INCORPORATION OF AMINO ACIDS INTO POLYRIBOSOME-ASSOCIATED PROTEIN IN CYTOPLASMIC EXTRACTS OF POLIOVIRUS-INFECTED HELA CELLS

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Received February 27, 1964

Recent reports have made clear that aggregates of ribosomes (polyribosomes) are functional units for protein synthesis both in the intact cell (e.g., Warner et al. 1962) and in cell-free systems (Goodman & Rich, 1963). These findings have prompted a further study of the incorporation of amino acids into ribosome-associated protein in cytoplasmic extracts of HeLa cells infected with poliovirus, a phenomenon first described by Attardi and Smith (1962).

Methods

Strain S_3 HeLa cells were cultivated in suspension, treated for 1 hour with medium containing 10 μg of actinomycin D per ml and then infected with Type 1 poliovirus in the same medium, according to methods which have been given in detail (Levintow & Darnell, 1960; Scharff et al. 1963). Actinomycin progressively inhibits cellular RNA and protein synthesis, but has no effect on the replication of the viral macromolecules (Darnell, 1962). Virus maturation begins at about 2-1/2 hours under these conditions, and is complete between 5 and 6 hours.

At 4 hours after infection unless otherwise stated, cells were collected by centrifugation, washed as previously described, and suspended at 0° for 5 minutes at 4×10^7 cells/ml in RSB (10^{-2}M tris HCl pH 7.4, 10^{-2}M KCl and 1.5×10^{-3}M $MgCl_2$) (Scharff et al. 1963). The cells were ruptured

with 4 strokes of a tight-fitting, 7 ml-capacity Dounce homogenizer (Kontes Glass Co.), and the debris and nuclei removed by centrifugation at 1600 rpm for 10 minutes. The preparations, from either infected or uninfected cells, contained about 1 mg of protein N per ml.

Two parts of such extracts were added to one part of a mixture made up so as to yield final concentrations as follows: tris HCl buffer pH 7.6, 0.1M; $MgCl_2$, 1.5×10^{-2}M; KCl, 5×10^{-2}M; β-mercaptoethanol, 6×10^{-3}M; sodium phosphoenolpyruvate, 7.5×10^{-3}M; phosphoenolpyruvate kinase, 10 μg/ml; GTP, 3×10^{-5}M; ATP, 1×10^{-3}M; L-amino acids, including 5 μC of L-threonine uniformly labeled with C^{14}, 2×10^{-5}M each. The pH of the incubation mixture was 7.6. The composition of the mixture is essentially that described by Matthaei et al. (1962). For determination of total acid-precipitable radioactivity, 0.2 ml portions of the mixture were withdrawn at intervals during incubation at 37° and made 10% with respect to trichloroacetic acid. The resulting precipitates were collected by centrifugation and treated twice for 20 minutes at 90° with 5 ml portions of 5% trichloroacetic acid. The residues were dissolved in 1% NH_4OH and plated for the measurement of radioactivity.

For analysis by sucrose density gradient centrifugation, 2 ml portions of reaction mixtures, with 10 μC each C^{14}-labeled threonine, valine and phenylalanine, were chilled and sodium desoxycholate was added to a concentration of 0.25%. The material was immediately layered over a 15-30% w/v linear sucrose gradient, and centrifuged at 25,000 rpm in a Spinco SW-25 rotor for 105 minutes at 0°. Samples of the gradient were collected and analyzed as previously described (Scharff et al. 1963) for total radioactivity precipitable with trichloroacetic acid and for radioactivity specifically precipitable by antibody to subunits of the poliovirus capsid (S antigen).

Results

Properties of the cell-free system. The cytoplasmic extracts employed in the present experiments differ from those described by Attardi and Smith (1962) in that the cells were ruptured under conditions designed to preserve the polyribosomes. Under the present conditions, incorporation of radioactive amino acids into protein was maintained at a nearly constant rate for about 45 minutes. If the rate of incorporation with extracts of growing cells is taken as a standard, considerable inhibition was brought about by pre-treating the cells for 5 hours with 10 μg per ml of actinomycin D, but the rate with extracts of actinomycin-treated cells 4 hours after infection with poliovirus was usually enhanced (Fig. 1). As the data in Table 1 show, the process of infection first accelerates the inhibitory effect of actinomycin on amino acid incorporation, but a stimulation, corresponding to the period of rapid synthesis of viral protein, then ensues.

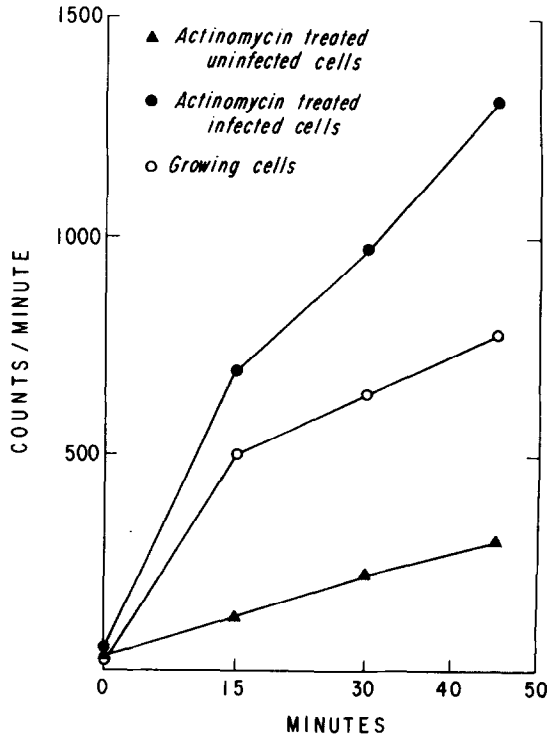

Fig. 1. Time course of incorporation of threonine into protein in cytoplasmic extracts of HeLa cells. See text for experimental details.

