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Rhinovirus RNA Polymerase: Products and Kinetics of Appearance in Human Diploid Cells

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The kinetics of appearance of an RNA-dependent RNA polymerase activity obtained from human embryo lung cells infected with rhinovirus type 2 have been followed by analysis of the RNA synthesized by the polymerase preparation in vitro. Little single-stranded RNA was synthesized and the proportion of replicative intermediate to replicative form was over threefold greater than obtained in vivo. The polymerase activity in vivo declined in the presence of cycloheximide showing that continued protein synthesis was necessary to maintain RNA replication.

Virus-induced RNA-dependent RNA polymerase activity was first found in cells infected with mengovirus (4). This enzyme activity has been identified in other picornavirus-infected cells (3, 9, 12) and most recently in rhinovirus-infected cells (13).

Rhinoviruses form the largest subgroup of the picornaviruses with at least 89 serotypes (5). Though causing predominantly a mild upper respiratory infection, rhinoviruses are also associated with more severe illness of the lower respiratory tract (8). Normal methods of control through immunization are precluded by the number of serotypes so that chemotherapy appears a more likely prospect, and the viral RNA-dependent RNA polymerase is a possible target for chemotherapeutic investigation.

In this paper we present data about the kinetics of appearance of a rhinovirus RNA polymerase activity in human diploid cells. In all experiments polymerase activity was estimated by the extraction of the RNAs synthesized in vitro and their analysis by polyacrylamide electrophoresis.

MATERIALS AND METHODS

Virus and cells. Rhinovirus type 2 was grown in diploid human embryo lung (HEL) cells as previously described (10). HEL cells at 33 C were infected at a multiplicity of 5 to 10 PFU per cell. Time after infection was measured from the start of incubation.

Preparation of polymerase-containing fraction. The procedure was similar to that described by Yin and Knight (13). Material from disrupted cells which sedimented at 800 × *g* was discarded and a second pellet was obtained by centrifuging at 30,000 × *g*. The majority of polymerase activity was found in this pellet which was resuspended in 0.05 M Tris and 0.01 M NaCl at pH 8.0.

Polymerase assay. The in vitro assay for RNA-dependent RNA polymerase was essentially that described by Yin and Knight (13). The enzyme sample (0.2 ml) was incubated at 33 C for 30 min with 0.2 ml of a mixture containing 0.5 μmol each of ATP, CTP, and UTP, 0.004 μmol of GTP, 20 μmol of Tris buffer, pH 8.0, 2 μmol of $MgCl_2$, 0.5 μmol of phosphoenolpyruvate, 10 μg of phosphoenolpyruvate kinase, 2 μg of actinomycin D, 2.6 μmol of dithiothreitol, and 50 μCi of [^{3}H]GTP (specific activity 12 Ci/mmol; Radiochemical Centre, Amersham).

Extraction and analysis of RNA products. The in vitro reaction was terminated by addition of 2 ml of cold buffered saline (50 mM Tris, 100 mM NaCl, 2.5 mM EDTA, pH 7.5) and 5 ml of buffer-saturated phenol. No detergent was used in the extraction. The extraction and subsequent analysis of RNA on polyacrylamide gel has already been described (10).

RESULTS

Analysis of RNA synthesized in vitro. One half of each enzyme preparation was incubated with a complete polymerase assay mixture, the other with an assay mixture from which the three cold triphosphates were missing. The RNA from these incubations was extracted with phenol and was analyzed by polyacrylamide gel electrophoresis. The results in Fig. 1 show that with a polymerase preparation taken at 8 h after infection three species of RNA were synthesized with the complete assay mixture (Fig. 1b) but no radioactivity above background was present when the three cold nucleoside triphosphates were omitted (Fig. 1a). Optical density amounts of rRNA were found in the polymerase fraction. In another experiment [^{3}H]guanosine-labeled RNA synthesized in vitro was coelectrophoresed with [^{14}C]uridine RNA labeled in vivo. The replicative intermediate (RI), replicative form

(RF), and single-stranded (SS) RNA coincided with the peaks of RNA from the in vitro reaction. However, a much smaller proportion of SS RNA was obtained in vitro than in vivo.

