MOLECULAR AND CELLULAR BIOLOGY, Nov. 1987, p. 3880–3887
0270-7306/87/113880-08$02.00/0

Vol. 7, No. 11

Inhibition of Host Cell RNA Polymerase III-Mediated Transcription by Poliovirus: Inactivation of Specific Transcription Factors

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Received 17 April 1987/Accepted 27 July 1987

The inhibition of transcription by RNA polymerase III in poliovirus-infected cells was studied. Experiments utilizing two different cell lines showed that the initiation step of transcription by RNA polymerase III was impaired by infection of these cells with the virus. The observed inhibition of transcription was not due to shut-off of host cell protein synthesis by poliovirus. Among four distinct components required for accurate transcription in vitro from cloned DNA templates, activities of RNA polymerase III and transcription factor TFIIIA were not significantly affected by virus infection. The activity of transcription factor TFIIIC, the limiting component required for transcription of RNA polymerase III genes, was severely inhibited in infected cells, whereas that of transcription factor TFIIIB was inhibited to a lesser extent. The sequence-specific DNA-binding of TFIIIC to the adenovirus VA1 gene internal promoter, however, was not altered by infection of cells with the virus. We conclude that (i) at least two transcription factors, TFIIIB and TFIIIC, are inhibited by infection of cells with poliovirus, (ii) inactivation of TFIIIC does not involve destruction of its DNA-binding domain, and (iii) sequence-specific DNA binding by TFIIIC may be necessary but is not sufficient for the formation of productive transcription complexes.

Infection of susceptible cultured cells with poliovirus or other members of the picornavirus family leads to marked inhibition of host cell RNA synthesis (3, 26, 45). The integrity of the incoming viral RNA genome is required for inhibition, and drugs that block translation prevent the virus-mediated inhibition of host cell RNA synthesis presumably by precluding the expression of a protein encoded or induced by the virus (3).

The timing of inhibition is dependent on both the virus and the host cell (31), and it is not yet obvious that all picornaviruses inhibit transcription by a similar mechanism. Each of the three transcription systems, defined by the sensitivity of the respective polymerase to the fungal toxin α-amanitin, is affected. During infection of HeLa cells with poliovirus, RNA polymerase II (polII)-specific initiation in isolated nuclei is inhibited approximately 3 h postinfection (19). Inhibition of preribosomal RNA synthesis by RNA polymerase I (polI) has been reported to occur very early during poliovirus infection (12). However, in encephalomyocarditis virus-infected L cells polI- and RNA polymerase III (polIII)-specific initiations in vivo are inhibited somewhat later than polII-specific initiation (36).

Among polymerases isolated from picornavirus-infected cells, the ability of all three classes to nonspecifically transcribe random DNA sequences is not impaired compared with that from mock-infected cells (1). No alterations of putative polII subunits from infected cells could be detected by two-dimensional gel analysis (2), further suggesting that the inhibition of transcription was not due to inhibition of the elongating polymerase.

Crawford et al. first showed that the in vivo inhibition by poliovirus of polII-mediated transcription could also be analyzed in vitro by using extracts from virus-infected cells (13). Extracts prepared from poliovirus-infected cells were found to be significantly less active than those from uninfected cells when assayed for the ability to transcribe from the adenovirus major late promoter. This inhibition could not be reversed by the addition of purified polII. However, a chromatographic fraction derived from uninfected cells, which contains a subset of factors required for specific initiation of transcription in vitro by polII, restored transcription in infected cell extracts. Crawford et al. concluded that one or more transcription factors required for specific initiation and not the elongating polII enzyme were inactivated in poliovirus-infected cells.

polIII transcribes a set of genes giving rise to small RNAs, some of which play a role in or modulate translation. This family includes the 5S RNAs, the tRNAs, the VA genes of adenovirus and the EBER genes of Epstein-Barr virus (11). In addition to polIII, three factors are required for specific transcription in vitro by polIII. TFIIIA, the only polIII-transcription factor purified to homogeneity (17, 32, 33) and characterized at the genetic level (22, 44), is required solely for transcription of the 5S genes (15, 39, 41). TFIIIB, TFIIIC, and polIII are required for transcription of all polIII genes (15, 39, 41). The factors form a complex with the gene in vitro (6, 8, 20, 30, 38, 40). The transcription complex is stable to dilution, changes in ionic strength, and competition with other DNA templates.

All polIII genes contain characteristic promoter elements located within the coding region of the gene. Two of these elements, the internal control region of the 5S gene (7, 17, 35) and the B block of the non-5S polIII-transcribed genes (23, 36), have been shown to be bound (4, 5, 10, 20, 21, 24, 30, 34, 42, 43) by proteins which copurify with two transcription factors, TFIIIA and TFIIIC, respectively.

Using in vitro transcription with both crude and fractionated mock- and poliovirus-infected cell extracts, we have assayed the activities of each of the four distinct components required for accurate transcription of polIII genes. Two of these components, TFIIIA and polIII, are not affected significantly during infection of cells with poliovirus. TFIIIC

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(and to a lesser extent TFIIIB) activity is severely depressed in extracts made from infected cells. This decrease in factor activity probably accounts for the 10- to 20-fold decrease in transcriptional activity of the crude, infected cell extract relative to mock-infected extract. TFIIIC derived from virus-infected cell extracts retains approximately the same specific activity for sequence-specific DNA binding as that derived from mock-infected extracts, thus leading us to conclude that the inactivation of TFIIIC by poliovirus does not necessarily involve destruction of its DNA-binding domain.

