Poliovirus-induced Inhibition of Host RNA Synthesis Studied in Isolated HEp-2 Cell Nuclei

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SUMMARY

Nuclei isolated from uninfected HEp-2 cells synthesized RNA for 60 to 90 min. The individual RNA polymerase activities were determined by α-amanitin differential inhibition and the RNA products characterized by electron microscope (EM) autoradiography and sucrose gradient centrifugation. In nuclei prepared from poliovirus-infected cells, the capacity to synthesize RNA in vitro decreased with time after infection. RNA polymerase II activity (hnRNA synthesis) was preferentially inhibited more than was the polymerase I activity (rRNA synthesis). Poliovirus-infected cytoplasm (S-30) inhibited in vitro RNA synthesis in uninfected nuclei by selectively affecting the polymerase II activity. Selective inhibition of hnRNA synthesis by the crude extracts could be monitored by EM autoradiography directly. Determinations of individual RNA polymerase activities by differential α-amanitin inhibition were done only after treatment of the infected cytoplasm with micrococcal nuclease to abolish virus RNA replication. Selective inhibition of hnRNA synthesis depended on preincubation of the nuclei together with the infected cytoplasm, indicating that inhibitory substances from the infected cytoplasm entered the nuclei. Isolated nuclei therefore provide a useful system for studying the nature of the inhibitor(s) and of host RNA synthesis inhibition by picornaviruses.

INTRODUCTION

Infection of mammalian cells with picornaviruses results in inhibition of host cell DNA, RNA and protein synthesis (Franklin & Baltimore, 1962). While the mechanism of host protein synthesis inhibition has been intensively studied, little is known about the mechanism of virus-induced inhibition of host cell nuclear functions. The inhibition of host RNA synthesis is not due to a decrease in the precursor pool size (Plagemann, 1971), DNA template destruction (Holland & Peterson, 1964) or rapid degradation of the RNA product (Franklin & Baltimore, 1962; Colby et al., 1974). Mengovirus-induced inhibition of host RNA synthesis is paralleled by a decrease in the DNA-dependent RNA polymerase activity (Baltimore & Franklin, 1962). It is characterized by a rapid loss of host RNA polymerase II activity (hnRNA synthesis) and a slower decrease in polymerase I activity (rRNA synthesis), while there is no apparent change in the activity of polymerase III (5S RNA and tRNA synthesis) (Miller & Penhoet, 1972). The pool sizes of polymerase II molecules are identical in uninfected and infected cells (Apriletti & Penhoet, 1974; Schwartz et al., 1974). The RNA polymerase itself, as well as the chain elongation rate, are also not changed in infected cells. However, a smaller number of polymerase II molecules are engaged in RNA synthesis in infected cells than in normal cells (Apriletti & Penhoet, 1978; Flores-Otero et al., 1982). Therefore, the virus interacts with the regulation of host RNA chain initiation. These results were recently confirmed in a reconstituted in vitro transcription system (Crawford et al., 1981).

Lipid or lipoprotein (Ho & Washington, 1971) as well as virus proteins (Balandin & Franklin, 1964) have been postulated to be the inhibiting agent(s). We found an accumulation of poliovirus proteins in the host cell nucleus as judged by PAGE and by EM autoradiography (Bienz et al., 1982). In the present report, we have investigated in vitro RNA synthesis in isolated HEp-2

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cell nuclei. Crude poliovirus-infected cytoplasm induced a selective inhibition of host RNA polymerase II activity in uninfected nuclei comparable to the inhibition observed in vivo or in nuclei isolated from infected cells.

**METHODS**

*Cells and viruses.* HEp-2 cells were grown in suspension cultures using Joklik's modified minimal essential medium (Gibco) supplemented with 10% calf serum. A partially purified stock solution containing $1.3 \times 10^8$ p.f.u./ml of poliovirus, type 1 Mahoney, was used for infection. The cells were infected at a multiplicity of 30 as previously described (Bienz et al., 1978).

