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Inhibition of Host Cell Transcription by Poliovirus: Cleavage of Transcription Factor CREB by Poliovirus-Encoded Protease $3C^{pro}$

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Host cell RNA polymerase II-mediated transcription is inhibited by poliovirus infection. Previous studies from our laboratory showed that activated transcription from a cyclic AMP-responsive element (CRE)-containing promoter was severely inhibited in extracts prepared from poliovirus-infected HeLa cells compared to those from mock-infected cells. Here we demonstrate that the CRE-binding protein, CREB, is specifically cleaved by the poliovirus-encoded protease $3C^{pro}$ both in vitro and in virus-infected cells. The proteolytic cleavage of CREB leads to a significant loss of its DNA binding as well as transcriptional activity. Additionally, we demonstrate that the phosphorylated, transcriptionally active form of CREB is cleaved by the viral protease in vitro. The results presented here suggest that a direct cleavage of CREB by the viral protease $3C^{pro}$ leads to inhibition of CREB-activated transcription in poliovirus-infected HeLa cells.

Infection of susceptible cells with members of the picornavirus family results in the rapid inhibition of host cell RNA synthesis (18). Infection of HeLa cells with poliovirus, for example, causes a severe decrease in transcription catalyzed by RNA polymerase I (pol I), pol II, and pol III (1, 2, 18). 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 HeLa cells (6). For each of the three polymerase systems, in vitro analysis has revealed that the inhibition of cellular transcription by poliovirus infection is a consequence of inactivation of specific transcription factors (11, 20, 28). Previous results from our laboratory have shown that the pol II transcription factor TFIID is inactivated in poliovirus-infected HeLa cells (20). More recently, we have shown that the TATA-binding protein (TBP), the DNA-binding subunit of TFIID, is proteolytically cleaved by the virus-specific protease $3C^{pro}$ (3). This cleavage leads to a loss of formation of the TBP-TATA box complex in vitro (32). We have shown recently that both TATA- and initiator element-mediated basal transcription is inhibited by the viral protease (32). Similarly, a pol III DNA-binding transcription factor, TFIIIC, is cleaved and inactivated by $3C^{pro}$ (4). The α subunit of TFIIIC, which actually contacts the pol III promoter, is the target of the viral protease $3C^{pro}$ (29). An unknown pol I transcription factor is also cleaved by the poliovirus protease, resulting in inhibition of pol I transcription in virus-infected cells (28).

While the TATA or initiator elements carry out low-level (basal) transcription of many genes, high-level (activated) transcription of many cellular and viral promoters involves interaction of multiple upstream transcription factors. These elements have been found to be required for induction by exogenous substrates, such as phorbol esters, serum, and cyclic AMP (cAMP) (25). One such factor, the cAMP-responsive element (CRE)-binding protein, CREB, binds to DNA elements required for induction by cAMP (24). A similar binding element, which binds a factor termed activating transcription factor (ATF), has been found in a variety of viral and cellular promoters (23). The observations that both ATF and CREB are induced by cAMP, are of similar molecular masses (43 kDa), and yield similar DNA-protein complexes in gel retardation assays suggest that these proteins are related. However, cloning of the CREB gene (14, 17) and the isolation of eight related but distinct cDNA clones encoding ATF binding factors (15) indicate that the CREB/ATF site is recognized by a family of proteins. The transcriptional activity of CREB has been shown to be modulated via phosphorylation-dephosphorylation events (33). It has been shown that mutation of a single serine residue (Ser-133) in a consensus sequence recognized by protein kinase A (PKA) abolished CREB transcriptional activity (13), indicating the importance of phosphorylation-dephosphorylation events in the regulation of CREB activity. More recently, only the phosphorylated form of CREB has been shown to interact with a protein called CBP (for CREB-binding protein); this interaction is likely to regulate CREB activity (27).

Previous studies from our laboratory showed that activated transcription from a CRE-containing promoter was severely inhibited in extracts prepared from poliovirus-infected cells (19). Gel retardation assays using CREB sequences and cellular proteins showed significant qualitative differences in the DNA-protein complexes formed with the CRE sequence in extracts prepared from virus-infected cells compared to those from mock-infected cells. Radioimmunoprecipitation reactions performed with antiserum against CREB revealed a severe reduction in a phosphorylated form of the protein present in poliovirus-infected cell extracts. Here we demonstrate that the CREB protein is specifically cleaved by the poliovirus-encoded protease $3C^{pro}$ both in vitro and in vivo. The proteolysis of CREB protein by $3C^{pro}$ leads to a significant loss of DNA binding activity of the protein as well as its transcriptional activity. The loss of phosphorylated CREB protein observed in virus-infected cells is also a consequence of proteolytic cleavage of the protein.

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MATERIALS AND METHODS

Cells and virus. HeLa cells grown in spinner culture in spinner modified essential medium supplemented with 6% newborn calf serum, 1 g of glucose, and 10^5 U (each) of penicillin and streptomycin per liter were infected at a multiplicity of infection of 25 with poliovirus type 1 (Mahoney) as described earlier (6). Where indicated, cells were incubated in the presence of cycloheximide (100 μg/ml) for 2 h before being harvested (28).

Preparation of extracts. Nuclear extracts were prepared from mock- and poliovirus-infected HeLa cells as previously described (8) with a slight modification. Instead of dialysis against buffer D, extracts were precipitated with ammonium sulfate at 50% saturation. The pellet was suspended in TM 0.1 buffer (50 mM Tris [pH 7.4], 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol [DTT], 20% glycerol, 100 mM KCl) and dialyzed against TM 0.1 buffer overnight at 4°C.