MATERIALS AND METHODS

Cells and viruses. HeLa and 293 (a human embryonic kidney cell line transformed by adenovirus type 5) cells were grown in spinner culture with SMEM medium (GIBCO Laboratories) supplemented with 1 g of glucose per liter and 5% newborn calf serum. Cells were infected with poliovirus type 1 (Mahoney strain) at a multiplicity of infection of 20 as previously described (14).

Preparation and fractionation of extracts. S100 was prepared from mock- and poliovirus-infected cells as described by Yoshinaga et al. (47). The S100 extracts were fractionated by chromatography on phosphocellulose as described by Segall et al. (39). The S100 extract was dialyzed against buffer A (20 mM HEPES [*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid] [pH 7.9], 0.2 mM EDTA, 0.5 mM dithiothreitol, and 20% glycerol) containing 100 mM KCl. A phosphocellulose column previously equilibrated with buffer A plus 100 mM KCl was used for chromatography of the proteins. The flow-through fraction and successive step elutions of 0.35 and 0.6 M KCl in buffer A were designated fractions A, B, and C, respectively. The fractions were dialyzed against buffer B (buffer A plus 5 mM $MgCl_2$ and 100 mM KCl).

In vitro transcription and polIII assays. Transcription reactions contained in a total volume of 40 μl the following: 30 mM HEPES (pH 8.0), 3 mM dithiothreitol, 7.5 mM $MgCl_2$, 8.25 μg of creatine phosphokinase per ml, 5 mM creatine phosphate, 1 μg of α-amanitin per ml, 500 μM each ATP, CTP, and UTP, and 25 μM GTP with 2 μCi [α-^{32}P]GTP (Amersham Corp.). Template DNA was added at a concentration of 6.25 μg/ml. S100 extract or phosphocellulose fraction A, B, or C was added as indicated in the figure legends. Templates used in this study were pVA1 (provided by Martin Schmidt) and pXbSF201 (provided by F. Razvi and A. Worcel). pVA1 contains the VA1 gene of adenovirus type 2 (cloned between *Sal*I and *Sma*I sites of pUC18). pXbSF201 contains the *Xenopus laevis* somatic-type 5S gene cloned in pUC9 (33). The elongation activity of polIII was assayed with a poly(dA-dT) template as previously described (37). Incubation of the specific transcription reaction was for 90 min at 30°C. RNA synthesis was terminated by the addition of sample buffer containing sodium dodecyl sulfate and urea. Labeled, specific RNA product was analyzed on 8% polyacrylamide gels containing 8 M urea. The synthesis of specific transcription products was quantitated by Cerenkov counting of the excised gel slices. TFIIIA, TFIIIB, and TFIIIC were assayed in reconstituted transcription assays by titration of the factor to be assayed into an excess of the other factors required for initiation. polIII is present mainly in the TFIIIB fraction and is in excess over TFIIIB in this fraction. This was determined by the inability of purified polIII to stimulate transcription in the presence of a limiting amount of the B factor and excess other factors (data not shown).

Labeling of proteins with [^{35}S]methionine. Cells were mock or poliovirus infected for 1 h, at which time the cell suspension received guanidine at a final concentration of 2 mM. Mock and poliovirus infections were continued for up to 4 h. Cells were then harvested by centrifugation, washed with phosphate-buffered saline, and suspended in minimal essential medium (containing no methionine) supplemented with 5% dialyzed new born calf serum. [^{35}S]methionine (specific activity, 1,000 Ci/mmol; ICN Pharmaceuticals) was then added to cells at a concentration of 10 μCi/ml; the infection was continued for up to 5 h and then was stopped by harvesting cells, washing with phosphate-buffered saline, and lysing in sodium dodecyl sulfate-gel sample buffer. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography.

DNase I footprinting. Footprinting was performed essentially as described by Yoshinaga et al. (46). Briefly, dephosphorylated *Sal*I-linearized pVA1 was 5′ end labeled with [γ-^{32}P]ATP and polynucleotide kinase, secondarily cut with *Eco*RI, and gel purified. The probe was preincubated for 30 to 60 min essentially under transcription conditions with 100 ng of poly(dI-dC), (Sigma Chemical Co.) and various amounts of phosphocellulose C fraction protein as indicated in the figure legend. After a 1-min digestion by DNase I (Worthington Diagnostics), the reaction was stopped, the preparation was phenol extracted, and labeled material was ethanol precipitated. Products were electrophoresed on a sequencing gel, and labeled fragments were visualized by autoradiography. *Msp*I-digested pBR322 and Maxam-Gilbert G+A reactions were used as markers to determine the location of protection.

RESULTS

Inhibition of polIII-specific transcription by poliovirus. To determine the kinetics of inhibition by poliovirus of polIII-mediated transcription, crude cell-free extracts (S100) (47) were prepared from mock- and poliovirus-infected cells harvested at hourly intervals postinfection. Specific polIII transcription was assayed by using a plasmid containing the VA1 gene of adenovirus type 2. Accurate transcription of the gene yields a 160-nucleotide VA1 transcript which can be visualized by autoradiography after its resolution by denaturing polyacrylamide gel electrophoresis.

