MOLECULAR AND CELLULAR BIOLOGY, Oct. 1990, p. 5106–5113
0270-7306/90/105106-08$02.00/0

Vol. 10, No. 10

# A Transcriptionally Active Form of TFIIIC Is Modified in Poliovirus-Infected HeLa Cells

MELODY E. CLARK AND ASIM DASGUPTA*

*Department of Microbiology and Immunology and Jonsson Comprehensive Cancer Center, University of California, Los Angeles, School of Medicine, Los Angeles, California 90024*

Received 11 January 1990/Accepted 29 June 1990

**In HeLa cells, RNA polymerase III (pol III)-mediated transcription is severely inhibited by poliovirus infection. This inhibition is due primarily to the reduction in transcriptional activity of the pol III transcription factor TFIIIC in poliovirus-infected cells. However, the specific binding of TFIIIC to the VAI gene B-box sequence, as assayed by DNase I footprinting, is not altered by poliovirus infection. We have used gel retardation analysis to analyze TFIIIC-DNA complexes formed in nuclear extracts prepared from mock- and poliovirus-infected cells. In mock-infected cell extracts, two closely migrating TFIIIC-containing complexes, complexes I and II, were detected in the gel retardation assay. The slower migrating complex, complex I, was absent in poliovirus-infected cell extracts, and an increase occurred in the intensity of the faster-migrating complex (complex II). Also, in poliovirus-infected cell extracts, a new, rapidly migrating complex, complex III, was formed. Complex III may have been the result of limited proteolysis of complex I or II. These changes in TFIIIC-containing complexes in poliovirus-infected cell extracts correlated kinetically with the decrease in TFIIIC transcriptional activity. Complexes I, II, and III were chromatographically separated; only complex I was transcriptionally active and specifically restored pol III transcription when added to poliovirus-infected cell extracts. Acid phosphatase treatment partially converted complex I to complex II but did not affect the binding of complex II or III. Dephosphorylation and limited proteolysis of TFIIIC are discussed as possible mechanisms for the inhibition of pol III-mediated transcription by poliovirus.**

Infection of HeLa cells with poliovirus leads to rapid inhibition of host cell RNA synthesis (see reference 18 for a review). Specific transcription mediated by RNA polymerases (pol) I, II, and III is inhibited. RNA pol I-mediated transcription is inhibited at 2 to 3 h postinfection, pol II-mediated transcription is inhibited at 3 to 4 h postinfection, and pol III-mediated transcription is inhibited at 4 to 5 h postinfection (11, 18, 22, 27). The inhibition of transcription observed in vivo with poliovirus-infected cells can be reproduced in vitro by using mock- and poliovirus-infected cell extracts (5). Previous studies from our laboratory have shown that the inhibition of transcription by poliovirus infection in all three polymerase systems is due to the inactivation of specific transcription factors (11, 22, 27).

All pol III genes, such as the genes for 5S RNAs, tRNAs, VA RNAs of adenovirus, and EBER RNAs of Epstein-Barr virus, contain characteristic promoter elements located within the coding region of the gene (see reference 14 for a review). In the VAI gene, a tRNA-type pol III gene, two essential promoter elements have been defined: the A box from nucleotides +14 to +40 and the B box from nucleotides +59 to +69 (10, 13, 15, 32). Two transcription factors, TFIIIB and TFIIIC, are needed in addition to the polymerase to accurately transcribe all pol III genes in vitro (23, 29). In the VAI gene, TFIIIC binds to the B-box sequence specifically. TFIIIB has not been shown to bind to the VAI gene directly, but recent studies have shown that TFIIIB from *Saccharomyces cerevisiae* can bind upstream of the transcriptional start site of a tRNA gene in a TFIIIC-dependent manner (20). Another transcription factor, TFIIIA, is required solely for transcription of 5S rRNA genes (29).

* Corresponding author.

Previous studies from our laboratory have shown that RNA pol III and TFIIIA activities are not affected by infection of HeLa cells with poliovirus (11). The activity of TFIIIC is severely reduced in infected cells, whereas the activity of TFIIIB is reduced to a lesser extent. The sequence-specific DNA-binding activity of TFIIIC, as assayed by DNase I footprinting, however, is not significantly altered by poliovirus infection. It was therefore concluded that transcriptional inactivation of TFIIIC in poliovirus-infected cells does not necessarily involve alteration of its DNA-binding domain.

To determine how poliovirus inactivates the transcriptional activity of TFIIIC, we used a gel retardation assay which allows us to look at DNA-protein complexes that contain TFIIIC. We found that infection of cells with poliovirus resulted in disappearance of a slowly migrating, transcriptionally active form of TFIIIC and an increase in intensity of a faster-migrating, transcriptionally inactive form of TFIIIC. Also, a new, quickly migrating form of TFIIIC appeared in virus-infected cell extracts. This form may have resulted from proteolysis of TFIIIC. These changes in TFIIIC-containing complexes correlated kinetically with the time that pol III transcription was inhibited by poliovirus infection. Treatment of the transcriptionally active form of TFIIIC with acid phosphatase resulted in partial conversion to a transcriptionally inactive form of the protein. The results presented here suggest dephosphorylation and limited proteolysis of transcriptionally active TFIIIC in poliovirus-infected cells.

## MATERIALS AND METHODS

**Cells and viruses.** HeLa cells were grown in spinner culture with minimal essential media (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 previously described (7). After suspension in minimal essential medium without serum, mock-infected cells were treated identically to poliovirus-infected cells.

**Extract preparation and fractionation.** All procedures were performed at 4°C unless otherwise stated. Nuclear extracts were prepared from mock- and poliovirus-infected cells by the procedure of Dignam et al. (9) except that extracts were not dialyzed against buffer D. Instead, extracts were precipitated by addition of solid ammonium sulfate to 50% saturation (36). Pellets were suspended in a small amount of buffer A (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9), 0.2 mM EDTA, 20% glycerol, 0.5 mM dithiothreitol [DDT], 0.5 mM phenylmethylsulfonyl fluoride, 2 μg of aprotinin [Sigma Chemical Co.] per ml) and desalted on a Sephadex G50-150 (Sigma) column equilibrated and eluted with buffer A plus 0.1 M KCl.

