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Inhibition of Basal Transcription by Poliovirus: a Virus-Encoded Protease ($3C^{pro}$) Inhibits Formation of TBP-TATA Box Complex In Vitro

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Host cell RNA polymerase II (pol II)-mediated transcription is inhibited by poliovirus infection. We demonstrate here that both TATA- and initiator-mediated basal transcription is inhibited in extracts prepared from poliovirus-infected HeLa cells. This inhibition can be reproduced by incubation of uninfected HeLa cell extracts with purified, recombinant poliovirus protease, $3C^{pro}$. Transient-transfection assays demonstrate that $3C^{pro}$, in the absence of other viral proteins, is able to inhibit cellular pol II-mediated transcription in vivo. Three lines of evidence suggest that inactivation of TATA-binding protein (TBP) is the major cause of inhibition of basal transcription by poliovirus. First, RNA pol II transcription in poliovirus-infected cell extract is fully restored by bacterially expressed TBP. Second, addition of purified TBP restores transcription in heat-treated nuclear extracts from mock- and virus-infected cells to identical levels. Finally, using a gel mobility shift assay, we demonstrate that incubation of TBP with the viral protease ($3C^{pro}$) inhibits its ability to bind TATA sequence in vitro. These results suggest that inhibition of pol II transcription in mammalian cells infected with poliovirus is, at least in part, due to the inability of modified TBP to bind pol II promoter sequences.

Infection of HeLa cells with poliovirus leads to dramatic inhibition of all three classes of host cell RNA polymerases known as host cell transcription shutoff (2, 3, 22). Earlier studies showed that RNA polymerases I, II, and III (pol I, II, and III) from poliovirus-infected cells were fully active, suggesting that transcriptional components other than the polymerases were inactivated in poliovirus-infected cells (1, 12, 37). Although previous studies identified DNA-binding transcription factors in all three polymerase systems to be affected in virus-infected cells, the precise mechanism by which these factors are inactivated remains to be elucidated (19, 24, 34).

Mammalian RNA pol II requires auxiliary factors known as general transcription factors (GTFs) to accurately initiate transcription from a promoter element. To date, eight GTFs have been identified and extensively purified; they are essential for RNA pol II-mediated transcription (11, 16, 18, 30, 31; for reviews, see references 32 and 45). These GTFs, along with RNA pol II, form a preinitiation complex at specific *cis*-acting elements on the DNA template. The most common *cis*-acting element or core promoter is the TATA box, which is situated approximately 25 nucleotides (nt) upstream of the transcription start site (5). One of the GTFs, TFIID, binds to the TATA box and nucleates the formation of a preinitiation complex (35, 36, 44). Some RNA pol II genes, most of which are housekeeping genes, have a second type of core promoter known as the initiator (Inr) element. The initiator sequence overlaps with the start site of transcription and directs accurate initiation of transcription in the absence of a TATA box (26, 39, 43). It also enhances the strength of a promoter that contains the TATA box. The protein that initiates assembly at the initiator is not yet known. Several proteins such as YY1, TFII I, HIP1, RNA pol II, USF, and TATA-binding protein (TBP)-associated factors (TAFs) have been implicated (4, 6, 7, 29, 33, 38). In vitro reconstitution experiments indicate that all the GTFs including TFIID are essential for Inr-mediated transcription. This led to the proposal that the assembly pathways for Inr- and TATA-mediated transcription converge after different initiation events (43). The general transcription factor TFIID plays an important role in initiation from the TATA box. It is a multiprotein complex containing the core TBP and TAFs. TBP, the DNA-binding component of TFIID, is sufficient for basal transcription, but TFIID (TBP plus TAFs) is required for activated transcription from the TATA box. In contrast, TFIID is required for both basal and activated transcription from the initiator element.

Previous studies from our laboratory correlated the decrease in transcription from the adenovirus (Ad) major late promoter (MLP) in poliovirus-infected cell extracts with a specific decrease in the transcriptional activity of partially purified TFIID (24). Recently, using Western immunoblot analysis, we have shown that TBP is proteolytically cleaved in poliovirus-infected cells at the same time postinfection when pol II transcription is inhibited (10). Furthermore, one of the cleaved forms of TBP seen in virus-infected cells can be reproduced in vitro by incubating purified TBP with cloned, purified poliovirus-encoded protease $3C^{pro}$. The normal function of $3C^{pro}$ in virus-infected cells is to process viral precursor proteins into mature polypeptides. The protease is remarkably specific in that it cleaves only Gln-Gly bonds in the viral polyprotein. The cleavage of TBP by $3C^{pro}$ occurs at the 18th glutamine-glycine bond from the N terminus (13). However, studies from other laboratories have shown that this N-terminal region of TBP is not essential for basal transcription, and there is controversy regarding its requirement for activated transcription (28, 47). Previous studies have focused on activated transcription from Ad MLP and the E3 promoters (25) in poliovirus-infected HeLa cells. However, whether poliovirus directly inhibits basal transcription in in-

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fected cells is still not known. Moreover, whether initiator-mediated transcription is also inhibited in poliovirus-infected cells has not been studied yet. In this report, we show that basal transcription mediated by both TATA and Inr elements is inhibited by poliovirus infection. The inhibition of basal transcription can also be reproduced in vitro by incubation of HeLa extracts with cloned, purified $3C^{pro}$. Three lines of evidence suggest that inactivation of TBP is the major cause of inhibition of basal transcription by poliovirus. First, RNA pol II transcription in poliovirus-infected cell extracts is fully restored by exogenous addition of bacterially expressed, purified TBP. Second, addition of purified TBP restores transcription to identical levels in heat-treated nuclear extracts from mock- and virus-infected cells, suggesting that TBP is the major transcription factor inactivated by poliovirus. Finally, using a gel mobility shift assay, we demonstrate that incubation of TBP with the viral protease ($3C^{pro}$) inhibits its ability to bind to the TATA sequence.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were grown in spinner culture in spinner modified essential medium supplemented with 5% newborn calf serum, 1 g of glucose per liter, and 10^5 U each of penicillin and streptomycin. HeLa cells were infected with poliovirus type 1 (Mahoney) (unless otherwise indicated) at a multiplicity of infection of 25 as previously described (8). A poliovirus mutant, sel 3C-02, was also used for infection at a multiplicity of infection of 25 in the experiment in Fig. 5A. 293 monolayer cells were grown in Dulbecco modified Eagle medium supplemented with 10% enriched calf serum.