When in vitro transcription assays were performed with extracts prepared from mock-infected cells harvested at hourly intervals, a relatively constant amount of VA1 transcripts was synthesized (Fig. 1, lanes 1 through 6). Similar amounts of transcripts were synthesized in cell extracts prepared from poliovirus-infected cells harvested at 0, 1, and 2 h postinfection (Fig. 1, lanes 7 through 9). Extracts prepared from cells infected with the virus for 3, 4, and 5 h, however, became increasingly less competent to generate VA1 transcripts, with no detectable activity remaining at 5 h (Fig. 1, lanes 10 through 12). Resistance to 1 μg and sensitivity to 320 μg of α-amanitin per ml characterized the synthesis of this transcript as being mediated by polIII (data not shown). Similar kinetics of inactivation were observed when transcription reactions were performed with a cloned tRNA gene (data not shown), indicating that this inhibition was not particular to the VA1 gene.

To examine whether the decrease of VA1 transcripts formed in the infected extracts was due to the presence of a nuclease, the stability of preformed transcripts was measured by using mock- and poliovirus-infected cell extracts. The labeled transcripts were stable over a 90-min period at

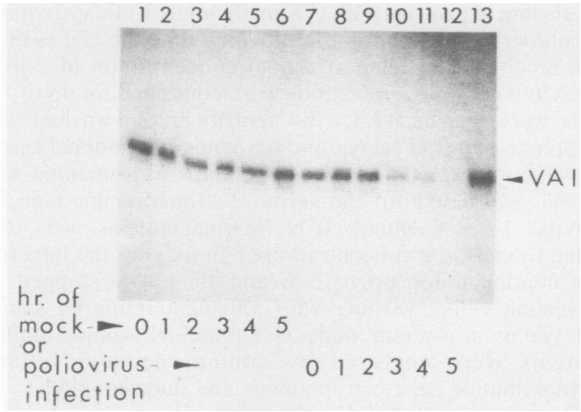

FIG. 1. Inhibition of polIII-mediated transcription by poliovirus. S100 extracts were prepared from mock- and poliovirus-infected HeLa cells harvested at hourly intervals after infection or mock infection as described in Materials and Methods. In vitro transcription reactions were performed with 20 μg of S100 extracts and the pVA1 template DNA as described in Materials and Methods. Incubation was for 90 min at 30°C. [α-^{32}P]GTP-labeled transcripts were electrophoresed on 8% polyacrylamide gels containing 8 M urea. Lanes: 1 through 6, VA1 transcripts synthesized by S100 extracts prepared from mock-infected cells harvested at 0, 1, 2, 3, 4, and 5 h after mock infection, respectively; 7 through 12, transcripts synthesized by S100 extracts prepared from poliovirus-infected cells harvested at 0, 1, 2, 3, 4, and 5 h postinfection, respectively; 13, VA1 RNA marker.

30°C when incubated with either mock- or poliovirus-infected extracts (data not shown). Therefore, we conclude that the inhibition of VA1 transcripts synthesized in extracts made from poliovirus-infected cells is due to a defect at the level of RNA synthesis and is not due to decreased stability of the transcript.

To determine whether an inhibitor was present in extracts prepared from poliovirus-infected cells, transcription reactions were performed by using a mixture of various ratios of extracts made from mock- and poliovirus-infected cells. The S100 extract does not titrate linearly over the range tested, as can be seen from the three-point titrations of either the mock- or the virus-infected cell extract (Fig. 2). Nonetheless, if an inhibitor were present in infected S100 extract, one would expect the amount of transcript in a mixed transcription reaction to be significantly less than additive at all ratios. At the two ratios tested, it is quite clear that the amount of transcripts formed is significantly more than additive (Fig. 2). At present we have no explanation for this finding. We note that a similar effect was seen by Crawford et al. (13) for the transcription of polII genes in mixed extracts. Further experiments involving preincubation of mixed extracts with ATP, both in the presence and absence of DNA, have also failed to detect a *trans*-acting inhibitory factor in infected extracts (data not shown). We were therefore unable to detect the activity of any soluble inhibitor in extracts from poliovirus-infected cells capable of inactivating transcription factor or polIII activity in the mock-infected extract.

Assay of individual components. To identify which component(s) required for in vitro transcription of polIII genes is inactivated in poliovirus-infected extracts, we assayed each component required for specific transcription at polIII promoters in vitro in a reconstituted system. We fractionated extracts made from both HeLa and 293 cells on phosphocellulose. We included 293 cells in addition to HeLa cells in our studies because (i) partially purified factors derived from 293 cells were more transcriptionally active than those from HeLa cells (47) and (ii) sequence-specific DNA binding by TFIIIC could not be performed with partially purified TFIIIC derived from HeLa cells (our unpublished results). The kinetics of inhibition by poliovirus of polIII-specific transcription in 293 cells parallel those for HeLa cells (data not shown). Both mock- and poliovirus-infected S100 extracts were fractionated in parallel by phosphocellulose chromatography. TFIIIA, TFIIIB, TFIIIC, and RNA polIII activities were assayed as described in Materials and Methods.

