in vitro.

The CREB protein binds to DNA elements required for induction by cyclic AMP (cAMP) (36). A similar binding element, which binds a factor termed activating transcription factor (ATF), has been found in a variety of viral and cellular promoters and shown to confer responsiveness to the adenovirus E1A transactivating protein (30). The observations that an ATF-binding site can confer induction by both cAMP and E1A and that ATF and CREB are of similar molecular masses (43 kilodaltons [kDa]) and yield similar complexes in gel retardation assays suggested that CREB and ATF are either related or identical proteins (9, 17, 32, 33, 42). However, the cloning of the CREB gene (14, 18) and, more recently, the isolation of eight related but distinct cDNA clones encoding ATF-binding factors (16) indicate that the CREB/ATF site is recognized by a family of proteins. The roles of the different family members in mediating the cellular response to cAMP and E1A remain unclear.

The transcriptional activity of CREB has been shown to be modulated via phosphorylation-dephosphorylation events

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(46). The CREB gene encodes a protein containing a cluster of potential phosphorylation sites and a leucine zipper motif (14, 18). It was recently shown that mutation of a single serine residue in a consensus sequence recognized by protein kinase A abolished CREB transcriptional activity (13), indicating the importance of phosphorylation-dephosphorylation events in the regulation of CREB activity.

In this report we examine the mechanism of inhibition of pol II-mediated transcription by poliovirus infection by using the adenovirus E3 promoter. We show that transcription factor CREB/ATF is modified by poliovirus infection. Our data are consistent with a model in which poliovirus infection results in the inhibition of transcription from the E3 promoter, at least in part, through the dephosphorylation of CREB/ATF.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were grown in Spinner culture in minimal essential media (MEM) (GIBCO Laboratories) supplemented with 6% newborn calf serum and 1 g of glucose per liter. Cells were infected for 4 h as previously described (7) with poliovirus type 1 (Mahoney strain) at a multiplicity of infection of 100. After suspension in MEM without serum, mock-infected cells were treated identically to poliovirus-infected cells.

Plasmids and transient transfection assays. The CREB expression vector was constructed by inserting a 2.3-kilobase *EcoRI* fragment containing the CREB gene (18) into a previously described Rous sarcoma virus (RSV) expression vector (11). The protein kinase A (PKA) expression vector (generously provided by Richard Maurer, University of Iowa, Iowa City) contained the PKA alpha subunit in an RSV expression vector (34). The wild-type E3-CAT construct has been previously described (12). Embryonal carcinoma F9 cells were plated in Dulbecco modified Eagle medium supplemented with 5% newborn calf serum. A 5- μ g portion of the E3-CAT construct and 5 μ g of the CREB and/or PKA expression vectors were transfected onto a single plate of F9 cells by the calcium phosphate transfection procedure. Cells were harvested after 36 h, and chloroamphenicol acetyltransferase (CAT) assays were performed as previously described (15).

In vivo labeling of cells with [32 P]phosphate. HeLa cells (2×10^5) to be labeled with [32 P]phosphate were washed four times in MEM without phosphate and serum, resuspended in 2 ml per liter of MEM without phosphate and serum, and either mock infected or poliovirus infected as previously described (7). After viral adsorption, the cells were suspended in MEM containing 500 μ Ci per ml of carrier-free [32 P]phosphate (Amersham Corp.) and 8% dialyzed newborn calf serum. Infections and mock infections were allowed to proceed for 4 h before harvesting. After harvesting, nuclear extracts were prepared from the [32 P]phosphate-labeled cells by a previously described procedure (29).

Antisera and radioimmunoprecipitations. To prepare antiserum against CREB, a full-length CREB cDNA was cut with *PvuII* and *NsiI* and the 850-base-pair fragment was end filled and fused to *trpE* in a Path 2 vector which had been cut with *SmaI*. Induction and preparation of TrpE-CREB fusion protein and injection schedules were performed as previously described (45). Immunoprecipitations were done with 75 μ g of [32 P]phosphate-labeled nuclear extracts and either 10 μ l of control preimmune serum or 10 μ l of CREB/ATF antiserum. Antigen-antibody complexes were collected by addition of protein A-Sepharose (Sigma Chemical Co.), and

the immunocomplexes were washed in RIPA buffer (10 mM Tris [pH 7.9], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) and prepared for SDS-polyacrylamide gel electrophoresis analysis. Immunoprecipitated complexes were resolved on 12.5% gels, which were then dried and subjected to autoradiography.

