

## Infection of HeLa Cells with Poliovirus Results in Modification of a Complex That Binds to the rRNA Promoter

SHERYL J. RUBINSTEIN,<sup>1</sup> THOMAS HAMMERLE,<sup>2</sup> ECKARD WIMMER,<sup>2</sup> AND ASIM DASGUPTA<sup>1\*</sup>

*Department of Microbiology and Immunology and Jonsson Comprehensive Cancer Center, UCLA School of Medicine, and Molecular Biology Institute, University of California at Los Angeles, Los Angeles, California 90024-1747,<sup>1</sup> and Department of Microbiology, School of Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794<sup>2</sup>*

Received 29 October 1991/Accepted 15 February 1992

**In HeLa cells, RNA polymerase I (Pol I)-mediated transcription is severely inhibited soon after infection with poliovirus. We have developed a gel retardation assay to analyze DNA-protein complexes formed at the Pol I promoter. We show here that two complexes (A and C) formed by nuclear extracts from uninfected cells disappear after infection of cells with poliovirus. In contrast, a new, rapidly migrating complex (D) is formed in virus-infected cell extract. This change in the mobility of gel-retarded complexes correlates well with the kinetics of inhibition of rRNA transcription in virus-infected cells. Incubation of nuclear extracts from mock-infected cells with bacterially expressed, purified poliovirus protease 3C results in the disappearance of complexes A and C with concomitant generation of complex D. A partially purified transcription factor fraction derived from uninfected cells that contains complex A is able to restore Pol I transcription when added to virus-infected cell extracts, suggesting that this complex plays an important role in Pol I transcription. These results suggest that poliovirus proteinase 3C may have an important role in the shutoff of Pol I transcription in cells infected with poliovirus.**

Poliovirus, a member of the picornavirus family, contains a positive-sense single-stranded RNA genome. This messenger-active RNA encodes a single long open reading frame (35). The reading frame is translated, yielding a large polyprotein that is cleaved by poliovirus-encoded proteases, producing all structural and nonstructural proteins. To date, two poliovirus-specific proteases, 2A and 3C, have been identified (for a review, see references 18 and 26). Protease 3C has been expressed in *Escherichia coli* and purified (30). Protease 3C cleaves viral precursor proteins specifically at glutamine-glycine junctions (17). Infection of cells by poliovirus causes extreme cytopathic effects. The rate of cellular protein and RNA synthesis decreases rapidly after infection. It is believed that the full inhibition of cellular macromolecular metabolism requires the synthesis of viral proteins, but these proteins have not yet been identified (for a review, see reference 35).

When HeLa cells are infected with poliovirus, host cell RNA synthesis is inhibited. All three classes of transcription, which are catalyzed by polymerases (Pol) I, II, and III, are inhibited (8, 10, 42). This inhibition is due to the decrease in activity of factors involved in the specific initiation of transcription; nonspecific Pol elongation activity is not affected (9, 14, 19, 23, 34). The development of *in vitro* transcription systems has allowed the analysis of this inhibition; the transcription activity observed *in vitro* with extracts prepared from infected cells mimics the inhibition observed *in vivo* for all three transcription systems (9, 34).

In a previous report we examined the inhibition of transcription of rRNA in poliovirus-infected HeLa cells. We found that transcription could be restored in extracts from poliovirus-infected cells by the addition of a factor closely associated with Pol I. Although restoration activity coeluted with Pol I nonspecific chain elongation activity, extracts

prepared from mock- and poliovirus-infected cells contained the same levels of elongation activity (34). In this report, we further characterize the effects of virus-mediated inhibition of rRNA transcription by examining the modification of factors involved in rRNA initiation complex formation.

Three factors have been identified as being necessary for transcription by Pol I (36). The first, RNA Pol I, cannot initiate transcription specifically at the promoter without the aid of accessory factors (29). Pol I is capable only of nonspecific chain elongation. It has been proposed that another activated form of RNA Pol I, which is involved in the specific initiation of transcription, exists. These results, obtained by fractionation of extracts from *Acanthamoeba* (33) and mouse (39) cells, have yet to be corroborated in the human rRNA transcription system. A second transcription factor, the upstream binding factor (UBF), protects a region of the class I promoter, the upstream control element (UCE) which is located between nucleotides -200 and -107 on the human gene coding for rRNA (rDNA gene). UBF also associates with the core promoter element, located between nucleotides +20 and -45 relative to the site of initiation of transcription (1, 27). UBF activity has been extensively purified and has recently been cloned (1, 20). The third activity, named SL1, has not yet been purified to homogeneity (28). While human SL1 alone does not footprint over the promoter when assayed in conjunction with UBF, the footprinted region over the UCE is extended. This extension is due either to the binding of SL1 to the promoter in the presence of UBF or to a conformational change in the UBF binding domain induced by an interaction with SL1 (1, 27).

Recent experiments in our laboratory have indicated that the inhibition of RNA Pol III and II transcription seen in poliovirus-infected cells may be induced by poliovirus protease 3C. The inhibition of RNA Pol III transcription mediated by poliovirus infection involves the inhibition of transcription factor TFIIC activity (14). TFIIC isolated from mock- and poliovirus-infected cells forms differently migrat-

\* Corresponding author.

ing complexes with the VAIRNA gene when analyzed by using a gel retardation assay (5). TFIIC purified from poliovirus-infected cells forms a rapidly migrating complex with the VAIRNA gene; in our laboratory, it has been recently determined that this change in complex conformation can be duplicated by *in vitro* treatment of TFIIC with purified poliovirus protease 3C (7). In the Pol II system, we have also shown that the TATA box-binding general transcription factor, TFIID, is specifically cleaved by purified 3C proteinase *in vitro* (6). Therefore, a possible mechanism involved in the inhibition of Pol II- and Pol III-mediated transcription by poliovirus seems to involve cleavage of sequence-specific DNA-binding transcription factors (TFIID and TFIIC) by a virus-encoded protease.