RNA polIII activity in the S100 was comparable between mock- and poliovirus-infected extracts (Fig. 3A and Table 1). Likewise, TFIIIA activity displayed approximately the same specific activity when TFIIIA was fractionated from either mock- or poliovirus-infected extracts (Fig. 3B). TFIIIB showed an approximately threefold decrease in specific activity when isolated from infected cells compared to that from mock-infected cells (Fig. 3C). TFIIIC purified from virus-infected S100 extract had a sixfold lower specific activity than that from mock-infected S100. The data in Table 1 summarize results from this experiment utilizing 293 cells and an analogous fractionation of mock- and poliovirus-infected HeLa cells. The extent of transcriptional inhibition observed with S100 extracts is clearly much greater than that seen in assays with any of the individual transcription factors. Furthermore, the effects of inhibition of TFIIIB and TFIIIC activity are not simply additive or multiplicative when the ratios of activities from the individual factor assays are compared with that of the S100 extracts. This is not surprising in that the formation of a transcription complex involves an unknown stoichiometry of components, some of which may interact synergistically through cooperative interactions. Nevertheless, it is apparent that the activities of both TFIIIB and TFIIIC are significantly inhibited in ex-

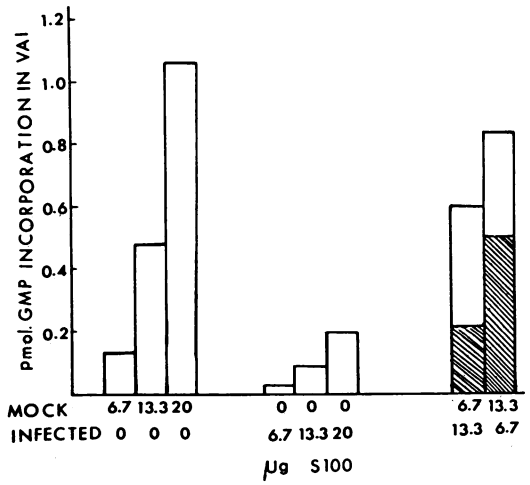

FIG. 2. In vitro transcription in mixed extracts. In vitro transcription of the pVA1 plasmid was performed with various amounts of mock S100 alone, infected S100 alone (4 h postinfection), and the indicated amounts of mixed mock-infected and infected S100 extracts. Labeled transcripts were visualized by gel electrophoresis and autoradiography and quantitated by counting Cerenkov counts per minute of the excised gel slices. The open bars indicate the amount of transcripts formed during in vitro transcription with mock-infected, infected, or mixed extracts. The shaded areas indicate the anticipated amount of transcript formed if transcription were additive in mixed extracts.

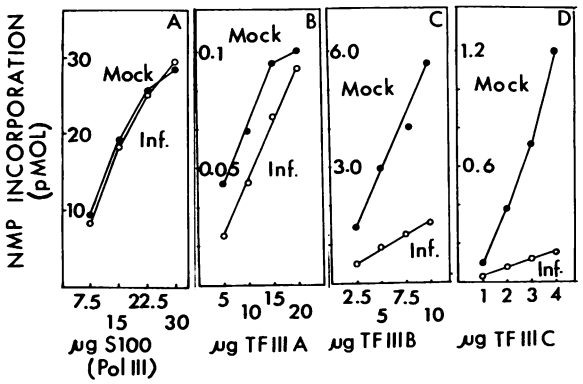

FIG. 3. Assay of individual protein components required for transcription. (A) Comparison of RNA polIII activities between S100 extracts prepared from mock- and poliovirus-infected cells. The indicated amounts of S100 extracts from mock- and poliovirus-infected cells (4 h postinfection) were assayed for polIII activity by using poly(dA-dT) template, [α-^{32}P]UTP, and unlabeled ATP as described previously (37). [^{32}P]UMP-labeled product was collected on membrane filters and counted by using Bray's scintillation fluid. (B, C and D) Quantitation of TFIIIA (B), TFIIIB (C), and TFIIIC (D) activities isolated from mock- and poliovirus-infected cells (4 h postinfection). Partially purified TFIIIA, TFIIIB, and TFIIIC were prepared from mock- and virus-infected cells as described in Materials and Methods. The indicated amounts of TFIIIA were assayed in the presence of saturating concentrations of TFIIIB, and TFIIIC, and purified polIII with pXbSF201 plasmid containing the 5S gene. TFIIIB and TFIIIC were assayed in the presence of saturating amounts of TFIIIC and polIII, and TFIIIB and polIII, respectively, with the pVA1 plasmid as a template. All in vitro-synthesized transcripts were analyzed on polyacrylamide gels and quantitated by counting Cerenkov counts per minute of the excised gel slices.

tracts derived from virus-infected cells, unlike those of TFIIIA and polIII.

To determine whether the activities of both TFIIIB and TFIIIC are reduced in the unfractionated extract derived from virus-infected cells, we tried to restore transcription in S100 from infected cells by the addition of individual transcription factors prepared from uninfected cells. The addition of TFIIIB to infected S100 extract only slightly stimulated transcription (Fig. 4, lane 6). The addition of TFIIIC significantly stimulated transcription in S100 prepared from infected cells (Fig. 4, lane 7), whereas the addition of purified polIII had no significant effect (Fig. 4, lane 8). Although this result would suggest that TFIIIC activity is reduced in virus-infected cell extract, this conclusion is clouded by the fact that TFIIIC also greatly stimulated transcription in mock-infected S100 extract (Fig. 4, lane 3). In contrast, transcription of pVA1 in mock-infected S100 extract was not stimulated by the addition of TFIIIB or polIII (Fig. 4, lanes 2 and 4). This result was expected, since it is known that TFIIIC is the limiting component required for transcription of polIII genes. We were therefore unable to determine unambiguously whether TFIIIC activity was reduced in virus-infected extracts by simple add-back experiments. Furthermore, this assay did not detect the decrease in activity of the nonlimiting factor (TFIIIB) which is seen in the reconstitution assay (Fig. 3). Similar experiments with the 5S gene as a template showed that TFIIIA was unable to restore transcription in infected extracts (data not shown).