Desalted nuclear extract was fractionated by chromatography on phosphocellulose (Whatman P11) equilibrated in buffer A plus 0.1 M KCl as described by Segall et al. (29). Step elutions of 0.35 and 0.60 M KCl in buffer A were designated fractions B and C, respectively. Fraction B, which contained TFIIIB and pol III, was further fractionated for the in vitro transcription reactions shown in Fig. 2A. The phosphocellulose B fraction was dialyzed to 0.1 M KCl in buffer A, and 5 mg was loaded onto a Mono Q HR 5/5 column (Pharmacia). Protein was eluted with a 20-ml 0.1 to 1 M KCl linear gradient in buffer A, using a flow rate of 1 ml/min at room temperature. Fractions with TFIIIB activity eluted at about 400 mM KCl, whereas pol III eluted at about 480 mM KCl. Pol III elongation activity was assayed with poly(dA-dT) as previously described (28). Pools of Mono Q-purified TFIIIB (4 mg) were loaded directly onto a 2-ml Cibacron blue-3GA agarose (Sigma) column equilibrated in buffer A plus 0.4 M KCl. The column was step eluted with buffer A plus 1 M KCl, and TFIIIB was eluted with buffer A plus 2 M KCl–5 M urea. Bovine serum albumin (0.1 mg/ml) was added to the pooled TFIIIB fractions, which were then dialyzed to a salt concentration of 0.1 M KCl and stored at −70°C (21). Separation of complexes I, II, and III by gradient elution of the phosphocellulose column was performed by the procedure of Hoeffler et al. (16) except that 80 mg of nuclear extract was incubated with 8 ml of phosphocellulose resin at 4°C for 1 h. The resin was then packed into a Pharmacia HR 10/10 column and eluted at room temperature with a 70-ml 0.1 to 1 M KCl gradient in buffer A. Cold (4°C) buffer A was used, and fractions were collected onto ice.

**Gel retardation analysis.** The gel retardation reaction mixture (15 μl) contained 7.5 μl of protein (1 to 3 μg) plus buffer A. The total salt concentration was adjusted to 75 mM KCl per reaction. The reactions were started by addition of a premix containing (per reaction) 2 μg of poly(dI-dC) (Pharmacia), 1.5 μl of binding buffer (100 mM Tris [pH 7.6], 5 mM EDTA, 30 mM $MgCl_2$, 50 mM DTT, 500 mM KCl), and 20,000 cpm of end-labeled gel retardation probe (4). To make the gel retardation probe, dephosphorylated, *Sal*I-linearized pVAI (which contains the VAI gene of adenovirus type 2 cloned between the *Sal*I and *Sma*I sites of pUC18) was digested with *Bst*EII and 5' end labeled with [γ-$^{32}$P]ATP. A 135-base-pair (bp) gel-purified fragment was isolated and electroeluted. Complete gel retardation reactions were incubated at room temperature for 30 min, loaded, with current on, into a 4% polyacrylamide (50:1.5 acrylamide/bisacrylamide) gel in 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA), and electrophoresed at room temperature for 3 to 4 h at 100 V or 7 h if free probe was run off the gel. Gels were prerun for 10 min before the samples were loaded (16). Competition reactions were performed by preincubating protein with a 100-fold molar excess of unlabeled B-box or HTLV oligonucleotides for 10 min [with poly(dI-dC) and binding buffer] before addition of the end-labeled gel retardation probe. Self-complementary oligonucleotides were synthesized (Applied Biosystems), hybridized, and ligated according to procedures described by Kadonaga and Tijan (19). The B-box oligonucleotide DNA contained the VAI B-box sequence GGATCCGGGGTTCGAACCCC (8). The HTLV oligonucleotide DNA sequence was CTTTCTAC AAGGGACTTTCCGCT (provided by R. Gaynor).

**In vitro transcription analysis.** Transcription reaction mixtures (40 μl) contained 30 mM HEPES (pH 7.9), 3 mM DTT, 7.5 mM $MgCl_2$, 8.25 μg of creatine phosphokinase per ml, 500 mM each ATP, CTP, and UTP, and 25 μM GTP with 2 μCi of [α-$^{32}$P]GTP (Amersham Corp.). pVAI template DNA was added to a concentration of 6.25 μg/ml. Protein was added as indicated in the figure legends. Reactions were terminated after 90 min of incubation at 30°C by addition of 160 μl of stop buffer (10 mM Tris [pH 7.9], 10 mM EDTA, 7 M urea, 100 mM $LiCl_2$, 0.5% sodium dodecyl sulfate, carrier tRNA) and extracted with phenol-chloroform. The RNA was ethanol precipitated, suspended in 15 μl of loading buffer (90% formamide, 0.01% xylene cyanol, and 0.01% bromophenol blue in 1× TBE) and loaded onto an 8% acrylamide–8 M urea gel. After electrophoresis, gels were dried and subjected to autoradiography. Densitometry was performed by using an LKB Ultrascan XL densitometer.

**Protease and phosphatase treatment.** Fractions containing separated complexes from the phosphocellulose gradient were incubated with 45 ng of chymotrypsin (*N*-α-*p*-tolyl-L-lysine chloromethyl ketone treated; Sigma). The protein was digested for 10 min at 30°C. The gel retardation reaction premix was then added, and the reaction was processed as described above. The resulting complexes were no different when chymostatin (Sigma) was added to the digested protein before the addition of premix (data not shown). Phosphatase treatment was performed essentially as described by Hoeffler et al. (16). Acid phosphatase from potato (grade I; Boehringer Mannheim Biochemicals) was spun out of $(NH_4)_2SO_4$ suspension and dissolved in 10 mM piperazine-*N*,*N'*-bis(2-ethanesulfonic acid) (PIPES) hydrochloride (pH 6.0). Approximately 0.03 U of phosphatase was added to each TFIIIC-containing fraction (0.5 μg). After 10 min at room temperature, sodium vanadate was added to a final concentration of 0.3 mM. The gel retardation reaction premix was then added, and the reaction was processed as described above. For in vitro transcription reactions, vanadate was first added to acid phosphatase-treated TFIIIC and then a TFIIIB-pol III fraction was added. The transcription reaction was then performed as described above. For some reactions, acid phosphatase was heat inactivated at 90°C for 10 min before addition of TFIIIC.