To investigate whether a similar mechanism is responsible for the shutoff of Pol I transcription by poliovirus, we have developed a gel retardation assay specific to the class I promoter. This allowed us to look at DNA-protein complexes formed at the UCE presumably containing the UBF. We have found that two such complexes normally present in mock-infected HeLa cells disappear after infection by poliovirus with concomitant generation of a faster-migrating complex. These changes in gel-retarded complexes correlate kinetically with the time Pol I transcription is inhibited by poliovirus infection. The faster-migrating complex found in virus-infected cells can be generated *in vitro* by treating extracts of mock-infected cells with bacterially expressed, purified poliovirus protease 3C<sup>pro</sup>. Results presented here suggest that poliovirus protease 3C<sup>pro</sup> plays a role in the shutoff of Pol I transcription in infected cells.

## MATERIALS AND METHODS

**Cells.** HeLa cells were grown in spinner culture and infected with poliovirus type 1 (Mahoney strain) as described previously (37). For treatment with cycloheximide, cells were concentrated to  $4 \times 10^9$  per liter in minimal essential medium (GIBCO Laboratories) supplemented with 1 g of glucose per liter and 6% newborn calf serum. Cells were incubated in the presence of cycloheximide (100  $\mu$ g/ml, final concentration) for 1.5 h before harvesting.

**Preparation and fractionation of extracts.** Nuclear extracts were prepared essentially as described by Dignam et al. (11), with the exception that extracts were dialyzed against buffer II (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.9], 20% [vol/vol] glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride, 5 mM MgCl<sub>2</sub>) plus 0.1 M KCl.

Nuclear extract was fractionated through heparin-agarose chromatography as previously described (34).

**Transcription assays.** Specific transcription assays and nonspecific Pol I elongation assays were performed as previously described (34).

**Gel mobility shift assay.** The gel retardation reactions were performed in a final volume of 15  $\mu$ l, containing 0.1 M KCl. Three-fifths of the reaction volume consisted of protein plus buffer E (40 mM HEPES [pH 7.9], 3.5 mM MgCl<sub>2</sub>, 10% [vol/vol] glycerol, 0.1 mM EDTA, 1 mM DTT, 0.15 M KCl), and the remaining 6  $\mu$ l adjusted the reaction to 1 mM DTT and 0.1 M KCl and contained all added DNA. Protein was preincubated with 2.5  $\mu$ g of poly(dI-dC) (Pharmacia) for 15 min at 30°C. A <sup>32</sup>P-labeled oligonucleotide (20,000 to 40,000 cpm) containing the UCE binding site was then added, and the reactions were incubated for 20 min. These reactions were loaded, with the current on, onto a prerun (10-min) 4% polyacrylamide (80:1, acrylamide-bisacrylamide) gel in 1 $\times$

TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) and electrophoresed at room temperature at 40 mA. Electrophoresis continued for 1.5 h or for 3 h if free probe was run off of the gel.

The self-complementary oligonucleotides (Applied Biosystems) were hybridized (21) and labeled with the large fragment of DNA Pol I, in the presence of [ $\alpha$ -<sup>32</sup>P]dCTP and [ $\alpha$ -<sup>32</sup>P]dGTP (specific activity, 3,000 Ci/mmol; Amersham). The oligomers contained the following UCE-specific sequences (1):

5'CAGGT GTCCG TGTCC CGCGT CGCCT GGGCC GGCGG CG3'  
5'ACCTG CGCCG CCGGC CCAGG CGACG CGCGA CACGG AC3'

Competition reactions were performed by preincubating protein with a fifty- to one hundred-fold molar excess of ligated (21) unlabeled UCE oligonucleotide or a nonspecific human T-cell leukemia virus (HTLV) oligonucleotide (kindly provided by J. Garcia and R. Gaynor) containing the following sequence:

5'GGGGT AATCG TCAGA GCCTT CCC3'  
5'AAGGC TCTGA CGATT ACCCC GGG3'

After electrophoresis, gels were dried, and then autoradiography was performed.

**Protease treatment.** Poliovirus 3C (30) and mutant protease (16) were expressed in *E. coli* and purified as previously described. The mutant protease differs in one cysteine residue and is inactive for cleavage of the poliovirus polyprotein (16). Nuclear extract in buffer E was incubated with up to 10  $\mu$ g of protease for 2 h at 30°C. Poly(dI-dC) was added, and the gel retardation assays were performed as described above.

The human UBF clone (pT $\beta$ GUBF1) (20) was kindly provided by H.-M. Jantzen, S. Bell, and R. Tjian. The plasmid contains the UBF cDNA sequence downstream of the T7 Pol promoter and the  $\beta$ -globin gene leader. It was cut with *Eco*RI, transcribed with T7 Pol, and translated in reticulocyte lysate (Promega) in the presence of [<sup>35</sup>S]methionine (specific activity, 1,333 Ci/mmol; Amersham). *In vitro*-translated UBF (3  $\mu$ l of the 50- $\mu$ l translation reaction mix) was incubated for 2 h at 30°C in buffer A (20 mM HEPES [pH 7.9], 0.2 mM EDTA, 0.5 mM DTT, 20% [vol/vol] glycerol, 0.5 mM phenylmethylsulfonyl fluoride) with up to 50  $\mu$ g of protease 3C in a 15- $\mu$ l final volume. *In vitro*-translated human TFIID (19) (kindly provided by P. Lieberman and A. Berk) was treated with 10  $\mu$ g of 3C protease as a control. These proteins were analyzed on a 12.5% polyacrylamide-sodium dodecyl sulfate (SDS) gel, which was followed by fluorography.

