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Modification of RNA Polymerase IIO Subspecies after Poliovirus Infection

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Infection of HeLa cells with poliovirus results in a shutdown of host transcription. In an effort to understand the mechanism(s) that underlies this process, we analyzed the distribution of RNA polymerase IIO before and after viral infection. Analysis of free and chromatin-bound enzyme indicated that there is a significant reduction in RNA polymerase IIO following infection. This observation, together with increasing evidence that transcription is catalyzed by RNA polymerase IIO, supports the hypothesis that poliovirus-induced inhibition of host transcription occurs at the level of RNA chain initiation and involves the direct modification of RNA polymerase II.

Picornavirus infection of mammalian cells is followed by rapid inhibition of host cell RNA synthesis (29, 30). Infection with poliovirus inhibits transcription when assayed by using nuclei or crude nucleoprotein complexes (5, 13, 20, 21, 35). For cell-free transcription systems, it has been shown that at least one factor needed for specific initiation of transcription is missing from poliovirus-infected cell extracts (7). In agreement with these results, we were able to show, using the detergent Sarkosyl (CIBA-GEIGY Corp.) (14, 17), that there is a reduction in the number of transcriptionally active DNA-bound RNA polymerase II molecules following poliovirus infection (13). It is not known whether the reduced number of the RNA polymerase II molecules engaged in transcription is due to a block in enzyme binding, to the premature release of polymerase from the template, or to a modification of the engaged enzyme.

Viral infection results in no detectable change in the activity of solubilized RNA polymerase II when the activity is assayed under conditions of nonselective transcription (1, 2, 31). Furthermore, neither the chromatographic behavior of RNA polymerase II nor the electrophoretic mobility of polymerase subunits is altered when enzymes isolated from mengovirus-infected or control cells are compared (2).

RNA polymerase II purified from a variety of eucaryotic cells consists of a mixture of three distinct subspecies designated IIO, IIA, and IIB (28; for a review, see reference 32). Each enzyme is composed of about 10 subunits and differs in the apparent molecular mass of its largest subunit (IIo, 240,000 daltons; IIa, 214,000 daltons; IIb, 180,000 daltons, for the calf thymus enzyme). Subunits IIo, IIa, and IIb have been shown to be the products of a single gene in *Saccharomyces cerevisiae* (23), *Drosophila melanogaster* (22), and mice (J. Corden, personal communication). Subunit IIc (140,000 daltons) is structurally distinct from subunits IIo, IIa, and IIb (9, 28) and is present in all three forms in equimolar stoichiometry with the largest subunit. RNA polymerases IIO and IIA are naturally occurring forms of the enzyme and are present in different proportions in various mammalian cells (13a, 24). RNA polymerase IIB is not detected in whole-cell extracts and appears to arise from the limited proteolysis of subunits IIo and IIa (6, 11, 18). Limited proteolysis results in the removal of the C-terminal domain encoded by the terminal exon of the largest RNA polymerase II subunit gene (6). The C-terminal domain of subunit IIa, and presumably IIo, contains 52 tandem repeats of a 7-amino-acid block with the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. Modification of this repeat, including phosphorylation, is thought to result in the conversion of subunit IIa to IIo (6, 8). Monoclonal antibody directed against RNA polymerase IIA (IIO) is capable of blocking promoter-dependent transcription in vitro from both viral (adenovirus type 2 major late promoter) and cellular (conalbumin and ovalbumin) promoters (10). In addition, RNA polymerase IIO appears to be the major form of the enzyme associated with simian virus 40 minichromosomes (13a) and cellular chromatin (P. Gariglio and L. M. Rangel, manuscript in preparation).

Previous studies indicate that the shutoff of host transcription following poliovirus infection occurs at the level of chain initiation (7, 13). However, RNA polymerase IIO is very labile in cellular extracts, and alterations of RNA polymerase II, in particular subspecies IIO, would probably not have been detected in these studies. In this work, we present evidence of a decrease in the amount of both total and chromatin-bound RNA polymerase IIO following poliovirus infection.

