JOURNAL OF VIROLOGY, Aug. 1987, p. 2480–2488
0022-538X/87/082480-09$02.00/0

Vol. 61, No. 8

Restriction of Translation of Capped mRNA In Vitro as a Model for Poliovirus-Induced Inhibition of Host Cell Protein Synthesis: Relationship to p220 Cleavage

RICHARD E. LLOYD,[1]* HOLLY G. JENSE,[1] AND ELLIE EHRENFELD[1,2]

Departments of Cellular, Viral and Molecular Biology,[1] and Biochemistry,[2] University of Utah Medical Center, Salt Lake City, Utah 84132

Received 6 February 1987/Accepted 5 May 1987

Poliovirus infection of HeLa cells results in a rapid inhibition of host protein synthesis by a mechanism that does not affect the translation of poliovirus RNA. It has been suggested that this virus-induced translational control results from inactivation of the cap-binding protein complex, and it has been shown that the 220-kilodalton component(s) (p220) of the cap-binding protein complex is cleaved in infected HeLa cells to form antigenically related polypeptides of 100 to 130 kilodaltons. We have previously described an activity in infected cells that specifically restricts translation of capped mRNA in rabbit reticulocyte lysates. Here, we describe further refinements and characterization of the restriction assay. We determined that the assay is a good in vitro model for study of host cell shutoff by several criteria: (i) translation was inhibited in both instances at the step involving mRNA binding to ribosomes; (ii) translation of capped mRNA was specifically inhibited, whereas translation of poliovirus RNA was not; (iii) restriction activity appeared in infected cells with kinetics which parallel host cell shutoff; and (iv) restriction activity, like the specific inhibition of host translation, appeared in cells infected in the presence of guanidine-HCl. The restricting activity was partially purified from poliovirus-infected cells and was compared with the virus-induced p220 cleavage activity. Both activities copurified through numerous cell fractionation and biochemical fractionation procedures. However, specific restriction of capped mRNA translation in reticulocyte lysates occurred without complete cleavage of the endogenous p220.

Infection of HeLa cells with poliovirus quickly results in marked inhibition of translation of cellular mRNA, an event referred to as host cell shutoff (for recent reviews, see references 9 and 32). Failure to synthesize host cell proteins results from a specific block at the initiation step of translation (19, 24) and is characterized by the cessation of ribosomal initiation complex formation with cellular mRNAs (10). However, all protein synthesis is not inhibited, since viral mRNA continues to be translated efficiently.

Experiments with fractionated extracts from poliovirus-infected HeLa cells have demonstrated that initiation factor (IF) preparations from infected cells could not support the translation of host or other capped mRNAs in vitro but did stimulate poliovirus mRNA translation (15, 19), reflecting the specificity of translational restriction seen in vivo. All other infected cell fractions, including ribosomes, did function in the translation of capped mRNA in this system. These crude IF preparations were made as ribosomal salt washes (RSWs) from poliovirus-infected HeLa cells. Extensive purification of individual IFs from the infected-cell RSW was undertaken in an effort to identify the defective or inhibitory component in material from infected cells. This approach, together with detailed analyses of IFs in rabbit reticulocytes, soon led to the discovery of a multi-subunit complex, referred to variously in the literature as eIF-4F, CBP II, or the cap-binding protein (CBP) complex (8, 13, 34). This complex has been identified as being both structurally and functionally altered in the infected cell (12, 22, 34). Several other IFs have also been examined (5, 7, 11, 22) but were unchanged in infected cells. Therefore, the CBP complex is the only known IF altered in poliovirus-infected HeLa cells, although other unknown virus-directed events may contribute to the shutoff state.

The CBP complex has been identified by several laboratories as a rapidly sedimenting complex that contains the 24- to 28-kilodalton (kDa) protein (CBP 1) which binds the m^7GTP cap group on mRNA and can be chemically cross-linked to the cap group (14, 23, 34). The CBP complex also contains a 220-kDa polypeptide of unknown function. Other polypeptides have variably been reported to be associated with or be an integral part of the complex, including eIF-4B and some of the complement of eIF-4A in the cells (13, 34). The function of the CBP complex is not known, but it has been shown to be required for translation of globin mRNA and other mRNAs in vitro (13). Some evidence suggests that the CBP complex aids the melting or unwinding of the 5′ ends of certain mRNAs with extensive 5′ secondary structure (8, 20, 31, 33) and/or interacts with eIF-3 (14, 35) and facilitates binding of the eIF-3-ribosome complex to the mRNA.