## RESULTS

**Multiple DNA-binding forms of TFIIIC.** Previous results from our laboratory showed that the transcriptional activity of TFIIIC isolated from poliovirus-infected cells was approximately eightfold lower than that from mock-infected cells at 5 h postinfection (11), yet partially purified TFIIIC prepared from mock- and poliovirus-infected cells was equally capable of protecting the B-box sequence of the VAI gene in a DNase I footprinting assay. To determine whether

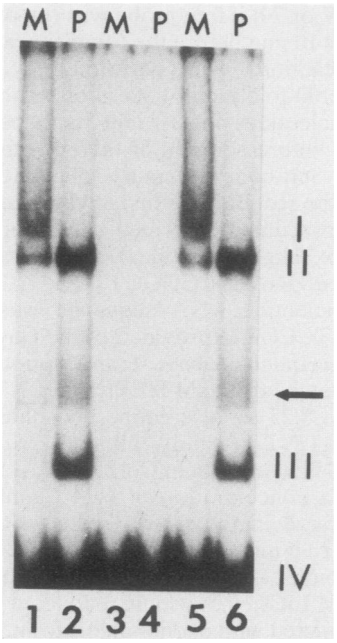


FIG. 1. TFIIIC-containing complexes in mock- and poliovirus-infected HeLa cells. Gel retardation assays were performed by using the TFIIIC fractions (3 μg) prepared from cells either mock infected (M) (lanes 1, 3, and 5) or poliovirus infected (P) for 5 h (lanes 2, 4, and 6) and the 135-bp VAI gene fragment. Complexes I, II, III, and IV are labeled along with an intermediate complex (arrow). Competitions were performed with a 100-fold molar excess of specific B-box oligonucleotide (lanes 3 and 4) or nonspecific HTLV oligonucleotide (lanes 5 and 6). Gel electrophoresis was done for 7 h. The free probe was run off the gel and is not shown.

domains of TFIIIC other than the DNA-binding domain are altered by poliovirus infection, we performed gel retardation assays. Equal amounts of phosphocellulose-purified TFIIIC (0.35 to 0.60 M KCl eluate) derived from cells either mock or poliovirus infected for 5 h were used in the assay. Reactions were performed by using a fragment of the VAI gene that contains both the A- and B-box promoter elements as the labeled probe.

When TFIIIC isolated from mock-infected cells was incubated with the VAI gene fragment, several DNA-protein complexes were detected (Fig. 1, lane 1). Only DNA-protein complexes designated I and II were specifically competed for with an excess of B-box oligonucleotide but not with an excess of unrelated HTLV oligonucleotide (lanes 3 and 5). The free probe and non-B-box competable complexes (data not shown) were run off the gel to accentuate differences in the mobility of complexes that were competable with the B-box oligonucleotide and thus were the result of TFIIIC binding. The complex labeled IV footprinted over the VA-binding protein region (data not shown) and was not competable by the B-box or HTLV oligonucleotide (lanes 3 to 6). The interaction of VA-binding protein with a region upstream of the B box has been detected previously in HeLa cell nuclear extracts by DNase I footprinting of the VAI gene, but its role in transcription of the gene is unclear (31).

When TFIIIC derived from poliovirus-infected cells was used in the gel retardation assay, complex I was not detected, and the intensity of complex II was increased relative to the amount of complex II present in mock-infected cell extract (Fig. 1, lane 2). Also, a new complex, designated complex III, was detected with TFIIIC from poliovirus-infected cells. Both complexes II and III were competed for specifically with the B-box oligonucleotide but not with the HTLV oligonucleotide (lanes 4 and 6). Another B-box competable complex (arrow in Fig. 1) also appeared more intensely in poliovirus-infected cell extract. We believe that this complex is an intermediate in the formation of complex III because of the kinetics of appearance of this complex (see Fig. 2B). The modifications of TFIIIC seen in poliovirus-infected cell extracts were not an in vitro artifact of extract preparation, since when mock- and poliovirus-infected cells were mixed before cell lysis, the resulting nuclear extract contained all three TFIIIC-DNA complexes (complexes I, II, and III) in a gel retardation assay (data not shown).

In summary, two B-box-specific complexes, I and II, were detected with mock-infected cell-derived TFIIIC. Upon infection of cells with poliovirus for 5 h, complex I disappeared, the intensity of complex II was increased, and a new B-box-specific complex, complex III, was generated.

**Kinetics of transcriptional inhibition correlate with changes in gel retardation patterns.** To determine whether the changes in gel retardation pattern correlated with the time course of inhibition of pol III transcription, we prepared nuclear extracts from mock- and poliovirus-infected cells harvested at 1, 3, 4, and 5 h postinfection. These extracts were fractionated in parallel on phosphocellulose columns, and the step-eluted fractions containing TFIIIC (0.35 to 0.60 M KCl) were used for both in vitro transcription and gel retardation assays. Saturating amounts of highly purified TFIIIB and pol III prepared from uninfected HeLa cells were added to the phosphocellulose TFIIIC fractions in a reconstituted in vitro transcription assay (Fig. 2A). No inhibition in the level of VAI transcription was evident up to 3 h postinfection (Fig. 2A, lanes 1 to 4). However, at 4 h postinfection, the level of VAI transcription was reduced to approximately 50% of the level from TFIIIC prepared from mock-infected cells, as measured by densitometry (lanes 5 and 6). At 5 h postinfection, the level of transcription was reduced to 20% of the level from TFIIIC prepared from mock-infected cells (lanes 7 and 8). This inhibition of pol III transcription was due to the decrease in TFIIIC activity alone, since TFIIIB and pol III from uninfected cells were used in the in vitro transcription assay.

When these same phosphocellulose TFIIIC fractions from mock-infected cells were used in the gel retardation assay, the pattern of DNA-protein complexes did not change with time of mock infection (Fig. 2B, lanes 1, 3, 5, and 7). Also, infection of cells with poliovirus for up to 3 h did not change this pattern (lanes 2 and 4). However, at 4 and 5 h postinfection, the slower-migrating complex I was greatly reduced in abundance, whereas complex II was increased (lanes 6 and 8). In addition, complex III appeared at 4 h postinfection, and the intensity of this complex increased at the 5-h time point. In contrast, the intensity of the intermediate complex (arrow in Fig. 2B) was reduced at the 5-h time point (compared with the 4-h time point), with a concomitant increase in the intensity of complex III (lanes 6 and 8). We do not know why the additive intensity of the poliovirus complexes II and III seems to be greater than the intensity of mock complexes I and II at the 5-h time point. We believe that this result may be due to changes that occur in the nucleus at later times postinfection, which allows more bound TFIIIC (complexes II and III) to be extracted during the preparation of nuclear extracts (1). An alternate possibility, that complexes from poliovirus-infected cells bind to the VA probe more stably than do complexes from mock-infected cells, is not likely, since the off rates of all four