MATERIALS AND METHODS

Cell growth and infection. Poliovirus type 1 (Mahoney strain) was grown and purified after serial passages. HeLa S3 cells were propagated as spinner cultures in Joklik modified minimal essential medium containing 10% fetal calf serum, at a density of 3×10^5 to 6×10^5 cells per ml. Cell cultures were infected as follows. The medium was removed by centrifugation, and poliovirus stock in fresh minimal essential medium (without serum and glutamine) was added at a multiplicity of infection of 100 PFU per cell to the suspension culture at a density of 10^7 cells per ml. After 45 min, the medium was removed and the cells were suspended, at a density of 5×10^6/ml, in fresh medium containing 5% fetal calf serum and glutamine. Cells were incubated at 37°C and at different times after infection, as described in the figure legends, aliquots were removed for the assay of RNA polymerase II. Uninfected (mock-infected) cell cultures were processed identically, except for omission of the poliovirus in the initial step of infection.

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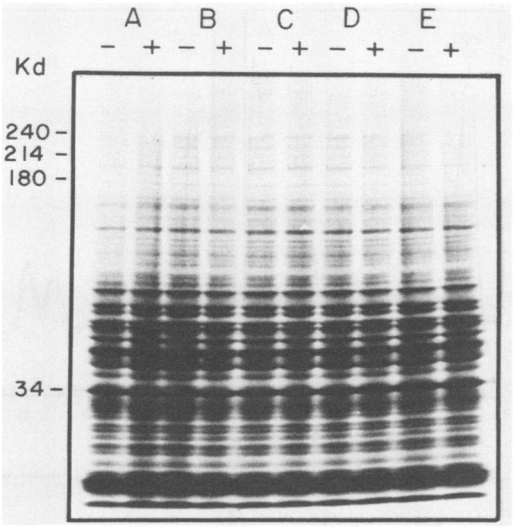

FIG. 1. SDS-PAGE of proteins from control and poliovirus-infected HeLa cells. Aliquots containing lysates of the equivalent of 6×10^5 uninfected and poliovirus-infected HeLa cells were prepared, fractionated by SDS-PAGE, and analyzed by Coomassie blue staining. Lysates from control cells (−) were prepared at 0 h (lanes A), 1 h (lanes B), 2 h (lanes C), 3 h (lanes D), and 4 h (lanes E) post-mock-infection. Extracts from poliovirus-infected HeLa cells (+) were prepared at the times indicated above.

The rate of transcription in mock- and poliovirus-infected HeLa cells was determined in vivo by a 5-min pulse with [^{3}H]uridine as previously described (13).

Separation of free and chromatin-bound RNA polymerase II. Free and chromatin-bound RNA polymerase II were separated as previously described (34), with the following modifications. Cells were pelleted and suspended in buffer B (25 mM Tris hydrochloride [pH 7.9], 2 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride). This whole-cell suspension was either analyzed directly by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) or layered and centrifuged in a discontinuous sucrose gradient, formed with 4 ml of 0.8 M sucrose dissolved in buffer C (20 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride), 2 ml of 0.5 M sucrose (in buffer C containing 0.25% Triton X-100), and 2 ml of 0.3 M sucrose dissolved in buffer C (16). After centrifugation (10 min, 8,700 × *g*), the pellet containing isolated nuclei was gently suspended (homogenized) in 0.34 M sucrose and incubated for 15 min on ice. The homogenate (whole nuclear extract) was either analyzed by SDS-PAGE or centrifuged for 10 min at 700 × *g* at 4°C to separate free RNA polymerase II. After centrifugation, the supernatant, which contains the solubilized enzyme, was carefully removed, and the nuclear pellet containing the chromatin-bound population of RNA polymerase II was suspended in 10 mM Tris hydrochloride (pH 7.9)–1 M sucrose–5 mM $MgCl_2$–1 mM dithiothreitol–0.2 mM phenylmethylsulfonyl fluoride.

PAGE. SDS-PAGE was carried out by the method of Laemmli (27). The resolving gel was a linear gradient of 5 to 17.5% polyacrylamide. After the tracking dye reached the bottom, the gel was either stained with Coomassie blue or the proteins were directly transferred to nitrocellulose and probed with antibody as described below.