Extract preparation and protein purification. Nuclear extracts from mock- and poliovirus-infected HeLa cells were prepared as described by Dignam et al. (15) with minor modifications. Instead of dialysis against buffer D, the extracts were precipitated with 50% ammonium sulfate and then centrifuged at 30,000 × *g* for 30 min. The precipitate was dissolved in dialysis buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 12.5 mM $MgCl_2$, 100 mM KCl) and dialyzed against the same buffer overnight. These extracts were aliquoted and stored at −70°C.

Plasmid DNAs. The circular plasmid DNAs 2038 (only TATA), 1634 (TATA plus Inr), A126 (Sp1 plus TATA), 2121 (Sp1 plus Inr), B98 (Sp1 plus Inr), and B96 (Sp1 plus TATA) were a generous gift from Steven Smale, University of California Los Angeles. These plasmids are identical to the plasmids described previously (40, 41, 46). Plasmid 2038 is identical to plasmid II described previously (41). It contains the Ad MLP TATA box inserted into the *Eco*RI and *Sac*I sites of pSP72. Plasmid 1634 is identical to plasmid IV described previously (41). It contains the terminal deoxytransferase (TdT) Inr sequence inserted into pSP72. Plasmid A126 is similar to plasmid V described previously (41). It contains simian virus 40 (SV40) 21-bp repeats (Sp1-binding sites) inserted into the *Bgl*II site of pSP72. This plasmid has 12 Sp1-binding sites instead of 6 Sp1-binding sites as found in plasmid V (41). It also contains the Ad MLP TATA box inserted into *Eco*RI and *Sac*I sites of pSP72. The sequences of the TATA box and regions around it are identical to those of plasmid 3A described previously (46). Plasmid 2121 is similar to plasmid VI as described previously (41). It contains SV40 21-bp repeats inserted into the *Bgl*II site of pSP72 and the TdT Inr sequence inserted into the *Sac*I and *Bam*HI sites. The transcriptional start sites and the length of the accurately initiated transcript from these plasmids have been mapped and characterized, respectively, in references 21, 40, 41, and 46. The promoters described above are diagrammed in Fig. 1A.

Plasmids B96 and B98, used for in vivo transfection, were prepared by cleaving the pSP72-based plasmids described above with *Hpa*I and *Bam*HI and inserting these fragments into pSVPyTK (40) digested with *Cla*I (for blunt ending) and *Bgl*II. Plasmid B96 has SV40 21-bp repeats and the Ad MLP TATA box in pSVPyTK. Plasmid B98 contains SV40 21-bp repeats and the TdT Inr sequence in pSVPyTK. pSVPyTK contains an SV40 origin of replication, a polyomavirus origin of replication, a large T-antigen gene, and the herpes simplex virus thymidine kinase gene. Plasmids B96 and B98 were used for transient transfections.

In vitro transcription assay. Transcription reaction mixtures contained 450 μg of template DNA, 150 μg of HeLa nuclear extracts (mock or poliovirus infected), 2.5 mM ribonucleoside triphosphates (rNTPs), and 100 U of RNasin, and the reaction volume was made up to 50 μl with TM 0.1 buffer (50 mM Tris [pH 7.4], 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 100 mM KCl). These reaction mixtures were incubated at 30°C for 90 min, and the reactions were terminated by the addition of 90 μl of stop solution (200 mM NaCl, 20 mM EDTA, 1% sodium dodecyl sulfate, 250 μg of tRNA per ml). The RNA was extracted with phenol-chloroform followed by ethanol precipitation. The RNA was annealed with 50,000 cpm of Sp6 primer in 10 μl of annealing buffer (250 mM NaCl, 5 mM Tris [pH 7.4], 1 mM EDTA) for 90 min. This annealed mix was analyzed by the primer extension method. The primer extension reaction mixture contained 10 μl of the above annealed mix, 1 mM dithiothreitol, reverse transcriptase buffer (50 mM Tris [pH 8.3], 8 mM $MgCl_2$, 50 mM KCl, 1.5 mM dNTPs) and 40 U of avian myeloblastosis virus reverse transcriptase (Life Science Inc.) 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), loaded onto an 8% acrylamide–8 M urea gel, and subjected to electrophoresis.

Transient transfections. $3C^{pro}$ was cloned into a replication-competent eukaryotic vector, pcDNA (Invitrogen) (9). 293 cells were cotransfected with 5 μg of B98 or B96 and 5 μg of $3C^{pro}$ or pcDNA by the calcium phosphate method. Total RNA was isolated from transfected cells 48 h posttransfection with Tri-Reagent (Molecular Sciences Inc.). The RNA was annealed with 50,000 cpm of thymidine kinase (TK) primer and analyzed by primer extension as indicated.

Protease reactions. $3C^{pro}$ and the $3C^{pro}$ C147S mutant were overexpressed and purified as previously described (20, 27). For $3C^{pro}$ reaction, 150 μg of HeLa nuclear extract was incubated with 4 μg of $3C^{pro}$ or $3C^{pro}$ C147S for 2 h at 30°C. These extracts were then used in an in vitro transcription assay.