One of the first reports indicating that a specific IF was inactivated by poliovirus infection was by Trachsel et al. (35), who examined the ability of IF preparations to restore translation of capped mRNAs in extracts from poliovirus-infected cells. The restoring activity obtained by these workers was unstable but copurified through several steps with the 24-kDa CBP, although it was shown soon after that the CBP complex isolated by m^7GDP affinity chromatography was required for restoration of capped mRNA translation and the 24-kDa CBP alone did not have restoring

* Corresponding author.

activity (34). These data, taken together, imply that the CBP complex is somehow inactivated as a consequence of poliovirus infection.

Several additional observations suggest that the CBP complex is structurally altered in poliovirus-infected cells. Hansen et al. (14) demonstrated that all of the 24-kDa CBP from infected cells sedimented slowly in sucrose gradients, indicating that the large protein complex present in uninfected cells was disrupted. Subsequently, Etchison et al. (12) found that the 220-kDa polypeptide (p220) normally found in the CBP complex is apparently cleaved to immunologically related proteins of 100 to 130 kDa. These data, together with the functional data, became the basis for an attractive hypothesis for the mechanism of host cell shutoff, which stated that cleavage of p220 leads to inactivation of the CBP complex and thereby blocks efficient initiation of capped mRNA translation. The rest of the ribosomal machinery is otherwise unaltered and free to translate uncapped poliovirus mRNA (16, 27), which presumably initiates translation via a distinct (and unknown) cap-independent mechanism.

Despite the plausibility of the hypothesis that p220 cleavage is the cause of host cell shutoff, the bulk of supporting evidence is rather circumstantial and correlative. The mediator of the cleavage event has not been identified, although both of the known poliovirus proteinases (polypeptides 2A and 3C) have been excluded as being directly responsible for p220 cleavage in vitro (21, 25, 26). The biochemical function of p220 is completely unknown, so its presumed requirement for the initiation reaction is unexplained. Furthermore, and most importantly, the relationship between p220 cleavage and the inability to translate capped mRNA has not been investigated thoroughly.

In summary, the question still remains whether cleavage of p220 is directly responsible for host cell shutoff. If this hypothesis is correct, it would be predicted that cleavage of p220 in any in vitro translation system would render the system functionally similar to a poliovirus-infected cell, i.e., the system would fail to translate capped mRNA but would still translate poliovirus mRNA. Such translational restriction would also constitute a functional assay system for p220 cleavage activity. We have previously described an activity in infected cells that specifically restricts translation of capped mRNA in reticulocyte lysates (3). In the present report, we describe the development of the restriction assay and present the assay as a suitable in vitro model for the study of host cell shutoff. The restriction assay and a previously described HeLa p220 cleavage assay were used together to partially purify both restriction and p220 cleavage activities from infected-cell material. We determined that both activities extensively copurified. However, we also determined that specific restriction of capped mRNA translation in rabbit reticulocytes could occur in the absence of complete cleavage of the endogenous reticulocyte p220.

MATERIALS AND METHODS

Cells and virus. HeLa S3 cells were grown in suspension in Joklik modified minimum essential medium (Irvine Scientific Sales Co., Inc., Santa Ana, Calif.) containing penicillin and streptomycin and supplemented with 7% calf serum. Growth and purification of the Mahoney strain of poliovirus has been previously described (18).

mRNA preparation. Vesicular stomatitis virus (VSV) mRNA was prepared from infected HeLa cells as described by Brown and Ehrenfeld (3). Poliovirus RNA was prepared from purified virions by extraction with phenol and precipitation with 70% ethanol-0.2 M sodium acetate as previously described (3).

