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Inhibition of rRNA Synthesis by Poliovirus: Specific Inactivation of Transcription Factors

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Synthesis of rRNA by RNA polymerase I is almost completely inhibited soon after infection of human cells with poliovirus. We show that extracts prepared from poliovirus-infected HeLa cells are severely inhibited in their ability to transcribe from a human rDNA promoter compared with extracts from mock-infected cells. Two lines of evidence presented here suggest that a specific transcriptional activity required for rDNA transcription in vitro is impaired in virus-infected cells. First, fractionation of individual transcriptional components by phosphocellulose chromatography and subsequent reconstitution experiments showed that the specific transcriptional activity of fraction C (0.8 M KCl eluate) from virus-infected cells was reduced three- to fourfold relative to that isolated from mock-infected cells. The activities of other transcription factors needed for in vitro transcription from the rDNA promoter were unaffected. Second, fraction C derived from mock-infected cells specifically restored transcription in extracts prepared from virus-infected cells. Fraction C contained both a nonspecific RNA polymerase I elongation activity and a specific factor activity which was needed for accurate transcription initiation. It is the specific transcriptional activity and not the nonspecific chain elongation activity of fraction C that is affected in cells infected with poliovirus.

Infection of HeLa cells by poliovirus causes a rapid inhibition of host cell RNA synthesis. Specific transcription by RNA polymerases I, II, and III is inhibited (9, 12, 39). Synthesis of pre-rRNA by RNA polymerase I accounts for almost half of the transcriptional capacity of the cell (35); this synthesis is rapidly inhibited in vivo after poliovirus infection (9, 12, 39). The development of new in vitro transcription systems directed by RNA polymerase I enables us to examine the factors involved in this inhibition.

When polymerases are solubilized from picornavirus-infected cells, the ability of all three classes of enzymes, RNA polymerases I, II, and III, to synthesize RNA from a nonspecific, random DNA template is not impaired (1, 19). Also, partially purified RNA polymerases prepared from infected and uninfected cells do not display different chromatographic properties (2, 34). Finally, comparison by two-dimensional gel electrophoresis revealed no differences in subunit structure between purified polymerase II from uninfected and picornavirus-infected cells (2). Together, these results suggested that a transcriptional component other than the elongating polymerase was inactivated in picornavirus-infected cells. Indeed, Crawford et al. (10), using an in vitro system, showed that highly purified polymerase II was unable to specifically restore transcription in infected-cell extracts. Instead, a partially purified preparation of a polymerase II transcription factor was found to restore transcription from the adenovirus major late promoter (10). More recently, Kliewer and Dasgupta, using the same promoter, demonstrated that this transcription-restoring activity copurified with transcription factor TFIID, which interacts with the TATA motif on polymerase II genes (21). In the polymerase III system, a recent report by Fradkin et al. (14) showed that another DNA-binding factor, TFIIIC, which recognizes the internal promoter of all tRNA-type polymerase III genes, was transcriptionally inactive when isolated from poliovirus-infected cells. Its ability to specifically bind sequences in the internal promoter, however, was not impaired (14). While in vitro transcription systems capable of accurate initiation from cloned rDNA templates have been developed (16, 25), the virus-mediated shut-off mechanism for polymerase I transcription has yet to be examined in vitro.

The sole product of synthesis by RNA polymerase I is a 45S rRNA precursor. This transcript is later processed to the 18S, 28S, and 5.8S rRNAs. Its synthesis is highly regulated; transcription by polymerase I is extremely sensitive to growth conditions and is generally species specific (17). Cells that are in stationary phase, treated with cycloheximide, or exposed to glucocorticoids are extremely deficient in their ability to produce the rRNA transcript (7, 38). When cell extracts prepared from normal and growth-arrested cells are assayed for their ability to synthesize a transcript from an rDNA promoter, differential levels of transcription mimic those found in vivo. Extracts from these growth-arrested cells are inhibited in their ability to direct transcription of the rRNA precursor (6, 16). Furthermore, mouse rDNA cannot direct transcription in a human cell extract, which confirms the species selectivity seen in vivo (17).

The in vitro rDNA transcription systems derived from human and mouse cells have been extensively studied by many groups in order to identify specific transcription factors. Fractionation of cellular extracts over a variety of chromatographic matrices has allowed the identification of factors required in addition to polymerase I for the specific initiation of rDNA transcription. By using initial fractionation of mouse and human cell extracts over phosphocellulose, factors C and D were identified (29, 36). Factor D binds to the promoter, initiating a stable transcription complex, and is involved in determining species specificity (27, 36). Extracts prepared from growth-arrested cells are deficient in factor C (5, 8, 15). Factor C has been extensively purified from mouse cells and is believed to be an activated form of the polymerase itself (37). Another set of factors was identified in human cells by using a heparin-agarose column as

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the first fractionation step. Two factors along with RNA polymerase I, UBF1 and SL1, have been isolated (23, 24). UBF1 binds to the upstream control element in the human rDNA gene as well as to the promoter itself (3, 24). SL1 is believed to guide the recognition of the promoter by the polymerase and bind to the promoter and upstream element in the presence of UBF1 (3). How these two sets of transcription factors isolated from mouse and human cells correlate is as yet unknown; all of the factors must be purified to homogeneity to determine this.