*Isolation of nuclei.* Nuclei from HEp-2 cells were isolated by a modified procedure originally described by Baltimore & Franklin (1962) and Penman (1966). Cells ($3 \times 10^7$) were washed once with phosphate-buffered saline (PBS) and allowed to swell on ice for 10 min in 0.125 M sucrose and 0.5 mM MgCl$_2$. After addition of 0.05% sodium deoxycholate, the cells were disrupted by Dounce homogenization. Isotonic conditions were restored by addition of 2 × Tris-NaCl buffer (50 mM-Tris-Cl pH 7.4, 280 mM-NaCl, 10 mM-KCl, 1 mM-Na$_2$HPO$_4$, 2H$_2$O) and mercaptoethanol to 5%. The nuclei were sedimented through 0.25 M sucrose and 1 mM MgCl$_2$ by centrifugation at 800 g for 5 min, suspended in 1 × Tris-NaCl buffer with 5% mercaptoethanol and subjected to a second centrifugation. The pellet consisted of purified nuclei with less than 5% contamination by intact cells. Uninfected nuclei were usually free of adhering cytoplasm. As time increased after infection, however, limited contamination due to cytoplasm sticking to the nuclei could not be avoided. The nuclei were suspended in 1 × Tris-NaCl buffer with 10 mM-dithiothreitol (DTT) and added to the *in vitro* transcription reaction mixture.

*Preparation of cytoplasmic extracts.* Uninfected as well as poliovirus-infected HEp-2 cells at 3 h and 5 h post-infection were harvested by centrifugation, washed once with PBS and swollen for 10 min on ice in a buffer containing 10 mM-Tris-Cl pH 7.4, 100 mM-KOAc, 1.5 mM-Mg(OAc)$_2$ and 2 mM-DTT. The cells were disrupted by Dounce homogenization, and the homogenate was centrifuged at 30000 g for 20 min. The supernatant (S-30) was divided into portions of 0.2 ml and fast-frozen in liquid nitrogen. Standard preparations contained 12 to 18 mg protein/ml as determined by the method of Rubin & Warren (1977). Portions of these cytoplasmic extracts were treated with micrococcal nuclease (P-L Biochemicals) according to Pelham & Jackson (1976).

In *in vitro* RNA synthesis. Isolated nuclei were incubated in a reaction mixture containing 50 mM-Tris-Cl pH 8.4, 10 mM-DTT, 0.02% Nonidet P40 (Shell), 2 mM-Mg(OAc)$_2$, 3 mM-Mn(OAc)$_2$, 60 mM-KOAc, 10 μM-S-adenosyl-L-methionine (Sigma), 2 mM each of ATP, GTP and CTP, 0.02 mM-UTP (all nucleoside triphosphates from Sigma) and 50 μCi/ml $[^3H]$UTP (Amersham International). For autoradiography, the unlabelled UTP was omitted and $[^3H]$UTP increased to 150 μCi/ml, which increased the radioactivity incorporated by a factor of 7. Standard transcription reactions were carried out with $1 \times 10^6$ to $2 \times 10^6$ nuclei/ml reaction mixture. Incubation was done at 30°C. In some experiments, 2 μg/ml actinomycin D (Merck, Sharp & Dohme) or various concentrations of α-amanitin (Boehringer, Mannheim) were included in the transcription reaction mixture. Cytoplasmic extracts were added to a final concentration of 1 mg/ml additional protein. Together with cytoplasmic extracts, 40 μg/ml tRNA (Boehringer, Mannheim) was included in the reaction mixture. Trichloroacetic acid (TCA)-insoluble material was precipitated from 50 μl amounts of the reaction mixture, filtered and counted in an Isocap-300 liquid scintillation counter (Searle).

*Analysis of RNA products.* At the end of *in vitro* incubation, the nuclei were washed twice with cold PBS and separated by ultrasonication into nucleolar and nucleoplasmic fractions as described by Vesco & Penman (1968). The RNA of each fraction was phenol-extracted and analysed on 15 to 30% sucrose gradients as previously described (Bienz et al., 1978). Centrifugation was done in the Spinco SW27.1 rotor for 16 h at 21000 rev/min.