## RESULTS

**Protein-UCE complexes from mock- and poliovirus-infected cells exhibit different mobilities.** To investigate whether there exists a qualitative difference in the type of DNA-factor complexes formed at the UCE (Pol I promoter region) in mock- and poliovirus-infected cells, we developed a gel retardation assay specific to the Pol I upstream promoter region. Since UBF is the only known promoter-binding Pol I transcription factor, we assumed any such interaction will indicate binding of UBF to the promoter (27). Extracts were prepared from 4-h mock-infected cells and from poliovirus-infected cells at 2, 3, and 4 h postinfection. Extracts were incubated with an internally labeled UCE oligomer probe, and the complexes formed were analyzed by nondenaturing polyacrylamide gel electrophoresis. Two DNA-protein complexes, named A and C, were detected with mock-infected

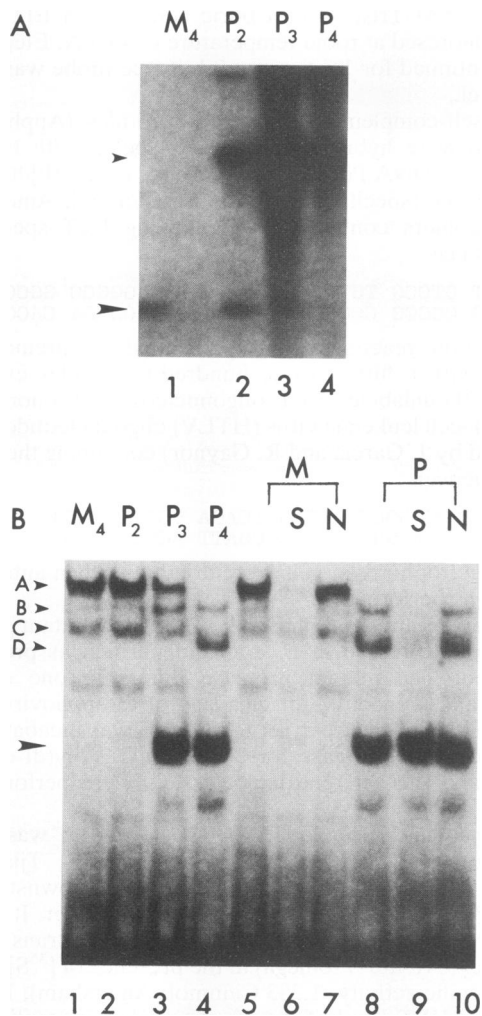


FIG. 1. Kinetics of inhibition of rRNA transcription correlate with an alteration in UCE complex mobility. (A) Cells were infected with poliovirus and harvested at 2 (lane 2), 3 (lane 3), and 4 (lane 4) h postinfection. Mock-infected cells were incubated for 4 h with minimal essential medium (lane 1). Nuclear extracts were prepared and assayed for specific transcription activity *in vitro*, as described in Materials and Methods. Reaction mixes contained 60  $\mu$ g of extract. The large arrowhead indicates the position of correctly initiated *in vitro*-synthesized transcript. The small arrowhead indicates the position of poliovirus-specific RNA synthesized by infected-cell extracts. (B) [<sup>32</sup>P]UMP-labeled oligonucleotide probe containing the UCE sequence was incubated with 30  $\mu$ g of mock-infected cell extract (lanes 1, 5, 6, and 7) or poliovirus-infected cell extract prepared at 2 (lane 2), 3 (lane 3), or 4 (lanes 4, 8, 9, and 10) h postinfection. For competition assays, reaction mixes contained a 100-fold excess of specific UCE (lanes 6 and 9) or nonspecific HTLV (lanes 7 and 10) unlabeled ligated oligonucleotides. Arrowheads labeled A to D indicate the positions of specific UCE-protein complexes. The large arrowhead indicates the position of a complex that appeared at later times of infection; the appearance of this band was not always reproducible. This band was not competed with by the specific UCE oligonucleotide (lane 9). The migration of free probe is shown at the bottom of the autoradiogram.

extracts (Fig. 1B, lane 1). At 2 h postinfection, virus-infected extracts formed the same complexes as the mock-infected extracts (lane 2). At 3 h postinfection, there was a decrease in the intensities of bands A and C as well as an increase in

band B (lane 3). By 4 h postinfection, bands A and C disappeared and a new band, D, appeared (lane 4). These four bands represent the only specific protein-DNA complexes (A to D), as indicated by competition experiments (Fig. 1B, lanes 5 to 10). Preincubation of nuclear extract with unlabeled UCE probe prevented subsequent binding of protein to the labeled probe (lanes 6 and 9). When extracts were preincubated with a nonspecific oligonucleotide, the amount of binding to the labeled UCE probe was not changed compared with that in the control (lanes 7 and 10). Incubation of extracts made from 3- and 4-h poliovirus-infected cells with labeled UCE oligonucleotide resulted in the generation of a high-intensity band (Fig. 1B, lanes 3 and 4, larger arrowhead). However, this band was nonspecific, as the homologous oligonucleotide was unable to compete with this band (lanes 9 and 10). As UBF is the only protein recognized as being able to bind to the UCE (27), these results indicate the probable presence of UBF in these complexes.

*In vitro* transcription assays with these mock- and poliovirus-infected nuclear extracts showed a correlation in the kinetics of the decrease of Pol I transcription in infected-cell extracts and the changes in the gel-retarded complexes (Fig. 1A). There was no significant change in the intensity of the Pol I transcript at 2 h of infection compared with that in the mock-infected extracts. However, transcription was completely inhibited at 4 h, at which time both bands A and C disappeared. As pointed out in our previous report (34), the high-molecular-weight RNA in Fig. 1A (indicated by a smaller arrowhead) represents elongation of preinitiated viral RNAs and is due to the presence of viral replication complexes in the infected-cell extract.