In this report, we demonstrate that the in vivo inhibition of rDNA transcription observed in HeLa cells infected with poliovirus can be mimicked in vitro. Cell extracts prepared from poliovirus-infected HeLa cells are severely depressed in their ability to catalyze specific transcription from a human rDNA promoter. Further chromatographic fractionation of these extracts into individual transcription factor-containing fractions and subsequent transcription reconstitution studies suggest that an activity associated with RNA polymerase I is impaired by virus infection. The transcription initiation activity of the RNA polymerase I transcription complex but not its RNA chain elongation activity is affected. The same fractions that contain RNA polymerase I derived from uninfected cells are able to restore specific transcription in extracts prepared from virus-infected cells.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were grown in Spinner culture with minimum essential medium (MEM) (GIBCO Laboratories) supplemented with 1 g of glucose per liter and 6% newborn calf serum. Cells were infected with poliovirus type 1 (Mahoney strain) at a multiplicity of infection of 20 as described previously (13). Mock-infected cells were incubated with SMEM without serum during the adsorption period.

Preparation and fractionation of extracts. S100 extracts were prepared from mock- and poliovirus-infected HeLa cells as described by Miller and Sollner-Webb (28).

Fractionation of the transcription components by step elution from phosphocellulose was performed essentially as described by Tower et al. (36). Cell extract (S100, approximately 12 mg of protein per ml) was diluted with buffer I (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) adjusted to 0.1 M KCl and loaded onto a phosphocellulose (P-11; Whatman) column preequilibrated with buffer I–0.1 M KCl at 10 to 15 mg of protein per ml of bed volume. The column was washed with buffer I–0.1 M KCl; this flowthrough constituted the A fraction. Fractions B, C, and D were eluted with buffer I containing 0.4, 0.8, and 1.2 M KCl, respectively. Fraction C was pooled based on protein concentration (Bio-Rad Laboratories). Fractions containing factor D were determined by assaying their in vitro transcriptional activity when supplemented with fractions A and C. The peak fractions were pooled, dialyzed against buffer II (buffer I plus 5 mM $MgCl_2$ and 0.1 M KCl), and stored at −70°C. Fraction D was concentrated approximately fivefold with a Centricon filter (Amicon) before storage.

Alternatively, S100 extracts were applied to a heparin-agarose column (Sigma Chemical Co.), preequilibrated with 0.1 M KCl as described before (23), with the substitution of buffer II for TM buffer. The column was washed with buffer II–0.1 M KCl and then eluted stepwise with buffer II containing 0.2, 0.4, and 1.0 M KCl. The resulting fractions (h.2, h.4, and h1.0, respectively) were pooled, dialyzed, and stored as above.

Phosphocellulose fraction C derived from S100 extract was loaded onto a DEAE Sephadex A25 (Pharmacia) column preequilibrated with 0.05 M KCl in buffer II. Protein was eluted with a 0.05 to 0.6 M KCl gradient. Acetyl bovine serum albumin (0.1 mg/ml) was added to the fractions before freezing and storage.

Plasmid constructs. The plasmid $prHU_3$ was kindly donated by R. Tjian (25). The complete 2.0-kilobase (kb) *Eco*RI-*Bam*HI insert, which includes the transcription initiation region of a human rRNA gene, was subcloned into pUC18 between the *Eco*RI and *Bam*HI sites for greater plasmid preparation yields. The two templates, $prHU_3$ and $pUCHU_3$, are equivalent in directing in vitro transcription activity.

Transcription assays. Specific transcription assays were performed basically as described by Tower et al. (36). The 25-μl reaction mixtures contained a final concentration of 70 to 80 mM KCl, 5 mM $MgCl_2$, 0 to 10 mM ammonium sulfate, 10 mM HEPES, pH 7.9, 1 mM DTT, 0.1 mM EDTA, 500 μM each ATP, CTP, and GTP, 3 μCi of [α-^{32}P]UTP (specific activity, 3,000 Ci/mmol; Amersham), 100 μg of α-amanitin per ml, and 2.5 μg of rDNA template $pUCHU_3$ per ml. The template was truncated with *Bam*HI so that correct initiation of transcription at the promoter produces a 1,500-nucleotide run-off transcript. Reaction mixtures were incubated at 30°C for 45 min, reactions were terminated, and RNA was isolated, denatured with glyoxal and dimethyl sulfoxide, electrophoresed on 1.4% agarose gels, and autoradiographed as described by Manley et al. (26).

Nonspecific RNA polymerase I activity was assayed as described by Culotta et al. (11) with 100 μg of calf thymus DNA per ml as the template, at concentrations of 80 mM KCl and 10 mM ammonium sulfate. To control for chain elongation catalyzed by the poliovirus replication complex, each reaction was performed in duplicate with one containing 200 μg of dactinomycin per ml. Polymerase I activity was measured by determination of dactinomycin-sensitive activity.

Labeling of proteins with [^{35}S]methionine. At appropriate times postinfection, samples containing 8×10^6 cells were washed with methionine-free MEM. The cells were suspended in methionine-free MEM supplemented with 8% dialyzed newborn calf serum and [^{35}S]methionine (specific activity, 1,000 Ci/mmol; ICN) at 300 μCi/ml. Cells were stirred at 37°C for 10 min, harvested, and washed with cold phosphate-buffered saline. The cells were then lysed in phospholysis buffer as described elsewhere (4), chromatin was removed by centrifugation in a microcentrifuge, and the supernatants were stored at −70°C. Proteins were analyzed on a 12.5% polyacrylamide–sodium dodecyl sulfate (SDS) gel, followed by fluorography.