Purification of CREB. CREB was purified from mock- and poliovirus-infected nuclear extracts as described earlier (24) with some modifications. Synthetic CRE oligonucleotides with *Bam*HI cohesive ends on either side were generated. These oligonucleotides were ligated with DNA ligase. Forty micrograms of ligated DNA was attached to 1 ml of CNBr-activated CL-4B Sepharose (Pharmacia). Ten milligrams of nuclear extract was passed over a 1-ml DNA affinity column equilibrated in buffer Z (25 mM HEPES [pH 7.8], 12.5 mM $MgCl_2$, 1 mM DTT, 20% glycerol, 0.1% Nonidet P-40) as described earlier (24). CREB was eluted with 0.4 ml of 1 M KCl. This was used as purified CREB for transcription reactions (see Fig. 4). The flowthrough nuclear extract was passed over the CRE column a second time. This flowthrough was designated CREB-depleted extracts.

Plasmid DNAs. pGem/CREB was a gift from R. Gaynor (University of Texas). Cloning of CREB cDNA into pGem2 was described earlier (12). pE3CAT DNA was used for in vitro transcription reactions. This construct was also described earlier (19).

Protease reactions. $3C^{pro}$ was cloned into pQE 30 (Qiagen) as described earlier (29). pGem/CREB was digested with *Hin*dIII, in vitro transcribed with Sp6 RNA polymerase, and translated in rabbit reticulocyte lysates. The indicated amount of $3C^{pro}$ was added to [^{35}S]methionine-labeled in vitro-translated CREB or mock-infected extracts, and the reaction mixtures were incubated at 30°C for 4 h. These samples were then separated by sodium dodecyl sulfate (SDS)–14% polyacrylamide gel electrophoresis (PAGE).

Western blot (immunoblot) analysis. The proteins separated by SDS-PAGE were electrophoretically transferred onto nitrocellulose. The nitrocellulose membrane was blocked with blocking reagent (Bio-Rad). Mouse monoclonal antibody against an epitope corresponding to amino acids 254 to 327 of CREB (Santa Cruz Biotech) was used as a primary antibody against CREB. The secondary antibody was anti-mouse immunoglobulin G coupled to horseradish peroxidase. The immunoblot was developed by using the chemiluminescence detection system (Bio-Rad).

UV cross-linking analysis. The UV cross-linking experiments were performed as described earlier (24) with a few modifications. A uniformly body-labeled probe was generated by using a synthetic oligonucleotide with CRE sequence and 19 bp of Sp6 promoter sequence. The Sp6 promoter primer was annealed to this oligonucleotide by heating to 90°C for 5 min and cooling to room temperature overnight. The fill-in reaction was started by adding 50 μM (each) dGTP, dATP, and bromodeoxyuridine triphosphate, 100 μCi of [α-^{32}P]dCTP (3,000 Ci $mmol^{-1}$), and 25 U of Klenow fragment. The reaction was allowed to proceed at 16°C for 90 min. Then it was ethanol precipitated and dissolved in 50 μl of water. The binding reaction mixtures contained 50,000 cpm of body-labeled CRE fragment, 50 μg of nuclear extract in 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, and 1 μg of poly(dI-dC). The samples were incubated at room temperature for 20 min and then irradiated by 300-nm UV for 45 min. To these samples, 10 mM $CaCl_2$ was added, and they were digested at 37°C for 20 min with 2.5 μg of DNase I and 50 U of micrococcal nuclease. The reactions were stopped by addition of 20 μl of SDS-PAGE buffer and analyzed by SDS–14% PAGE.

In vitro transcription assays. The transcription reaction mixtures (50 μl) contained 450 ng of pE3CAT template DNA, 150 μg of HeLa extracts, purified CREB (as indicated), 2.5 mM (each) ribonucleoside triphosphates, 100 U of RNasin, 10 mM $(NH4)_2SO_4$, 2.5 mM Tris (pH 7.4), 6.25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol, and 50 mM KCl. The reaction mixtures were incubated at 30°C for 90 min and terminated by addition of 90 μl of stop solution (20 mM NaCl, 20 mM EDTA, 1% SDS, 250 μg of RNA per ml). The RNA was extracted with phenol chloroform, ethanol precipitated, and analyzed by primer extension. The RNA pellets were dissolved in 5 μl of annealing buffer (250 mM NaCl, 5 mM Tris [pH 7.4], 1 mM EDTA). Fifty thousand counts per minute of CAT primer (24-nucleotide primer hybridizes to a sequence 115 bp downstream of start site) was added to the mix described above, and the final volume was adjusted to 10 μl with water. The primer was annealed at 65°C for 5 min and at 50°C for 90 min. To the annealed mix, 1 mM DTT, 400 U of avian myeloblastosis virus reverse transcriptase (Life Science Inc.), and reverse transcriptase buffer (50 mM Tris [pH 8.7], 8 mM $MgCl_2$, 50 mM KCl, 1.5 mM [each] deoxynucleoside triphosphates) were added in a 20-μl reaction volume. These reaction mixtures were incubated at 40°C for 90 min, the reactions were stopped by addition of 15 μl of stop dye (80% formamide, 0.01% xylene cyanol, 0.01% bromophenol blue) and loaded on an 8% acrylamide–8 M urea gel, and products were separated by electrophoresis.

MTMESGAENQQSGDAAVTEAENQQMTVQAQPQIATLAQVS

MPAAHATSSAPTVTLVRCPMGNSQVHGVIQAAQPSVIQSP

QVQTVQISTIAESEDSQESVDSVTDSQKRREILSRRPSYR

KILNDLSSDAPGVPRIEEEKSEEETSAPAITTVTVPTPIY

QTSSGQYIAITQGGAIQLANNGTDGVQGLQTLTMTNAAAT

QPGTTILQYAQTTDGQQILVPSNQVVVQAASGDVQTYQIR

TAPTSTIAPGVVMASSPALPTQPAEEAARKREVRLMKNRE

AARECRRKKKEYVKCLENRVAVLENQNKTLIEELKALKDLY

CHKSD

FIG. 1. Predicted amino acid sequence and potential $3C^{pro}$ cleavage sites in CREB. The two glutamine-glycine (QG) sites are boxed.