A transcription factor fractionation scheme has been developed by Learned et al. to identify factors necessary for Pol I transcription in human whole-cell extracts (28). In this scheme the whole-cell extract is loaded to heparin-agarose at 0.1 M (h.1) KCl and eluted stepwise with 0.2 (h.2), 0.4 (h.4), and 1.0 M (h1.0) KCl. Learned et al. found that h.4 and h1.0 fractions were sufficient for *in vitro* RNA synthesis from the Pol I promoter. The h.4 fraction contains UBF and Pol I, whereas h1.0 fraction contains SL1. In our previous report (34), we showed that the transcriptional activity of the h1.0 fraction was not altered by poliovirus infection. However, h.4 fraction isolated from virus-infected cells was transcriptionally inactive compared with the same fraction derived from mock-infected cells. We wished to determine whether gel-retarded complexes A and C could be found in heparin-agarose fraction h.4. Extracts prepared from 4-h mock- and poliovirus-infected cells were fractionated in parallel on heparin-agarose columns. The h.4 fractions recovered from each column were incubated with the labeled probe and analyzed by gel electrophoresis. As shown in Fig. 2, both complexes A and C were detected in the h.4 fraction from mock-infected cells (lane 6); however, these complexes were missing in the h.4 fraction derived from poliovirus-infected cells. The h.4 fraction from poliovirus-infected cells contained complex D activity (lane 7). In a transcription restoration assay, in which the transcription-inhibited virus-infected cell extract is rescued by addition of an uninfected cell-derived fraction, the mock h.4 fraction was found to be capable of restoring transcription activity in poliovirus-infected cell extracts (Fig. 2, lanes 1 to 3). This restoration of transcription was not due to addition of a limiting factor present in the h.4 fraction, since this fraction did not stimulate transcription in mock-infected extract (data not shown). Thus, the difference in the complexes formed be-

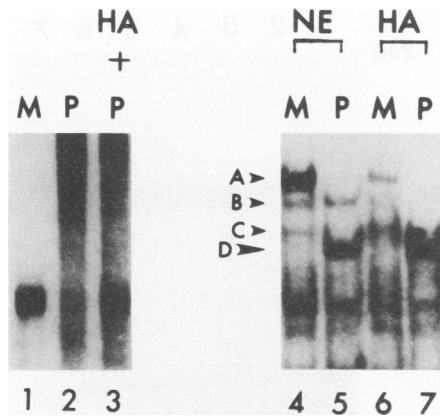


FIG. 2. Heparin-agarose-derived fraction h.4 restores transcription and also exhibits an altered gel retardation pattern. Fraction h.4 was prepared from uninfected cells as described in Materials and Methods. Specific transcription assays were as follows. Fraction h.4 was assayed for its ability to restore specific transcription in poliovirus-infected cell extracts. Reaction mixes contained 9  $\mu$ g of mock (lane 1) or poliovirus-infected (lanes 2 and 3) cell extracts. Lane 3 also contained 4  $\mu$ g of the h.4 fraction. Fraction h.4 itself did not have any transcriptional activity (data not shown). Gel retardation assays were as follows. Gel retardation reaction mixes contained the  $^{32}$ P-labeled UCE oligonucleotide and either 28  $\mu$ g of nuclear extract prepared from mock- (lane 4) or poliovirus (lane 5)-infected cells or 13  $\mu$ g of fraction h.4 prepared from mock- (lane 6) or poliovirus (lane 7)-infected cell extracts. The small arrowheads indicate the positions of bands A to C. The large arrowhead indicates the position of the poliovirus-specific band D.

tween mock- and poliovirus-infected cell extracts is reflected in a fraction that contains restoration activity. Taken together, the results presented in Fig. 1 and 2 suggest that formation of complexes A and C may be important in Pol I transcription, whereas complexes B and D appear to be transcriptionally inactive, and that infection of cells with poliovirus somehow converts complexes A and C to mainly complex D (and small amounts of complex B).

**Extracts from cycloheximide-treated cells form the same gel-retarded complex as extracts from mock-treated cells.** Cells that are treated with various growth and protein synthesis inhibitors exhibit a decrease in Pol I-catalyzed transcription activity (4, 40). This decrease is reflected in vitro with extracts prepared from these inhibited cells (3, 15). In mouse cells, treatment with cycloheximide results in the depletion of a transcriptionally active subform of RNA Pol I (named activated Pol I) (39). We wished to compare the cycloheximide-inhibited extracts with poliovirus-inhibited extracts for their abilities to form the UCE binding complex. Cells were mock treated, cycloheximide treated, or infected with poliovirus. Nuclear extracts were prepared from these cells and used in in vitro transcription and gel retardation assays. Extracts from both cycloheximide-treated and poliovirus-infected cells were inhibited for in vitro transcription compared with those from mock-infected cells (Fig. 3, lanes 1, 2, and 3). However, inhibition of transcription was more pronounced in extracts from cycloheximide-treated cells than from poliovirus-infected cells. When analyzed by the gel retardation assay, extracts from both mock- and cycloheximide-treated cells formed approximately equal amounts of complex A (Fig. 3, lanes 5 and 6). The complexes formed with poliovirus-infected extract (B and D), however, lacked complex A. Thus, the effect that poliovirus has on the UCE

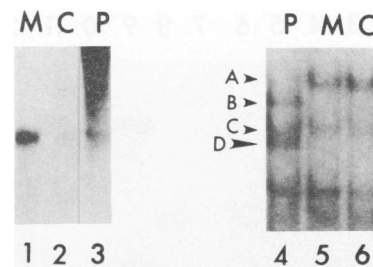


FIG. 3. Comparison of extracts prepared from poliovirus-infected and cycloheximide-treated cells. Cells were treated with cycloheximide or infected with poliovirus and nuclear extracts were prepared as described in Materials and Methods. Specific transcription assays were as follows. A total of 50  $\mu$ g of mock-infected (lane 1), cycloheximide-treated (lane 2), or poliovirus-infected (4 h of infection [lane 3]) nuclear extract was assayed for its ability to specifically initiate transcription at the rRNA promoter. Gel retardation assays were as follows. Reaction mixes contained 40  $\mu$ g of nuclear extract prepared from poliovirus-infected (lane 4), mock-infected (lane 5), or cycloheximide-treated (lane 6) cells. The small arrowheads indicate the positions of bands A to C, and the large arrowhead indicates the position of the poliovirus-specific band D. Free labeled probe was run off of the gel.

binding complex is not reflected in all cells deficient in Pol I transcription activity.