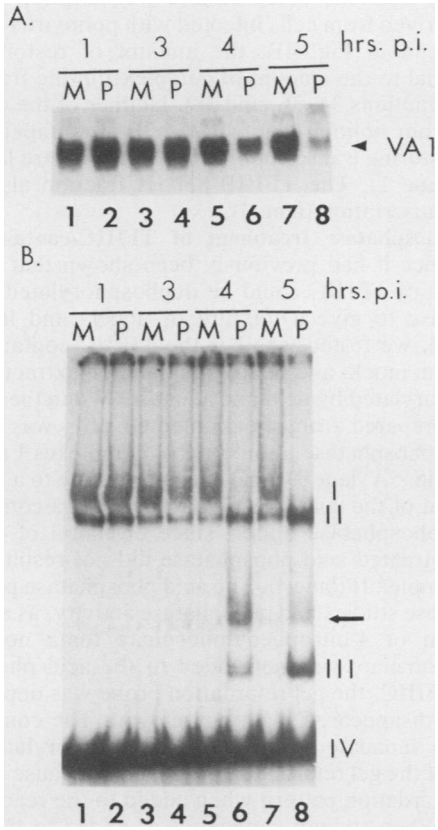


FIG. 2. In vitro transcription and gel retardation analysis of TFIIIC prepared at different times postinfection. (A) Equal amounts of phosphocellulose-purified TFIIIC (4 μg) prepared from mock-infected (lanes 1, 3, 5, and 7) and poliovirus-infected (lanes 2, 4, 6, and 8) cells at 1, 3, 4, and 5 h postinfection were added to partially purified TFIIIB (3 μg) and pol III (0.3 μg) in a reconstituted in vitro transcription system. The position of correctly initiated VAI transcript is indicated by the arrowhead. (B) Gel retardation reactions were performed by using equal amounts (3 μg) of the TFIIIC fractions used for panel A and a 135-bp VAI probe. The free probe was run off the gel and is not shown.

complexes were similar (data not shown). In summary, the loss of complex I and the increase in complexes II and III in gel retardation analysis correlated kinetically with the decrease in TFIIIC transcriptional activity.

**TFIIIC complex I is transcriptionally active and restores transcription in poliovirus-infected cell extracts.** Hoeffler et al. used gradient elution from a phosphocellulose column followed by gel retardation analysis to separate an upper band and a lower-band form of TFIIIC from HeLa nuclear extract (16). To separate complexes II and III from poliovirus-infected cell extracts and compare their transcriptional activity with those of complexes I and II from mock-infected cell extracts, we used a similar gradient elution procedure. Figure 3A shows resolution of complexes I and II from mock-infected cell nuclear extract by a linear salt gradient elution off a phosphocellulose column. Fractions were examined by the gel retardation assay described earlier. To keep the gel retardation reaction at the correct salt concentration, different amounts of the various fractions were used. Complex II eluted from the column first at 350 to 380 mM KCl, followed by fractions with both binding activities and then fractions with complex I activity alone at 430 to 470 mM KCl (Fig. 3A). The same chromatographic conditions were used to separate complexes II and III from poliovirus-infected cell nuclear extract. Complex II from virus-infected cell extract eluted at approximately the same salt concentration (350 to 400 mM) as its counterpart from mock-infected cell extract, whereas the bulk of virus-specific complex III eluted from the column at a lower salt concentration (240 to 320 mM) (Fig. 3B). These fractions with the chromatographically distinct B-box-binding activities were tested for transcriptional activity in a reconstituted in vitro transcription assay.

Phosphocellulose gradient fractions containing either mock complexes I or II or poliovirus complexes II or III were assayed in the presence of a phosphocellulose-purified fraction containing TFIIIB and pol III (0.1 to 0.35 M KCl eluate), which alone did not efficiently reconstitute pol III transcription (Fig. 4A, lane 3). Fraction 30, which contained only complex II, was transcriptionally inactive in the pres-

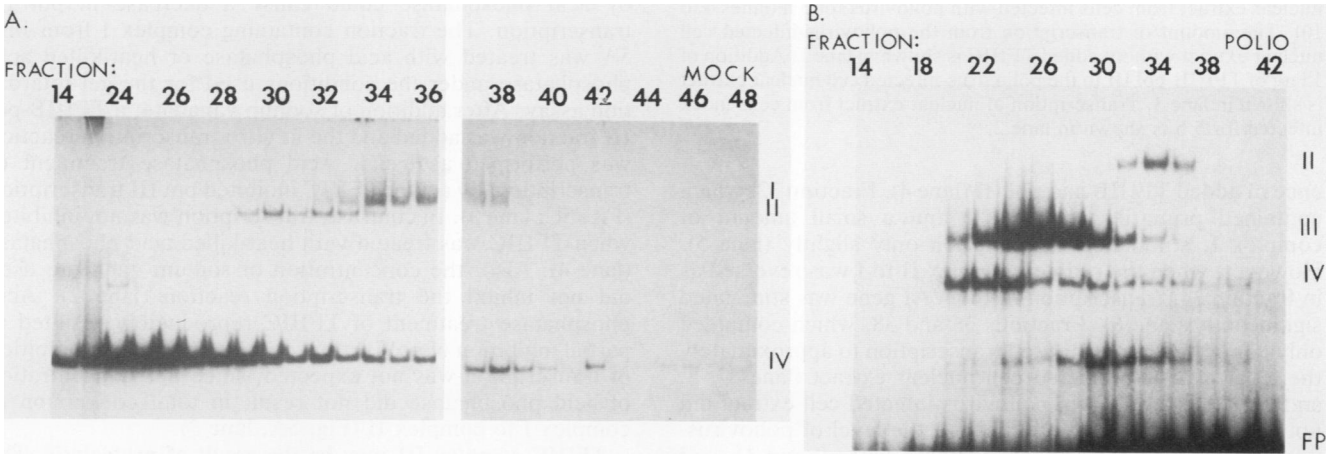


FIG. 3. Resolution of complexes I, II, and III by phosphocellulose chromatography. (A) Nuclear extract from cells mock infected for 5 h was eluted with a 0.1 to 1 M KCl linear salt gradient from a phosphocellulose column as described in Materials and Methods. Gel retardation analysis of each fraction was performed with the 135-bp VAI probe. Different amounts of the various fractions were used to keep the salt concentration of the gel retardation reaction constant. The free probe was run off the gel. (B) Nuclear extract from cells infected with poliovirus for 5 h was eluted from a phosphocellulose column by the procedure used for fractions in panel A. Gel retardations were performed as for panel A. Free probe (FP) is shown at the bottom of the gel.