In vitro kinase reactions. CREB was phosphorylated in vitro by a modification of a previously described procedure (19). Unlabeled CREB synthesized in vitro with nonradioactive amino acids was incubated in the presence of 50 mM orthophosphate, 10 mM $MgCl_2$, 10 mM DTT, 30 μCi of [γ-^{32}P]ATP, and 63 U of catalytic subunit of cAMP-dependent protein kinase (Promega). Reaction mixtures were incubated at 30°C for 10 min, and immunoprecipitation reactions were performed as described earlier (7) with an antibody raised against amino acids 254 to 327 of CREB protein.

Site-directed mutagenesis. A PCR-based method was used to mutate glutamine and glycine at amino acids 172 and 173, respectively, for mutant C1 and glutamine and glycine at amino acids 187 and 188, respectively, for mutant C2. The same method was used for mutants C1 and C2, except that the primers annealed at amino acid positions 172 and 173 for C1 and 187 and 188 for C2. Initially, two PCRs were performed with pGem/CREB as the template for C1. One reaction consisted of primer A annealing to nucleotides 1014 to 1021 of CREB (nucleotide 1 corresponds to the first nucleotide of initiator Met codon) with a *Hin*dIII site attached and primer B annealing to nucleotides 558 to 573. Primer B had changes so that a *Sal*I site was generated and glutamine and glycine were changed to valine and aspartate. The second PCR had upstream primer D annealing to nucleotides 1 to 15 of CREB with a custom *Bam*HI site attached and primer E annealing to nucleotides 547 to 558. Primer E had changes so that a *Sal*I site was generated and glutamine and glycine at nucleotides 558 and 564 were changed to amino acids valine and aspartate, respectively. The two PCR products were gel purified and digested with *Sal*I and *Bam*HI or *Hin*dIII. A three-way ligation was performed with the two gel-purified PCR products and *Bam*HI-*Hin*dIII-digested pGem3. The mutation was verified by digestion with *Sal*I.

RESULTS

The CREB protein is cleaved by the viral protease $3C^{pro}$ in vitro. Poliovirus mRNA is translated as a large polyprotein which is then proteolytically cleaved by two viral encoded proteases, $3C^{pro}$ and $2A^{pro}$ (22). These proteases are remarkably specific in cleaving specific peptide bonds within the viral precursor proteins. While $3C^{pro}$ cleaves only glutamine-glycine bonds, $2A^{pro}$ cleaves tyrosine-glycine bonds in viral precursor proteins. Recently, we have shown that specific glutamine-glycine bonds of TBP and the α subunit of TFIIIC are cleaved by $3C^{pro}$ in virus-infected cells (3, 29). A single-amino-acid change in the cleavable glutamine-glycine site of TBP renders the polypeptide resistant to proteolytic cleavage by $3C^{pro}$ (5). This observation prompted us to examine the amino acid sequence of the CREB protein. As can be seen in Fig. 1, two glutamine-glycine sites are present in the protein. To determine whether the viral protease $3C^{pro}$ is able to cleave CREB, in vitro-translated, [^{35}S]methionine-labeled CREB was incubated with highly purified, recombinant $3C^{pro}$ or a mutant protease in which the active-site cysteine moiety was changed to alanine. This mutation in $3C^{pro}$ renders the protease inactive (16). As can be seen in Fig. 2A, when the protease concentration in the reaction mixture was increased, the amount of the intact CREB protein was reduced, with concomitant