**The mobility shift differences are due to proteolytic cleavage.** We next examined whether poliovirus 3C protease could be involved in the alteration of initiation complex formation in infected-cell extracts. Treatment of the Pol III transcription factor TFIIC with protease 3C results in the conversion of the TFIIC-VAIRNA complex to a rapidly migrating conformation as assayed by gel retardation (7). Protease 3C also cleaves in vitro-synthesized Pol II transcription factor TFIID (7) (see Fig. 5). The glutamine-glycine site necessary for 3C protease activity is also found in the UBF sequence. This site is located between two regions of the protein sequence, the probable DNA binding domain and the putative activation domain (20).

Nuclear extracts prepared from mock- and poliovirus-infected cells were incubated with bacterially expressed, purified 3C protease (30) for 2 h at 30°C. These extracts were then assayed for gel retardation activity in the presence or absence of competitor DNA. Figure 4, lanes 1 to 3, demonstrates that in the untreated mock nuclear extract, complexes A to C are specific to the UCE. In this experiment, approximately half the amount of unlabeled competitor UCE oligomer was used, as in the experiment shown in Fig. 1. This explains why labeled band C (Fig. 4, lane 2) did not completely disappear in the competition assays. Incubation of mock-infected extracts in the presence of increasing amounts of 3C protease (lanes 4, 5, and 6) resulted in a loss of complexes A to C and the appearance of a complex which comigrates with complex D formed by poliovirus-infected cell extracts (lane 12). This protease-treated complex D activity also was UCE specific (lanes 6 to 8). Mock extracts were also treated with a mutant 3C protease, created through substitution of a critical cysteine residue with a serine at amino acid 147 (18). This change completely inactivates the protease (16). Incubation of mock-infected extract with the purified mutant protease did not result in the formation of complex D (lanes 9 to 11). Lane 11 exhibits reduced overall band formation activity because of the extremely high concentrations of salt added in the ammonium sulfate precipitate of the enzymes (3C protease). Treatment of the poliovirus-

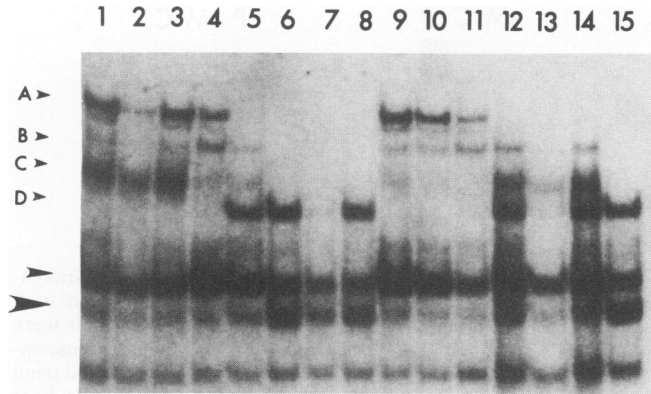


FIG. 4. Complex D is formed by the protease 3C-catalyzed cleavage of factors contained in complexes A to C. Nuclear extracts (23  $\mu$ g) were incubated with purified protease 3C and then assayed for gel retardation activity, as described in Materials and Methods. Specific and nonspecific competitor oligonucleotides were added to certain reactions to verify the specificity of binding to the UCE. Mock-infected extracts were also treated in parallel with a mutant protease in order to control for nonspecific alterations in UCE complex formation. Lanes 1 to 11 contained mock-infected extract, and lanes 12 to 15 contained poliovirus-infected extract, with the following additions. Lanes: 1, no additions; 2, 50-fold specific HTLV competitor; 3, 50-fold nonspecific HTLV competitor; 4, 0.1  $\mu$ g of protease 3C; 5, 1  $\mu$ g of protease 3C; 6, 10  $\mu$ g of protease 3C; 7, 10  $\mu$ g of protease 3C plus UCE competitor; 8, 10  $\mu$ g of protease 3C + HTLV competitor; 9, 0.1  $\mu$ g of mutant protease; 10, 1  $\mu$ g of mutant protease; 11, 10  $\mu$ g of mutant protease; 12, no additions; 13, UCE competitor; 14, HTLV competitor; 15, 10  $\mu$ g of protease 3C. The small arrowhead indicates the position of a nonspecific band that is decreased in activity upon addition of increasing salt present in the protease preparations. The large arrowhead indicates an additional specific UCE complex that was not observed in all of our assays but might indicate an additional cleavage product.

infected cell extract with protease 3C reduced the signal of all of the upper bands so that only band D was present (lane 15). The largest arrowhead indicates the appearance of another UCE-specific band that correlates with the increase in band D activity. This band was not detected in all of our assays, but might be caused by another factor cleaved by the protease. Complex B activity also seemed to increase when very low amounts of the protease were added (lane 4); this might indicate the presence of an intermediate step between complex A and complex D. These initial results demonstrate, therefore, that the alteration in complex formation, seen in poliovirus-infected extracts, can be duplicated *in vitro* by treatment of uninfected cell extracts with purified poliovirus protease 3C.