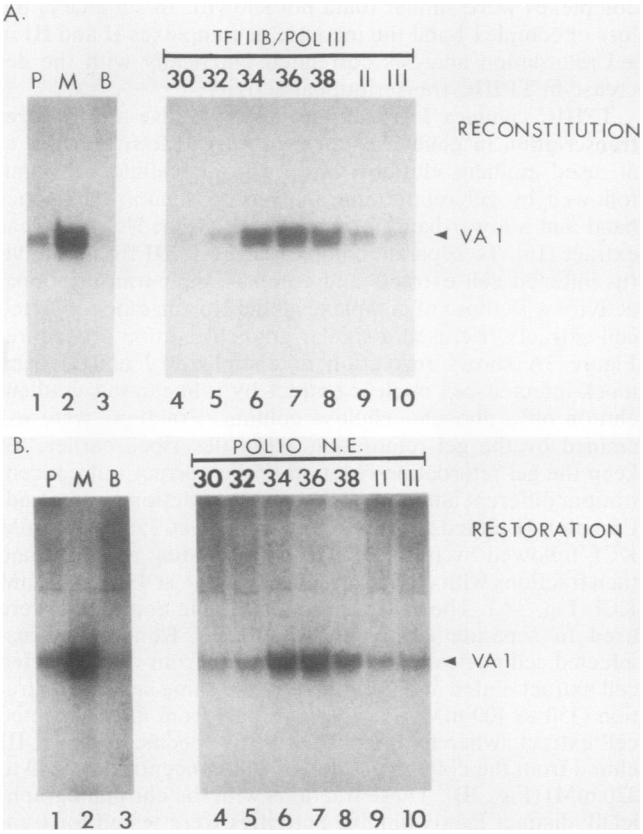


FIG. 4. In vitro transcription analysis of TFIIIC-containing complexes from mock- and poliovirus-infected cells. (A) In vitro transcription assays were performed with TFIIIC-containing fractions 30 to 38 from mock-infected cells (lanes 4 to 8) and complexes II and III from poliovirus-infected cells (lanes 9 and 10) (1.1 μg each) added to 15 μg of a TFIIIB-pol III fraction (0.1 to 0.35 M KCl eluate) from a phosphocellulose column. Lane 3 shows the transcriptional activity of the TFIIIB-pol III fraction without any added TFIIIC. In vitro transcription with nuclear extracts from 5-h mock- and poliovirus-infected cells is shown in lanes 2 and 1, respectively. (B) In vitro transcription assays were performed with the fractions used for panel A. Each TFIIIC-containing fraction was added to 10 μg of nuclear extract from cells infected with poliovirus for 5 h (lanes 4 to 10). The amount of transcription from the poliovirus-infected cell nuclear extract without added TFIIIC is shown in lane 1. Addition of 15 μg of TFIIIB-pol III to the poliovirus-infected cell nuclear extract is shown in lane 3. Transcription of nuclear extract from cells mock infected for 5 h is shown in lane 2.

ence of added TFIIIB and pol III (lane 4). Fraction 32, which contained primarily complex II and a small amount of complex I, stimulated transcription only slightly (lane 5). However, when the ratio of complex II to I was reversed as in fraction 34, transcription of the VAI gene was stimulated significantly (lane 6). Fractions 36 and 38, which contained only complex I, reconstituted transcription to approximately the level of mock-infected cell nuclear extract (lanes 7, 8, and 2). Complex II from poliovirus-infected cell extract did not reconstitute transcription above the level of poliovirus-infected cell nuclear extract (compare lanes 9 and 1), and complex III did not reconstitute transcription at all (compare lanes 10 and 3). Thus, only the form of TFIIIC that constitutes complex I was capable of reconstituting transcription in the presence of TFIIIB and pol III.

We next examined which, if any, of the different TFIIIC-containing fractions could restore transcription in nuclear extract derived from cells infected with poliovirus for 5 h. As can be seen in Fig. 4B, the amount of restoration was proportional to the amount of complex I in the fraction (see Fig. 3A, fractions 34, 36, and 38). Neither of the complexes derived from poliovirus-infected cells was capable of efficiently restoring transcription (Fig. 4B; compare lanes 9 and 10 with lane 1). The TFIIIB-pol III fraction also did not restore transcription (lane 3).

**Acid phosphatase treatment of TFIIIC-containing complexes.** Since it had previously been shown that an upper-band form of TFIIIC could be dephosphorylated with acid phosphatase to give a mixture of upper- and lower-band forms (16), we tested whether the TFIIIC-containing complexes from mock- and poliovirus-infected extracts could be dephosphorylated by acid phosphatase. When the complex I fraction prepared from mock-infected cells was incubated with acid phosphatase, a mixture of complexes I and II was formed (Fig. 5A, lane 3). The change was due to a heat-labile component of the acid phosphatase and not a component of the acid phosphatase buffer, since digestion of complex I with heat-treated acid phosphatase did not result in formation of complex II (lane 4). The acid phosphatase preparation used in these studies had phosphatase activity, as assayed by conversion of 4-nitrophenylphosphate (data not shown). Also, if vanadate was not added to the acid phosphatase-treated TFIIIC, the gel retardation probe was dephosphorylated and disappeared (data not shown). The concentration of sodium vanadate used to stop dephosphorylation before addition of the gel retardation probe did not cause changes in the gel retardation pattern when added to the reaction alone (lane 2). When sodium vanadate was added to the reaction before the addition of acid phosphatase, there was a partial inhibition in the formation of complex II (data not shown). Although acid phosphatase digestion did cause changes in the gel retardation pattern of the mock complex I, it did not affect the binding of the mock complex II or poliovirus complex II or III (Fig. 5A, lanes 5 to 8; Fig. 5B). To minimize the possibility that proteolysis of complex I was occurring, a protease-free grade of acid phosphatase was used in these studies and multiple protease inhibitors were used in all reaction buffers.

We next examined whether dephosphorylation of TFIIIC by acid phosphatase could cause a decrease in pol III transcription. The fraction containing complex I from Fig. 5A was treated with acid phosphatase or heat-killed acid phosphatase under the conditions used for the gel retardation assay. After addition of sodium vanadate, a TFIIIB-pol III fraction was added and the in vitro transcription reaction was performed as usual. Acid phosphatase treatment of transcriptionally active TFIIIC inhibited pol III transcription (Fig. 5C, lane 3). In contrast, transcription was not inhibited when TFIIIC was treated with heat-killed acid phosphatase (lane 4). Also, the concentration of sodium vanadate used did not inhibit the transcription reaction (lane 2). Acid phosphatase treatment of TFIIIC reproducibly resulted in partial inhibition of pol III transcription. Complete inhibition of transcription was not expected, since this concentration of acid phosphatase did not result in total conversion of complex I to complex II (Fig. 5A, lane 3).