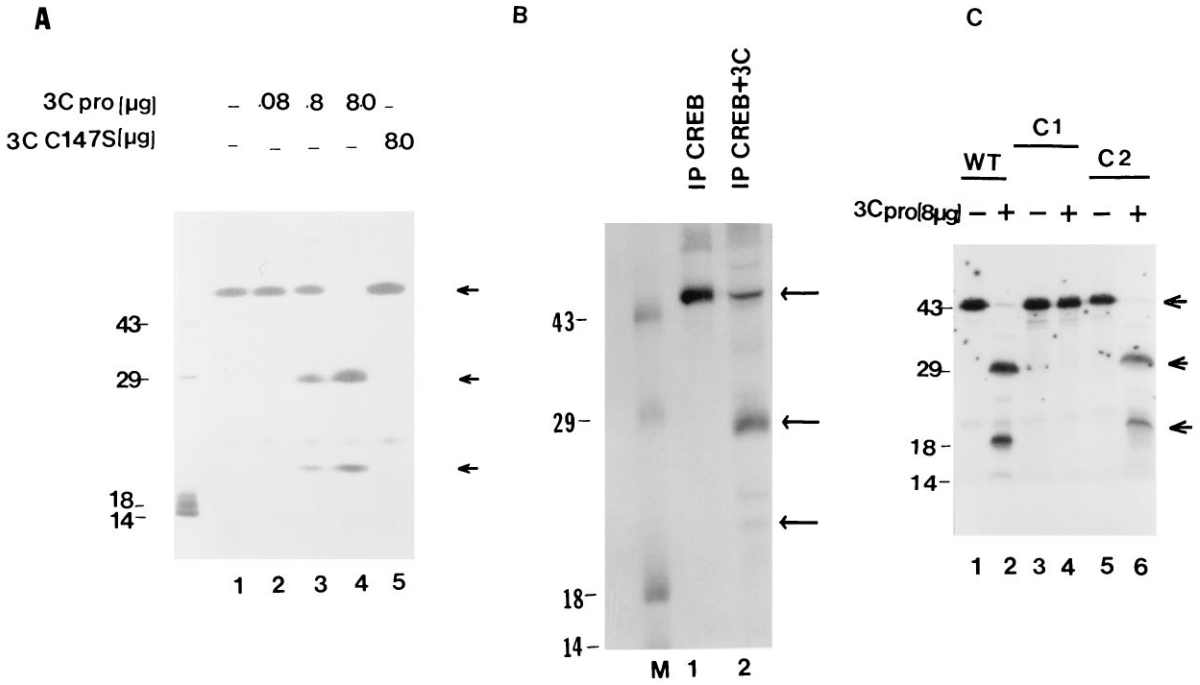

FIG. 2. Cleavage of CREB by $3C^{pro}$. (A) Cleavage of in vitro-translated CREB by $3C^{pro}$. Human CREB was in vitro translated in the presence of [^{35}S]methionine. One microliter of ^{35}S-labeled in vitro-translated CREB was incubated with the indicated amount of $3C^{pro}$ or 3C C147S for 4 h at 30°C. The reactions were analyzed by SDS–14% PAGE. The sizes of ^{14}C markers (in kilodaltons) are indicated on the left. The positions of full-length CREB and cleaved products are indicated by arrows on the right. (B) Cleavage of immunoprecipitated CREB by $3C^{pro}$. CREB was translated in vitro in the presence of [^{35}S]methionine and was immunoprecipitated with an antiserum raised against the peptide between amino acids 254 and 327 of CREB. The antigen-antibody complex was purified by its binding to a protein A-Sepharose bead. Then the bound complex (IP CREB) was incubated at 30°C for 6 h in the absence (lane 1) or presence (lane 2) of $3C^{pro}$ (0.8 μg) and run on an SDS–15% polyacrylamide gel. Lane M, ^{14}C-labeled protein molecular mass (in kilodaltons) markers. (C) Identification of the $3C^{pro}$ cleavage site in CREB. Site-directed mutagenesis was used to replace the glutamine-glycine pairs at positions 172 and 187 with valine-aspartate pairs. The wild type (WT; lanes 1 and 2) and mutants C1 (position 172; lanes 3 and 4) and C2 (position 187; lanes 5 and 6) were translated in the presence of [^{35}S]methionine. The in vitro-translated proteins were incubated with buffer (−; lanes 1, 3, and 5) or purified $3C^{pro}$ (+; lanes 2, 4, and 6). Labeled products were analyzed by SDS-PAGE. The positions of molecular mass (in kilodaltons) markers are indicated on the left, and the positions of full-length CREB and cleaved products are indicated by arrows on the right.

formation of two proteolytic fragments with approximate molecular masses of 28 and 20 kDa (Fig. 2A, lanes 1 through 4). In the reaction mixture, containing the highest amount of protease, no full-length CREB was detected; all of the intact CREB was converted to two lower-molecular-weight polypeptides. In contrast, the purified mutant protease was unable to cleave the CREB protein even at a very high concentration (Fig. 2A, lane 5). These results suggest that the viral protease, not a contaminating bacterial protease, was responsible for cleavage of CREB.

To determine whether the CREB protein is directly cleaved by $3C^{pro}$, in vitro-translated, [^{35}S]methionine-labeled CREB was immunoprecipitated from the reticulocyte lysate by anti-CREB antibody. The immunoprecipitate was thoroughly washed with buffer containing no SDS to remove contaminating host cell proteins, and the protein A-bound antigen-antibody complex was incubated with purified $3C^{pro}$. The cleaved products were then analyzed by SDS-PAGE. As can be seen in Fig. 2B, a significant amount of the full-length CREB disappeared with the concomitant appearance of cleaved products. In this reaction a relatively small amount of the 20-kDa fragment of CREB was detected. The reason for this is not clear at present. This result suggests that $3C^{pro}$ directly cleaves the CREB protein.

Two glutamine-glycine sites appear in CREB, at amino acids 172 and 187 (Fig. 1). To determine which glutamine-glycine site is cleaved by $3C^{pro}$, one glutamine-glycine site at a time was altered by site-directed mutagenesis. The glutamine-glycine pairs at positions 172 and 173 and positions 187 and 188 were replaced by valine-aspartic acid pairs. The resulting mutants were designated C1 (position 172) and C2 (position 187). Proteins translated from these plasmids in rabbit reticulocyte lysate were incubated with purified $3C^{pro}$. As can be seen in Fig. 2C, when the glutamine-glycine pair at positions 172 and 173 (C1) was changed to valine-aspartic acid, the resulting CREB was not cleaved by $3C^{pro}$ (Fig. 2C, lanes 3 and 4). However, both wild-type CREB (Fig. 2C, lanes 1 and 2) and the C2 (position 187) mutant (lanes 5 and 6) were readily cleaved by $3C^{pro}$. These results demonstrate that the glutamine-glycine site at amino acids 172 and 173 is cleaved by $3C^{pro}$.

Cleavage of CREB in virus-infected cells. To determine whether CREB is cleaved during infection of HeLa cells with poliovirus, cell extracts were prepared from mock- and poliovirus-infected cells and examined by Western blot analysis using a mouse monoclonal antibody against an epitope corresponding to amino acids 254 to 327 of CREB. The antibody recognized the CREB protein from mock-infected cells (Fig. 3, lane 1). In poliovirus-infected cell extract, the CREB protein was almost totally absent (Fig. 3, lane 4). Although the intensity of the CREB protein was drastically reduced, we were unable to detect the cleaved products in infected extracts (Fig. 3, lane 4). The reason for this is not known. The proteolytic fragments of CREB may be unstable in HeLa cell extracts. Alternatively, the monoclonal antibody may have somehow failed to detect the C-terminal fragment of CREB. That one of