RESULTS

Inhibition of rDNA transcription by poliovirus in vitro. Upon infection of HeLa cells by poliovirus, synthesis of rRNA is rapidly inhibited (9, 12, 39). To determine whether the inhibition of polymerase I transcription observed in vivo can be mimicked in vitro, cell extracts were prepared from mock- and poliovirus-infected HeLa cells at 3 h postinfection. Specific RNA polymerase I activity was assayed by addition of a linearized rDNA template, $pUCHU_3$, which contains the 5′-terminal regulatory sequences of a human

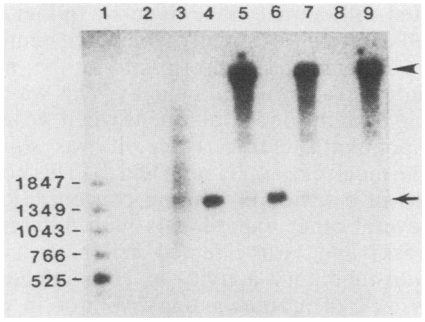

FIG. 1. Inhibition of polymerase I-mediated transcription in poliovirus-infected-cell extracts. Transcriptions from a human rDNA template (pUCHU$_3$) were performed as described in Materials and Methods; 50 μg of S100 extract prepared from mock-infected (lanes 2, 3, 4, 6, 8) or poliovirus-infected (3 h of infection; lanes 5, 7, 9) cells was added to each reaction mix, with the following additions: lane 2, no template; lane 3, uncut template; lanes 4 to 9, linearized template; lanes 6 and 7, 300 μg of α-amanitin per ml; lanes 8 and 9, dactinomycin (100 μg/ml). Lane 1 contains *Hin*fI-cut pSVS DNA size markers (32); the size of the markers is indicated on the left (in nucleotides). The arrow indicates the position of correctly initiated in vitro transcript (approximately 1,500 nucleotides). The arrowhead indicates the position of poliovirus-specific RNA synthesized by infected-cell extracts.

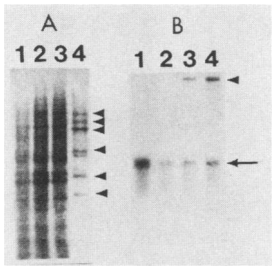

FIG. 2. Kinetics of inhibition of rRNA transcription by poliovirus. Cells were infected with poliovirus and harvested at the indicated times postinfection. (A) Samples were removed before harvest, and cellular and virus-specific proteins were labeled with [^{35}S]methionine as described in Materials and Methods. Cells were lysed, and labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Lanes 1 to 4 represent cells harvested at 0, 2, 2.5, and 3 h postinfection, respectively. The arrowheads indicate poliovirus-specific proteins synthesized during infection. (B) S100 extracts were prepared and assayed for specific transcription activity in vitro; 30 μg of extract was added to each reaction mix. Lanes 1 to 4 represent cells harvested at 0, 2, 2.5, and 3 h postinfection, respectively. The arrow indicates the position of correctly initiated in vitro transcript. The arrowhead indicates the position of poliovirus-specific RNA.

rDNA gene. Specific transcription from this template, correctly initiated at the rDNA promoter, generates an approximately 1,500-nucleotide-long transcript which can be resolved after glyoxal treatment on an agarose gel. As evident from the results presented in Fig. 1, synthesis of the 1,500-nucleotide transcript was absolutely dependent on added *Bam*HI-digested template DNA (lanes 2, 3, and 4) and was insensitive to concentrations of α-amanitin (up to 300 μg/ml) required to specifically inhibit polymerase II- and polymerase III-catalyzed transcription (lane 6). This insensitivity to high concentrations of α-amanitin is characteristic of polymerase I transcription. As expected, synthesis of the transcript was inhibited by dactinomycin (lane 8). Transcription of the rDNA template in the infected-cell extract was greatly reduced compared with that in mock-infected cell extracts (compare lanes 4 and 5). Reaction mixes containing infected-cell extracts consistently produced a high-molecular-weight RNA (indicated by an arrowhead). This virus-specific product has previously been shown to be due to the presence of viral replication complexes capable of incorporating ribonucleoside triphosphates to preinitiated viral RNA chains (10; Ransone and Dasgupta, unpublished observation). Synthesis of the virus-specific RNA was not inhibited by dactinomycin (lane 9), indicating RNA-dependent RNA synthesis, nor did it require the presence of the rDNA template (data not shown).

We next examined possible nonspecific mechanisms by which transcription in extracts from poliovirus-infected cells could be inhibited. Altering the concentrations of various components of the in vitro reaction mix, such as protein concentration, amount of template added, or concentration of ribonucleoside triphosphates, did not reverse the inhibition of transcription in virus-infected cell extracts (data not shown). To examine whether the decrease in the level of transcripts synthesized in virus-infected cell extracts was due to RNA degradation, [^{32}P]UMP-labeled, in vitro-synthesized transcript was incubated for 60 min with extracts prepared from either mock- or virus-infected cells. There was no significant difference in the amount of labeled transcript remaining after incubation in the two extracts; therefore, there was no increased activity of nonspecific nucleases in the infected-cell extract (data not shown).

Another possible mechanism of inhibition would be a covalent modification of the template caused by incubation in poliovirus-infected cell extracts. To investigate this possibility, the pUCHU$_3$ template was preincubated with extracts from either mock- or poliovirus-infected cells. The DNA was then isolated and used in a standard transcription assay with extract from mock-infected cells. There was no observed difference between the levels of transcript synthesis directed by templates that had been preincubated in either extract. Thus, the capacity of the template to direct synthesis of the rRNA transcript was not altered by incubation in extracts from virus-infected cells (data not shown).