DNase I footprinting analysis. Nuclear extracts prepared from either mock- or poliovirus-infected HeLa cells (8) were loaded onto a heparin-agarose column, washed with 5 column volumes of buffer D (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.9], 20% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride) containing 100 mM KCl, and eluted with buffer D containing 500 mM KCl. The eluted protein was dialyzed into buffer D containing 100 mM KCl and used in DNase I footprinting assays. DNase I footprinting assays were performed with a construct containing the E3 promoter region (−236 to +31) cloned into *SmaI*-*SacI*-cut pUC19. The coding strand was labeled with T4 kinase and [γ - 32 P]ATP at the *HindIII* site in the polylinker, and after cutting with *PvuII*, a 400-base-pair fragment was isolated by gel purification and electroelution. Two to five nanograms of end-labeled probe was used in each 50- μ l footprinting reaction, which contained 10 mM HEPES (pH 7.9), 50 mM KCl, 10 mM (NH₄)₂SO₄, 6.25 mM MgCl₂, 1 mM DTT, 10% glycerol, 1 mM EDTA, 1 μ g of poly(dI-dC), and various amounts of heparin-purified HeLa cell extract. The reaction mixes were incubated for 30 min at room temperature, the reaction volume was increased to 100 μ l with H₂O, and DNase I (final concentration of 1 μ g per ml) was added. Reactions were stopped after 1 min with phenol-chloroform, and the DNA probe was ethanol precipitated and loaded onto an 8% polyacrylamide-8 M urea sequencing gel. Gels were dried and subjected to autoradiography.

Gel shift analysis. Gel retardation assays (20 μ l) contained 10 mM HEPES, 6 mM MgCl₂, 50 mM KCl, 0.5 mM DTT, 2 μ g of poly(dI-dC), and 4 μ g of nuclear extract prepared from either mock- or poliovirus-infected cells. Reaction mixes were incubated for 20 min at 4°C. Synthetic double-stranded oligonucleotides containing E3 binding region II (GGCGGCTTCGTCACAGGGTG), III (GAAGTTCAGATGACTAAC TCA), or IV (TAGTTGGCCCGCTTCCCTGGTGTA) were end labeled with [γ - 32 P]ATP and T4 polynucleotide kinase. End-labeled oligonucleotides (25,000 cpm) were added to the preincubated reaction mixes, and incubation was allowed to continue for an additional 10 min. Competition reactions were performed by including in the preincubation reaction mix a 100-fold molar excess of specific oligonucleotide containing either region II, III, or IV or a nonspecific, heterologous oligonucleotide of similar length (CTAGCCC TGACGTGTCCCCC). DNA-protein complexes were resolved on an 8% polyacrylamide gel (40:1, acrylamide: bisacrylamide) in 0.5 $\times$ TBE (1 $\times$ TBE is 89 mM Tris, 89 mM boric acid, 2 mM EDTA). Gels were dried and subjected to autoradiography.

In vitro transcription analysis. Nuclear extracts were prepared from mock- and poliovirus-infected cells at 4 h post-infection by a previously described procedure (8). Transcription reactions (50 μ l) contained 10 mM HEPES (pH 7.9), 50 mM KCl, 10 mM (NH₄)₂SO₄, 6.25 mM MgCl₂, 1 mM DTT, 10% glycerol, 500 μ M each ribonucleoside-5'-triphosphates, 500 ng of supercoiled E3-CAT DNA, and 100 μ g of HeLa nuclear extract (approximately 20 μ l). Reactions were incubated for 60 min at 30°C and terminated by the addition of 150 μ l of stop buffer (200 mM NaCl, 20 mM EDTA, 1% SDS,