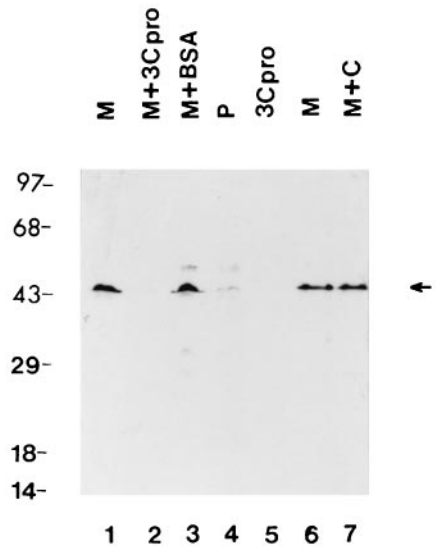

FIG. 3. Western blot analysis of CREB from mock- and poliovirus-infected extracts and mock extracts treated with $3C^{pro}$ or BSA. One hundred micrograms of mock (M)- or poliovirus (P)-infected extracts (lanes 1 and 4) or mock extracts treated with $3C^{pro}$ or BSA (lanes 2 and 3) at 30°C for 4 h was analyzed by SDS–14% PAGE. In addition, mock extracts or extracts from cells treated with cycloheximide (C) for 2 h were run on the same gel (lanes 5 and 6). The proteins were transferred to nitrocellulose. The blot was probed with a mouse monoclonal antibody against an epitope corresponding to amino acids 254 to 327 of CREB (Santa Cruz Biotech). The bands were visualized by using the chemiluminescence detection system. (Bio-Rad). The positions of the prestained markers (in kilodaltons) are shown on the left. The position of CREB is indicated by an arrow on the right.

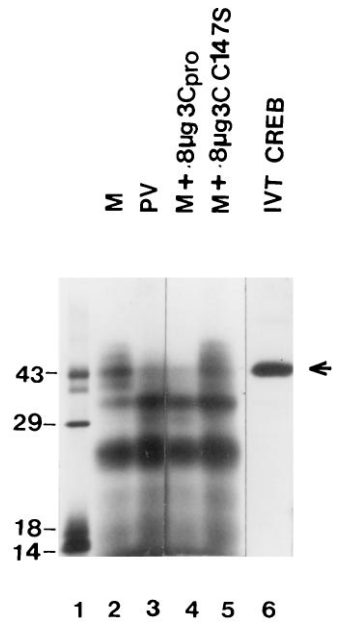

FIG. 4. Cross-linking analysis of the binding activity of CREB to CRE. A uniformly labeled probe was used for cross-linking reactions. This probe was obtained by annealing a 47-bp synthetic oligonucleotide containing a high-affinity binding site (CRE) for CREB protein with a complementary 19-bp synthetic oligonucleotide. This hybridized complex was used as a substrate for the Klenow fragment, and the probe was labeled with [α-^{32}P]dCTP. The labeled probe (10^6 cpm) was incubated with 50 μg of HeLa nuclear extracts and 2 μg of poly(dI-dC). The protein-DNA complex was UV cross-linked for 45 min at 25°C. The samples were then digested with 2.5 U of micrococcal nuclease and 1.25 μg of DNase I at 37°C for 30 min. The samples were analyzed by SDS–14% PAGE. Lane 1, molecular mass (in kilodaltons) marker proteins; lane 2, mock (M)-infected nuclear extract (50 μg) incubated with body-labeled CRE; lane 3, Poliovirus (PV)-infected nuclear extract (50 μg) incubated with CRE; lane 4, mock-infected extract treated with 0.8 μg of $3C^{pro}$ at 30°C for 4 h and incubated with CRE; lane 5, mock-infected extract treated with 0.8 μg of 3C C147S at 30°C for 4 h and incubated with CRE; lane 6, in vitro-translated (IVT) CREB. The position of CREB is indicated by an arrow on the right.

these two possibilities was correct was confirmed by analyzing products generated by incubation of mock-infected cell extracts with exogenously added $3C^{pro}$ (Fig. 3, lanes 1 through 3). Incubation of mock-infected extracts with $3C^{pro}$ (0.8 μg) resulted in the total loss of the intact CREB protein without the generation of cleaved fragments (Fig. 3, lane 2). Incubation of the extract with an equivalent amount of bovine serum albumin (BSA) did not result in the disappearance of CREB. It should be noted that similar experiments with in vitro-translated CREB showed the generation of cleaved products when analyzed directly on an SDS gel without immunoprecipitation (Fig. 2).

Poliovirus shuts off host cell protein synthesis after 2.5 to 3 h postinfection. It was possible that CREB could not be detected in infected cell extracts due to inhibition of host cell protein synthesis. To prove that this was not the case, cells were treated with cycloheximide, a protein synthesis inhibitor, for a period sufficient to inhibit cellular protein synthesis (data not shown). Extracts prepared from both mock-treated and cycloheximide-treated cells were examined by Western blot analysis. As can be seen (Fig. 3, lanes 6 and 7), approximately equal amounts of CREB could be detected from mock- and cycloheximide-treated cells. These results suggest that the disappearance of intact CREB during infection of cells with poliovirus is not due to the decrease in the steady-state level of CREB and is most likely due to cleavage of CREB by $3C^{pro}$.