**TFIIIC complex III may be the result of proteolysis.** The faster mobility of complex III suggested that it could be a proteolyzed fragment of TFIIIC that still retained its DNA-binding domain. Poliovirus encodes two proteases necessary for cleavage of the viral precursor polyprotein (26). Boulanger et al. have shown that chymotrypsin will cleave a

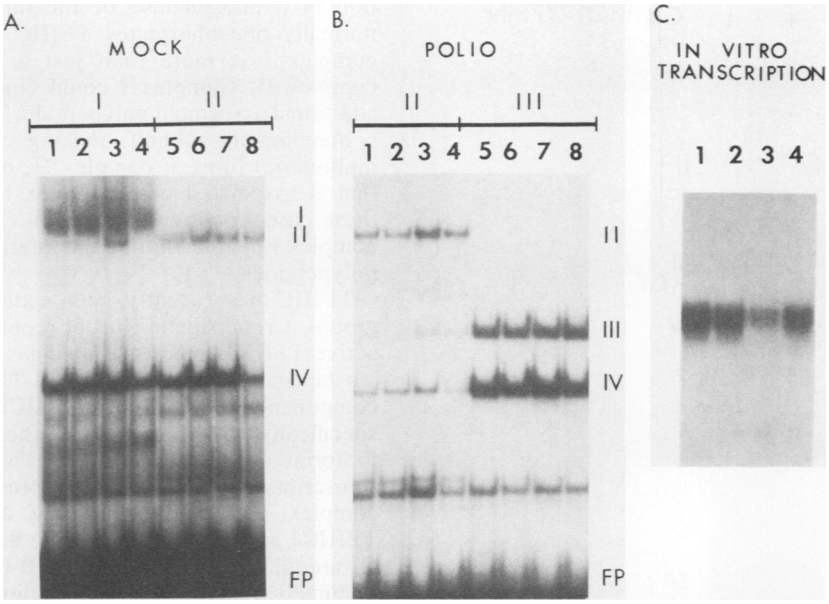


FIG. 5. Acid phosphatase treatment of TFIIIC-containing complexes. (A) Gel retardation reactions were performed with complexes I and II from mock-infected cells either untreated (lanes 1 and 5), treated with 0.3 mM sodium vanadate (lanes 2 and 6), treated with 0.03 U of acid phosphatase for 10 min followed by the addition of sodium vanadate (lanes 3 and 7), or treated with 0.03 U of heat-treated acid phosphatase followed by the addition of sodium vanadate (lanes 4 and 8). (B) Gel retardation reactions were performed as for panel A with complexes II and III from poliovirus-infected cells untreated (lanes 1 and 5), treated with sodium vanadate (lanes 2 and 6), treated with acid phosphatase (lanes 3 and 7), or treated with heat-treated acid phosphatase (lanes 4 and 8). (C) A TFIIIB-pol III fraction was added to complex I that was untreated (lane 1), treated with sodium vanadate (lane 2), treated with acid phosphatase and then sodium vanadate (lane 3), or treated with heat-treated acid phosphatase (lane 4) in an in vitro transcription reaction. FP, Free probe.

component of TFIIIC into a fragment of faster mobility which still binds to the B box of the VAI gene (3). This fragment is no longer transcriptionally active in a reconstituted in vitro transcription assay. We treated complexes I and II from mock-infected cells with chymotrypsin and compared the mobility of the resulting complexes with that of complex III from poliovirus-infected cells (Fig. 6). After treatment of either complex I or complex II with chymotrypsin, two new complexes appeared in the gel retardation assay (Fig. 6, lanes 4 and 6). The upper complex, indicated as CT, was of slightly faster mobility than complex III (lanes 1, 4, and 6). A fraction containing complex II from poliovirus-infected cells also resulted in the formation of this complex (data not shown). Treatment of complex III with chymotrypsin did not result in formation of this complex (lane 2). The weaker binding of the chymotrypsin-cleaved complex may have been due to the fast on-off rate (less than 1 min) of this complex (3; data not shown). We did not expect complex III and the chymotrypsin-cleaved complex to coelectrophorese, since it is unlikely that poliovirus infection would result in proteolysis of a TFIIIC complex at exactly the same site as chymotrypsin treatment. The two complexes were of very similar mobility, though, which suggests an exposed region in TFIIIC between a DNA-binding and transcriptional activating domain which is accessible to proteolytic cleavage. Experiments to determine whether a poliovirus protease is responsible for the generation of complex III are in progress.

We believe that the other complex in the chymotrypsin-treated lanes (indicated with an asterisk in Fig. 6) was the result of proteolysis of complex IV, since a fraction from the phosphocellulose gradient that contained only complex IV resulted in generation of this complex after chymotrypsin treatment (Fig. 6, lanes 7 and 8). Also, the amount of this complex was comparable to the amount of complex IV present in the fractions before treatment.

## DISCUSSION

We have shown in this report that inhibition of pol III transcription as a result of poliovirus infection can be correlated with modifications of protein-DNA complexes containing TFIIIC. Upon poliovirus infection, the transcriptionally active form of TFIIIC, mock complex I, disappeared and two transcriptionally inactive complexes containing TFIIIC, the poliovirus complexes II and III, were increased in abundance. These changes occurred at the same time postinfection that pol III transcription was first inhibited. Only the complex I form was able to restore VAI transcription when added to virus-infected cell extract. Since transcriptionally active complex I was partially converted to the inactive complex II by acid phosphatase treatment, we believe that poliovirus infection may result in dephosphorylation of some of complex I to complex II. At about the same time postinfection (4 h), poliovirus complex III may be formed by limited proteolysis of mock complex I or II.