*Electron microscopy (EM) and EM autoradiography.* Samples for electron microscopy were fixed, embedded and processed for autoradiography as previously described (Bienz, 1977; Bienz et al., 1978). For routine screening of the samples, autoradiographs of 0.2 μm sections were investigated by light microscopy. Important findings were confirmed by EM autoradiography. The samples of nuclei selected for autoradiography were taken from pools of nuclei which were also analysed by biochemical methods.

**RESULTS**

*RNA synthesis in uninfected HEp-2 cell nuclei.*

The kinetics of $[^3H]$UTP incorporation by uninfected HEp-2 cell nuclei is shown in Fig. 1. Incorporation was linear for 30 to 45 min and then levelled off. The initial rate of transcription was approximately $2 \times 10^4$ nucleotides/s/nucleus. The $^3$H counts at the end of incubation represented 20 to 30 pmol of UTP incorporated per 50 μl reaction mixture (about $10^5$ nuclei). Gross contamination of the reaction mixture with RNase could be ruled out since the TCA-precipitable radioactivity stayed fairly stable and decreased only by 10 to 20% in 5 h incubation
RNA synthesis in isolated nuclei

Fig. 1. Total incorporation of $[^3H]UTP$ in nuclei prepared from uninfected HEp-2 cells. Reaction mixture contained 0.02 mM unlabelled UTP, 50 $\mu$Ci/ml $[^3H]UTP$. Standard reaction: ●, +1 $\mu$g/ml α-amanitin; ○, +150 $\mu$g/ml α-amanitin.

at 30 °C. However, incorporation of $[^3H]UTP$ was slightly enhanced by addition of 40 $\mu$g/ml tRNA to the reaction mixture. The tRNA probably simply served as substrate for any residual RNase, thus preserving the newly synthesized RNA from being extensively degraded. In later experiments, tRNA was routinely included in the reaction mixture used to study the effects of cytoplasmic extracts on RNA synthesis in uninfected HEp-2 cell nuclei.

The relative amounts of the three RNA polymerases were determined by using α-amanitin. Seifart & Benecke (1975) reported that in extracts from HeLa cells the polymerase II activity is completely inhibited at 1 $\mu$g/ml α-amanitin, the polymerase III activity at 150 $\mu$g/ml, whereas polymerase I is completely resistant to 200 $\mu$g/ml. Using these concentrations of α-amanitin, we estimated polymerase I to represent 40%, polymerase II 55% and polymerase III about 5% of the total activity. These measurements had to be done within the linear range of $[^3H]UTP$ incorporation, i.e. not later than 30 min incubation.

RNA was shown by EM autoradiography to be synthesized in the nucleolus as well as randomly in the nucleoplasm of isolated nuclei (Fig. 2a), characterizing the products as rRNA and hnRNA respectively (Bernhard & Granboulan, 1968; Fakan & Bernhard, 1971). A 1 $\mu$g/ml amount of α-amanitin abolished the synthesis of hnRNA in the nucleoplasm (Fig. 2b). Occasionally, however, a few silver grains showed up over the nucleoplasm even in the presence of 1 $\mu$g/ml α-amanitin, probably due to polymerase III activity.

The RNA synthesized in vitro was extracted from nucleolar and nucleoplasmic fractions and analysed on 15 to 30% sucrose gradients (Fig. 3). The nucleolar RNA exhibited sedimentation characteristics of ribosomal RNA precursors. The nucleoplasmic RNA was polydisperse with no major peaks.

RNA synthesis in nuclei isolated from poliovirus-infected HEp-2 cells

Nuclei were isolated from poliovirus-infected HEp-2 cells at 1·5 h, 3·5 h, 6 h and 9 h post-infection and tested for their RNA-synthesizing capacity (Fig. 4). With time after infection, it became increasingly difficult to prepare pure nuclei, free of adhering cytoplasm, from the infected cells. These nuclei exhibited cellular RNA synthesis within the nucleus, but also virus-specific RNA replication in the contaminating cytoplasm, as confirmed by autoradiography. Virus RNA replication was resistant to 2 $\mu$g/ml actinomycin D whereas cellular RNA synthesis was completely abolished by actinomycin D. To calculate net cellular RNA synthesis, the
Fig. 2. EM autoradiograph of an uninfected HEp-2 cell nucleus. Reaction mixture contained no unlabelled UTP but 150 μCi/ml [3H]UTP. Incubation in vitro was for 60 min in the absence (a) or presence (b) of 1 μg/ml α-amanitin. Nuclei were processed for EM by conventional methods. Bar marker represents 2 μm for (a) and (b).
RNA synthesis in isolated nuclei