TABLE 1. Ratios of transcription factor activity from mock- and poliovirus-infected HeLa and 293 cell extracts[a]

Factor	Ratio of activity[b]	
	HeLa (4 h)[c]	293 (5 h)[c]
S100	19	8
PolIII	1	1
TFIIIA	1.4	1.5
TFIIIB	4	3
TFIIIC	8	6

[a] In vitro transcription reactions were performed either with S100 extracts or in a reconstituted system utilizing TFIIIA, TFIIIB and TFIIIC from mock- and poliovirus-infected cells as described in Materials and Methods. RNA polIII elongation activity from mock- and poliovirus-infected cells was measured as previously described (37).

[b] Ratio of activity in mock-infected cells to that in poliovirus-infected cells.

[c] Time postinfection.

Transcriptionally inactive TFIIIC binds to DNA. An activity which protects the A- and B-block sequences of tRNA and VA genes from DNase digestion has been found to copurify with the TFIIIC factor required for in vitro transcription of these genes (20, 42, 43). Transcription, both in vitro and in vivo, of mutants with sequence alterations in the two conserved sequence blocks indicates the importance of both regions in the initiation of transcription (5, 21, 24). Studies of protein-DNA complexes formed with wild-type and mutant DNAs and partially purified TFIIIC indicate the primary interactions required for stable binding of TFIIIC are those with the B block (4). We wished to determine the ability of partially purified TFIIIC from mock- and poliovirus-infected cells to bind to the control region of the VA1 gene.

TFIIIC-containing protein pools from phosphocellulose fractionation of mock- and poliovirus-infected 293 cells were incubated with an end-labeled probe containing the VA1 gene. After incubation, the protein-DNA complexes were subjected to a brief digestion by DNase 1, leading to the

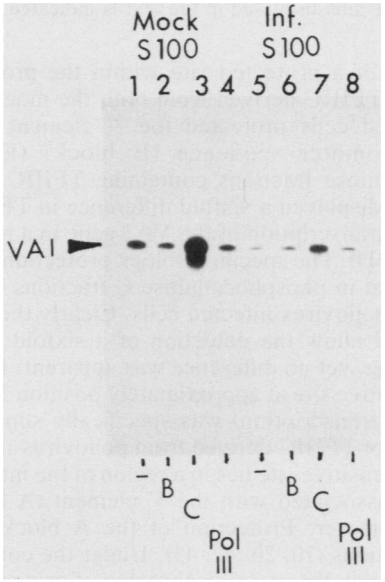

FIG. 4. Restoration of polIII-specific transcription by purified transcription factors. The pVA1 template was transcribed in vitro with 20 μg of either mock-infected (lanes 1 through 4) or poliovirus-infected (4 h postinfection, lanes 5 through 8) HeLa cell extract. Phosphocellulose-fractionated, uninfected cell-derived transcription factors were added as follows: no addition (lanes 1 and 5), 12 μg of TFIIIB (lanes 2 and 6), 6 μg of TFIIIC (lanes 3 and 7), and 12 U of polIII (lanes 4 and 8). Transcription products were resolved on denaturing polyacrylamide gels and detected by autoradiography.

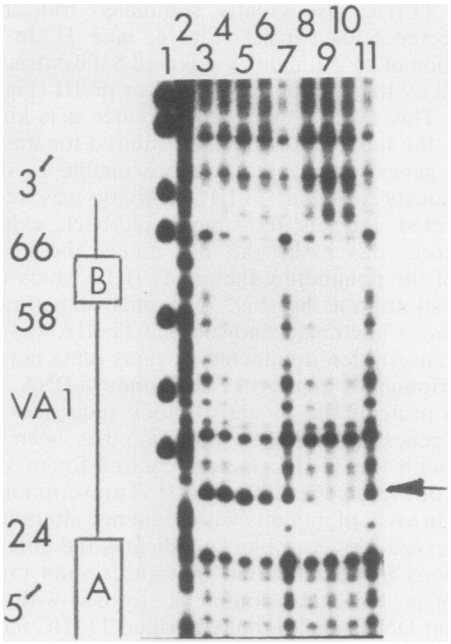

FIG. 5. DNase I footprint analysis of TFIIIC derived from mock- and poliovirus-infected 293 cells. The 5′ end-labeled *Sal*I-*Eco*RI fragment of pVA1 was incubated with TFIIIC purified from either mock-infected (lanes 4 through 6) or poliovirus-infected (lanes 8 through 10) cells. Amounts of protein in preincubation were as follows: no added protein (lanes 3, 7, and 11); 10 μg of protein (lanes 4 and 8); 17.5 μg of protein (lanes 5 and 9); and 25 μg of protein (lanes 6 and 10). Protein-DNA complexes were digested and processed as described in Materials and Methods. End-labeled *Msp*I-digested pBR322 (lane 1) and the Maxam-Gilbert G+A reaction (lane 2) were used to determine the regions of protection. The location of the A and B blocks is indicated on the side panel. The hypersensitive site discussed in the text is indicated by an arrow.