FIG. 1. Inhibition of in vitro transcription from the E3 promoter in poliovirus-infected cell extracts. (A) E3-CAT constructs containing either the wild-type promoter (WT) (lanes 1, 2, and 5) or a promoter with site-directed mutations in the TATA element (Δ TATA) (lanes 3 and 4) were transcribed in vitro by using nuclear extracts prepared from either mock-infected (m) (lanes 1, 3, and 5) or poliovirus-infected (p) (lanes 2 and 4) HeLa cells. The transcription reaction shown in lane 5 contained 2 μ g of alpha-amanitin per ml. In a parallel experiment, the E3 transcript synthesized in vitro in mock-infected cell extract was analyzed by primer extension analysis (lane 7) along with 122 and 115 base pairs of standard DNA markers (lane 6). The position of the correctly initiated 115-base-pair primer-extended product is indicated by the arrowhead. (B) The nucleotide sequence of the E3 promoter from -237 to +1 is shown. The boxes (sites I to IV) indicate regions protected in a DNase I footprinting assay by using partially purified HeLa cell extracts. Mutations introduced in the TATA element through site-directed mutagenesis are indicated by underlined and substituted nucleotides.

250 μ g of tRNA per ml). Each reaction was extracted twice with phenol-chloroform and precipitated with 0.3 M sodium acetate-ethyl alcohol. The pellets were rinsed with 70% ethyl alcohol and dried. Primer extension was performed by a modification of a previously described protocol (23). Each pellet was suspended in 8 μ l of 10 mM Tris hydrochloride (pH 7.9)–1 mM EDTA containing approximately 50 fmol of 5'-end-labeled primer (the 24-nucleotide primer hybridizes to sequences 4945 to 4968 of RSV-CAT). A 2- μ l portion of 10 mM Tris (pH 7.9)–1 mM EDTA–1.25 M KCl was added, and the primer was annealed to the RNA for 60 min at 63°C. A buffer (25 μ l) containing 20 mM Tris (pH 7.9), 10 mM MgCl₂, 5 mM DTT, 500 μ M deoxynucleoside triphosphate, and 50 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Inc.) was added, and the primer extension reaction was incubated for 90 min at 37°C. Each reaction was extracted with phenol-chloroform, precipitated with 0.3 M sodium acetate-ethyl alcohol, suspended in 10 μ l of loading buffer (80% formamide, 0.01% xylene cyanol, 0.01% bromophenol blue, in 1 $\times$ Tris-borate-EDTA), and loaded onto 8% acrylamide-8 M urea gels. After electrophoresis, gels were dried and subjected to autoradiography. Densitometry was performed with an LKB Ultra-scan XL densitometer.

In vitro kinase reactions. CREB/ATF was phosphorylated in vitro by a modification of a previously described procedure (36). Heparin-purified nuclear extract (20 μ g) prepared from either mock- or poliovirus-infected cells was incubated in the presence of 50 mM potassium orthophosphate–10 mM MgCl₂–5 mM NaF–2 μ Ci of [γ -³²P]ATP–40 U of the catalytic subunit of cAMP-dependent protein kinase (Sigma). Reactions were incubated at 30°C for 10 min, and immunoprecipitation reactions were performed as described above.

RESULTS

In vitro transcription from the E3 promoter is inhibited in poliovirus-infected cell extracts. It was previously shown that

transcription from the adenovirus major late promoter is inhibited in extracts prepared from poliovirus-infected HeLa cells (6, 26). To determine whether transcription from the E3 promoter would be inhibited by viral infection, nuclear extracts were prepared from HeLa cells which had been either mock infected or poliovirus infected for 4 h. Transcription from the wild-type E3 promoter was severely reduced in extracts prepared from poliovirus-infected cells relative to mock-infected cells (Fig. 1A, lanes 1 and 2).

In previous work, we had shown that transcription from the major late promoter could be restored in poliovirus-infected cell extracts by addition of partially purified TFIID, the TATA-binding transcription factor (26). Transcription from the major late promoter in vivo as well as in vitro has been shown to require an intact TATA element (4, 5, 21). Transcription from the E3 promoter, however, has been shown to be less dependent on the presence of a TATA element (12, 27, 31). Site-directed mutations in the TATA element reduce transcription approximately 10-fold in in vitro reactions performed with HeLa cell nuclear extracts and have no significant effect on transcription in vivo. Efficient transcription from the E3 promoter in vivo and in vitro is strongly dependent on binding sites II (CREB/ATF) and III (AP-1). A double mutation in sites II and III effectively abolishes transcription from the E3 promoter in vitro as well as in vivo (27). The sequence of the E3 promoter and the regions which are protected in a DNase I footprinting assay with HeLa cell extracts are shown in Fig. 1B.