Fig. 3. Sucrose gradient sedimentation patterns of RNA synthesized in isolated nuclei. Incubation in vitro was for 60 min. The nuclei were separated into nucleoplasmic (○) and nucleolar (○) fractions. The RNA was extracted and centrifuged on 15 to 30% sucrose gradients in the Spinco SW27.1 rotor for 16 h at 21000 rev/min. Sedimentation is from right to left.

Fig. 4. Total incorporation of $[^3H]$UTP in nuclei prepared from poliovirus-infected HEp-2 cells at the times post-infection indicated. Incubation in vitro was for 30 min. Reaction mixture as described, contained no unlabelled UTP but 150 µCi/ml $[^3H]$UTP. ○, Standard reaction; ▲, +1 µg/ml α-amanitin; ◯, +2 µg/ml actinomycin D.

Fig. 5. Inhibition of total $[^3H]$UTP incorporation by 1 µg/ml α-amanitin in nuclei prepared from poliovirus-infected HEp-2 cells at the times post-infection indicated. Incubation in vitro was for 30 min. The '100% controls' (ordinate) refer to the untreated standard reaction of each preparation of nuclei, absolute ct/min of which can be read from Fig. 4.

amount of virus-specific RNA synthesized in the presence of 2 µg/ml actinomycin D was deducted from the total amount of RNA synthesized by untreated nuclear fractions.

The capacity of isolated nuclei to synthesize RNA in vitro decreased with time after infection at which the nuclei were prepared from poliovirus-infected cells. The kinetics of the inhibition of in vitro RNA synthesis were slightly faster than is the virus-induced inhibition of host RNA synthesis in intact HEp-2 cells (Bienz et al., 1978). This could be due to an increased leakage of transcription components out of the nuclei during fractionation of virus-infected cells in addition to the virus shut-off mechanism.

Parallel to the reduction in synthesizing capacity, in vitro RNA synthesis in infected nuclei was increasingly resistant to inhibition by α-amanitin (Fig. 5). In nuclei isolated at 3-5 h post-infection RNA polymerases I and III already represented 83% and polymerase II only 17% of the total activity expressed in vitro. Nuclei prepared at 6 h or 9 h post-infection expressed
polymerase I and III activities almost exclusively. RNA polymerase III activity always stayed at a level of about 5% and is omitted in the graph. The synthesis of hnRNA directed by polymerase II, therefore, was much more sensitive to the virus-induced inhibition mechanism than was the polymerase I activity. This finding was confirmed by EM autoradiography (Fig. 6). After incubation of nuclei isolated at 3.5 h post-infection silver grains showed up mainly over the nucleoli and scarcely over the nucleoplasm. EM autoradiographs of nuclei prepared at 6 h post-infection or later showed silver grains only over the nucleoli, and thus resembled pictures of uninfected nuclei after α-amanitin treatment (Fig. 2b).

Effects of infected cytoplasm on RNA synthesis in uninfected nuclei

Cytoplasmic extracts (S-30) were prepared from uninfected and poliovirus-infected HEp-2 cells at 3 h and 5 h post-infection. Uninfected HEp-2 cell nuclei and infected cytoplasm were preincubated for 10 min before addition of [3H]UTP. Fig. 7 shows the effects of crude infected cytoplasm on subsequent in vitro RNA synthesis in isolated uninfected nuclei. Synthesis of hnRNA in the nucleoplasm was preferentially inhibited by infected cytoplasm more than was rRNA synthesis in the nucleoli. Quantitative measurements, however, could not be made since endogenous virus RNA replication occurred in the infected cytoplasmic extracts, thus masking cellular RNA synthesis when added to uninfected nuclei. Therefore, some cytoplasmic extracts were treated with micrococcal nuclease according to Pelham & Jackson (1976) prior to addition to the uninfected nuclei. The nuclease treatment was found to abolish the endogenous virus RNA replication in the infected S-30 (data not shown).