Gel shift analysis. Gel retardation assay mixtures (12.5 μl) contained 50 mM HEPES (pH 7.9), 0.05 mM EDTA, 5% glycerol, 75 mM $MgCl_2$, 140 mM β-mercaptoethanol, 0.1 μg of poly(dI-dC), 40 mM KCl, 5 μg of bovine serum albumin (BSA), and 100 ng of purified TBP as previously described (5a, 23). TBP was cloned into pQE 30 (Qiagen) and overexpressed in *Escherichia coli*. TBP was purified by heparin-Sepharose and nickel chromatography and used in gel shift analysis. Synthetic double-stranded oligonucleotides containing the TFIID-binding region (Santa Cruz Biotech, Inc.) were end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. End-labeled oligonucleotides (25,000 cpm) were added to the above reaction mixes, which were then incubated at 30°C for 40 min. DNA-protein complexes were resolved on a 6% polyacrylamide gel (acrylamide/bis acrylamide ratio, 60:1) in 0.5× TBE (1× TBE is 89 mM Tris, 89 mM boric acid, plus 2 mM EDTA). The gels were dried and subjected to autoradiography.

RESULTS

Poliovirus infection inhibits basal transcription. Previous studies from our laboratory showed that infection of HeLa cells with poliovirus resulted in drastic inhibition of TATA-mediated USF-activated transcription from the Ad MLP (10, 24). This could be due to either inhibition of basal transcription or inhibition of activated transcription or both. To find whether basal transcription is directly affected by poliovirus infection of mammalian cells, cell extracts were prepared from mock- and virus-infected cells and their ability to initiate accurate transcription from both basal and activated promoters was examined by a primer extension assay. Constructs containing only TATA and TATA plus Inr sequence were used to measure basal transcription (Fig. 1A). Activated transcription was examined by using constructs containing either Sp1 plus TATA or Sp1 plus Inr sequences (Fig. 1A). HeLa cells were either mock or poliovirus infected for 1.5, 3, 4, and 5 h, and extracts prepared from these time points were used in in vitro transcription reactions. Accurate transcription from pl2038 (TATA only) and plA126 (Sp1 plus TATA) produces a 70-nt transcript, while the correctly initiated transcript from pl1634 (Inr plus TATA) and pl2121 (Sp1 plus Inr) is 79 nt long. As can be seen in Fig. 1B, significant inhibition of synthesis of the 70-nt transcript was apparent from the TATA-containing promoter at 3 h postinfection (lane 3). Transcription from this promoter was almost totally inhibited at 4 h postinfection (lane 4). A similar result was obtained with the promoter containing both TATA and the Inr element (Fig. 1C). As expected, basal transcription from the TATA plus Inr promoter was much stronger than from the TATA promoter alone. In both cases, transcription was totally inhibited at 4 h postinfection. We were unable to detect transcription from the promoter containing the Inr element alone (data not shown). Thus, poliovirus infection resulted in the inhibition of basal transcription mediated by TATA and Inr elements. Although not shown here, the kinetics of inhibition of basal transcription in vitro correlated with the time course of shutoff of cellular transcription observed in vivo (reference 12 and data not shown).

A similar kinetics of transcription inhibition was observed when Sp1-activated transcription was examined from the

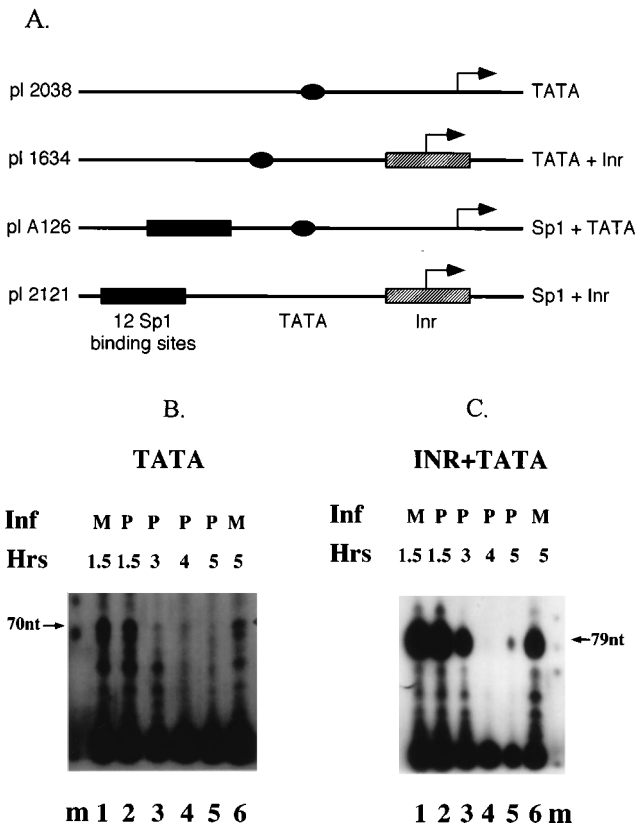

FIG. 1. Inhibition of TATA- and Inr-mediated transcription in poliovirus-infected cell extracts. In vitro transcription reactions were performed with HeLa cell extracts prepared at different times postinfection. The template DNAs had either Ad MLP TATA or TdT Inr sequence inserted into plasmid pSP72 (see Materials and Methods) (40). The transcribed RNA was analyzed by primer extension with a ^{32}P-labelled Sp6 promoter primer (Promega). The products were analyzed on an 8% denaturing gel. (A) The promoters used for in vitro transcription were as described in Materials and Methods. Plasmid 2038 contains only TATA box (solid oval). Plasmid 1634 contains both TATA and initiator (Inr, shaded rectangle). Plasmid A126 contains TATA and 12 Sp1 sites upstream of the TATA box (solid rectangle). Plasmid 2121 contains Inr and Sp1-binding sites. These plasmids are described in reference 41. The arrows indicate transcription start sites from these promoters. (B) Effect of poliovirus on TATA-mediated transcription: The template DNA used for these reactions was pl2038. This plasmid has the Ad MLP TATA box inserted into pSP72 (Promega) and directs basal transcription from the TATA promoter (40). In vitro transcription reactions were performed with mock-infected (M) HeLa cell extracts prepared 1.5 and 5 h p.i. (lanes 1 and 6) or with poliovirus-infected (P) HeLa cell extracts prepared 1.5, 3, 4, and 5 h p.i. (lanes 2 through 5). RNA was analyzed by primer extension, and the size of the extension product was 70 bp. The arrow indicates the size of the expected products. The DNA marker used was pBR322/HpaII labelled with ^{32}P (lane m). The sizes of the DNA marker fragments are 67 and 76 bp from bottom to top. (C) Effect of poliovirus on basal transcription from TATA and Inr elements. The template used for these reactions was pl1634. This plasmid has Ad MLP TATA and TdT Inr sequences inserted into pSP72 (Promega) and directs basal transcription. In vitro transcription reactions were performed with mock-infected (M) cell extracts prepared 1.5 and 5 h p.i (lanes 1 and 6) or with poliovirus-infected (P) HeLa cell extracts prepared 1.5, 3, 4, and 5 h p.i. (lanes 2 through 5). RNA was analyzed by primer extension, and the size of the extended products was 79 bp (40). The arrow indicates the size of correctly initiated transcription products. The DNA marker used was pBR322/HpaII labeled with 32P (lane m). The lengths of the markers are 67, 76, and 90 bp from bottom to top.