The severity of the decrease in in vitro transcription from the E3 promoter in poliovirus-infected cell extracts suggested that factors in addition to TFIID might be inactivated by poliovirus infection. As shown in Fig. 1A, in vitro transcription reactions performed with an E3 construct containing mutations in the TATA element (TATAA mutated to TTAAC) showed that transcription from the mutant, while reduced relative to the wild-type promoter, was still

FIG. 2. DNase I footprinting analysis of the E3 promoter in mock- and poliovirus-infected HeLa cell extracts. DNase I footprinting analysis of the coding strand of the E3 promoter was performed in the absence (lanes 0) or presence of heparin-purified HeLa cell extracts prepared from either mock-infected (lanes 1 and 2) or poliovirus-infected cells (lanes 3 and 4). Reactions contained 50 μ g (lanes 1 and 3) or 100 μ g (lanes 2 and 4) of cell extract. Protected regions I, II, III, and IV are indicated. The hypersensitive site at position -135 is indicated by the arrowhead. G+A sequencing reactions are shown.

severely reduced in poliovirus-infected cell extracts compared with mock-infected cell extracts (lanes 3 and 4). These data suggest that transcription factors in addition to the TATA factor are altered by poliovirus infection. However, we are unable to exclude the possibility that inactivation of the TATA factor by poliovirus infection interferes with the function of factors binding to regulatory regions II and III.

DNase I footprinting pattern is not altered in poliovirus-infected cell extracts. We were next interested to determine whether the dramatic differences we observed in the transcriptional activity of extracts prepared from either mock- or poliovirus-infected cells would be reflected in changes in the DNA-binding activity of transcription factors. DNase I footprinting assays were performed with the E3 promoter by using partially purified extracts prepared from mock- and virus-infected cells. As shown in Fig. 2, no significant differences were observed in the pattern of protection from DNase I over binding sites I, II, III, and IV with mock- or virus-infected cell extracts. These data indicate that inhibition of transcription from the E3 promoter by poliovirus infection is not a consequence of significant alterations in the regions protected by transcription factors which bind to E3 regulatory regions I, II, III, and IV.

One difference was observed in the DNase I footprinting

pattern obtained with extracts prepared from either mock- or poliovirus-infected cells: footprinting reactions performed with virus-infected cell extracts resulted in the appearance of a hypersensitive site at nucleotide -135 (Fig. 2, lanes 3 and 4, indicated by arrowhead) and partial protection from DNase I digestion between nucleotides -135 and -155. This hypersensitive site and partial protection are the result of the binding of a factor to an NF- κ B consensus sequence (43) located between -143 and -152; mutation of this region has no significant effect on basal levels of transcription from the E3 promoter (J. Garcia and R.G., unpublished observations). As protection from DNase I is observed over this region in extracts prepared from HeLa cells treated with cycloheximide (25 μ g per ml) for 2 h (S.K. and A.D., unpublished observations), we believe the induction of NF- κ B-binding activity to be a consequence of viral-mediated shut off of host cell translation.

CREB/ATF gel-retarded complexes are altered in poliovirus-infected cell extracts. Because complete clearing over the binding sites was observed at the lower protein concentration tested in the DNase I footprinting assay, differences in the amount of binding activity present in extracts prepared from either mock- or virus-infected cells might not have been detected in this experiment. In order to examine more carefully whether poliovirus infection resulted in a decrease in the binding activity of transcription factors which interact with the E3 promoter regions, gel retardation assays were performed with increasing amounts of nuclear extracts prepared from mock- or poliovirus-infected cells and oligonucleotides containing binding sites II (CREB/ATF), III (AP-1), and IV (NF-1). As shown in Fig. 3, DNA-protein complexes were formed with each of the three oligonucleotides. The specificity of these DNA-protein interactions was demonstrated by competition experiments performed with an excess of either specific (Fig. 4, lanes 3, 7, and 11) or nonspecific (Fig. 4, lanes 4, 8, and 12) oligonucleotides. No specific complexes were formed with an oligonucleotide containing region I (data not shown). No difference in the amount of AP-1- or NF-1-binding activity was observed in extracts prepared from either mock- or poliovirus-infected cells (Fig. 3, B and C). A slight decrease in the amount of DNA-binding activity was observed in virus-infected cell extracts at higher protein concentrations when gel retardation assays were done with a region III (CREB/ATF) oligonucleotide (Fig. 3A). However, overall, the severe decrease in transcriptional activity of extracts prepared from poliovirus-infected cells was not reflected by marked decreases in the amount of DNA-binding activity of factors which interact with the E3 promoter elements.