Fig. 8 shows the effects of nuclease-treated cytoplasmic extracts on RNA synthesis in uninfected HEp-2 cell nuclei. Infected cytoplasm induced a strong inhibition of total [3H]UTP incorporation in uninfected nuclei. The degree of inhibition depended upon the amount of S-30, reaching 70 to 80% at about 0.5 mg additional protein per ml reaction mixture (data not shown). However, uninfected cytoplasm also inhibited RNA synthesis in uninfected nuclei but to a lesser extent and only at higher protein concentrations. Concentrations of 1 mg/ml additional
RNA synthesis in isolated nuclei

Fig. 7. Selective inhibition of in vitro hnRNA synthesis in isolated uninfected HEp-2 cell nuclei by cytoplasmic extracts prepared from poliovirus-infected HEp-2 cells at 3 h post-infection. Nuclei and cytoplasm were preincubated for 10 min before addition of [³H]UTP (1-3 mCi/ml). Incubation in vitro was for 60 min. Bar marker represents 2 µm.

protein, at which infected S-30 inhibited nuclear RNA synthesis by 70 to 80% whereas only 30% inhibition was caused by uninfected S-30, proved to be optimal for further experiments. The extracts made 3 h and 5 h post-infection were about equally effective in inhibiting RNA synthesis in uninfected nuclei. To test the assumption that an inhibitory substance migrated from the cytoplasmic extracts into the nuclei, isolated nuclei were preincubated with the extracts prior to addition of [³H]UTP. Preincubation of nuclei in the reaction mixture without UTP rapidly decreased the capacity of isolated nuclei to synthesize RNA. However, the ratios of [³H]UTP incorporation by nuclei without S-30, with uninfected S-30 or with infected S-30 in the reaction mixture remained fairly constant during the whole preincubation time.

The relative proportions of RNA polymerase II and polymerase I/III activity are shown in Fig. 9. Polymerase III represented about 5% of the total activity under any conditions and, again, was omitted from the graph. Addition of uninfected cytoplasm to the isolated nuclei changed the ratio of polymerase II to polymerase I/III activities only slightly and independent of preincubation of the nuclei in the reaction mixture. The decrease of RNA synthesis in isolated nuclei induced by uninfected cytoplasm, therefore, is a non-specific inhibition. On the other hand, infected cytoplasm did not affect the relative synthesis of rRNA and hnRNA when [³H]UTP was present from the beginning of incubation. However, infected cytoplasm selectively inhibited the polymerase II activity more than the polymerase I activity when the nuclei were preincubated with extracts prior to addition of [³H]UTP. The selectivity of inhibition was highest after 5 to 10 min preincubation. The extract prepared at 3 h post-infection was more effective in selectively inhibiting the polymerase II activity than was the extract prepared at 5 h post-infection.
The effects on uninfected nuclei induced by infected cytoplasm \textit{in vitro} within a few minutes were identical with the events occurring in nuclei of intact poliovirus-infected cells (Miller & Penhoet, 1972; Apriletti & Penhoet, 1978; Bienz et al., 1978). Therefore, we think that isolated nuclei will be useful for investigating picornavirus-induced host RNA synthesis inhibition.

DISCUSSION

The main goal of our experiments was to set up an \textit{in vitro} system to study the host cell RNA synthesis inhibition induced by picornaviruses. Isolated nuclei rather than intact cells were chosen for this investigation to overcome difficulties due to changes in the intracellular ionic and energetic pools (Egberts et al., 1977) as well as the impermeability of the cell membrane to various compounds. \(\alpha\)-Amanitin, for example, had no effect on RNA synthesis in intact HEp-2 cells (data not shown), but proved to be important for investigating RNA synthesis in isolated nuclei. Testing the effects of infected cytoplasm on RNA synthesis in isolated nuclei assumes that the nuclear membrane is permeable to larger molecules such as proteins. Preliminary results indicate that poliovirus proteins enter the nuclei during \textit{in vitro} incubation as judged by liquid scintillation counting, EM autoradiography and gel electrophoresis (W. Bossart et al., unpublished observations).