$3C^{pro}$ inhibits binding of CREB to the CRE. To determine whether treatment of CREB with $3C^{pro}$ inhibits the sequence-specific DNA-binding activity of CREB, a ^{32}P-labeled, double-stranded DNA probe containing a high-affinity CRE binding site was incubated with HeLa cell extracts. The protein-DNA complexes were UV cross-linked and digested with micrococcal nuclease and DNase I, and the resulting complexes were analyzed by SDS-PAGE. When mock-infected extract was used in the cross-linking reaction, three distinct complexes were visualized. The slowest-migrating complex was heterogeneous in nature, and the band (indicated by an arrow) comigrated with in vitro-translated CREB protein (Fig. 4, lanes 2 and 6). This complex appears to be specific for CREB since it can be competed with the unlabeled homologous probe but not with heterologous probes (data not shown). Approximately 52% inhibition of this complex formation was observed when extracts from virus-infected cells were used in cross-linking compared to mock-infected extracts (Fig. 4; compare lanes 1 and 2). Fifty-five percent inhibition of the formation of this complex was observed when mock-infected extracts were treated with 0.8 μg of purified $3C^{pro}$ (Fig. 4, lane 3). Incubation of mock-infected extract with the enzymatically inactive, purified mutant protease did not significantly inhibit the formation of this complex (Fig. 4, lane 4). These results suggest that proteolytic cleavage of CREB by $3C^{pro}$ inhibits its ability to bind CRE-binding sequences.

$3C^{pro}$ inhibits transcriptional activity of CREB. To examine whether virus infection and $3C^{pro}$ treatment inhibit the transcriptional activity of CREB, it was necessary to generate cell extracts depleted of CREB. This was achieved by repeated passage of nuclear extract through a DNA affinity column containing the CRE-binding sequence (24). CREB bound to the column was eluted with buffer containing high levels of salt

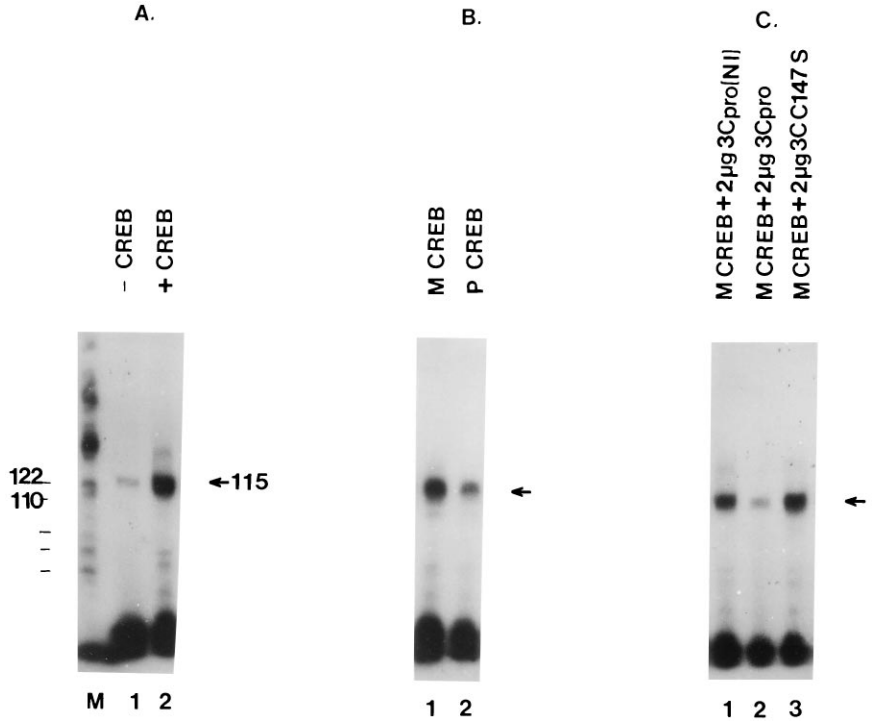

FIG. 5. In vitro transcriptional efficacy of CREB protein purified from mock- and poliovirus-infected cells or CREB treated with $3C^{pro}$. (A) Primer extension analysis of CREB-depleted extract (−; lane 1) and CREB-depleted extract supplemented with 5 μl of CREB purified from mock-infected cells (+; lane 2). Lane M, markers (in base pairs). The plasmid used for primer extension was pE3CAT, as described in Materials and Methods. One hundred fifty micrograms of nuclear extract was used for in vitro transcription analysis, and the E3 transcript synthesized was analyzed by primer extension using a [γ-^{32}P]-labeled primer. The position of the correctly initiated 115-bp product is indicated by an arrow. (B) Transcriptional efficacy of CREB purified from mock (M)- or poliovirus (P)-infected cells. CREB-depleted nuclear extracts from mock-infected cells were supplemented with 5 μl of CREB purified from mock-infected cells (lane 1) or 5 μl of CREB purified from poliovirus-infected cells (lane 2). The E3 transcript synthesized was analyzed as described for panel A. We were unable to determine the protein concentrations of CREB purified from mock- and poliovirus-infected cells. (C) Transcriptional efficacy of CREB treated with $3C^{pro}$. Purified CREB from mock (M)-infected cells was incubated with 2 μg of $3C^{pro}$ (lane 2) or 2 μg of 3C C147S (lane 3) for 4 h at 30°C. Two micrograms of $3C^{pro}$ was added to a reaction just before the start of transcription as a control to study the effects of $3C^{pro}$ on other transcription factors during transcription (lane 1). Appropriately treated CREB was added to CREB-depleted extracts, and in vitro transcription reactions were performed as described for panel A.