**Poliovirus protease 3C does not cleave *in vitro*-synthesized UBF.** We next examined the possibility that 3C protease might cleave UBF, which is known to bind directly to the UCE. The human UBF clone (20) (the kind gift of H.-M. Jantzen and R. Tjian) was translated *in vitro* and analyzed on an SDS-polyacrylamide gel electrophoresis gel. When treated with cloned, purified 3C protease, the mobility of the *in vitro*-synthesized UBF was not altered (Fig. 5, lanes 1 to 5). Even at a very high concentration of protease (lane 5), no alteration of UBF was apparent. As a positive control, *in vitro*-synthesized human TFIID (22) (kindly provided by P. Lieberman and A. Berk) was also treated in parallel with the protease. As demonstrated in Fig. 5, lanes 6 and 7, treatment with 3C protease cleaved human TFIID into a faster-migrat-

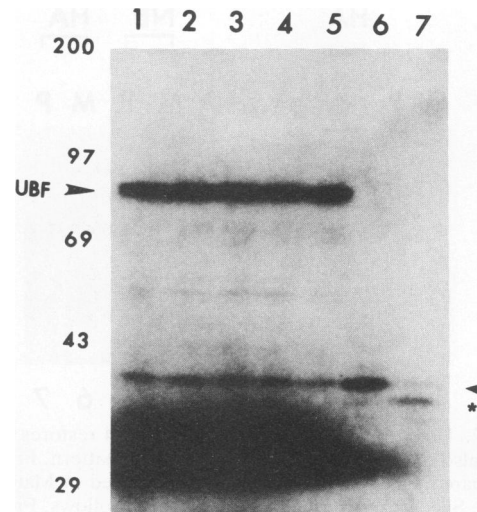


FIG. 5. *In vitro*-synthesized UBF is not cleaved by protease 3C. A plasmid containing the human UBF clone was translated *in vitro* in the presence of [ $^{35}$ S]methionine. The labeled UBF protein was incubated with purified poliovirus 3C protease, and the proteins were then analyzed on an SDS-polyacrylamide gel, as described in Materials and Methods. As a control for protease 3C activity, a known substrate, *in vitro*-translated human transcription factor TFIID, was treated with the enzyme. Lanes: 1, human UBF incubated on ice; 2, human UBF incubated at 30°C; 3, human UBF plus 1  $\mu$ g of protease 3C at 30°C; 4, human UBF plus 10  $\mu$ g of protease 3C at 30°C; 5, human UBF plus 40  $\mu$ g of protease 3C at 30°C; 6, human TFIID incubated at 30°C; 7, human TFIID plus 10  $\mu$ g of protease 3C at 30°C. The presence of a band migrating with human TFIID in all of the lanes is due to background activity in the reticulocyte lysates. The large arrowhead indicates the position of human UBF. The small arrowhead indicates the position of human TFIID. The asterisk indicates the cleavage product of human TFIID.

ing peptide. Therefore, protease 3C is incapable of cleaving the *in vitro*-synthesized UBF.

## DISCUSSION

In this series of experiments, we have presented evidence that the kinetics of inhibition of transcription activity upon infection of cells with poliovirus corresponds to a conversion of one UCE binding complex to another. Poliovirus infection of cells results in the disappearance of one UCE binding complex, complex A, and the appearance of a new, rapidly migrating complex, complex D. This change in complex conformation was not apparent in extracts prepared from cycloheximide-treated cells, indicating that the two mechanisms of inhibition of rRNA transcription are not identical. While the factors involved in the gel retardation activity have yet to be identified, at least one of the factors involved is UBF. UBF is known to bind to the UCE sequence (1, 27), and complexes A to D are specific for proteins binding to the UCE, as shown by competition with specific and nonspecific unlabeled probes. When we have tried to further purify the activity that forms complexes A to C, this activity has been lost after more than one fractionation step, suggesting that more than one factor is involved in the UCE binding complex. While UBF binds directly to the UCE, as measured by a footprinting assay (27), other factors may be associating with UBF, allowing us to detect this complex through the gel mobility shift assay. Indeed, SL1 has been

shown to associate with the UBF-UCE complex through footprinting assays (1, 27). Initial experiments examining the migration of complex A-associated proteins through a glycerol gradient suggest the presence of more than one factor in this fraction.

Treatment of nuclear extract with the poliovirus protease 3C caused a definite alteration of the UCE binding complex. This treatment resulted in the formation of a complex that comigrated with band D, representing the complex formed with poliovirus-infected extracts. Complex D could be formed by the cleavage of a factor that remains in the complex, causing a shift in mobility. This cleaved factor could prevent another normally associated factor from binding, because of the loss of a domain required for association with other factors. Alternatively, cleavage could result in a factor no longer being able to associate with the complex, because of disruption of a binding domain.

While the poliovirus-specific protease was not able to cleave the *in vitro*-translated cloned human UBF, we did not rule out the possibility that this cleavage could occur *in vivo*. The *in vitro*-translated UBF also was not able to form the gel-retarded complex, suggesting that this synthetic UBF might not be correctly modified and was thus inactive (data not shown). *In vitro*-translated UBF is capable of binding to the UCE as demonstrated by using oligonucleotide-affinity column binding assays (20). However, the gel-retarded complex that we have seen might be due to more than one factor associating with the UCE, because the complex formation activity is only evident in crude nuclear or heparin-agarose-purified fractions. Once the activity is fractionated on more than one column, the gel-retarded complexes are no longer visible (data not shown). Therefore, while the possibility remains that protease 3C cleaves UBF, the protease could instead be responsible for cleavage of another complex-associated factor.

Cleavage by the 3C protease is specific to a glutamine-glycine site (17) in the processing of viral precursor polyproteins. Not all glutamine-glycine sites are recognized by this enzyme, suggesting the importance of the surrounding sequence environment (31, 32, 41). *In vitro*-synthesized UBF might not be in the correctly folded conformation, because of a possible missing posttranslational modification. For example, while it is known that active natural UBF is phosphorylated, *in vitro*-synthesized UBF is most probably not phosphorylated. In the presence of other factors or modifications, the conformation of UBF might change, exposing the glutamine-glycine site to the protease. We would like to examine whether UBF purified with the vaccinia virus vector containing the UBF sequence (20) would be cleaved by protease 3C. Also, an antibody to UBF would help in determining whether this cleavage is apparent *in vivo*.