TATA- and Inr-containing promoter constructs (Fig. 2). In both cases, transcription inhibition was complete by 4 h postinfection. Although correlative, these results are consistent with the notion that inhibition of activated transcription may be due at least in part to poliovirus-induced inhibition of basal transcription.

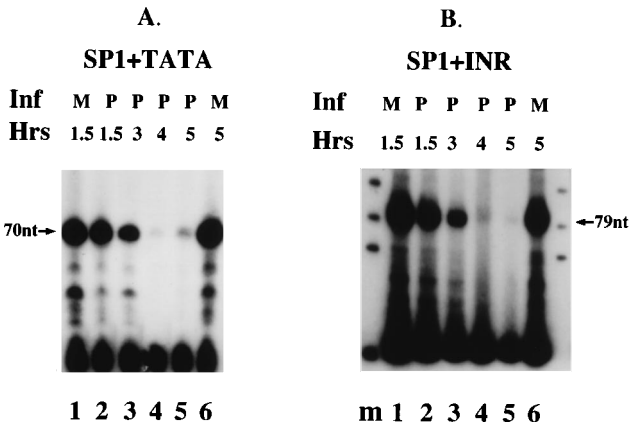

FIG. 2. Inhibition of Sp1-activated transcription by poliovirus infection. In vitro transcription reactions were analyzed by primer extension as described in the legend to Fig. 1 and reference 40. (A) Effect of poliovirus on Sp1-activated transcription from the TATA element. The template DNA used for transcription reactions was plA126. This plasmid has an Ad MLP TATA box and SV40 21-bp repeats (Sp1-binding sites) inserted into pSP72 (40). In vitro transcription reactions were performed with mock-infected (M) HeLa cell extracts prepared 1.5 and 5 h p.i. (lanes 1 and 6) or with poliovirus-infected (P) HeLa cell extracts prepared 1.5, 3, 4, and 5 h p.i. (lanes 2 through 5). The RNA transcript synthesized was analyzed by primer extension analysis, and the position of the correctly initiated transcript (70 bp) is indicated by the arrow. (B) Effect of poliovirus on Sp1-activated transcription from Inr. The template DNA used was pl2121. This plasmid has TdT Inr and SV40 21-bp repeats (Sp1-binding sites) inserted into pSP72. In vitro transcription reactions were performed with mock-infected (M) HeLa cell extracts prepared 1.5 and 5 h p.i. (lanes 1 and 6) or with poliovirus-infected (P) HeLa cell extracts prepared 1.5, 3, 4, and 5 h p.i. (lanes 2 through 5). The size of the correctly initiated product was 79 bp, as indicated by an arrow. The above-mentioned lanes are flanked by pBR322/HpaII markers of 67, 76, and 90 bp (lane m).

Poliovirus-encoded protease 3C^{pro} inhibits basal transcription. In a previous study, we demonstrated inhibition of Ad MLP-mediated, USF-activated transcription when cell extracts were treated with bacterially expressed, purified poliovirus 3C^{pro} (10). Here we have used both genetic and biochemical approaches to examine whether TATA- and Inr-mediated basal transcription is inhibited by the viral protease 3C^{pro}.

A mutant poliovirus (sel 3C-02), which has a valine-to-alanine substitution at amino acid 54 of 3C^{pro}, was used to determine if it was defective in inhibiting basal transcription. Upon infection of HeLa cells, this virus produces very little active 3C^{pro} and consequently is a small-plaque mutant (14). It still grows to nearly wild-type titers because poliovirus overproduces 3C^{pro} and other viral proteins (14). The amount of viral RNA synthesized in cells infected with the mutant sel 3C-02 was approximately 40% of that of the wild-type virus. Initial plaque assays revealed that the mutant virus grew to titers of approximately 1 log unit/ml less than that observed for transfection-derived wild-type stocks (14). It is important to note that this mutant protease is not totally defective but is much less active than the wild-type protease (14). Earlier studies from our laboratory demonstrated that this mutant virus was defective in shutting off cellular RNA pol III transcription (9). HeLa cells were either mock infected or infected with the wild-type or mutant virus. Extracts were prepared from mock- and virus-infected cells and assayed for transcription from various promoter constructs. As can be seen in Fig. 3, transcription from both the TATA and TATA-plus-Inr constructs was almost totally inhibited as a result of infection with the wild-type virus (Fig. 3, lanes 1). However, the mutant virus was much less effective in inhibiting basal transcription from these promoters (lanes 2). Quantitation of these results showed 0 to

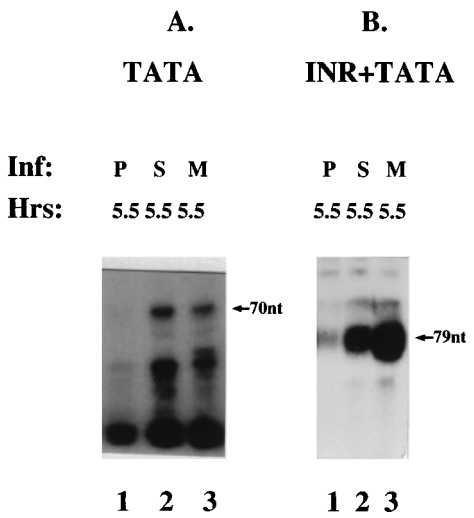

FIG. 3. Inhibition of basal transcription by poliovirus mutant sel 3C-02. Shown is in vitro transcriptional analysis of HeLa cell extracts prepared 5.5 h p.i. The DNA templates used for each reaction are indicated at the top. Transcription from the TATA (A) and Inr-plus-TATA (B) promoter was analyzed by primer extension as described in the legend to Fig. 1. Transcription reactions were performed following 5.5 h of mock infection (lane 3) and virus infection with the wild type (lane 1) or the sel 3C-02 mutant (lane 2). The arrow indicates the correctly initiated product.