Immunoblotting. After SDS-PAGE, the proteins were transferred to nitrocellulose filters as described by Towbin et al. (33) for 5 h at 170 V (600 to 900 mA). Following transfer, the filters were blocked overnight with washing buffer (WB: 10 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 0.5% Triton X-100, 0.2% SDS) containing 3% bovine serum albumin. The filters were then incubated with antibody directed against calf thymus RNA polymerase II (1:1,000 dilution) in WB containing 1% bovine serum albumin for 3 h at room temperature, washed three times with WB, and incubated with ^{125}I-labeled protein A (about 10^6 cpm) in WB for 2 h at room temperature. The filters were washed as indicated above and exposed to Kodak X-Omat AR X-ray film with an intensifying screen.

RESULTS

Characterization of RNA polymerase II subspecies in HeLa cell lysates at different times after poliovirus infection. In light of the observation that mammalian cells contain RNA polymerases IIO and IIA and of increasing evidence that promoter-dependent transcription is catalyzed by RNA polymerase IIO (see Discussion), we analyzed extracts from poliovirus-infected cells for changes in the distribution of RNA polymerase II subspecies.

Stained SDS-polyacrylamide gel patterns of proteins from whole-cell extracts of mock- and poliovirus-infected HeLa cells are shown in Fig. 1. In agreement with previous results (25, 26), such analysis indicates that there is no detectable degradation of host cell proteins immediately following poliovirus infection. Furthermore, stained gel patterns of different preparations of extracts from mock- and poliovirus-infected cells, obtained even at 5 h postinfection (p.i.), did not differ appreciably from one another (data not shown). From this analysis of the protein pattern, it is possible to conclude that no major degradation of the cellular proteins or major cell loss is taking place after infection.

The development of polyclonal antibody against calf thymus RNA polymerase II provides a tool for analysis of the cellular enzyme for possible alterations following poliovirus infection. This antiserum cross-reacts with HeLa RNA polymerase II (24) and reacts more strongly with subunit IIo than with subunits IIa and IIb. Low-molecular-weight subunits as well as subunit IIc also react with antibody. The whole-cell extract obtained from mock- and poliovirus-infected cells was resolved by SDS-PAGE (Fig. 1), and the proteins were electrophoretically transferred to nitrocellulose paper. Specific RNA polymerase II subunits were located by a two-step antibody reaction involving the use of specific antisera followed by ^{125}I-labeled protein A as described in Materials and Methods. The pattern of immunoreactive polypeptides obtained from whole HeLa cell extracts, at different times after infection, is shown in Fig. 2 (lanes marked as +). The positions of calf thymus RNA polymerase subunits IIo, IIa, and IIb are indicated on the left. Mock-infected cells served as controls (lanes marked as −) and were run for each time point. The autoradiogram shows a prominent protein of M_r 240,000 as well as a number of smaller-M_r polypeptides. This 240,000-M_r polypeptide corresponds to RNA polymerase subunit IIo. In an effort to quantitate the changes that occur following infection, transfer autoradiograms were scanned and peak areas were determined. A comparative analysis of the scanning pattern of the autoradiogram shown in Fig. 2 is presented in Fig. 3A (2 h p.i.) and B (4 h p.i.). The relatively constant amount of RNA polymerase II subunits in control cells and the specific difference in subunit IIo in poliovirus-infected HeLa cells are apparent. The similar intensities of subunit IIo in sam-

ples from control cells indicate that the content of this polymerase subunit did not change from 0 to 4 h post-mock-infection (Fig. 3C). In contrast, in cells infected with poliovirus at a multiplicity of infection of 100 PFU per cell, a reduction in subunit IIo intensity was visible by about 2 to 3 h p.i. By 4 h p.i., there was a 75% reduction in subunit IIo intensity relative to that of control cells (Fig. 2, lane E, and Fig. 3B and C). These results indicate that subunit IIo is either degraded or modified in such a way that it no longer reacts with antibody. The relative amount of the 34,000-M_r subunit of the RNA polymerase II, plotted versus time after infection, is shown in Fig. 3D. No significant difference in the amount of this subunit in control and poliovirus-infected cells was observed.

Analysis of chromatin-bound and total RNA polymerase IIO relative to poliovirus infection. RNA polymerase II is known to exist both in a soluble form (free enzyme) and bound to the cellular chromatin (34). Recently, Garcia-Carranca et al. (13a) observed that RNA polymerase IIO is associated with transcriptionally active simian virus 40 minichromosomes as well as cellular chromatin. In light of this observation and of previous reports that poliovirus infection results in a reduction in the number of chromatin-bound molecules (13), it is of interest to know whether the poliovirus-induced reduction in RNA polymerase IIO results from changes in the level of chromatin-bound enzyme. To investigate this possibility, we analyzed the disappearance of the chromatin-bound polymerase IIO relative to total cellular RNA polymerase at 4 h p.i.