Preparation of cell extracts and purification of restriction activity. Approximately 2×10^9 HeLa cells were infected with poliovirus at a multiplicity of infection of 100 PFU per cell, and the virus was adsorbed for 30 min at 37°C before calf serum was added to a concentration of 5%. The cells were incubated for 4 h at 37°C, harvested, washed twice in cold Earle salts solution, and disrupted by Dounce homogenization. Postmitochondrial cytoplasmic extracts and RSW were obtained as previously described (2). All other purification procedures were done at 4°C. Infected-cell RSW was precipitated in 40% (RSW-A) and 70% (RSW-B) ammonium sulfate as described previously (2). The precipitates were suspended in buffer A (20 mM Tris hydrochloride [pH 7.4], 10% (vol/vol) glycerol, 7 mM 2-mercaptoethanol, 0.1 mM EDTA) containing 10 mM KCl and dialyzed against the same buffer. The RSW-A material was applied to a DEAE column (Whatman DE-52; 1.5 by 7 cm), and fractions were eluted in steps with buffer A containing 10 mM KCl, 100 mM KCl, and then a continuous gradient of 100 to 500 mM KCl. Fractions containing restriction or p220 cleavage activity were pooled and dialyzed against buffer B (50 mM potassium phosphate [pH 7.0], 10% (vol/vol) glycerol, 7 mM 2-mercaptoethanol) containing 10 mM KCl. The protein sample was applied to a phosphocellulose column (Whatman P-11; 1.5 by 4 cm) and eluted in steps with the same buffer containing 10, 100, and 500 mM KCl. The eluted fractions were dialyzed against buffer a with 100 mM KCl before assay and analysis.

In vitro protein synthesis. Rabbit reticulocyte lysates were purchased from Green Hectares Custom Laboratory Animal Work, Oregon, Wis. Portions (500 μl) were treated with micrococcal nuclease (Pharmacia) as described by Pelham and Jackson (28). Translation reaction mixtures (25 μl) contained 10 μl of nuclease-treated lysate, a 25 μM concentration of each of 19 amino acids (less methionine), 1.0 mM ATP, 0.2 mM GTP, 25 mM creatine phosphate, 5 μg of creatine phosphokinase (Boehringer Mannheim Biochemicals Indianapolis, Ind.), 2.0 mM dithiothreitol, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5), 2.0 mM magnesium acetate, 2.5 μg of calf liver tRNA (Boehringer Mannheim), 10 μCi of [^{35}S]methionine (1,400 Ci/mmol; New England Nuclear Corp., Boston, Mass.), and 80 mM KCl. mRNA was used at 0.5 to 2 μg per reaction mixture. Translation reaction mixtures were incubated at 37°C for 90 min. When specified, translation reaction mixtures were supplemented with buffers or dialyzed IF preparations. The translation reaction mixtures used for the experiment for which results are shown in Fig. 1 were the same except that 5 μl of nuclease-treated HeLa cell postmitochondrial cytoplasmic extract was added before preincubations. The products of the translation reactions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography, as previously described (3).

Antibody and immunoblot analysis. Immunoblot analysis was performed with mouse monoclonal antibody directed against p220 (25) or a rabbit polyclonal antiserum directed against the p220 degradation products from poliovirus-infected cells. The antiserum was raised against antigen prepared by one-step affinity purification of the 24-kDa CBP together with p220 degradation products on m^{7}GTP-Sepharose 4B (Pharmacia). This procedure routinely allowed isolation of these two proteins together but totally free of contaminating proteins. This material was further purified

and concentrated on DEAE-cellulose and used directly as the immunogen. Rabbits were administered approximately 50 μg of protein in Freund complete adjuvant subcutaneously in several locations on the back. They were twice boosted with the same dose of protein in Freund incomplete adjuvant at 2-week intervals. Serum was harvested 1 week later by venipuncture. Immunoblot analysis was performed as previously described (25).

p220 cleavage activity. p220 cleavage activity was assayed in vitro as previously described (25). Briefly, RSW from uninfected cells (containing intact p220) was incubated at 37°C for 60 to 120 min with fractions purified from poliovirus-infected cells. p220 was then analyzed by SDS-PAGE and immunoblotting.