In order to examine for more subtle changes in the number and/or mobility of DNA-protein species formed with regions II, III, and IV, gel retardation assays were performed with longer periods of electrophoresis. No significant differences in gel-retarded complexes were observed between extracts prepared from mock- and virus-infected cells with oligonucleotides containing binding site III or IV (Fig. 4, lanes 5 and 6, 9, and 10). However, differences were observed in the pattern of DNA-protein complexes formed with mock- or virus-infected cell extracts when an oligonucleotide containing region II (CREB/ATF) was used. Assays performed with mock-infected cell extracts resulted in the resolution of five discrete, specific complexes (Fig. 4, lane 1). A sixth complex (the complex with the lowest mobility, lane 1) was not competed reproducibly with an excess of unlabeled region II oligonucleotide. Two of the specific DNA-protein complexes formed in mock-infected cell extracts were severely reduced

FIG. 3. Gel retardation analysis of binding activity to E3 regulatory regions in mock- and poliovirus-infected cell extracts. Gel retardation assays were done with oligonucleotides containing E3 promoter regions II (CREB/ATF) (A), III (AP-1) (B), and IV (NF-1) (C) and increasing amounts of nuclear extracts prepared from either mock-infected (M) (lanes 1 through 4) or poliovirus-infected (PV) (lanes 5 through 8) cells. Assays contained 1.3 μ g (lanes 1 and 5), 2.5 μ g (lanes 2 and 6), 5.0 μ g (lanes 3 and 7), and 10 μ g (lanes 4 and 8) of nuclear extract.

in abundance in poliovirus-infected cell extracts (Fig. 4, compare lanes 1 and 2, complexes indicated by arrowheads). The three other specific complexes were also slightly reduced in abundance in poliovirus-infected cell extracts. Thus, poliovirus infection results in a reduction in the intensity of discrete DNA-protein complexes which form with a CREB/ATF-binding site.

FIG. 4. DNA-protein complexes formed with E3 regulatory regions in mock- and poliovirus-infected HeLa cell extracts. Gel retardation assays were done by using nuclear extracts (5 μ g) prepared from either mock (lanes 1, 3, 4, 5, 7, 8, 9, 11, and 12) (M)- or poliovirus (lanes 2, 6, and 10) (P)-infected cells and oligonucleotides containing binding site II (CREB/ATF) (lanes 1 through 4), III (AP-1) (lanes 5 through 8), or IV (NF-1) (lanes 9 through 12). Competition experiments were performed with a 100-fold molar excess of specific (lanes 3, 7, and 11) or nonspecific (lanes 4, 8, and 12) competitor oligonucleotide. Specific complexes formed with the region II oligonucleotide in mock-infected cell extracts which were severely reduced in abundance in poliovirus-infected cell extracts are indicated by arrowheads. Gel electrophoresis was done for 2.5 h. The free probe was run off the gel and is not shown.

By using a similar gel retardation assay, Yamamoto et al. (46) recently demonstrated the presence of transcriptionally active dimeric (lower mobility) and relatively inactive monomeric (higher mobility) forms of the CREB/ATF-DNA complex. Treatment of highly purified CREB/ATF with alkaline phosphatase resulted in a dramatic reduction in the levels of the dimeric complex; in contrast, dimer formation was enhanced by treatment with protein kinase C. By using nuclear extracts prepared from mock-infected HeLa cells, we have been unable to specifically reduce formation of any of the five complexes by treatment with alkaline phosphatase; all five forms appear equally sensitive to phosphatase treatment (data not shown). This could be due to the fact that we are using crude HeLa cell extracts as opposed to highly purified CREB/ATF from PC12 cells or to differences in the gel retardation conditions.