HeLa cells were infected at a multiplicity of infection of 100 PFU per cell, and at 4 h p.i., nuclei were isolated and homogenized in 0.34 M sucrose. Chromatin was isolated by low-speed centrifugation as described in Materials and Methods. The sample volume loaded onto each lane was adjusted so that each contained extract from an equivalent number of cells. The results of the immunoblot analysis presented in Fig. 4 show that in control samples (marked as −), the relative amount of RNA polymerase IIO is almost equal in the whole-cell extract (lane A), the nuclear extract (lane B), and the cellular chromatin fraction (lane C). The free enzyme (lane D) accounts for a small fraction of the total RNA polymerase IIO. Similarly, in poliovirus-infected cells (marked as +), although there is a dramatic reduction in the amount of RNA polymerase IIO, the relative amount is comparable in the whole-cell extract (lane A), the nuclear extract (lane B), and the cellular chromatin fraction (lane C). In addition, there is no significant accumulation of free RNA polymerase II following poliovirus infection (lane D+). Consequently, the majority of RNA polymerase IIO in HeLa cells appears to be bound to cellular chromatin. Furthermore, poliovirus infection does not appear to significantly change the distribution of RNA polymerase IIO in the cell.

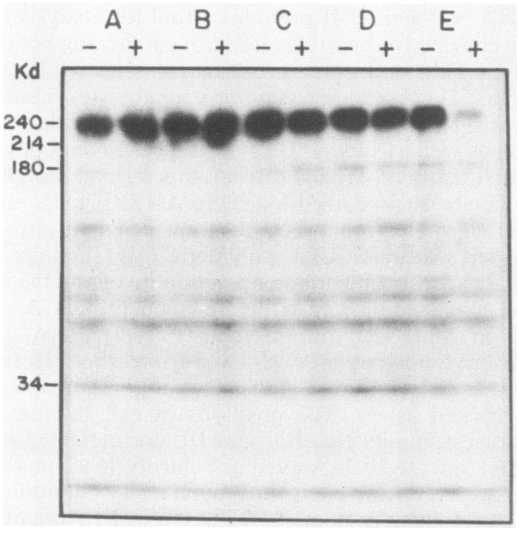

FIG. 2. Effect of poliovirus infection on distribution of RNA polymerase II subunits in whole-cell extracts. HeLa cells were grown as described in Materials and Methods. The cell pellet was suspended in buffer B, and the samples were processed as described in the legend to Fig. 1. After SDS-PAGE, the proteins were transferred to a nitrocellulose filter and reacted with antibody against RNA polymerase II and then with ^{125}I-labeled protein A (10^6 cpm). The exposure was for 4 days. Lysates were prepared at 0 h (lanes A), 1 h (lanes B), 2 h (lanes C), 3 h (lanes D), and 4 h (lanes E) as in Fig. 1. Lanes marked (−) and (+) refer to mock- and poliovirus-infected cell extracts, respectively.