RNA synthesis in uninfected HEp-2 cell nuclei proceeded for a short time; 40\% of the total activity was due to RNA polymerase I, 55\% to polymerase II and 5\% to polymerase III activity. Synthesis of rRNA and hnRNA was confirmed by EM autoradiography and sucrose gradient analysis. The results are in good agreement with results on RNA synthesis in HeLa cell nuclei (Busiello & DiGirolamo, 1975; Vennstroem & Philipson, 1977; Wydro et al., 1980). In nuclei isolated from poliovirus-infected cells, the capacity to synthesize RNA \textit{in vitro} decreased with
time after infection. In addition, the RNA polymerase II activity was selectively inhibited more than was the polymerase I activity, but there was no apparent change in the polymerase III activity. The virus-induced inhibition of host RNA synthesis could not be overcome under the in vitro assay conditions. The kinetics of inhibition as well as the preferential inhibition of hn RNA synthesis in the in vitro system were comparable to picornavirus-induced effects on cellular RNA synthesis in intact cells (Miller & Penhoet, 1972; Apriletti & Penhoet, 1978; Bienz et al., 1978; Flores-Otero et al., 1982).

Poliovirus-infected cytoplasm (S-30) was able to inhibit in vitro RNA synthesis in isolated uninfected HEp-2 cell nuclei in a very specific manner. Shortly after mixing cytoplasm and nuclei in vitro, the RNA polymerase II activity became selectively inhibited. Selective inhibition of hnRNA synthesis depended upon preincubation of nuclei and cytoplasm prior to addition of [3H]UTP. Uninfected cytoplasm also inhibited cellular RNA synthesis, but to a lesser extent and in a non-specific manner. Selective inhibition by crude infected cytoplasm was detectable by EM autoradiography. For quantitative measurements, virus RNA replication in the cytoplasmic extracts had to be destroyed by micrococcal nuclease treatment prior to addition to the uninfected nuclei.

The above results are in contrast to those in previous reports, which have failed to demonstrate any inhibition of in vitro RNA synthesis by extracts from picornavirus-infected cells (Holland, 1962; Schwartz et al., 1974; Crawford et al., 1981). However, there are many discrepancies in the type of in vitro systems, cytoplasmic extract preparations and even virus–host systems used by these authors, making it difficult to compare the results. In our system, preservation of the structural integrity of the nuclei turned out to be very important. At present, we do not know the mechanism by which infected cytoplasm affects in vitro RNA synthesis in isolated nuclei. Crawford et al. (1981) presented evidence that poliovirus interacts with the regulation of host RNA transcription. The extent of preservation of regulation mechanisms in isolated nuclei is not known.

Since preincubation of nuclei and cytoplasm is a prerequisite for selective hnRNA synthesis inhibition, we assume that an inhibitor moves from the infected cytoplasm into the nuclei. Balandin & Franklin (1964) postulated that in mengovirus-infected L cells, a virus protein synthesized in the cytoplasm migrates into the host cell nucleus and interacts with host nuclear functions. In fact, we have demonstrated an accumulation of poliovirus proteins in the host cell nucleus (Bienz et al., 1982). According to our results, isolated nuclei provide a sensitive system for testing individual components of the infected extracts for inhibitory activities.

However, there are also disadvantages connected with the system of isolated nuclei. First, the activity of isolated nuclei compared to intact cells is low. Second, preincubation, which is a prerequisite for virus-specific inhibition of hnRNA synthesis in vitro, also rapidly decreased the capacity of isolated nuclei to synthesize RNA. Therefore, a balance between the penetration of macromolecules into the nuclei and the decrease in RNA-synthesizing capacity had to be worked out. Within these limitations, we will use the isolated nucleus system for further studies on picornavirus-induced inhibition of host RNA synthesis.

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