appearance of a protected site within the probe sequence (footprint). TFIIIC derived from both the mock- and poliovirus-infected cells protected the 3′ element of the VA1 internal promoter sequence (B block) (Fig. 5). The phosphocellulose fractions containing TFIIIC used in this experiment displayed a sixfold difference in TFIIIC specific activity for transcription of the VA1 gene in a reconstitution assay (Fig. 3D). The specific B-block protection was approximately equal in phosphocellulose C fractions derived from mock- and poliovirus-infected cells. Clearly the footprinting assay would allow the detection of a sixfold difference in DNA binding, yet no difference was apparent. Interestingly, a hypersensitive site at approximately position 30 (relative to the start of transcription) was specifically suppressed by a component of TFIIIC derived from poliovirus-infected cells. This hypersensitive site lies in a region of the internal control sequences associated with the 5′ element (A block) of the internal promoter. Protection of the A block varies with assay conditions (10, 20, 42, 43). Under the conditions used here, we do not observe a clear region of protection near the A block with the fractions derived from either the mock- or the virus-infected cells. Clearly, however, TFIIICs from both mock- and virus-infected cells contain components capable of roughly equal protection of the B block of the VA1 gene. We obtained a similar result, i.e., equivalent specific activity between mock-infected and infected cell C fractions for binding to the VA1 gene B block, with the restriction enzyme site protection assay devised by Lassar et al. (30; data not shown). Therefore, on the basis of both footprinting and restriction enzyme protection assays, we conclude that transcriptionally inactive TFIIIC derived from poliovirus-infected cell extracts retains its ability to bind to its cognate sequence.

We were unable to detect any DNA-binding activity of phosphocellulose-purified TFIIIC isolated from either mock- or poliovirus-infected HeLa cells with either assay (data not shown). We suspect this was due to a lower specific activity or amount of TFIIIC in extracts from HeLa cells relative to those from 293 cells (unpublished data) (47).

Inhibition of transcription is not due to inhibition of translation. A possible explanation for the inhibition of polIII-mediated transcription by poliovirus is that the shutoff of host cell protein synthesis by the virus (16, 29), maximal at 3 h postinfection, prevents the de novo synthesis of a transcription factor(s) that has a relatively short half-life. A recent report indicated that the transcription machinery of both polI and polIII requires a component which turns over relatively rapidly in a lymphosarcoma cell line (23). We have used two approaches to examine whether the inhibition of polIII-mediated transcription is a consequence of the inhibition of translation.

First, we used guanidine, a drug that specifically blocks initiation of viral RNA replication but still allows the virus to shut off host cell protein synthesis (9). The objective of this experiment was to determine whether inhibition of cellular transcription by poliovirus would still occur when cells were treated with 2 mM guanidine during infection, a condition that allows general inhibition of synthesis of host cell proteins by the virus. HeLa cells were infected (or mock infected) for 1 h. Then both the mock- and infected-cells were divided in half. One half was left untreated, and guanidine was added to 2 mM final concentration to the other half. The infections (or mock infections) were continued for a further 3 h, [^{35}S]methionine was added to the cells, and the infections were continued for up to 5 h. Cells were then harvested and washed, and a crude cell extract was prepared. Labeled proteins were visualized by fluorography after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Guanidine did not inhibit synthesis of cellular proteins (compare lanes 1 and 2, Fig. 6B) during mock infection. A distinct set of virus-specific polypeptides was synthesized in untreated cells infected with poliovirus (Fig. 6B, lane 3). Treatment of infected cells with guanidine at 1 h postinfection greatly reduced the yield of viral proteins due to inhibition of viral RNA replication and consequently reduced production of viral mRNA (Fig. 6B, lane 4). Synthesis of cellular proteins was inhibited to a great extent in infected cells in both the absence and presence of guanidine (Fig. 6B, lanes 3 and 4). These results therefore indicated that the virally induced inhibition of host cell protein synthesis still occured when guanidine was added to cells at 1 h postinfection.

Identical experiments were carried out as described above, except that guanidine was added to cells at 0, 1, or 2 h postinfection and infections were continued for up to 5 h without the addition of [^{35}S]methionine. Crude extracts were then prepared from mock- and virus-infected cells, and in vitro transcription reactions were performed. The ability to transcribe the VA1 gene by the mock- and poliovirus-infected extracts prepared at 5 h postinfection without added guanidine is shown in lanes 4 and 5, respectively, of Fig. 6A. The addition of guanidine to cells at either 0 or 1 h after infection prevented the inhibition of polIII-mediated transcription by poliovirus (lanes 1 and 2, Fig. 6A). However,

when the infection was allowed to proceed for 2 h before the addition of guanidine, the inhibition of transcription was manifested by 5 h (lane 3, Fig. 6A). Two conclusions can be drawn from the results presented in Fig. 6A and B. First, it is clear that guanidine added at 1 h postinfection prevents inhibition of polIII-mediated transcription by poliovirus, yet allows virally induced shutoff of cellular translation to occur. This suggests that overall cellular translation can be blocked by viral infection without causing inhibition of polIII-mediated transcription. Second, the results presented in Fig. 6A define an approximate time after infection at which a change leading to the inhibition of polIII-mediated transcription occurs. Guanidine added to infected cells at or after this time is unable to block inhibition of transcription by poliovirus.