20% inhibition with the mutant whereas the wild-type virus inhibited transcription almost 100% compared with that in mock-infected cells (Fig. 3). Thus, a mutant poliovirus defective in $3C^{pro}$ activity was unable to fully inhibit basal transcription.

To confirm the results of the genetic studies, biochemical studies were performed with bacterially expressed, purified poliovirus $3C^{pro}$. In these experiments, nuclear extracts prepared from mock-infected cells were incubated with either the wild-type protease or a mutant $3C^{pro}$ (C147S) as a negative control. This mutant protease has a single substitution (cysteine to serine) at amino acid 147 and has no protease activity. Both the wild-type and mutant proteases were overexpressed in *E. coli* and purified to near homogeneity (20, 27). Nuclear extracts treated with these proteases were examined for their ability to support TATA- and Inr-mediated basal and activated transcription. As can be seen in Fig. 4, basal transcription from both the TATA and TATA-plus-Inr constructs was almost totally inhibited by the wild-type, purified protease whereas the mutant protease had no significant effect on transcription (Fig. 4A and B, compare lanes 2 and 3). Similarly, activated transcription from Sp1-plus-TATA and Sp1-plus-Inr constructs was significantly inhibited by the wild-type protease but not the mutant protease (Fig. 4C and D). Thus, poliovirus-encoded $3C^{pro}$ inhibits both basal and activated transcription from TATA- and Inr-containing promoters both in vitro (Fig. 4) and in vivo (Fig. 3).

Expression of the $3C^{pro}$ gene in animal cells is sufficient to inhibit TATA- and Inr-mediated transcription. To examine whether $3C^{pro}$ is sufficient to cause inhibition of host cell transcription seen in poliovirus-infected cells, a transient-transfection assay was used. $3C^{pro}$ was cloned into the eukaryotic expression vector pcDNA. In this construct ($pP3C^{pro}$), the $3C^{pro}$ gene was placed downstream of a promoter sequence from the immediate-early gene of human cytomegalovirus. To study the effect of $3C^{pro}$, reporter plasmids (B98 and B96) that support RNA pol II transcription in vivo were used. These reporter plasmids contain the initiator element (B98) or TATA promoter (B96) inserted into plPSVTy PK with the TK reporter gene and SV40 origin of replication. The reporter plasmids were transfected into 293 cells along with $pP3C^{pro}$ or pcDNA (vector), and after 48 h, RNA was isolated and analyzed by primer extension with TK primer. Cells transfected with B98 or B96 support Sp1-activated transcription from the Inr element or TATA box to give TK RNA (Fig. 5, lanes 1 and 5). The transcriptional start site of TATA promoter is 9 nt downstream of Inr element. Therefore, the transcript synthesized by Inr is 9 nt longer than that from the TATA promoter. When cells were transfected with the plasmid containing the $3C^{pro}$ gene, transcription from both the TATA- and Inr-containing constructs was significantly inhibited (lanes 2 and 6). No significant inhibition of transcription was observed in cells

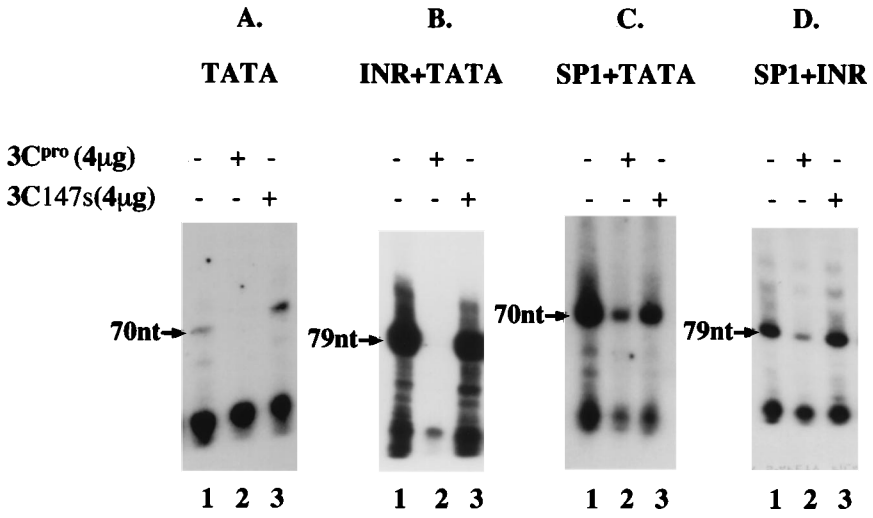

FIG. 4. Effect of purified $3C^{pro}$ on basal and activated transcription. Nuclear extracts prepared from uninfected HeLa cells were treated with *E. coli*-expressed and purified $3C^{pro}$ before being used in an in vitro transcription reaction. In vitro transcription reactions were performed as indicated in the legend to Fig. 1. The DNA templates used for the transcription reactions were plasmids 2038 (A), 1634 (B), A126 (C), and 2121 (D). TATA- (A), TATA- plus Inr- (B), Sp1- plus TATA- (C), and Sp1- plus Inr (D)-mediated transcription was determined in the absence (lanes 1) or presence of bacterially expressed purified wild-type protease $3C^{pro}$ (lanes 2) or an inactive protease mutant, 3C 147s (lanes 3).