Whereas poliovirus inhibits pol III transcription in large part by decreasing TFIIIC activity, adenovirus stimulates pol III transcription by increasing the activity of TFIIIC (17, 35). The results presented here are consistent with those of Hoeffler et al., who recently separated two forms of TFIIIC by using a gel retardation assay (16). The slower-mobility form was shown to be transcriptionally active when assayed in a reconstituted in vitro transcription system, whereas the faster-mobility form was transcriptionally inactive. Wild-type adenovirus-infected HeLa cells had a greater amount of the transcriptionally active form of TFIIIC than did cells infected with a mutant adenovirus that did not express the

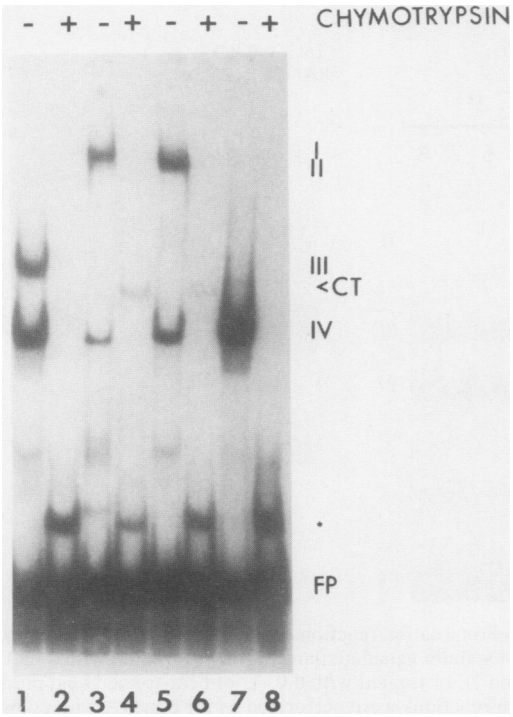


FIG. 6. Chymotrypsin treatment of complexes I, II, III, and IV. Gel retardation reactions were performed by using fractions containing complex III (lanes 1 and 2), complex I (lanes 3 and 4), complex II from mock-infected cell extracts (lanes 5 and 6), and complex IV from mock-infected cell extracts (lanes 7 and 8). Lanes 2, 4, 6, and 8 were incubated in the presence of 45 ng of chymotrypsin. The two complexes generated after chymotrypsin treatment are indicated with an arrowhead and an asterisk. Free probe (FP) is shown at the bottom of the gel.

E1A gene products. We have used in our gel retardation reactions a fragment of the VAI gene similar to that used by Hoeffler et al., but our reaction and gel conditions were significantly different. Using the reaction and gel conditions of Hoeffler et al. with adenovirus-infected and mock- and poliovirus-infected cell extracts, we observed that the upper-band form of TFIIIC ran at the same mobility as mock complex I and that the lower-band form ran at the same mobility as mock complex II (data not shown).

In Fig. 1, both mock and poliovirus complexes are designated as complex II sine they had the same mobility in the gel retardation assay, were competable with the B-box oligonucleotide, eluted at similar salt concentrations from a phosphocellulose gradient, and were transcriptionally inactive. Although mock complex II and poliovirus complex II appeared the same, there may still be subtle differences between these two complexes.

Although we do not know exactly what constitutes the differences between complexes I and II, results of the acid phosphatase experiments imply that proteins which form complex I are phosphorylated whereas proteins which form complex II are not phosphorylated. Many other transcription factors are phosphoproteins, and the activities of some of these factors have been shown to be regulated by phosphorylation state (2, 25, 30, 33). If complex I is a phosphorylated form of complex II, then poliovirus infection could result in the shutoff of pol III transcription by dephosphorylating complex I to transcriptionally inactive complex II. This could occur in poliovirus-infected cells by the activation of a phosphatase or the inhibition of a kinase that normally phosphorylates TFIIIC. It is also possible that complex I is more than just a phosphorylated form of complex II. Complex I could contain complex II plus an additional associated polypeptide. This polypeptide could be a homologous subunit, making complex I a dimeric (or multimeric) form of complex II, or a heterologous subunit that is associated with complex II to form complex I. In these cases, poliovirus infection could result in the loss of complex I by interrupting the interaction of these associated polypeptides.

TFIIIC has recently been extensively purified by two groups. Cromlish and Roeder reported that transcriptionally active TFIIIC consists of a single polypeptide (6), whereas Yoshinaga et al. have separated TFIIIC into two functional components: TFIIIC1 and TFIIIC2 (34, 36). TFIIIC2 binds specifically to the B box of the VAI gene in DNase I footprint assays, and TFIIIC1 is needed to reconstitute transcriptional activity in the presence of TFIIIC2. Since complex I is transcriptionally active, it contains both TFIIIC1 and TFIIIC2 activities. Since complexes I, II, and III are all competable with the B-box oligonucleotide, they minimally contain the DNA-binding domain of TFIIIC2.

Both the loss of complex I and the appearance of complex III correlated kinetically with the shutoff of pol III transcription in poliovirus-infected cells. Whereas treatment of complex I with acid phosphatase resulted in formation of complex II, complex III was not formed by acid phosphatase treatment (Fig. 5A and B). Thus, complex III is likely generated by a different mechanism than complex II. Since proteases had been used to define the DNA-binding domains of TFIIIC2 (3) and tau (12, 24), the yeast equivalent to TFIIIC, we treated phosphocellulose-purified TFIIIC with chymotrypsin. Although this treatment did not result in the formation of complex III, it did result in formation of a complex similar in mobility to complex III (Fig. 6). It is therefore possible that TFIIIC has a protease-sensitive hinge or exposed region that separates a DNA-binding domain from a transcriptional activating domain. Further experiments are needed to determine whether complex III is the result of a viral protease or a virus-induced cellular protease.

The cell may normally regulate pol III transcription by controlling the interconversion of TFIIIC-containing complexes I and II by phosphorylation. Interestingly, adenovirus infection could activate pol III transcription by shifting the equilibrium to the complex I state, and poliovirus could inhibit pol III transcription by shifting the equilibrium to the complex II state (16). Future studies will be aimed at elucidating the relationship between the TFIIIC-containing complexes by assaying for interconversion of complexes I and II. Also, further investigation of the formation of complex III and its role in shutoff of transcription is needed. Finally, development of an antibody to TFIIIC will be important to look for differences in the phosphorylation state of TFIIIC from mock- and poliovirus-infected cells.

## ACKNOWLEDGMENTS

We thank Richard Gaynor for the HTLV oligonucleotide and Noelle L'Etoile for chymotrypsin and chymostatin. We are indebted to Steve Kliewer, Lee Fradkin, and members of the Dasgupta laboratory for helpful discussion throughout the course of this work.

This work was supported by Public Health Service grants AI-18272 and AI-27451 from the National Institutes of Health. A.D. is supported by a Faculty Research Award from the American Cancer Society, and M.E.C. is supported by Public Health Service National Research Award GM-07185 from the National Institutes of Health.