Kinetics of inhibition of rRNA transcription and cellular protein synthesis shut-off during the course of infection. In order to determine the kinetics of inhibition of RNA polymerase I transcription, HeLa cells were infected with poliovirus and infections were terminated at 0, 2, 2.5, and 3 h postinfection. S100 extracts were then prepared and assayed for their ability to catalyze transcription from the rDNA promoter. At 2 h postinfection, synthesis of the rRNA transcript was almost completely inhibited (Fig. 2B, lane 2). Although the intensity of the labeled transcript appeared to increase slightly at 2.5 and 3 h postinfection (lanes 3 and 4, Fig. 2B) compared with that at 2 h (lane 2), this result was not reproducible. In separate experiments, we have not seen inhibition of transcription at 1 h postinfection (data not shown). Among preparations of extracts from different infections, the time of onset of complete inhibition varied between 2 and 3 h postinfection. This is probably due to slightly different infection conditions with separate preparations of virus and to the condition of the cells at the time of infection. This disparity in the time at which inhibition is first detected after infection has previously been observed in vivo; the time of onset of inhibition has ranged from 1 h (9) to 2 h (12, 39) and 4.5 h (19) postinfection.

It has been demonstrated that treatment of cells with cycloheximide, which inhibits protein synthesis, results in inhibition of rRNA transcription (30). Treatment of mouse

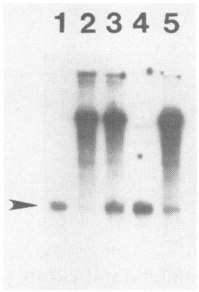

FIG. 3. In vitro transcription in mixed extracts. In vitro transcription from the $pUCHU_3$ template was performed by using various amounts of mock-infected S100 alone, infected S100 alone (3 h of infection), and after mixing indicated amounts of mock-infected and infected S100 extracts. Lanes: 1, 50 μg of mock-infected-cell extract; 2, 50 μg of poliovirus-infected-cell extract; 3, 50 μg of mock-infected extract plus 50 μg of virus-infected extract; 4, 100 μg of mock-infected-cell extract; 5, 100 μg of poliovirus-infected-cell extract. The arrowhead indicates the position of correctly initiated in vitro transcript.

cells with cycloheximide affects the activity of transcription factor C; its activity in extracts from treated cells is greatly reduced compared with that in extracts from untreated cells (15, 37). Infection of cells with poliovirus results in the inhibition of cellular translation and the exclusive synthesis of viral proteins by 2 to 3 h postinfection. Since the onset of the shut-off of translation and the inhibition of RNA polymerase I-catalyzed transcription occur early in the infection process, we examined the kinetics of each inhibition mechanism in the same set of infections. At the time of harvest of the cells for extracts used in Fig. 2B, smaller samples were removed, washed, and pulse labeled with [^{35}S]methionine for 10 min. These cells were then harvested, lysed, and analyzed by SDS-polyacrylamide gel electrophoresis. The inhibition of translation of the majority of cellular proteins occurred at 3 h postinfection (Fig. 2A). Synthesis of virus-specific proteins was also detected at this time, as indicated by the arrows. The inhibition of RNA polymerase I-catalyzed transcription, analyzed in vitro, occurred at 2 h postinfection (Fig. 2B). This experiment demonstrates that the inhibition of rRNA transcription occurs 1 h prior to the inhibition of cellular translation and therefore is a primary result of infection, not a secondary result of the inhibition of protein synthesis. This experiment makes the assumption, however, that all cellular proteins have the same lability and that the shut-off of translation occurs all at once. The possibility remains that a limiting factor required for transcription could have greater lability than the majority of cellular proteins and be inactivated much earlier in the infection.

Extracts from infected cells do not contain a *trans*-acting inhibitor. To determine whether the extract from poliovirus-infected cells contained a *trans*-acting catalytic inhibitor capable of inhibiting RNA polymerase I transcription when added to the extract from mock-infected cells, equal amounts of S100 extract from both mock- and poliovirus-infected cells were mixed and assayed for transcription activity. We would expect that the presence of a catalytic inhibitor in the S100s prepared from poliovirus-infected cells would cause the level of transcript synthesized by the mixed extracts to be less than the additive amount produced individually by each extract (Fig. 3, lanes 1 and 2). The level of transcript synthesized by the mixed extracts (lane 3) was not reduced compared with the level synthesized by the mock-infected-cell extract alone (lane 1). In fact, transcription in mixed extracts was slightly stimulated compared with the additive amount of transcription in the mock- and poliovirus-infected extracts individually. This was not due to an increase in protein concentration, as when we assayed the amount of poliovirus extract twice, it was still inhibited relative to the mock extract (lanes 4 and 5). This could reflect the presence of a factor in limiting concentrations in cell extracts. Several other experiments with different proportions of mock- and virus-infected cell extracts failed to detect a *trans*-inhibitory activity in virus-infected-cell extracts. This lack of a *trans*-acting inhibitor in poliovirus-infected cell extracts has previously been shown for RNA polymerase II and polymerase III transcription (10, 14).