The poliovirus proteases exhibit highly selective substrate specificity, and one would expect that they would be specific only for the poliovirus polyprotein. However, protease 2A has been shown to be involved in inhibition of cellular translation by poliovirus. Protease 2A, although not directly, catalyzes the cleavage of a component of the cap binding complex, thus inhibiting translation of mRNAs containing a 5' cap structure (25, 37). Recently it has been found that protease 3C from a related picornavirus, foot-and-mouth disease virus, can cleave histone 3 (12, 38). Poliovirus-specific protease 3C, however, is unable to cleave histone (12). Thus, the results obtained in our laboratory indicating the cleavage of host cell RNA Pol I, II, and III transcription factors are the first evidence of the ability of poliovirus protease 3C to cleave cellular proteins. While these tran-

scription factors are present in the nucleus and poliovirus replication occurs exclusively in the cytoplasm, the entry of protease 3C into the nucleus during infection has been observed (2, 13).

Infection of cells with poliovirus results in a conformational change in a complex that binds to the rDNA promoter. The exact effect of this change on transcriptional activity remains to be determined. Unfortunately, our initial experiments to evaluate the effect of the viral protease 3C<sup>pro</sup> on Pol I transcription have failed. Relatively high concentrations of ammonium sulfate in the protease preparation, needed for stabilization of the purified enzyme, were found to nonspecifically inhibit *in vitro* transcription by Pol I. Ultimately, the treatment of individual components of a reconstituted system with protease 3C would help determine the identity of the affected factor as well as the effect that proteolytic cleavage has on transcription. In order to perform these experiments, a specific protease 3C inhibitor is necessary.

#### ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI27451 to A.D. and AI15122 and CA28146 to E.W. from the National Institutes of Health. T.H. is a recipient of a fellowship from Deutsche Forschungsgemeinschaft, Bonn, Germany.

We thank H. M. Jantzen and R. Tjian for the human UBF clone and P. Lieberman and A. Berk for the human TFIID clone. We are indebted to members of the Dasgupta laboratory for helpful discussion throughout the course of this work.

#### REFERENCES

- Bell, S. P., R. M. Learned, H.-M. Jantzen, and R. Tjian. 1988. Functional cooperativity between transcription factors UBF1 and SL1 mediates human ribosomal RNA synthesis. *Science* **241**:1192-1197.
- Bienz, K., D. Egger, Y. Rasser, and W. Bossart. 1982. Accumulation of poliovirus proteins in the host cell nucleus. *Intervirology* **18**:189-196.
- Cavanaugh, A. H., P. K. Gokal, R. P. Lawther, and E. A. Thompson, Jr. 1984. Glucocorticoid inhibition of initiation of transcription of the DNA encoding rRNA (rDNA) in lymphosarcoma P1798 cells. *Proc. Natl. Acad. Sci. USA* **81**:718-721.
- Cavanaugh, A. H., and E. A. Thompson, Jr. 1983. Hormonal regulation of transcription of rDNA. *J. Biol. Chem.* **258**:9768-9773.
- Clark, M., and A. Dasgupta. 1990. A transcriptionally active form of TFIIC is modified in poliovirus-infected HeLa cells. *Mol. Cell. Biol.* **10**:5106-5113.
- Clark, M., and A. Dasgupta. Unpublished data.
- Clark, M., T. Hammerle, E. Wimmer, and A. Dasgupta. 1991. Poliovirus proteinase 3C converts an active form of transcription factor IIIC to an inactive form: inhibition of host cell polymerase III transcription by poliovirus. *EMBO J.* **10**:2941-2947.
- Contreras, G., D. F. Summers, J. V. Maizel, and E. Ehrenfeld. 1973. HeLa cell nucleolar RNA synthesis after poliovirus infection. *Virology* **53**:120-129.
- Crawford, N., A. Fire, M. Samuels, P. A. Sharp, and D. Baltimore. 1981. Inhibition of transcription factor activity by poliovirus. *Cell* **27**:555-561.
- Darnell, J. E., M. Girard, D. Baltimore, D. F. Summers, and J. V. Maizel. 1967. The synthesis and translation of poliovirus RNA, p. 375-401. *In* J. Colter and W. Paranchych (ed.), *The molecular biology of viruses*. Academic Press, Inc., New York.
- Dignam, J. D., R. M. Lebovitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**:1475-1489.
- Falk, M. M., P. R. Grigera, I. E. Bergmann, A. Zibert, G. Multhaup, and E. Beck. 1990. Foot-and-mouth disease virus