and was used to reconstitute transcription from an adenovirus E3 promoter (pE3CAT 24]) in CREB-depleted extracts by primer extension assay, as described in Materials and Methods. Very little transcription was observed in CREB-depleted extract (Fig. 5A, lane 1). Addition of 5 μl of purified CREB completely reconstituted transcription (Fig. 5A, lane 2). We were unable to accurately measure the protein concentrations in the highly purified fractions of CREB. The level of transcription seen in the reconstituted reaction was approximately 95% of that seen with nondepleted nuclear extracts prepared from uninfected cells (data not shown). Thus, efficient transcription in depleted extracts was highly dependent on added CREB. To determine whether poliovirus infection results in the inactivation of CREB transcriptional activity, CREB was purified by DNA affinity chromatography from mock- and virus-infected cell extracts. Figure 5B shows transcriptional activity of CREB, as determined by its ability to reconstitute transcription in CREB-depleted extracts. The transcriptional activity of affinity-purified CREB isolated from poliovirus-infected cells was approximately fivefold lower than that isolated from mock-infected cells (Fig. 5B). The same assay was used to determine the effect of $3C^{pro}$ on CREB transcriptional activity (Fig. 5C). Incubation of purified CREB derived from mock-infected cells with the inactive $3C^{pro}$ mutant did not change significantly its ability to stimulate transcription compared to the level seen with untreated CREB (compare Fig. 5C, lane 3, with B, lane 1). However, drastic (~ sevenfold) inhibition of transcription was observed when CREB isolated from mock-infected cells was incubated with $3C^{pro}$ prior to being added to the transcription reaction (Fig. 5C, lane 2). To examine the possibility that $3C^{pro}$ inactivates transcription factors other than CREB, a reaction in which $3C^{pro}$ was added to the reaction just before the start of transcription was performed. Slight inhibition of transcription was observed in this reaction (Fig. 5C, lane 1) compared to those containing untreated or mutant $3C^{pro}$-treated CREB. These results clearly suggest that infection of cells with poliovirus results in a significant reduction of CREB transcriptional activity; this is most probably due to cleavage of CREB by the viral protease.

Phosphorylated CREB is cleaved by $3C^{pro}$. Phosphorylation of CREB by PKA is necessary for its transcriptional activity. Previous results from our laboratory showed that there was a dramatic reduction in the phosphorylated form of CREB in poliovirus-infected cells compared to that in mock-infected cells (19). To examine whether phosphorylated CREB is susceptible to cleavage by the viral protease $3C^{pro}$, CREB translated in vitro in the presence of unlabeled methionine was phosphorylated with [γ-^{32}P]ATP by the catalytic subunit of PKA (PKA-C) and then subjected to digestion by the purified recombinant $3C^{pro}$. ^{32}P-labeled CREB was then immunoprecipitated with anti-CREB antibody as previously described (19). In parallel experiments, in vitro-translated [^{35}S]methionine-labeled CREB and that also treated with $3C^{pro}$ were analyzed; however, these reactions were not immunoprecipi-

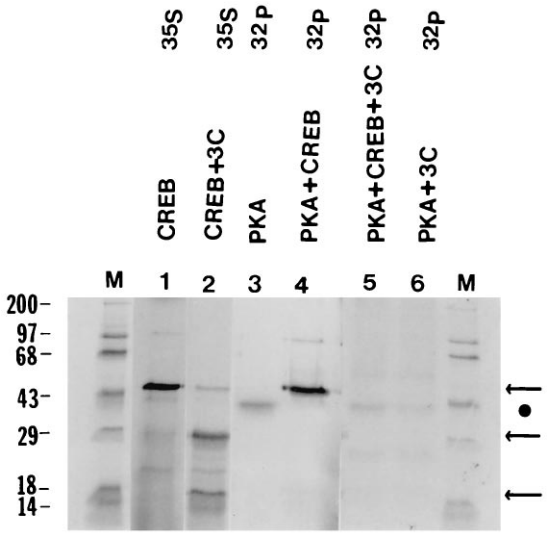

FIG. 6. Cleavage of phosphorylated CREB by $3C^{pro}$. In vitro-translated, [^{35}S]methionine-labeled CREB was analyzed by SDS-PAGE before (lane 1) and after (lane 2) digestion with 0.8 μg of $3C^{pro}$. In lanes 3 and 4, in vitro-translated, unlabeled CREB was phosphorylated by the catalytic subunit of PKA in the presence of [γ-^{32}P]ATP. Then, ^{32}P-labeled CREB was immunoprecipitated from these reactions and analyzed by SDS-PAGE. Lane 3, PKA alone; lane 4, PKA and CREB; lane 5, PKA, CREB, and $3C^{pro}$ lane 6, PKA and $3C^{pro}$. Arrows indicate full-length and proteolytic fragments of CREB. The dot indicates the position of the autophosphorylated catalytic subunit of PKA nonspecifically precipitated by anti-CREB antibody. Lanes M, molecular mass (in kilodaltons) markers.

tated. As expected, a significant amount of ^{35}S-labeled CREB was cleaved by $3C^{pro}$ to two smaller polypeptides (Fig. 6, lanes 1 and 2). The ^{32}P-labeled CREB phosphorylated by PKA-C totally disappeared when digested with $3C^{pro}$ (Fig. 6, lanes 4 and 5). The smaller proteolytic fragments of ^{32}P-labeled CREB were not detected in immunoprecipitates (Fig. 6, lane 5) because the cleaved products recognized by the antibody have no PKA phosphorylation sites. Some background bands (Fig. 6, lanes 5 and 6) consisted of phosphorylated PKA-C and nonspecific bands which originated most probably from the $3C^{pro}$ preparation. The autophosphorylated PKA was nonspecifically bound to anti-CREB antibody (Fig. 6, lane 3). The reason for this is not clear. These results suggest that phosphorylated CREB is susceptible to cleavage by $3C^{pro}$.