Assay of individual chromatographic fractions. Transcription of the rDNA gene involves at least two factors, C and D. These factors were derived from phosphocellulose fractionation of S100 extracts. The C fraction contains factor C as well as RNA polymerase I (29, 36). Factor C from mouse cells has been further purified. It contains an activated form of the polymerase, capable of initiating transcription specifically at the promoter (37). Factor D, purified from the D fraction, contains a promoter-binding activity; its binding initiates the formation of the transcription complex (27, 36). S100 extracts prepared from mock- and poliovirus-infected cells were loaded onto a phosphocellulose column and eluted as described in Materials and Methods. Fraction A consists of the 0.1 M KCl flowthrough; fractions B, C, and D were step eluted at 0.4, 0.8, and 1.2 M KCl, respectively. Fraction B does not contain any necessary transcription factors for the in vitro system (35). The A, C, and D fractions derived from mock- and poliovirus-infected-cell extracts were assayed for transcription activity in the presence of saturating concentrations of the remaining required factors from mock-infected cells. Although one group has successfully replaced the A fraction with commercially prepared RNasin (36), we were unable to use this substitute, having tested four different batches of RNase inhibitor from the supplier cited in the report. Other laboratories, examining the formation of the transcription complex on the human rDNA gene, have shown a requirement for a factor present in the A fraction (20, 27). Therefore, the activity in fraction A was assayed along with the C and D fraction activities. All three fractions were required for synthesis of the rDNA transcript. Specific transcriptional activity in fraction A derived from mock- and poliovirus-infected cells was comparable (Fig. 4A). The top band present in these assays is nonspecific and has been seen previously by Learned et al. (23). It was present even in the absence of exogenous rDNA template (data not shown). Fraction D activity also was comparable in extracts prepared from both mock- and poliovirus-infected cells (Fig. 4B). In the absence of added fraction D, no specific transcription was observed (data not shown).

The major difference in transcription-stimulatory activity between the two extracts was seen in fraction C. Upon titration of fraction C in the presence of fractions A and D from mock-infected cells, we found a threefold difference in RNA polymerase I-specific transcription activity (Fig. 4C). We note that total transcription appeared to be considerably more reduced in virus-infected cell extracts (Fig. 2) than was the abundance of factor C (Fig. 4). Efficient reinitiation of transcription in crude extracts compared with that in the reconstituted system may have contributed to the discrepancy observed between these two systems. Alternatively, it is conceivable that a factor(s) other than the known polymerase I transcription factors is affected in virus-infected

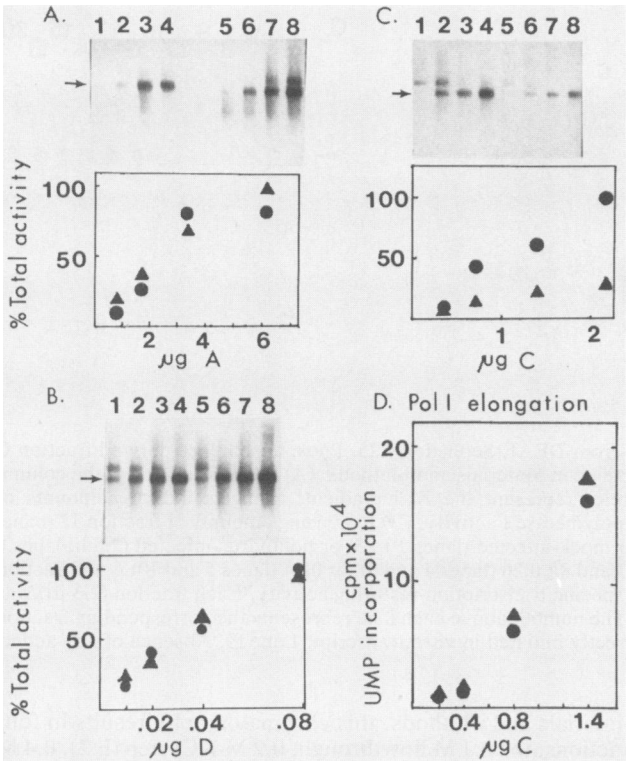

FIG. 4. Assay of individual phosphocellulose column-derived fractions. Fractions A, C, and D were prepared from mock-infected (lanes 1 to 4) and poliovirus-infected (lanes 5 to 8) cells at 3 h postinfection as described in Materials and Methods. Each fraction was titrated into an excess of the other two fractions prepared from mock-infected cells. Circles and triangles represent the amount of [^{32}P]UMP-labeled product catalyzed by mock- and poliovirus-infected cell-derived fractions, respectively. Total activity is defined as the amount of labeled product synthesized with saturating amounts of each fraction. The arrows indicate the position of correctly initiated in vitro transcript. (A) Comparison of fraction A: 0.9 (lanes 1 and 5), 1.8 (lanes 2 and 6), 3.6 (lanes 3 and 7), and 6.8 (lanes 4 and 8) μg of fraction A. The lower band represents the specific transcription product in lanes 5 to 8. (B) Comparison of fraction D: 0.02 (lanes 1 and 5), 0.04 (lanes 2 and 6), 0.08 (lanes 3 and 7), and 0.1 (lanes 4 and 8) μg of fraction D. (C) Comparison of fraction C: 0.4 (lanes 1 and 5), 0.7 (lanes 2 and 6), 1.4 (lanes 3 and 7), and 2.2 (lanes 4 and 8) μg of fraction C. (D) Comparison of RNA polymerase I chain elongation activities between fraction C prepared from mock- and poliovirus-infected cells. Indicated amounts of fraction C were assayed for dactinomycin-sensitive RNA polymerase I (Pol I) activity by using sonicated calf thymus DNA as the template, as described in Materials and Methods.

cells. Nevertheless, it is clear that the loss of transcription activity in extracts from poliovirus-infected cells is probably due to the loss of activity in the C fraction and not in the A and D fractions. As evident in Fig. 4D, there was no significant difference in the RNA chain elongation activity (on a nonspecific DNA template) of polymerase I present in mock- or virus-infected cell-derived fraction C.