Time course of development of polymerase activity. The RNA-dependent RNA polymerase activity induced in HEL cells after rhinovirus infection was measured at various times after infection by analyzing the products of the in vitro assay by polyacrylamide gel electrophoresis (Fig. 2). RNA polymerase activity was just detectable at 6 h after infection. RI and RF were clearly present in the later samples, taken at 7.5, 9.25, and 11 h but only trace amounts of SS RNA were found. Total radioactivity in the peaks of viral RNA was normalized with respect to the unlabeled 28*S* rRNA present in each incubation mixture (Fig. 3). Polymerase activity increased linearly until 9 h postinfection. Subsequently there was a slight decrease which coincided with extensive cytopathic effects.

Requirement for protein synthesis for continued polymerase activity. Cycloheximide inhibits protein synthesis efficiently in HEL cells and has no effect on the polymerase activity in vitro (unpublished observations). The possibility that continued protein synthesis was necessary to maintain polymerase activity was tested by preparing a polymerase fraction at 45 min and 3.5 h after adding 300 μg of cycloheximide per ml to infected cultures (this concentration of drug results in a drop of 97% of total protein synthesis after 10 min). Figure 4 shows that the polymerase activity had fallen after 45 min of incubation with cycloheximide to about 50% of the untreated culture. The proportion of RI to RF synthesized under these conditions was unaltered. After 3.5 h of treatment no polymerase activity could be detected.

DISCUSSION

The appearance of rhinovirus type 2 RNA-dependent RNA polymerase activity in human diploid cells was followed by electrophoretic analysis of the RNA species synthesized by infected cell extracts in vitro. Polymerase activity and RNA synthesis in vivo (10) are both first detected at 6 h after infection and increase linearly throughout the multiplication cycle. This is in contrast to the decline in polymerase activity late in infection which has been reported for poliovirus-infected cells (7). The decline in rhinovirus polymerase activity after the addition of cycloheximide indicates that it is unstable in vivo and thus resembles poliovirus polymerase activity (7). The latter had an apparently shorter half-life than rhinovirus polymerase activity but this may merely reflect the lower temperature at which the rhinovirus is grown.

The rhinovirus polymerase preparation synthesized RI and RF in vitro which were indistinguishable from species obtained in vivo. However, SS RNA was only occasionally synthesized in vitro and was always proportionately less than found in vivo. This situation is in contrast to a poliovirus polymerase preparation which was shown by electrophoresis of the in vitro

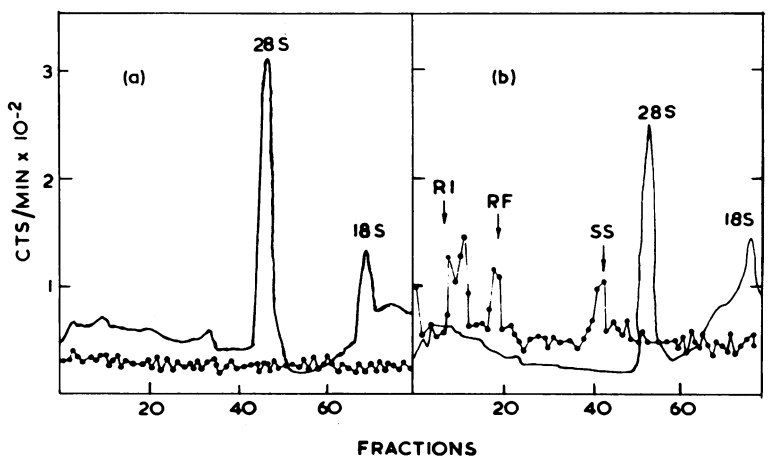

FIG. 1. *Polyacrylamide electrophoresis of the RNA synthesized in vitro by a RNA-dependent RNA polymerase fraction extracted from rhinovirus type 2-infected HEL cells. In (a) the three cold nucleoside triphosphates were omitted from the assay mixture, and in (b) the complete assay mixture was used. The replicative intermediate (RI), replicative form (RF) and single-stranded RNA are arrowed. The continuous line is the absorbance at 260 nm of 28 and 18S rRNA extracted from the polymerase reaction mixture.*