A second approach to this problem involved the inhibition of cellular translation by cycloheximide for a period reflecting the time between the virus-mediated shutoff of host cell protein synthesis (approximately 3 h postinfection) and inhibition of transcription (4 to 5 h postinfection). In other words, the objective of this experiment was to create a situation in which cellular protein synthesis would be shut off for the same length of time that it would have been shut off during a typical poliovirus infection. Uninfected cells were incubated with or without cycloheximide for 1.5 and 2 h, respectively, in two separate experiments. The S100 extracts were then prepared from these cells, and the ability of these extracts to catalyze transcription of the VA1 gene

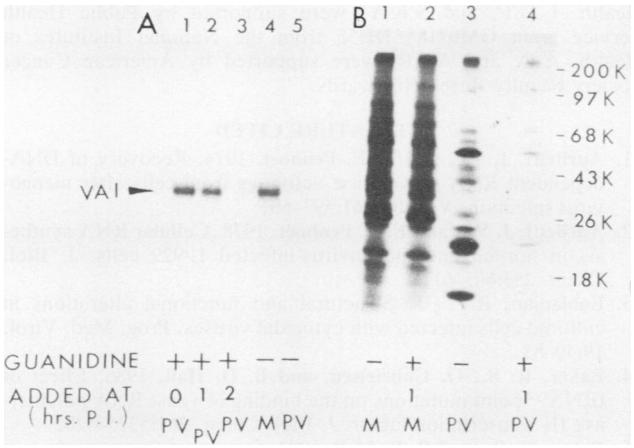

FIG. 6. Effect of guanidine on poliovirus-induced inhibition of polIII-mediated transcription. (A) Effect of guanidine on transcription. Guanidine (final concentration, 2 mM) was added to HeLa cells at 0, 1, or 2 h post infection, infections were continued for up to 5 h, cells were harvested, and S100 extracts were prepared. These extracts were then used for in vitro transcription of the VA1 plasmid. Lanes: 1, guanidine added at 0 h of infection; 2, guanidine added at 1 h postinfection; 3, guanidine added at 2 h postinfection; 4, mock infected to 5 h, no added guanidine; and 5, poliovirus infected to 5 h, no added guanidine. (B) Cellular and virus-specific proteins were labeled in vivo with [^{35}S]methionine as described in Materials and Methods. Guanidine was added at a final concentration of 2 mM at 1 h post infection (or after mock infection) as indicated. Mock and virus infections were stopped at 5 h, and crude extracts were prepared. Labeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by fluorography. Lanes: 1, mock-infected extract, no added guanidine; 2, mock-infected extract, guanidine added at 1 h after mock infection; 3, poliovirus-infected extract, no added guanidine; 4, poliovirus-infected extract, guanidine added at 1 h postinfection. Migration of standard protein markers is shown on the right.

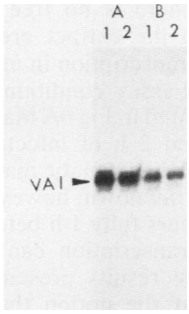

FIG. 7. Effect of cycloheximide treatment of cells on polIII-mediated transcription. Uninfected HeLa cells (4×10^8) were treated with 10 μg of cycloheximide per ml for 1.5 h (A) and 2 h (B) in two separate experiments. Crude S100 extracts were then prepared from these cells, and in vitro transcription reactions were performed as described in Materials and Methods with the VA1 plasmid. Lanes: 1, control cells not treated with cycloheximide; 2, cells treated with cycloheximide. The difference between the intensities of the VA1 transcripts formed in experiments A and B is due to variances between cell extracts prepared at different times and is routinely seen.

was examined. In both experiments, no significant difference in VA1 RNA synthesis could be detected between untreated and cycloheximide-treated extracts (Fig. 7A, lanes 1 and 2; Fig. 7B, lanes 1 and 2). In control experiments, we were able to show that cycloheximide treatment of cells for 1.5 and 2 h inhibited protein synthesis to greater than 95% (data not shown). These results further strengthen the idea that inhibition of cellular transcription in poliovirus-infected cells is not a consequence of inhibition of cellular protein synthesis by providing a second example in which host cell translation is inhibited without an inhibition of transcription necessarily following.

DISCUSSION

The in vivo inhibition of transcription by RNA polIII in cells infected with poliovirus can be demonstrated in vitro. The ability of S100 extracts prepared from poliovirus-infected cells to catalyze accurate transcription of cloned plasmids containing polIII promoters is severely inhibited compared to that from mock-infected cells. The observed in vitro inhibition of transcription cannot be explained by inactivation of RNA polIII, since the enzyme isolated from both mock- and virus-infected cells displays comparable elongation activity when assayed with added nonspecific DNA templates. We cannot exclude the possibility that polIII is altered during poliovirus infection of cells such that its ability to participate in specific transcription is somehow impaired. We find this possibility unlikely in that the addition of purified polIII does not restore transcription to that seen with mock-infected extracts. Fractionation of transcription factors from both mock- and poliovirus-infected S100 extracts and subsequent reconstitution studies show that the activity of the limiting transcription factor, TFIIIC, required for in vitro transcription of polIII genes is severely depressed in infected cells. Another transcription factor, TFIIIB, is inhibited to a lesser extent by virus infection of cells, whereas TFIIIA activity remains largely unaffected. It is therefore the loss of activity of TFIIIC and TFIIIB that explains the ability of poliovirus to inhibit host cell RNA synthesis by polIII.