Fraction C restores transcription activity in extracts from poliovirus-infected cells. Since fraction C activity was deficient in extracts from poliovirus-infected cells when assayed in a reconstituted system, the next question was whether addition of this fraction could restore transcription in the S100 extracts from poliovirus-infected cells to the level observed in extracts prepared from mock-infected cells.

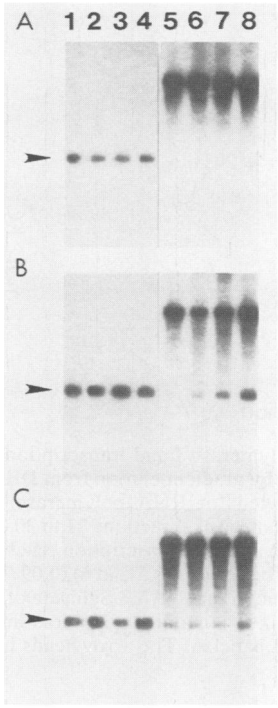

FIG. 5. Fraction C specifically restores transcription in poliovirus-infected cell extracts. Transcriptions were performed in vitro with 80 μg of either mock-infected (lanes 1 to 4) or poliovirus-infected (3 h of infection; lanes 5 to 8) cell extracts. Phosphocellulose-fractionated, mock-infected cell-derived transcription factors were added as follows. (A) Fraction A: 0 (lanes 1 and 5), 0.8 (lanes 2 and 6), 1.7 (lanes 3 and 7), and 4 (lanes 4 and 8) μg. (B) Fraction C: 0 (lanes 1 and 5), 0.4 (lanes 2 and 6), 0.7 (lanes 3 and 7), and 1.4 (lanes 4 and 8) μg. (C) Fraction D: 0 (lanes 1 and 5), 0.02 (lanes 2 and 6), 0.04 (lanes 3 and 7), and 0.05 (lanes 4 and 8) μg. Approximately half of the pellet in lane 3 was lost during processing; the decrease in activity for this point was not reproducible. The arrowheads indicate the position of correctly initiated in vitro transcript.

These restoration experiments consisted of adding phosphocellulose-purified fractions from mock-infected cells to S100s prepared from poliovirus-infected cells. To control for stimulation through addition of a limiting factor, the fractions were also added to the S100s from mock-infected cells. Addition of fraction A did not restore specific transcription from the rDNA promoter in the S100s from poliovirus-infected cells (Fig. 5A). Similarly, addition of the D fraction did not significantly stimulate specific transcription in virus-infected cell extracts compared with the control (Fig. 5C, compare lanes 6 and 7 with lane 5). The slight stimulation of transcription in infected-cell extracts seen at the highest concentration of fraction D (lane 8) was also observed when the same concentration of the factor was added to mock-infected-cell extracts (lane 4). Additionally, this stimulation could not be reproduced consistently in other experiments. In contrast, addition of fraction C derived from mock-infected cells restored specific transcription in the extract from poliovirus-infected cells to almost the level seen in the control extract (Fig. 5B). The stimulation of transcription in infected extracts was quite linear with increasing amounts of fraction C. This result is consistent with the data from the reconstitution assay; fraction C activity is impaired in extracts from poliovirus-infected cells.