- protease 3C induces specific proteolytic cleavage of host cell histone H3. *J. Virol.* **64**:748–756.
13. Fernandez-Tomas, C. 1982. The presence of viral-induced proteins in nuclei from poliovirus-infected HeLa cells. *Virology* **116**:629–634.
  14. Fradkin, L. G., S. K. Yoshinaga, A. J. Berk, and A. Dasgupta. 1987. Inhibition of host cell RNA polymerase III-mediated transcription by poliovirus: inactivation of specific transcription factors. *Mol. Cell. Biol.* **7**:3880–3887.
  15. Grummt, I. 1981. Specific transcription of mouse ribosomal DNA in a cell-free system that mimics control in vivo. *Proc. Natl. Acad. Sci. USA* **78**:727–731.
  16. Hammerle, T., C. U. T. Hellen, and E. Wimmer. 1991. Site-directed mutagenesis of the putative catalytic triad of poliovirus 3C proteinase. *J. Biol. Chem.* **266**:5412–5416.
  17. Hanecak, R., B. L. Semler, C. W. Anderson, and E. Wimmer. 1982. Proteolytic processing of poliovirus polypeptides: antibodies to polypeptide P3-7c inhibit cleavage at glutamine-glycine pairs. *Proc. Natl. Acad. Sci. USA* **79**:3973–3977.
  18. Hellen, C. U. T., H.-G. Krausslich, and E. Wimmer. 1989. Proteolytic processing of polyproteins in the replication of RNA viruses. *Biochemistry* **28**:9881–9890.
  19. Holland, J. J., and J. A. Peterson. 1964. Nucleic acid and protein synthesis during poliovirus infection of human cells. *J. Mol. Biol.* **8**:556–573.
  20. Jantzen, H.-M., A. Admon, S. P. Bell, and R. Tjian. 1990. Nucleolar transcription factor hUBF contains a DNA binding motif with homology to HMG proteins. *Nature (London)* **344**:830–836.
  21. Kadonaga, J. T., and R. Tjian. 1986. Affinity purification of sequence-specific DNA binding proteins. *Proc. Natl. Acad. Sci. USA* **83**:5889–5893.
  22. Kao, C. C., P. M. Lieberman, M. C. Schmidt, Q. Zhou, R. Pei, and A. J. Berk. 1990. Cloning of a transcriptionally active human TATA binding factor. *Science* **248**:1646–1650.
  23. Kliewer, S., and A. Dasgupta. 1988. An RNA polymerase II transcription factor inactivated in poliovirus-infected cells copurified with transcription factor TFIID. *Mol. Cell. Biol.* **8**:3175–3182.
  24. Kliewer, S., C. Muchardt, R. Gaynor, and A. Dasgupta. 1990. Loss of a phosphorylated form of transcription factor CREB/ATF in poliovirus-infected cells. *J. Virol.* **64**:4507–4515.
  25. Krausslich, H.-G., M. J. H. Nicklin, H. Toyoda, D. Etchison, and E. Wimmer. 1987. Poliovirus proteinase 2A induces cleavage of eukaryote initiation factor 4F polypeptide p220. *J. Virol.* **61**:2711–2718.
  26. Krausslich, H.-G., and E. Wimmer. 1988. Viral proteinases. *Annu. Rev. Biochem.* **57**:701–754.
  27. Learned, R. M., T. K. Learned, M. M. Haltiner, and R. T. Tjian. 1986. Human rRNA transcription is modulated by the coordinate binding of two factors to an upstream control element. *Cell* **45**:847–857.
  28. Learned, R. M., S. Cordes, and R. Tjian. 1985. Purification and characterization of a transcription factor that confers promoter specificity to a human RNA polymerase I. *Mol. Cell. Biol.* **5**:1358–1369.
  29. Mishima, Y., I. Financsek, R. Kominami, and M. Muramatsu. 1982. Fractionation and reconstitution of factors required for accurate transcription of mammalian ribosomal RNA genes: identification of a species-dependent initiation factor. *Nucleic Acids Res.* **10**:6659–6669.
  30. Nicklin, M. J. H., K. S. Harris, P. V. Pallai, and E. Wimmer. 1988. Poliovirus proteinase 3C: large-scale expression, purification, and specific cleavage activity on natural and synthetic substrates in vitro. *J. Virol.* **62**:4586–4593.
  31. Nicklin, M. J. H., H.-G. Krausslich, H. Toyoda, J. J. Dunn, and E. Wimmer. 1987. Poliovirus polypeptide precursors. Expression in vitro and processing by 3C and 2A proteinases. *Proc. Natl. Acad. Sci. USA* **84**:4002–4006.
  32. Pallai, P. V., F. Burkhardt, M. Skoog, K. Schreiner, P. Bax, K. A. Cohen, G. Hansen, D. E. H. Palladino, K. S. Harris, M. J. Nicklin, and E. Wimmer. 1989. Cleavage of synthetic peptides by purified poliovirus 3C proteinase. *J. Biol. Chem.* **264**:9738–9741.
  33. Paule, M. R., C. T. Iida, P. J. Perna, G. H. Harris, D. A. Knoll, and J. M. D'Alessio. 1984. In vitro evidence that eukaryotic ribosomal RNA transcription is regulated by modification of RNA polymerase I. *Nucleic Acids Res.* **12**:8161–8180.
  34. Rubinstein, S. J., and A. Dasgupta. 1989. Inhibition of rRNA synthesis by poliovirus: specific inactivation of transcription factors. *J. Virol.* **63**:4689–4696.
  35. Rueckert, R. R. 1985. Picornaviruses and their replication, p. 705–738. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, J. L. Melnick, B. Roizman, and R. E. Shope (ed.), *Virology*. Raven Press, New York.
  36. Sollner-Webb, B., and J. Tower. 1986. Transcription of cloned eukaryotic ribosomal RNA genes. *Annu. Rev. Biochem.* **55**:801–830.
  37. Sun, X.-H., and D. Baltimore. 1989. Human immunodeficiency virus tat-activated expression of poliovirus protein 2A inhibits mRNA translation. *Proc. Natl. Acad. Sci. USA* **86**:2143–2146.
  38. Tesar, M., and O. Marquardt. 1990. Foot-and-mouth disease virus protease 3C inhibits cellular transcription and mediates cleavage of histone H3. *Virology* **174**:364–374.
  39. Tower, J., and B. Sollner-Webb. 1987. Transcription of mouse rDNA is regulated by an activated subform of RNA polymerase I. *Cell* **50**:873–883.
  40. Warner, J. R. 1974. The assembly of ribosomes in eukaryotes, p. 461–483. *In* M. Nomura, A. Tissieres, and P. Lengyel (ed.), *Ribosomes*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
  41. Ypma-Wong, M. F., and B. L. Semler. 1987. In vitro molecular genetics as a tool for determining the differential cleavage specificities of the poliovirus 3C proteinase. *Nucleic Acids Res.* **15**:2069–2088.
  42. Zimmerman, E. F., M. Hecter, and J. E. Darnell. 1963. RNA synthesis in poliovirus-infected cells. *Virology* **19**:400–408.