It is not known how poliovirus infection of cells negatively affects the activity of these factors. Results of mixing exper-

iments suggest that there is no free and active catalytic inhibitory factor(s) in the extract prepared from infected cells that can inhibit transcription in mock-infected extracts under RNA synthesis assay conditions (Fig. 2). It is clear from the results presented in Fig 6A that an event takes place in cells between 1 and 2 h of infection which causes an inactivation of the polIII enzymatic machinery 3 h later. The nature of this event is unknown; however, it is interesting to note that this step occurs fully 1 h before the time at which inhibition of polIII transcription can first be detected in infected extracts. The results presented in Fig. 6 and 7 strongly argue against the notion that poliovirus-induced shutoff of host cell protein synthesis is the cause for inhibition of polIII-mediated transcription. It is also unlikely that the virus might cause sequestering of one or more transcription factors so they are not extractable by our methods. If this were true, at least for one factor, TFIIIC, both DNA binding and transcription-stimulatory activity should have been lost. It is the transcription-stimulatory activity of TFIIIC which is inactivated in the TFIIIC preparation. It is also possible that one or more poliovirus-encoded (or induced) proteases might cause decay of transcription factors. In such a case, however, one has to assume that the transcription-stimulatory domain and not the DNA-binding domain of TFIIIC is destroyed by protease action.

At present we cannot completely rule out the possibility that poliovirus RNA synthesis or the newly made poliovirus RNA itself is involved in the virus-induced inhibition of transcription. Guanidine added to cells at 2 h postinfection is unable to prevent inhibition of transcription. This result argues against the involvement of viral RNA in inhibition, because the bulk of viral RNA is synthesized after 2 h of infection and guanidine added at 2 h postinfection is known to block viral RNA synthesis almost completely (9). The most likely explanation of the guanidine experiment is that the addition of the drug at 2 h postinfection still allows accumulation of a viral protein in sufficient quantities to affect inhibition of transcription. The addition of guanidine at 0 or 1 h postinfection prevents accumulation of this protein in sufficient quantities because of early inhibition of viral RNA replication leading to insufficient amounts of translatable viral mRNA. It is worth mentioning that two poliovirus-specific polypeptides having approximate molecular weights of 75,000 and 65,000 accumulate in the nuclei just before the inhibition of cellular RNA synthesis (18).

Our results suggest that DNA binding by TFIIIC to the B block of the VA1 gene is not sufficient to allow transcription. We cannot exclude the possibility that poliovirus infection of cells causes changes in the DNA-binding activity too subtle to be seen in the assay used. We consider this possibility unlikely and favor the idea that there exist separable domains or subunits of TFIIIC conferring DNA-binding activity and transcriptional stimulation. It seems likely that the transcription-stimulatory domain could effect its activity through interaction with the other factors or polymerase. Recent studies on both eucaryotic and procaryotic DNA-binding transcription factors in Ptashne's laboratory (27) support this idea of a bifunctional factor. While these experiments were in progress, Yoshinaga et al. (46) demonstrated that TFIIIC derived from 293 cells could be chromatographically separated into two fractions. One of these fractions binds tightly to the B block of the VA1 gene (TFIIIC2) but is transcriptionally inactive in the absence of the other component (TFIIIC1). TFIIIC1 does not display DNA-binding characteristics alone but extends the protection by TFCIIIC2 over the 5′ half of the gene. Thus we hypothesize that infection of cells by poliovirus leads to a decrease in the activity of the TFIIIC component which does not bind DNA (TFIIIC1). Alternatively, alterations in a domain of TFIIIC2 involved in interactions with other factors, possibly including TFIIIC1, might lead to a DNA-binding yet transcriptionally inactive factor. Clearly, both of these possibilities will be investigated.

A number of viral proteins, e.g., the E1A protein of adenovirus, herpesvirus immediate-early protein, the large T antigens of both simian virus 40 and polyomaviruses, and a product of human T lymphotropic viruses stimulate transcription of genes in a *trans*-acting manner (28). The mechanism(s) by which these proteins activate transcription is not known. The adenovirus E1A protein, however, has been shown to stimulate transcription of polIII genes apparently by increasing the number of active TFIIIC molecules (25, 47). Whereas factor activity is positively regulated by adenovirus, the same factor is negatively affected by poliovirus. The elucidation of the mechanism of inactivation of TFIIIC in poliovirus-infected cells might give us clues as to how the transcription of polIII genes is regulated in both normal and transformed cells.

ACKNOWLEDGMENTS

We thank members of the Dasgupta and Berk laboratories for constructive criticism during the course of this work. We are grateful to P. Boulanger and S. Kliewer for a critical reading of the manuscript. We thank N. Tran and H. Hu for typing the manuscript.

This work was supported by Public Health Service grants AI18272 (to A.D.) and CA41062 (to A.J.B.) from the National Institute of Health. L.G.F. and S.K.Y. were supported by Public Health Service grant GM07185 DH55 from the National Institutes of Health. A.D. and A.J.B. were supported by American Cancer Society Faculty Research Awards.

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