Further purification of the restoring activity. We next

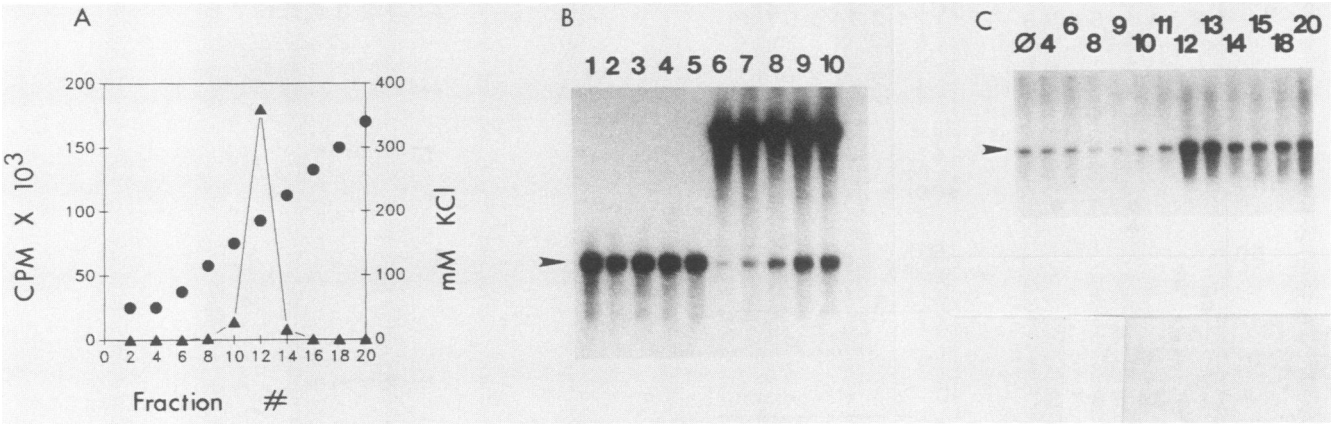

FIG. 6. RNA polymerase I and transcription-restoring activity coelute from DEAE-Sephadex A25. Phosphocellulose-derived fraction C was further purified by gradient elution from DEAE-Sephadex A25 as described in Materials and Methods. (A) Portions (10 μl) of the column fractions were assayed for RNA polymerase I elongation activity. Circles represent the KCl gradient; triangles indicate amounts of [^{32}P]UMP-labeled transcript. Fractions 21 to 30 did not contain detectable polymerase I activity. (B) Increasing amounts of fraction 12 (panel A) were titrated into specific transcription reaction mixes containing either mock-infected (lanes 1 to 5) or poliovirus-infected (2.5 h; lanes 6 to 10) cell extract. Then, 0 (lanes 1 and 6), 0.09 (lanes 2 and 7), 0.17 (lanes 3 and 8), 0.26 (lanes 4 and 9), or 0.34 (lanes 5 and 10) μg of fraction 12 was added. (C) Individual DEAE-Sephadex fractions were assayed for specific transcription-restoring activity. Each fraction (2.3 μl) was added to reaction mixes containing poliovirus-infected (2.5 h) cell extract. The number above each lane represents the corresponding fraction number as shown in panel A. The arrowheads indicate the position of correctly initiated in vitro transcript. Lane Ø, Absence of any added fraction.

wished to purify the restoring activity further in order to determine whether it could be separated from polymerase I activity. We decided to use a DEAE-Sephadex chromatographic step, as it has generally been used for RNA polymerase I purification (33). Phosphocellulose-derived fraction C from uninfected-cell extracts was loaded onto a DEAE-Sephadex A25 column at 0.05 M KCl and eluted with a 0.05 to 0.6 M gradient. Fractions were assayed for polymerase I elongation activity; the peak of activity occurred in fraction 12, at approximately 0.2 M KCl (Fig. 6A). When these fractions were assayed for their ability to restore transcription in poliovirus-infected-cell extracts, the peak of restoration activity was also present in fraction 12 (Fig. 6C). While some trailing of the restoration activity did occur after fraction 13, the two peaks of polymerase I elongation and restoration of specific transcription were coincident. Fraction 12 stimulated transcription in infected-cell extracts without increasing transcription in mock-infected-cell extracts; thus, it contains the true restoration activity and not simply a limiting factor (Fig. 6B). It is worth nothing that fraction 12 did not reconstitute transcription in the presence of phosphocellulose A and D fractions (data not shown), suggesting that an additional transcriptional component other than polymerase I, A, and D might have been separated during DEAE-Sephadex chromatography. Whatever this putative factor might be, it is clearly not required for restoration of transcription in extracts from virus-infected cells (Fig. 6).

Fractionation of extracts over heparin-agarose. Another fractionation scheme has been developed by Learned et al. to identify factors necessary for polymerase I transcription in human whole-cell extracts (23). Instead of phosphocellulose chromatography, a heparin-agarose column is used as the first fractionation step. How the two sets of factors derived from the two fractionation schemes are related to each other is not clear. We wished to determine where the specific transcription restoration activity eluted from a heparin-agarose column to determine how it might relate to previously discovered transcription factors. As described in Materials and Methods, this chromatography results in four fractions: the 0.1 M flowthrough, 0.2 M KCl step (h.2), 0.4 M KCl step (h.4, which contains RNA polymerase I and UBF1), and the 1.0 M KCl step (h1.0, which contains SL1). Learned et al. found that the h.4 and h1.0 fractions were sufficient for in vitro rRNA synthesis (23). Each fraction was titrated individually in order to compare the activities contained in mock- and poliovirus-infected-cell extracts. As with the phosphocellulose-derived fractions, in order to

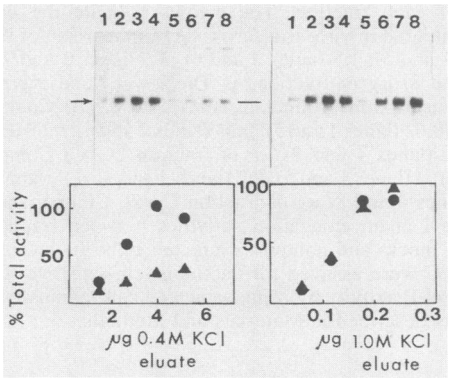

FIG. 7. Assay of individual heparin-agarose-derived fractions. Heparin-agarose fractions h.4 (0.4 M KCl eluate) and h1.0 (1.0 M KCl eluate) were prepared from mock-infected (lanes 1 to 4) and poliovirus-infected (lanes 5 to 8) cells as described in Materials and Methods. Each fraction was titrated into the specific transcription reaction mixes in the presence of an excess of the remaining fraction and the 0.1 M KCl flowthrough from the column. Circles and triangles represent the amount of transcript catalyzed by mock- and poliovirus-derived fractions, respectively, as in Fig. 4. For the 0.4 M KCl eluate: 1.4 (lanes 1 and 5), 2.8 (lanes 2 and 6), 4.2 (lanes 3 and 7), and 5.6 (lanes 4 and 8) μg of h.4 added. For the 1.0 M KCl eluate: 0.06 (lanes 1 and 5), 0.12 (lanes 2 and 6), 0.18 (lanes 3 and 7), and 0.24 (lanes 4 and 8) μg of h1.0 added. The arrow indicates the position of correctly initiated in vitro transcript. The percent total activity is defined as in the legend to Fig. 4.

synthesize a specific transcript, we required the addition of the flowthrough fraction. Fraction A from phosphocellulose could be used interchangeably with the heparin-agarose column flowthrough. When titrated into the reconstituted system, the heparin-agarose column flowthrough fraction exhibited the same amount of activity whether it was derived from mock- or poliovirus-infected cells (data not shown). Fraction h1.0 activity was not diminished in extracts prepared from poliovirus-infected cells (Fig. 7). The transcription-stimulating activity of fraction h.4, however, was reduced approximately threefold in poliovirus-infected-cell extracts.