Poliovirus infection has been shown to result in the shut off of host cell translation at approximately 2 h postinfection (see reference 24 for a review). To rule out the possibility that the changes we observed in gel mobilities for the CREB/ATF-DNA complexes were a consequence of translational shut off, nuclear extracts were prepared from HeLa cells treated with cycloheximide (25 μ g per ml) for 2 h. No differences in gel mobility patterns were seen between mock-infected and cycloheximide-treated extracts (data not shown), indicating that the altered mobilities of the CREB/ATF-DNA complexes in poliovirus-infected cell extracts were not a result of the inhibition of host cell translation.

Poliovirus infection results in a dramatic reduction in the phosphorylated form of CREB/ATF. The differences seen in the gel retardation pattern by using an oligonucleotide containing a CREB/ATF-binding site and extracts prepared from either mock- or virus-infected cells suggested that the transcription factors recognizing this site were modified by poliovirus infection. In order to further study these differences, antibodies were prepared against a TrpE-CREB fusion protein (14, 18). Immunoprecipitations were then

FIG. 5 Immunoprecipitation of CREB/ATF from mock-infected and poliovirus-infected HeLa cell extracts. (A) Immunoprecipitations were performed by using preimmune serum (lane 2) or CREB antiserum (lanes 3 and 4) and nuclear extracts prepared from [³²P]phosphate-labeled cells which were either mock infected (lanes 2 and 3) (M) or infected with poliovirus (lane 4) (P). The immunoprecipitated complexes were analyzed by SDS-polyacrylamide gel electrophoresis. In vitro-synthesized CREB labeled with [³⁵S]methionine (IV) is shown (lane 1). The CREB/ATF product is indicated. The positions of molecular mass markers, in kilodaltons, are shown on the right. The position of the 35-kDa product is indicated by an asterisk. (B) In vitro kinase reactions were done with heparin-purified nuclear extracts prepared from either mock (lanes 2 and 3) or poliovirus (lanes 4 and 5)-infected cells and immunoprecipitations subsequently done by using preimmune serum (lanes 2 and 4) or antiserum prepared against CREB/ATF (lanes 3 and 5). In vitro-synthesized CREB labeled with [³⁵S]methionine (IV) is shown (lane 1). The CREB/ATF product is indicated. The positions of molecular mass markers, in kilodaltons, are shown on the right. The position of the 35-kDa product is indicated by an asterisk.

performed with CREB/ATF antiserum, and [³²P]phosphate-labeled nuclear extracts were prepared from either mock- or poliovirus-infected cells. Immunoprecipitation of CREB/ATF from [³²P]phosphate-labeled, mock-infected cell extracts yielded a 43-kDa radiolabeled product (Fig. 5A, lane 3) which comigrated with [³⁵S]methionine-labeled CREB synthesized in vitro (lane 1). CREB/ATF has previously been shown to be a phosphoprotein of approximately 43 kDa (14, 36). The 43-kDa [³²P]phosphate-labeled product was not observed in extracts prepared from poliovirus-infected cells (lane 4) or when preimmune serum was used (lane 2). No 43-kDa product was detected in poliovirus-infected cell extracts in four separate labeling experiments. Thus, poliovirus infection resulted in a severe reduction in the phosphorylated form of CREB/ATF. In addition to the 43-kDa product, immunoprecipitation of CREB/ATF from [³²P]phosphate-labeled, mock-infected cell extracts also resulted in a labeled product of approximately 35 kDa (lane 3, indicated by asterisk; see also reference 27). This product was slightly reduced in intensity in poliovirus-infected cell extracts (lane 4) and was not observed when preimmune serum was used (lane 2). It is unclear at present whether the 35-kDa product is a proteolytic degradation product or a protein closely associated with or related to CREB/ATF. The 35-kDa size of this product suggests that it is not the alternately spliced Δ CREB protein recently described (47) that deletes a 14-amino-acid region of CREB.