## LITERATURE CITED


1. **Bablanian, R.** 1975. Structural and functional alteration in cultured cells infected with cytocidal viruses. Virology **19**:54–66.
2. **Bagchi, S., P. Raychaudhuri, and J. R. Nevins.** 1989. Phosphorylation-dependent activation of the adenovirus-inducible E2F transcription factor in a cell-free system. Proc. Natl. Acad. Sci. USA **86**:4352–4356.
3. **Boulanger, P. A., N. D. L'Etoile, and A. J. Berk.** 1989. A DNA-binding domain of human transcription factor IIIC2. Nucleic Acids Res. **17**:7761–7770.
4. **Boulanger, P. A., S. K. Yoshinaga, and A. J. Berk.** 1987. DNA-binding properties and characterization of human transcription factor TFIIIC2. J. Biol. Chem. **262**:15098–15105.
5. **Crawford, N., A. Fire, M. Samuels, P. A. Sharp, and D. Baltimore.** 1981. Inhibition of transcription factor activity by poliovirus. Cell **27**:555–561.
6. **Cromlish, J. A., and R. G. Roeder.** 1989. Human transcription factor IIIC: purification, polypeptide structure, and the involvement of thiol groups in specific DNA binding. J. Biol. Chem. **264**:18100–18109.
7. **Dasgupta, A.** 1983. Purification of host factor required for *in vitro* transcription of poliovirus RNA. Virology **128**:245–251.
8. **Dean, N., and A. J. Berk.** 1987. Separation of TFIIIC into two functional components by sequence specific DNA affinity chromatography. Nucleic Acids Res. **15**:9896–9907.
9. **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.
10. **Fowlkes, D. M., and T. Shenk.** 1980. Transcriptional control regions of the adenovirus VA1 RNA gene. Cell **22**:405–413.
11. **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.
12. **Gabrielsen, O. S., N. Marzouki, A. Ruet, A. Sentrnac, and P. Fromageot.** 1989. Two polypeptide chains interact with DNA. J. Biol. Chem. **264**:7505–7511.
13. **Galli, G., H. Hofstetter, and M. L. Birnstiel.** 1981. Two conserved sequence blocks within eukaryotic tRNA genes are major promoter elements. Nature (London) **294**:626–631.
14. **Geiduschek, E. P., and G. P. Tocchini-Valentini.** 1988. Transcription by RNA polymerase III. Annu. Rev. Biochem. **57**: 873–914.
15. **Guilfoyle, R., and R. Weinmann.** 1981. Control region for adenovirus VA RNA transcription. Proc. Natl. Acad. Sci. USA **78**:3378–3382.
16. **Hoeffler, W. K., R. Kovelman, and R. G. Roeder.** 1988. Activation of transcription factor IIIC by the adenovirus E1A protein. Cell **53**:907–920.
17. **Hoeffler, W. K., and R. G. Roeder.** 1985. Enhancement of RNA polymerase III transcription by the E1A gene product of adenovirus. Cell **41**:955–963.
18. **Kaariainen, L., and M. Ranki.** 1884. Inhibition of cell functions by RNA-virus infections. Annu. Rev. Microbiol. **38**:91–109.
19. **Kadonaga, J. T., and R. Tjian.** 1986. Affinity purification of sequence-specific DNA binding proteins. Proc. Natl. Acad. Sci. USA **83**:5889–5893.
20. **Kassavetis, G. A., D. L. Riggs, R. Negri, L. H. Nguyen, and E. P. Geiduschek.** 1989. Transcription factor IIIB generates extended DNA interactions in RNA polymerase III transcription complexes on tRNA genes. Mol. Cell. Biol. **9**:2551–2566.
21. **Klekamp, M. S., and A. Weil.** 1986. Partial purification and characterization of the *Saccharomyces cerevisiae* transcription factor TFIIIB. J. Biol. Chem. **261**:2819–2827.
22. **Kliewer, S., and A. Dasgupta.** 1988. An RNA polymerase II transcription factor inactivated in poliovirus-infected cells copurifies with transcription factor TFIID. Mol. Cell. Biol. **8**:3175–3182.
23. **Lassar, A. B., P. L. Martin, and R. G. Roeder.** 1983. Transcription of class III genes: formation of preinitiation complexes. Science **222**:740–748.
24. **Marzouki, N., S. Camier, A. Ruet, A. Moenne, and A. Sentenac.** 1986. Selective proteolysis defines two DNA binding domains in yeast transcription factor t. Nature (London) **323**:176–178.
25. **Mitchell, P. J., and R. Tjian.** 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245**:371–378.
26. **Reuckert, R. R., T. J. Matthews, O. M. Kew, M. Pallansch, C. McClean, and D. Omikanawski.** 1979. Synthesis and processing of picornaviral protein, p. 113–125. *In* R. Perez-Bercott (ed.), The molecular biology of picornaviruses. Plenum Publishing Corp., New York.
27. **Rubinstein, S. J., and A. Dasgupta.** 1989. Inhibition of rRNA synthesis by poliovirus: specific inactivation of transcription factors. J. Virol. **63**:4689–4696.
28. **Schwartz, L. B., V. E. F. Sklar, J. A. Jaehning, R. Weinmann, and R. G. Roeder.** 1974. Isolation and partial characterization of the multiple forms of deoxyribonucleic acid-dependent ribonucleic acid polymerase in the mouse myeloma, MOPC 315. J. Biol. Chem. **249**:5889–5897.
29. **Segall, J., T. Matsui, and R. G. Roeder.** 1980. Multiple factors are required for the accurate transcription of purified genes by RNA polymerase III. J. Biol. Chem. **255**:11986–11991.
30. **Sorger, P. K., and H. R. B. Pelham.** 1988. Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. Cell **54**:855–864.
31. **Van Dyke, M. W., and R. G. Roeder.** 1987. Multiple proteins bind to VA RNA genes of adenovirus type 2. Mol. Cell. Biol. **7**:1021–1031.
32. **Wu, G., J. F. Railey, and R. E. Cannon.** 1987. Defining the functional domains in the control region of the adenovirus type 2 specific VA RNA1 gene. J. Mol. Biol. **194**:423–442.
33. **Yamamoto, K. K., G. A. Gonzalez, W. H. Biggs III, and M. R. Montminy.** 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature (London) **334**:494–498.
34. **Yoshinaga, S. K., P. A. Boulanger, and A. J. Berk.** 1987. Resolution of human transcription factor TFIIIC into two functional components. Proc. Natl. Acad. Sci. USA **84**:3585–3589.
35. **Yoshinaga, S. K., N. Dean, M. Han, and A. J. Berk.** 1986. Adenovirus stimulation of transcription by RNA polymerase III: evidence for an E1A-dependent increase in transcription factor IIIC concentration. EMBO J. **5**:343–354.
36. **Yoshinaga, S. K., N. D. L'Etoile, and A. J. Berk.** 1989. Purification and characterization of transcription factor IIIC2. J. Biol. Chem. **264**:10726–10731.