DISCUSSION

We have shown that the inhibition of specific transcription by cellular RNA polymerase I after poliovirus infection can be examined in an in vitro system. The amount of transcript synthesized from a human rDNA template was significantly decreased in crude extracts prepared from virus-infected HeLa cells compared with that in extracts from mock-infected cells. Reconstitution experiments with partially purified transcription factors from a phosphocellulose column demonstrated that an activity in fraction C was deficient in the extracts from virus-infected cells. The specific transcription-stimulatory activity of virus-infected cell-derived fraction C was threefold less than that of fraction C derived from mock-infected cells. In addition, mock-infected cell-derived fraction C specifically restored transcription in virus-infected-cell extracts almost to the level seen in extracts from mock-infected cells. Fraction C contains both a nonspecific RNA polymerase I elongation activity and a specific activity (factor C) which is needed for accurate initiation of polymerase I transcription from the promoter. These two activities copurified upon gradient elution through a DEAE-Sephadex column (Fig. 6). However, we have shown that it is the specific transcriptional activity and not the nonspecific RNA chain elongation activity of fraction C that is affected in cells infected with poliovirus.

In addition, using a heparin-agarose column for the initial fractionation of the S100 extract, we found that the fraction that contains RNA polymerase I (h.4, 0.4 M KCl eluate) had reduced transcriptional activity when isolated from virus-infected cells (Fig. 7). It is unclear how the fractionation scheme used for human cell extracts by Learned et al. (23) corresponds to the fractionation developed by using mouse and human cell extracts by other groups (29, 36). The activated form of the polymerase purified from mouse cells by the Sollner-Webb group (37) has not yet been identified in human cell extracts. The 0.4 M KCl eluate from heparin-agarose contains a DNA-binding factor, UBF1. This protein binds upstream of the promoter and is needed for the efficient binding of another factor, SL1, which elutes from heparin-agarose in the 0.4 to 1.0 M KCl step. As shown in Fig. 7, the transcriptional activity of the fraction which presumably contains SL1 is comparable whether isolated from mock- or virus-infected cells. Since our results indicate that an activity in the h.4 fraction restores specific transcription, this activity could be UBF1. Our initial attempts to footprint heparin-agarose-purified UBF1 over the human rDNA upstream control element have failed. Once we have more extensively purified the transcription restoration activity, we will use the DNA footprint assay to determine whether it contains an upstream control element-binding activity. Further purification of the restoration activity will therefore be necessary before we can determine the nature of the factor deficient in virus-infected cells.

It should be pointed out that irrespective of whether phosphocellulose or heparin-agarose is used for fractionation of polymerase I transcription factors, the flowthrough fractions from these columns, in our hands, are required to reconstitute transcription in the presence of other fractions. For example, the phosphocellulose flowthrough (0.1 M KCl eluate) is required along with fractions C (0.8 M KCl eluate) and D (1.2 M KCl eluate) for specific polymerase I transcription (Fig. 4). Similarly, the flowthrough fraction from heparin-agarose is needed to reconstitute transcription with the 0.4 and 1.0 M KCl eluates from the column (Fig. 7). While this requirement has been observed by some (20, 27), other groups have not found this requirement (23, 36). As suggested by Tower et al. (36), we tried to replace the flowthrough fraction with commercially available RNasin, but failed. We do not know the reason for this disparity but suspect that it could be due to separation of one or more polypeptides from a multicomponent factor during fractionation of the extract under our conditions.

How poliovirus inhibits the specific transcriptional activity of one or more factors required for polymerase I transcription is not known. We were unable to detect an inhibitory activity in infected-cell extracts that was capable of inhibiting transcription in mock-infected cell extracts (Fig. 3). One possibility is that an extremely labile factor is affected earlier than the majority of host cell proteins during translational shut-off by the virus. Although the results presented in Fig. 2 suggest that this is unlikely, complete purification of the restoring factor and immunologic reagents will be necessary to determine with certainty whether the absolute amount of this factor is reduced in infected cells. Another possible mechanism is posttranslational modification of the restoring factor. Results obtained in this laboratory with the polymerase II and III systems suggest the modification of transcription factors in cells infected with poliovirus (14, 21). Possible modification could involve proteolytic cleavage or phosphorylation-dephosphorylation of transcription factors in infected cells. The poliovirus genome encodes proteases (22). Also, infection of cells with the virus has been shown to stimulate a number of host cell kinase activities (31). These could be involved in the regulation of activity of the transcription restoration factor.

These initial studies have determined that the inhibition of cellular transcription upon infection by poliovirus can be examined in an in vitro system, allowing identification of the factor(s) responsible for inhibition. We will concentrate on further purification of the restoration activity in order to determine how it is inhibited in infected cells. The examination of the mechanism of inhibition of activity in poliovirus-infected-cell extracts should provide important clues to the mechanism of RNA polymerase I transcriptional regulation.

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