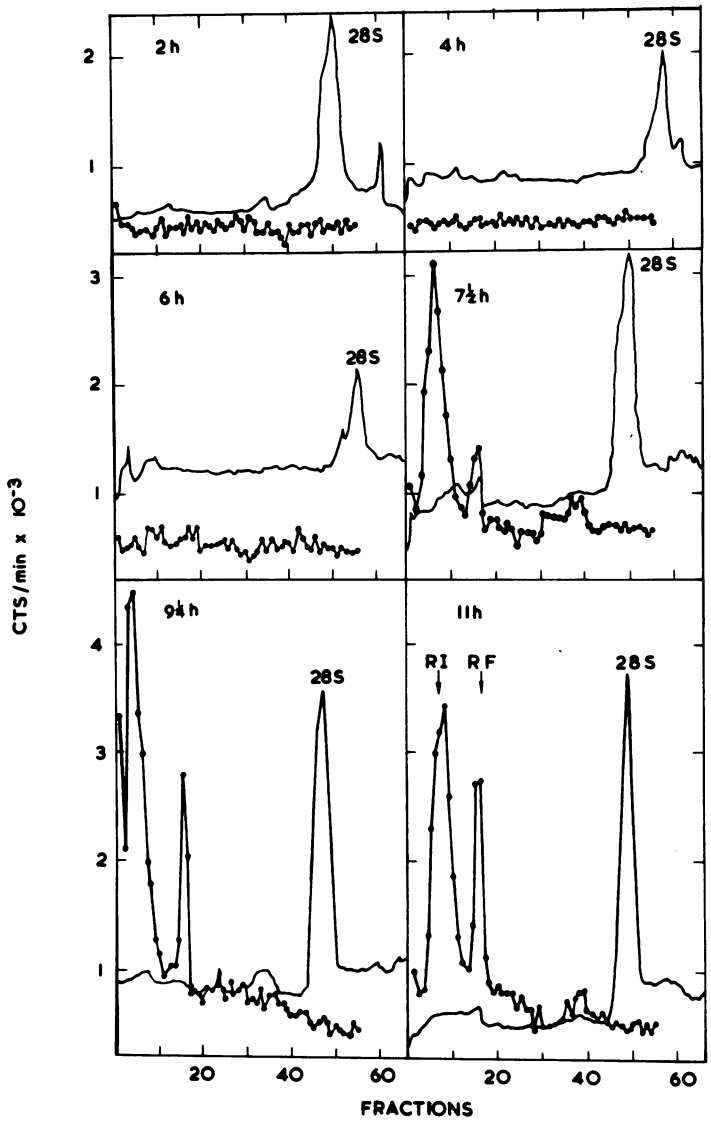

FIG. 2. *Increase in rhinovirus RNA-dependent polymerase activity with time after infection. A polymerase fraction was prepared at the indicated times and used to synthesise RNA under standard conditions in vitro. The products of the reaction were analyzed by polyacrylamide electrophoresis. The continuous line is the absorbance at 260 nm of 28S rRNA extracted from the polymerase reaction mixture.*