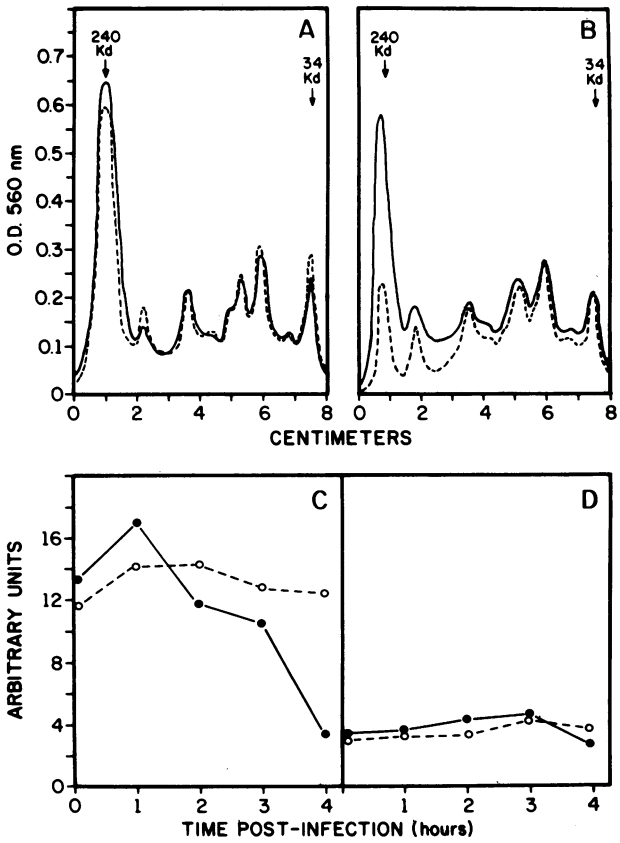

FIG. 3. Quantitation of RNA polymerase II subunits at different times after poliovirus infection. Different lanes of the autoradiogram described in the legend to Fig. 2 were scanned for optical density at 560 nm (O.D. 560 nm). (A) and (B) Samples taken 2 and 4 h p.i., respectively, from mock-infected (—) and poliovirus-infected (-----) cells. The arrows indicate the peak corresponding to RNA polymerase II subunits of M_r 240,000 and 34,000. (C) and (D) Quantitation of the 240,000- and 34,000-M_r polymerase subunits, respectively, plotted as a function of time after infection. Peak areas were estimated from autoradiogram scans of transfers presented in Fig. 2. Symbols: ○, mock-infected cells; ●, poliovirus-infected cells.

Analysis of RNA polymerase IIO relative to the inhibition of host RNA synthesis. To test the correlation between the disappearance of RNA polymerase IIO and the inhibition of

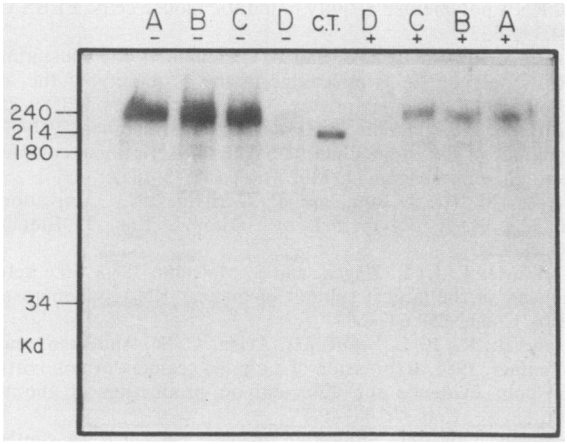

FIG. 4. Analysis of RNA polymerase IIO in HeLa whole-cell extracts, isolated nuclei, and free and chromatin-bound fractions. At 4 h p.i., poliovirus-infected (+) and mock-infected (−) HeLa cells were fractionated as described in Materials and Methods. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and reacted with antibody as described in Materials and Methods and the legend to Fig. 2. Lanes: A, whole-cell extract; B, isolated nuclei; C, chromatin-bound enzyme; D, free enzyme; C.T., calf-thymus-purified RNA polymerase II.

host cell RNA synthesis, we determined the rate of transcription in poliovirus-infected HeLa cells in vivo by a 5-min pulse with [^{3}H]uridine. The rate of [^{3}H]uridine incorporation is plotted in Fig. 5 along with the quantitation of subunit IIo. The modification of total cellular RNA polymerase IIO parallels, perhaps with a slight lag, the inhibition of host RNA synthesis.

DISCUSSION

The results presented here indicate that there is a significant reduction in both total and chromatin-bound RNA polymerase IIO following poliovirus infection. The decrease in subunit IIo was apparent within 2 h after infection and continued until, at 4 h, its level was about 25% that of control cells. During this period, there was no significant change in the level of the 35,000-M_r polymerase subunit. Consequently, poliovirus-induced modification of RNA polymerase II appears to be specific for the largest polymerase subunit (IIo) and does not result in complete polymerase degradation. This is the first demonstration of a specific structural change, resulting from poliovirus infection, in an enzyme involved in host cell transcription.