DISCUSSION

In this study, we examined the mechanism of poliovirus-induced inhibition of RNA pol II-mediated activated transcription. Transcription factor CREB, which binds to a DNA sequence element that is required for efficient in vivo and in vitro expression of adenovirus E3 promoter, was shown to be altered in HeLa cells infected with poliovirus. The contentions that modification of CREB in virus-infected cells involved proteolytic cleavage of CREB by the poliovirus-encoded protease $3C^{pro}$ and that this cleavage resulted in the inactivation of CREB activity were supported by several independent observations. First, in vitro-translated CREB is specifically proteolyzed when incubated with the purified, recombinant $3C^{pro}$ but not with an inactive protease mutant, C147S. Specific cleavage of CREB by $3C^{pro}$ generated two smaller polypeptides (Fig. 2). Secondly, Western blot analysis of poliovirus-infected cell extracts using anti-CREB antibody demonstrated that CREB was also proteolyzed in virus-infected cells (Fig. 3). Thirdly, specific binding of CREB to the CRE sequence was almost totally abolished by wild-type $3C^{pro}$ but not the inactive $3C^{pro}$ mutant protease (Fig. 4). Finally, using CREB-depleted HeLa cell extracts and purified CREB, we showed that $3C^{pro}$ treatment of CREB inhibits its transcriptional activity significantly. We also showed that the glutamine-glycine site at amino acids 172 and 173 of CREB was cleaved by $3C^{pro}$ (Fig. 2C). 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 proteolytic cleavage of pol II transcription factor CREB.

We were unable to detect cleavage products of CREB in poliovirus-infected cells (Fig. 3). This could have been due to instability of cleaved products in cell extracts. Alternatively, the monoclonal antibody (to C-terminal amino acids 254 to 327) may have failed to detect the cleaved C-terminal fragment of human CREB. The second possibility appears likely for the following reasons. The two cleaved products can be easily detected after cleavage of in vitro-translated CREB in cell lysates with $3C^{pro}$ (Fig. 2A, lanes 3 and 4). However, these cleaved fragments can be neither immunoprecipitated (data not shown) nor detected by Western blot analysis (Fig. 3, lane 2). Both cleaved fragments were detected, however, when full-length CREB was immunoprecipitated first and then the immunoprecipitate was digested with $3C^{pro}$ (Fig. 2B).

Our previous studies have shown that poliovirus inhibits TATA- and initiator-mediated basal transcription by proteolytic cleavage of TBP in infected cells (32). Why is it necessary to target transcription activator proteins? In other words, does poliovirus infection lead to inhibition of basal or activated transcription or both? Clearly, our previous results and those presented here suggest that both basal transcription and activated transcription are inhibited by infection of cells with poliovirus (32). It is worth noting that proteolysis of TBP is never complete in virus-infected cells; a significant amount of residual intact TBP is found in infected cells. Thus, basal transcription is not totally inhibited by virus infection. It is therefore advantageous to the virus to inhibit activated transcription to achieve maximal levels of inhibition of cellular transcription. Here we have shown that in addition to inhibiting basal transcription by proteolytic cleavage of TBP, the virus-specific protease $3C^{pro}$ is also capable of inhibiting CREB-mediated activated transcription.

Because basal transcription is inhibited by infection of cells with poliovirus, it was necessary to dissociate CREB-mediated activated transcription from basal transcription to examine the role of $3C^{pro}$ in activated transcription. This was achieved by purifying CREB away from other transcription factors that are affected by virus infection (e.g., TBP). The activities of purified CREB from mock- and poliovirus-infected cells were studied with CREB-depleted transcription extracts derived from mock-infected cells (Fig. 5). This system allowed us to directly study the effects of poliovirus infection and $3C^{pro}$ on CREB.

The results presented in Fig. 6 suggest that the disappearance of phosphorylated CREB in virus-infected cells is due to proteolytic cleavage of CREB by $3C^{pro}$. We also considered the possibility that PKA activity is inhibited by poliovirus infection. However, a comparison of PKA activity in virus-infected cell extract versus that in mock-infected cell extract did not show any significant difference. Nonspecific phosphatase activities were also comparable between mock- and virus-infected cell extracts (data not shown). Therefore, we suggest that both phosphorylated CREB and unphosphorylated CREB are proteolyzed by $3C^{pro}$ in poliovirus-infected cells.

Although poliovirus encodes three proteinases, poliovirus infection does not result in large-scale or random proteolysis of

viral or cellular proteins. Two-dimensional gel analysis of mock-infected cell extracts versus poliovirus-infected cell extracts demonstrates that fewer than 10 proteins are altered by poliovirus infection (31). Thus, the lack of large-scale modification of cellular proteins in poliovirus-infected cells argues that the proteolysis of CREB by $3C^{pro}$ is a specific event and is not due to generalized proteolysis in infected cells. To date, two RNA pol II factors (TBP and CREB), a pol III factor (TFIIIC [α subunit]), and an unknown pol I factor have been shown to be cleaved by the viral protease $3C^{pro}$ (3, 28, 29). Cleavage by $3C^{pro}$ of these factors invariably occurs at glutamine-glycine bonds (22). Many other transcription factors contain glutamine-glycine bonds; however, they are not cleaved by $3C^{pro}$. This may be because other determinants, such as accessibility of the cleavage site and subcellular localization of the proteins, are also necessary for cleavage. Immunohistochemical studies have shown that a precursor of $3C^{pro}$, 3CD, migrates to the nucleus after infection of cells with poliovirus (10). Thus, CREB and $3C^{pro}$ could interact in the nuclei of infected cells.

The homologous $3C^{pro}$ from another picornavirus, foot-and-mouth disease virus (FMDV), inhibits gene expression when transiently transfected into BHK cells (30). This inhibition is believed to be caused by cleavage of histone H3, although this has not been shown directly (9). Degradation of histone H3 is not observed in poliovirus-infected cells; thus, although poliovirus and FMDV are in the same virus family, they have different mechanisms for transcriptional inhibition. Interestingly, FMDV $3C^{pro}$ and poliovirus $3C^{pro}$ have different cleavage site specificities (21).

In summary, the results presented here demonstrate that CREB-mediated activated transcription is inhibited by poliovirus infection of human cells via proteolysis of CREB by a virus-specific protease, $3C^{pro}$.

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