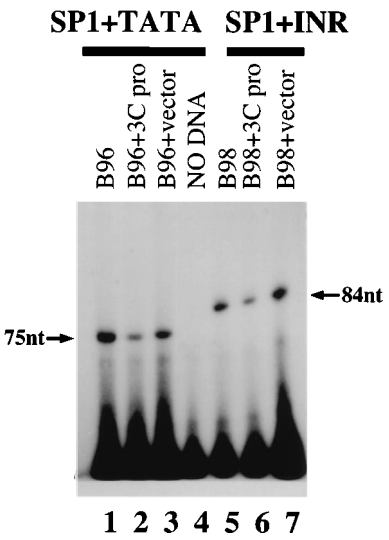

FIG. 5. In vivo analysis of the effect of $3C^{pro}$ on RNA pol II transcription. $3C^{pro}$ was inserted into the vector pcDNA (Invitrogen), a replication-competent plasmid. Sp1-plus-TATA or Sp1-plus-Inr sequences were inserted into plasmid pSVPyTK (40) to give plB96 and plB98, respectively. The transcriptional start site of TATA promoter was 9 nt downstream of the Inr element; hence, transcripts of differing mobilities are apparent from B98 and B96. The length of the transcript from B96 is 75 nt. The length of transcript from B98 is 84 nt. $3C^{pro}$ was cotransfected with plasmid B96 or plasmid B98 into 293 cells. After 48 h, cytoplasmic RNA was isolated and analyzed by primer extension analysis. Lanes: 1, transcription from plB96 (Sp1-activated transcription from TATA box); 2, B96 transcription in the presence of $3C^{pro}$; 3, B96 transcription in the presence of the vector pcDNA containing no $3C^{pro}$ insert; 4, no-DNA control; 5, transcription from plB98 (Sp1-activated transcription from the Inr element); 6, B98 transcription in the presence of $pc3C^{pro}$; 7, B98 transcription in the presence of the vector pcDNA containing no $3C^{pro}$ insert.

transfected with the vector alone (lanes 3 and 7). Cells transfected with the $3C^{pro}$-containing plasmid did not show any morphological differences from those transfected with the vector DNA alone. Earlier studies on inhibition of RNA pol III transcription by poliovirus in our laboratory showed that $3C^{pro}$ did not decrease the viability of transfected cells within the time frame used for these experiments (9). Our attempts to measure TATA- and Inr-mediated basal transcriptions in vivo have not been successful because of the very low level of transcription mediated by these promoters in the absence of activators (data not shown). Thus, we were unable to determine effect of $3C^{pro}$ on basal transcription in vivo by using transient-transfection assays.

Recombinant TBP restores TATA- and Inr-mediated transcription in poliovirus infected cell extract. Earlier studies from our laboratory correlated the decrease in pol II transcription in poliovirus-infected cell extracts with a specific decrease in the transcriptional activity of partially purified TFIID (24). TFIID is a multiprotein complex and contains TBP and TAFs. TBP is sufficient for basal transcription, while TBP and TAFs are both required for TATA-mediated activated transcription. To determine the specific component of TFIID inactivated in poliovirus-infected HeLa cells, bacterially expressed purified TBP was used to restore transcription in virus-infected cell extracts. With all three promoters tested (Sp1 plus TATA, TATA plus Inr, and Sp1 plus Inr), addition of purified TBP resulted in restoration of transcription in infected-cell extracts to the extent seen in mock-infected cell extracts (Fig. 6, compare lanes 6 with lanes 1). Transcription from the Sp1-plus-Inr construct in mock-infected cell was stimulated twofold by addition of TBP, suggesting that TBP was limiting in mock-infected extract for Sp1-activated transcription from the TATA promoter (Fig. 6A, lanes 1 and 3). However, transcription in infected-cell extracts was stimulated sixfold (lanes 4 and 6) by TBP addition. Transcription from Sp1-plus-Inr and TATA-plus-Inr promoters in mock-infected cell extracts was stimulated only 1.5-fold by TBP addition (Fig. 6B and C, lanes 1 to 3). However, the increase in transcription in poliovirus-infected cell extracts was ~15-fold by addition of TBP (lanes 4 to 6). These results are consistent with the idea that TBP is the major transcription factor inactivated in poliovirus-infected cells.

To further examine whether TBP is the only component inactivated in poliovirus-infected cells, transcription in heat-treated nuclear extracts was examined. Heat treatment of nuclear extracts at 47°C for 10 to 15 min preferentially inactivates TBP (44). Recombinant TBP restores both basal and activated transcription in heat-treated, uninfected cell extracts (28). If TBP was the only component inactivated in virus-infected cell

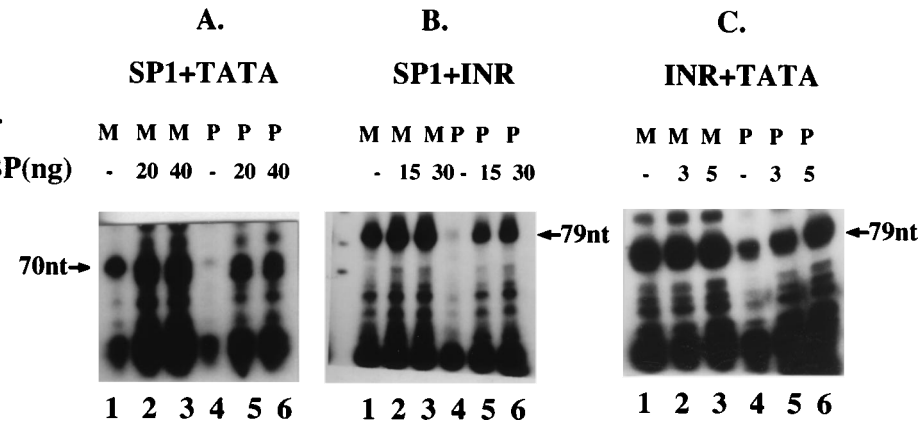

FIG. 6. Restoration of transcription by TBP. Recombinant TBP was added to mock-infected and poliovirus-infected cell extracts. These TBP-supplemented extracts were then used for in vitro transcriptional analysis as described in the legend to Fig. 1. The DNA templates used for transcription contained Sp1 plus TATA (A), Sp1 plus Inr (B), and Inr plus TATA (C) sequence elements. Transcription reactions were performed with mock-infected nuclear extract (lanes 1) and mock-infected nuclear extracts supplemented with increasing amounts of TBP (lanes 2 and 3). Transcription reactions were also performed with poliovirus-infected extracts prepared 4 h p.i. (lanes 4) and poliovirus-infected extracts supplemented with increasing amounts of TBP (lanes 5 and 6). The amount of TBP added is indicated at the top. Panel B has the pBR/HpaII marker in the far-left lane.