TABLE 1

Capacity of Cell Extracts to Bring About Amino Acid Incorporation

	cpm incorporated per 10 minutes					
Hours after infection	1	2	3	4	5	6
Uninfected	310	270	90	80	40	50
Infected	280	220	30	320	110	90

Actinomycin (10 μg per ml) was added to both cultures one hour before infection. Samples were withdrawn at the stated times after infection, cytoplasmic extracts were prepared, and the rate of amino acid incorporation was measured as described in the text.

It was not possible to demonstrate significant dependence of the system on added amino acids or nucleotides, doubtless because of their presence in the crude extracts, but omission of the energy-generating system reduced the rate of the reaction by two-thirds. Dependence on Mg^{++} was also demonstrable, with a sharp maximum rate at a concentration of 1.5×10^{-2} M. Addition of 5 μg per ml of RNase inhibited the reaction immediately and completely, and a similar concentration of DNase was without effect.

Polyribosomal site of amino acid incorporation. The polyribosomes concerned with the synthesis of viral protein in poliovirus-infected HeLa cells are enclosed within sedimentable structures, from which they can be released by 0.25% sodium desoxycholate (DOC) (Becker et al. 1963). The addition of this concentration of desoxycholate to cytoplasmic extracts reduced amino acid incorporation to one-fifth the original rate (cf. Kirsch et al. 1960). Accordingly, for the isolation of polyribosomes from the reaction mixture by sucrose gradient centrifugation, DOC was added at the

conclusion of the reaction, and immediately before centrifugation. An analysis of a sucrose gradient is compared in Fig. 2 with an analysis of a similar preparation from uninfected, actinomycin-treated cells. The major fraction of the amino acid incorporated with the extracts of infected cells was associated with the large polyribosomes (350-400S) characteristic of these cells (Penman et al. 1963), and of the total, acid-precipitable radioactivity, about 21% was specifically precipitable with antiserum to dissociated poliovirus protein (Scharff et al. 1963).

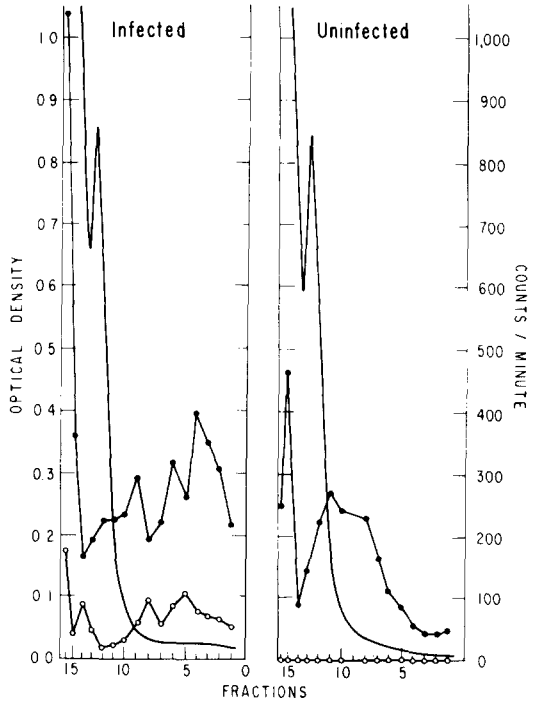

Fig. 2. Analysis by sucrose density gradient centrifugation of reaction mixtures after amino acid incorporation for 20 minutes in vitro. Optical density at 260 mμ, continuous line; total precipitable radioactivity, closed circles; radioactivity precipitable with antibody to subunits of poliovirus protein, open circles. The bottom of the gradient is to the right, and the sharp peak in the optical density trace represents single ribosomes (~ 74S). The degree of inhibition of amino acid incorporation in the uninfected, actinomycin-treated preparation was somewhat less than usual.

Extracts of infected cells were centrifuged for 20 minutes at 40,000 g, with a zone of 0.5 ml of 50% w/v sucrose at the bottom of the

tube, conditions which are sufficient to deposit the structures which enclose the polyribosomes at the interface (cf. Becker et al. 1963). The material at the interface was collected, and suspended in the original volume of either RSB or the supernatant from cell extracts centrifuged for 2 hours at 100,000 g. The latter preparation was as active as the original, unfractionated extract in bringing about amino acid incorporation, but there was minimal activity in the absence of the supernatant fraction.

Discussion and Summary

The characteristic large polyribosomes, which play a functional role in viral protein synthesis within poliovirus-infected HeLa cells also appear to be the principal, if not sole site of the amino acid incorporation catalyzed in vitro by cytoplasmic extracts of such cells. In analogy with the results in the intact cell, a portion of the polyribosome-associated protein labeled in vitro reacts with antibody directed against subunits of the virus capsid. At least the bulk of the capacity of extracts of infected cells to incorporate amino acids can be ascribed to polyribosomes which are associated with cytoplasmic structures sedimentable in 20 minutes at 40,000 g. Such structures also have been shown to contain most of the RNA polymerase responsible for the synthesis of viral RNA, and to be the site of viral RNA synthesis both in vitro and in the intact cell (D. Baltimore and J. E. Darnell, personal communication).

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