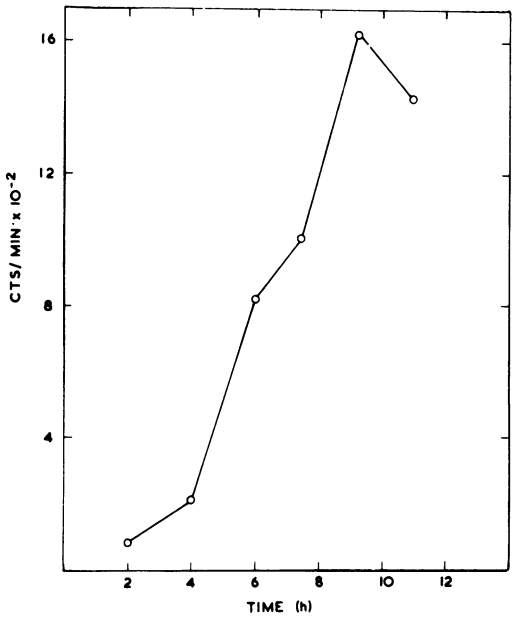

FIG. 3. *Development of rhinovirus polymerase activity. The peaks of radioactivity in Fig. 2 were summed and corrected with respect to the amount of 28S rRNA present in the reaction mixture.*

products to synthesize normal amounts of RI, RF, and SS RNA (11). It appears unlikely that rhinovirus SS RNA was synthesized and then degraded by RNase since RI and rRNA which are both susceptible to RNase (1, 6, 10) were extracted as intact molecules from the reaction mixture. It seems more likely that complete replication did not occur in vitro. In foot and mouth disease virus-infected cells two polymerase activities could be demonstrated, one of which synthesized double-stranded RNA and the other SS RNA (2). If this is true for rhinoviruses it is possible that rhinovirus-induced RNA polymerase activity in vitro was incompletely expressed. The lack of SS RNA may be correlated with the increased ratio (3.5-fold) of RI:RF obtained in vitro compared with that in vivo (10). This observation would be consistent with the failure of the RI to release SS RNA. The synthesis of RF indicates that both viral and complementary RNA are available in vitro to form this double-stranded structure. A determination of the symmetry of label-

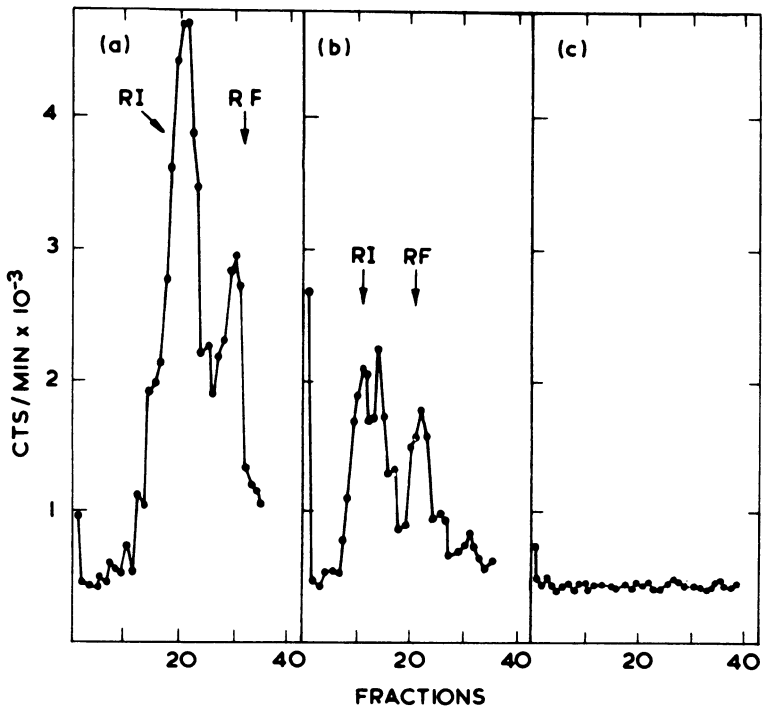

FIG. 4. *Decline in polymerase activity following the addition of 300 μg of cycloheximide per ml to infected cells. After incubation with cycloheximide for 45 min or 3.5 h polymerase fractions were prepared and assayed in vitro. The products of the reaction are shown. (a) No cycloheximide; (b) cycloheximide treated for 45 min; (c) cycloheximide treated for 3.5 h.*

ing will indicate whether or not both types of RNA are synthesized in vitro.

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