In the absence of a clear understanding of the relationship between subunit IIo and subunits IIa and IIb, it is not possible to define the biochemical basis for the reduction in RNA polymerase IIO. In principle, however, the reduction could result either from the dephosphorylation of subunit IIo, resulting in the formation of subunit IIa, or from limited proteolysis, resulting in the formation of subunit IIb. We cannot yet distinguish between these possibilities. The antibody used in these experiments reacts predominantly with the C-terminal repeat peptide of subunits IIo and IIa and consequently reacts only weakly with subunit IIb. The relative antibody reactivity to HeLa RNA polymerase subunits IIo, IIa, and IIb is approximately 3:1:0:3, respectively (24). Consequently, we would not expect to see an increase in subunit IIa or IIb, or both, as determined by antibody reactivity, proportional to the decrease in subunit IIo.

Apriletti and Penhoet (1, 2) and Schwartz et al. (31) have shown that the chromatographic behavior of RNA polymerase II and the nonselective transcriptional activity with an exogenous template are not altered in cells infected with the polio-related virus EMC and with mengovirus. It is unlikely, however, given the fact that RNA polymerase IIO is very unstable in cell extracts, that the differences observed here would have been detected in such experiments. On the other hand, Crawford et al. (7) have reported that poliovirus-infected cell extracts, prepared 3 h after infection, were unable to support selective transcription from the major late promoter of adenovirus type 2. Activity could, however, be restored by the addition of a partially purified S100 extract. The fact that activity could not be restored by the addition of purified RNA polymerase II suggests that modifications, in addition to that of subunit IIo, may be involved in poliovirus-induced repression of host transcription.

Although the modification of RNA polymerase IIO is paralleled by a shutoff of host transcription, it is not possible to conclude from these experiments whether this modification is the cause or the consequence of transcriptional changes. Indeed, the modification of the bulk of RNA polymerase IIO appears to lag behind the shutoff of host transcription. However, since only a relatively small fraction of the chromatin-bound enzyme appears to be engaged in transcription, we cannot eliminate the possibility that the early modification of a select fraction of RNA polymerase IIO is responsible for the shutoff of host transcription. Given the fact that RNA polymerase IIO is the predominant form in actively growing cells (13a, 24), is preferentially associated with cellular and viral chromatin templates (13a), and is transcriptionally more active than RNA polymerase IIA (3), it is not unreasonable that a modification of this enzyme form may bring about a change in transcriptional activity.

Future experiments will focus on defining the nature of the virus-induced products involved in the modification of RNA polymerase IIO. The following observations are consistent

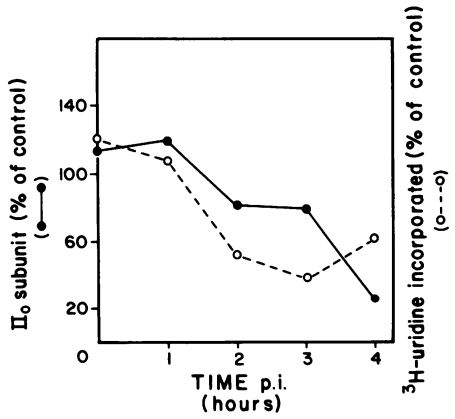

FIG. 5. Correlation of the disappearance of RNA polymerase IIO and poliovirus-induced inhibition of RNA synthesis. HeLa cells were mock or poliovirus infected (multiplicity of infection, 100) as described in Materials and Methods and the legend to Fig. 1 and 2. At the times indicated, 50-μl aliquots (2.5×10^5 cells) were removed and incubated with 5 μCi of [^{3}H]uridine (specific activity, 38 Ci/mmol) at 37°C. After 5 min, 40 μl of the suspension was analyzed for acid-insoluble radioactivity (○) as previously described (13). At the same times, RNA polymerase IIO from mock- and poliovirus-infected cells was analyzed as described in the legend to Fig. 2. After scanning of the autoradiogram, the percentage of polymerase subunit IIo from infected relative to control cells was determined for each time point (●).

with the idea that poliovirus-induced modifying enzymes are responsible for RNA polymerase IIO modification: (i) virus-induced products are required to inhibit host cell mRNA synthesis (for a review, see C. Fernandez-Tomas, *in* L. Carrasco, ed., *Mechanism of Viral Toxicity in Animal Cells*, in press), (ii) poliovirus-encoded proteases are present in infected cells (19, 25, 26), and (iii) poliovirus proteins are found in nuclei of infected cells (4, 12). Finally, an understanding of the modifications that occur in subunit IIo and the ways in which these changes influence transcriptional activity is important to our overall understanding of virus-induced repression of host transcription.

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