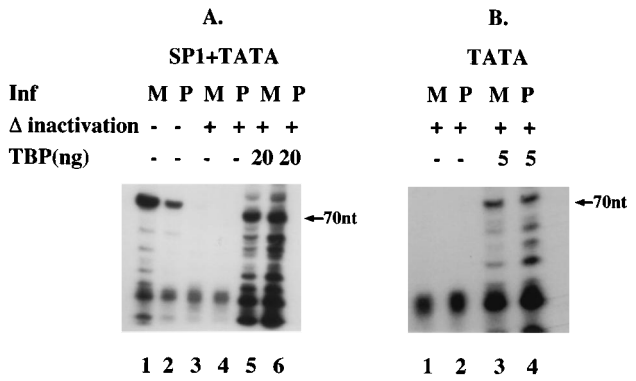

FIG. 7. Restoration of transcription by TBP in heat-inactivated poliovirus-infected extracts. Poliovirus-infected (P) and mock-infected (M) cell extracts (150 μg) were heated at 47°C for 15 min. Transcription-restoring activity of TBP was assayed in both poliovirus- and mock-infected cell extracts. (A) Restoration of Sp1-activated transcription from the TATA promoter. Lanes: 1, mock-infected cell extract prepared 4 h p.i.; 2, poliovirus-infected cell extract prepared 4 h p.i.; 3, heat-treated mock-infected extract; 4, heat-treated poliovirus-infected extract; 5, heat-treated mock-infected cell extract with 20 ng of TBP; 6, heat-treated poliovirus-infected extract with 20 ng of TBP. (B) Restoration of TATA-mediated basal transcription in heat-treated mock-infected (M) and poliovirus-infected (P) extracts. Lanes: 1, heat-treated mock-infected extract; 2, heat-treated poliovirus-infected extract; 3, heat-treated mock-infected extract plus 5 ng of TBP; 4, heat-treated poliovirus-infected extract plus 5 ng of TBP.

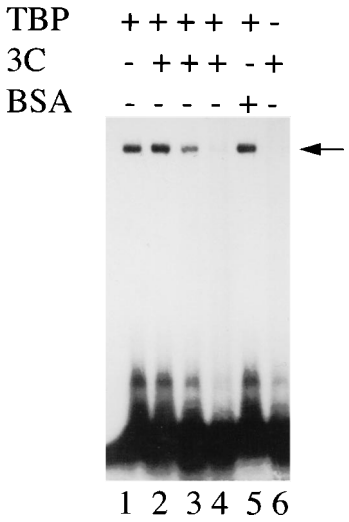

FIG. 8. Gel retardation analysis of TBP binding to the TATA box following $3C^{pro}$ treatment. Gel retardation assays were performed with 150 ng of TBP as described in Materials and Methods without $3C^{pro}$ (lane 1) or treated with 1, 4, or 8 μg of $3C^{pro}$ (lanes 2 through 4) or 8 μg of BSA (lane 5). Lane 6 contained 8 μg of $3C^{pro}$ but no TBP.

extract, it would be possible to restore transcription (by addition of TBP) in heat-treated, virus-infected cell extracts to the level observed in heat-treated mock-infected cell extracts. Figure 7A shows TATA-mediated Sp1-activated transcription in heat-treated mock- and poliovirus-infected cell extracts. As a control, transcription from mock- and poliovirus-infected extracts with no heat treatment is shown (Fig. 7A, lanes 1 and 2). Upon heat treatment, there was complete inhibition of transcription in both mock- and poliovirus-infected extracts (lanes 3 and 4). Transcription was restored to almost identical levels in heat-treated mock- and poliovirus-infected extracts upon addition of purified recombinant TBP (lanes 5 and 6). A similar result was obtained when a construct containing a TATA element was used in the transcription reaction (Fig. 7B). These results suggest that TBP is most probably the only transcription factor inactivated in virus-infected cells.

Poliovirus protease $3C^{pro}$ inhibits TBP binding to the TATA sequence. To determine how $3C^{pro}$ inhibits transcriptional activity of TBP in poliovirus-infected HeLa cells, the TATA box-binding ability of purified recombinant TBP was analyzed following its incubation with purified $3C^{pro}$. As shown in Fig. 8, a TBP-TATA box complex was clearly detected when TBP was incubated with a TATA box-containing oligonucleotide (Fig. 8, lane 1). This complex can be specifically competed out with an unlabeled TATA box containing oligonucleotide but not with a nonspecific oligonucleotide (data not shown). Formation of the DNA-protein complex was inhibited in a concentration-dependent manner by incubation of TBP with $3C^{pro}$ (lanes 2 to 4). No TATA box-binding activity was associated with $3C^{pro}$ alone (lane 6), and incubation of TBP with BSA did not interfere with its ability to form the retarded complex. A parallel experiment with identical levels of labeled TBP and various concentrations of unlabeled $3C^{pro}$ showed disappearance of full-length TBP with concomitant generation of two proteolytic products having approximate molecular masses of 40 and 29 kDa (data not shown). Together with our previous result that TBP is cleaved by $3C^{pro}$ both in vivo and in vitro (10), these results suggest that purified $3C^{pro}$ inhibits TBP binding to the TATA box by proteolytic cleavage of TBP.