We next wished to determine whether the decrease in [³²P]phosphate-labeled CREB/ATF in virus-infected cell extracts was due to a decrease in the steady-state levels of CREB/ATF or to dephosphorylation. However, as previ-

FIG. 6 Activation of the E3 promoter in vivo by CREB. F9 teratocarcinoma cells were transfected with E3-CAT (lanes 1 through 4) and expression vectors containing CREB and PKA-C (lane 2), CREB alone (lane 3), or PKA-C alone (lane 4). CAT assays were performed as described in Materials and Methods.

ously described (27), we were unable to detect the 43-kDa CREB/ATF product by either immunoprecipitation with [³⁵S]methionine-labeled cell extracts or by Western immunoblot analysis (data not shown). The reason for this is unclear. In an effort to circumvent this problem, we attempted to determine the steady-state levels of CREB/ATF in mock- and virus-infected cell extracts by performing in vitro phosphorylation reactions. Partially purified nuclear extracts prepared from either mock- or poliovirus-infected cells were incubated with the catalytic subunit of cAMP-dependent protein kinase in the presence of [γ -³²P]ATP as described previously (36). Radioimmunoprecipitations were then done with preimmune or CREB/ATF antiserum. As shown in Fig. 5B, approximately equal amounts of phosphorylated CREB/ATF product were present in reactions performed with mock- and virus-infected cell extracts (lanes 3 and 5). The CREB/ATF product was not detected when radioimmunoprecipitation reactions were done with preimmune serum (lanes 2 and 4). These results, in conjunction with our DNase I footprinting and gel retardation data, indicate that the decrease seen in the amount of the phosphorylated form of CREB/ATF in poliovirus-infected cell extracts is not a consequence of reductions in the steady-state levels of CREB/ATF but is instead likely a result of dephosphorylation of CREB/ATF by poliovirus infection.

CREB/ATF stimulates transcription from the E3 promoter in vivo. It was recently shown that cotransfection of constructs expressing CREB and the catalytic subunit of protein kinase A (PKA-C) into F9 teratocarcinoma cells resulted in a dramatic stimulation of expression from the somatostatin promoter (13). Efficient transactivation required the presence of both CREB and PKA-C, indicating that CREB needed to be phosphorylated to stimulate transcription (13). In order to determine whether CREB/ATF stimulates expression of the E3 promoter, similar experiments were performed by using CREB and PKA-C expression vectors and an E3-CAT reporter construct. As shown in Fig. 6, expression of either CREB or PKA-C alone failed to efficiently stimulate expression of E3-CAT in F9 cells (lanes 3 and 4). Cotransfection of the CREB and PKA-C expression vectors, however, resulted in activation of expression from the E3-CAT reporter construct (lane 2). The experiment

shown in Fig. 6 was the maximal amount of activation of the E3 promoter seen with CREB and PKA-C expression constructs. The level of this activation was highly dependent on the growth condition of the F9 cells. This was due to the fact that in some experiments the PKA-C expression construct resulted in activation of the E3 promoter, most likely because of the presence of constitutive amounts of CREB or other members of the ATF family in the F9 cells. These data suggest that CREB is able to induce expression from the E3 promoter and that this induction is dependent upon phosphorylation of CREB. Thus, our observation that the level of phosphorylated form of CREB/ATF is severely reduced in extracts prepared from poliovirus-infected cells is consistent with the inability of these extracts to catalyze transcription from the E3 promoter.

DISCUSSION

In this report we have examined the mechanism of poliovirus-induced inhibition of pol II-mediated transcription with the adenovirus E3 promoter. Transcription factor CREB/ATF, which binds to a DNA sequence element that is required for efficient *in vivo* and *in vitro* expression of the E3 promoter, was shown to be modified in HeLa cells infected with poliovirus. Poliovirus infection resulted in reduced levels of the phosphorylated form of CREB/ATF without significantly reducing steady-state levels of the protein. Thus, our results are consistent with a model in which poliovirus infection results in the inhibition of pol II-mediated transcription, at least in part, through the dephosphorylation of pol II transcription factors. Our observation that the dramatic inhibition of transcription from the E3 promoter in virus-infected cell extracts was not reflected in marked changes at the level of DNA-binding activity (as assayed by DNase I footprinting and gel retardation analysis) suggests that the modifications effected by poliovirus infection alter protein-protein interactions instead of protein-DNA interactions.

Our results with a transient transfection assay and F9 teratocarcinoma cells reveal the importance of phosphorylation of CREB/ATF for the activation of E3 expression *in vivo*. Transfection of constructs expressing either CREB/ATF or the catalytic subunit of PKA alone failed to stimulate E3 expression; cotransfection of these constructs, however, resulted in a strong activation of E3 expression (Fig. 6), indicating that CREB/ATF must be phosphorylated before it is capable of efficiently activating the E3 promoter. Thus, the loss of the phosphorylated form of CREB/ATF in poliovirus-infected cell extracts is consistent with the inability of these extracts to catalyze transcription from the E3 promoter.

The transcriptional activity of CREB/ATF has previously been shown to be modulated through the phosphorylation-dephosphorylation of the protein (46). Yamamoto et al. (46) recently showed that CREB exists as a mixture of transcriptionally active dimers and relatively inactive monomers. Dimerization and transcriptional activity were enhanced by treating CREB with protein kinase C and protein kinase A, respectively. Treatment of CREB with phosphatase, on the other hand, resulted in a dramatic reduction in dimer formation. The isolation and characterization of cDNAs encoding CREB has revealed a cluster of potential protein kinase A, protein kinase C, and casein kinase II recognition sites in the CREB protein (14, 18). The importance of phosphorylation for CREB function was shown directly in a recent report demonstrating that mutation of the serine residue within the protein kinase A recognition motif abolished the ability of

CREB to activate transcription from the somatostatin promoter (13).

Phosphorylation-dephosphorylation events have also been implicated as modulators of the activity of other transcription factors (35). The heat shock promoter of yeast contains binding sites for a specific DNA-binding transcription factor designated heat shock factor. Increased transcription of the heat shock genes correlates with an increase in the extent of phosphorylation of heat shock factor already bound to the heat shock promoter (44). It has been proposed that the addition of negatively charged phosphate groups might stimulate the activity of heat shock factor as well as other pol II transcription factors by creating analogs of the acidic activation sequences found in other pol II transcription factors such as GAL4 and GCN4 (44).

A role for phosphorylation has also been indicated in the regulation of nuclear proteins E2F and E4F, which interact with promoter elements important for E1A transactivation of the adenovirus E2 and E4 promoters, respectively (2, 39). The DNA-binding activity of E2F and E4F to their recognition elements is dramatically increased following adenovirus infection. It was recently shown that the increase in binding activity and subsequent transcriptional activation of the E2 and E4 promoters involves phosphorylation of inactive forms of E2F and E4F (2, 39). Adenovirus infection has also been shown to activate pol III-mediated transcription through an increase in the activity of transcription factor TFIIC (20, 48). Hoeffler et al. (19) recently demonstrated that the increase in TFIIC activity in adenovirus-infected cells was associated with increased phosphorylation of the factor. It is interesting that TFIIC activity has been shown to be severely reduced in extracts prepared from poliovirus-infected cells (10), an observation consistent with the idea that dephosphorylation of TFIIC may contribute to the inhibition of pol III-mediated transcription in poliovirus-infected cells.

A model in which poliovirus infection results in the dephosphorylation of pol II transcription factors is consistent with the results of Rangel et al. (38), who showed that levels of the IIO form of RNA pol II are significantly reduced by viral infection. Three forms of RNA pol II, termed IIO, IIA, and IIB, have been described and shown to differ in the molecular mass of their largest subunit (see reference 41 for a review). The large subunit can exist as a highly phosphorylated 240-kDa protein (IIO), a dephosphorylated 215-kDa protein (IIA), or a proteolytically degraded 180-kDa protein (IIB). The IIO species has been shown to be essential for pol II-mediated transcription *in vivo* (1, 3, 37, 49), although all three forms function efficiently *in vitro* (25, 49). The loss of the IIO form of pol II in poliovirus-infected cells could be explained if the IIO subunit of pol II were dephosphorylated during the course of infection.

The poliovirus-mediated inhibition of transcription from the E3 promoter and loss of the phosphorylated form of CREB/ATF are, at this point, correlative. Further studies in which highly purified CREB/ATF prepared from both mock- and poliovirus-infected cells and a CREB/ATF-dependent reconstituted transcription system are used will be required to directly demonstrate the loss of CREB/ATF transcriptional activity in poliovirus-infected cells. A model in which poliovirus infection disrupts pol II transcription by causing dephosphorylation of critical components of the transcriptional machinery is attractive. It remains possible, however, that poliovirus inhibits pol II transcription through multiple mechanisms. Future studies will focus on the characterization of other pol II transcription factors, including the

TATA-binding factor, from mock- and poliovirus-infected cells.

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