DISCUSSION

Soon after infection of mammalian cells with poliovirus, host cell transcription is almost totally inhibited. Transcriptions catalyzed by RNA pol I, II, and III are all inhibited by virus infection. We have demonstrated here that TATA- and Inr-mediated basal transcription, as well as Sp1-activated pol II transcription, is inhibited in virus-infected cells. We have provided three lines of evidence suggesting that a poliovirus-encoded protease ($3C^{pro}$) is involved in shutoff of basal and activated transcription. First, a virus with a mutation in the $3C^{pro}$ gene is defective in efficiently inhibiting host cell transcription compared with the wild-type virus. Second, in vitro transcription from TATA- and Inr-containing promoters in HeLa nuclear extracts was drastically inhibited by exogenous addition of recombinant, purified $3C^{pro}$ but not by a purified protease with a single amino acid mutation in the active site. Third, introduction of an active protease gene in transiently transfected cells caused significant inhibition of TATA- and Inr-mediated transcription in these cells. We have also provided multiple lines of evidence suggesting that TBP is the major transcription factor inactivated in poliovirus-infected cells. First, both TATA- and Inr-mediated basal and Sp1-activated transcription in infected cell extracts can be rescued by addition of bacterially expressed, purified TBP. Next, addition of purified TBP to TBP-deficient, heat-inactivated cell extracts results in restoration of transcription to identical levels in both mock- and poliovirus-infected extracts. All these results strongly suggest that TBP is the sole factor inactivated in virus-infected cells.

The role of the TATA box in basal and activated transcription has been clearly established by numerous studies from many laboratories. Previous results from our laboratory and data presented here clearly suggest that poliovirus inhibits TATA-mediated basal transcription by proteolytic cleavage of TBP (10). We assume, although we have not shown, that inhibition of Sp1-activated, TATA-mediated transcription is a result of inhibition of TATA-mediated basal transcription. This conclusion is supported by the fact that Sp1 itself is not proteolytically cleaved by $3C^{pro}$ (data not shown) and that

TAFs also remain unaltered following incubation with $3C^{pro}$ (13a).

The precise function of TBP in initiator-mediated transcription is not yet known. Studies have shown that a factor able to associate with TBP is rate limiting for TATA-less promoters. Our results suggest that poliovirus inhibits both TATA- and initiator-mediated transcription by inactivating TBP.

Earlier studies from our laboratory showed that TBP is directly cleaved by purified $3C^{pro}$ in vitro (10). This cleavage was shown to be at the N-terminal 18th amino acid (Gln-18–Gly-19) (13). This result was surprising, because almost the whole N-terminal half of TBP was found not to be essential for the ability of this protein to reconstitute in vitro transcription (28, 47). We have recently engineered and overexpressed a TBP mutant (Δ18N) that lacks its 18 N-terminal amino acids. This mutant (Δ18N) TBP was found to be fully transcriptionally active, suggesting that the cleavage of the Gln-Gly bond at the junction of amino acids 18 and 19 of TBP was not responsible for the inactivation of TBP seen in poliovirus-infected cells. The inactivation of TBP might be due to an additional cleavage that was not detected earlier. In fact, incubation of TBP with freshly prepared $3C^{pro}$ generated two distinct bands, a 40-kDa band and a 29-kDa band (44a). The 40-kDa band was shown to be Δ18N TBP. The identity of the 29-kDa band is not yet known. Future studies will determine whether generation of the 29-kDa band is the cause of transcriptional inactivation of TBP.

Results presented in Fig. 8 clearly demonstrate that treatment of purified TBP with recombinant, purified $3C^{pro}$ inhibits formation of TBP-TATA complex in vitro. This is consistent with our previously published data that $3C^{pro}$ proteolytically cleaves TBP both in vivo and in vitro. These results suggest that transcription inhibition by poliovirus is due to the inability of the protease-modified TBP to bind the TATA sequence. It must be pointed out, however, that we have not shown whether cleavage of TBP by $3C^{pro}$ leads to inhibition of TBP binding to the TATA sequence in vivo. Future studies will be directed toward examining whether TBP binding to the TATA box is impaired in virus-infected cells. It is worth mentioning that immunohistochemical studies have shown that the viral protease $3C^{pro}$ migrates to the nucleus after infection of cells with poliovirus (17). Thus, TBP and $3C^{pro}$ could interact in the nucleus of infected cells.

Recent evidence suggests a more active role of poliovirus-encoded protease $3CD^{pro}$, a precursor of $3C^{pro}$, in viral replication. The results presented here do not rule out the possibility that $3CD^{pro}$ may be involved in proteolytic inactivation of TBP in virus-infected HeLa cells. $3C^{pro}$, $3CD^{pro}$, or both may be involved in cellular transcription shutoff. However, bacterially expressed, proteolytically active 3CD was unable to cleave TBP in vitro (3a). We therefore think that it is unlikely that $3CD^{pro}$ is involved in host transcription shutoff.

Although poliovirus encodes three proteases, poliovirus infection does not result in large-scale or random proteolysis of viral or cellular proteins (42). The lack of large-scale modification of cellular proteins in poliovirus-infected cells argues that the proteolysis of TBP is a specific event and is not due to generalized proteolysis in infected cells. A protease designed to cleave viral polyproteins is not likely to engage itself in random cleavage of cellular proteins, because it may be detrimental to viral growth and replication. The primary cellular transcription targets of $3C^{pro}$ appear to be TBP and the pol III transcription factor TFIIIC (9, 10). In both cases, $3C^{pro}$ appears to cleave Gln-Gly bonds, as it normally does in the processing of viral polyprotein. However, a pol I transcription factor, UBF-1, which also contains Gln-Gly bonds, is not cleaved by $3C^{pro}$. Whether cleavage of TBP by the viral protease contributes to inhibition of pol I- and pol III-transcribed genes in poliovirus-infected cells remains an open question. In summary, the results presented here suggest a possible mechanism by which a pathogenic human RNA virus inhibits cellular pol II transcription, which eventually helps the virus to express its own genes.

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