RESULTS

Specificity of the restriction assay. Previous work in this laboratory demonstrated that poliovirus-infected cells contain an activity that can specifically restrict the translation of capped mRNAs in a rabbit reticulocyte lysate without affecting the ability of the lysate to translate poliovirus RNA (3, 4). The restriction occurs at the initiation step of protein synthesis; although initiator Met-tRNA^{Met} bound normally to the small ribosomal subunit, capped mRNAs failed to bind (3). To measure this restriction activity, a rabbit reticulocyte lysate was supplemented with fractions derived from poliovirus-infected HeLa cells, and the lysate was then tested for its ability to support translation of either poliovirus RNA or capped mRNAs. VSV mRNA was routinely used as the capped mRNA because it is easily obtained and is translated efficiently in vitro; however, translation of globin mRNA showed the same restriction. The results of a restriction assay demonstrating the specificity for restriction of translation of capped mRNAs check figure are shown in Fig. 1. In this experiment, the assay conditions were changed slightly: the poliovirus-infected cell material was preincubated with the reticulocyte lysate for 30 min at 37°C before the translation mixture was added and the reaction was allowed to proceed. The products of translation of the VSV mRNA preparation without preincubation are shown in lane b. These included the viral N, NS, and M proteins, as well as the faintly visible mixture of HeLa cell proteins whose synthesis was directed by the heterogeneous populations of poly(A)$^+$ RNA present in VSV-infected cells. Preincubation of the lysate with buffer for 30 min before translation caused a partial loss of translational activity (cf. lanes c and b); however, preincubation with an RSW from uninfected HeLa cells stimulated VSV translation slightly (lane d). Strikingly, a similar preparation from poliovirus-infected HeLa cells completely restricted all translation of VSV and contaminating cellular, capped mRNAs (lane e).

In contrast, Fig. 1, lanes f through i, show the effects of similar preincubation treatments on the translation of poliovirus RNA. Preincubation of the lysate with buffer had no effect on its subsequent translational activity (lane g), but preincubation with the RSW from either uninfected (lane h) or infected (lane i) cells stimulated translation. Absolutely no restriction of the translation of poliovirus RNA was produced by preincubation with infected-cell RSW. The translation products of poliovirus RNA shown in Fig. 1 demonstrate a close match with those synthesized in vivo in infected cells, indicating that both initiation and subsequent polypeptide processing occurred accurately in these lysates. Dorner et al. (6) and Phillips and Emmert (30) have presented evidence that the fidelity of poliovirus translation in

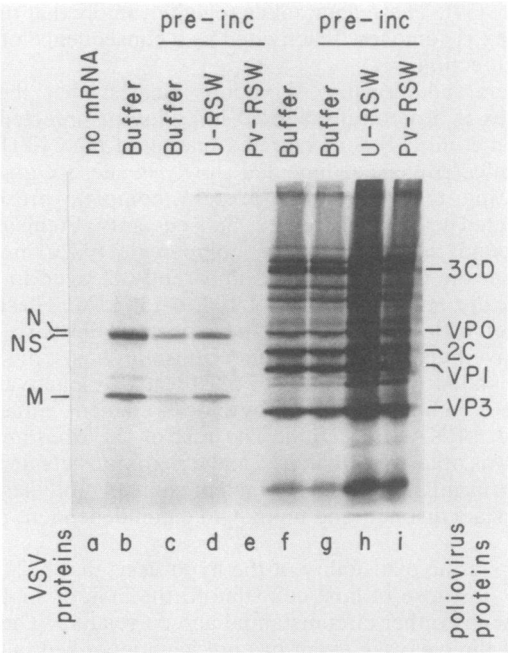

FIG. 1. Specificity of translational restriction in vitro. Translation of VSV mRNA or poliovirus RNA was performed in 10 μl of rabbit reticulocyte lysate supplemented with 5 μl of HeLa cytoplasmic extract. The lysate was preincubated (pre-inc) with 1 μl of buffer A (lanes a, c, and g), 1 μl of RSW from uninfected cells (lanes d and h), or 1 μl of RSW from infected cells (lanes e and i) or was not preincubated but contained 1 μl of buffer A (lanes b and f). VSV mRNA (lanes be to e) or poliovirus RNA (lanes f to i) was then translated as described in the text, and samples were subjected to SDS-PAGE and autoradiography. Poliovirus translation products were identified by comparison with [^{35}S]methionine-labeled proteins in a cytoplasmic extract from infected cells.

reticulocyte lysates is poor and that alternate, aberrant initiation sites are utilized, producing abnormal polypeptide products. In both of these studies, the aberrant initiation events are eliminated by the inclusion in the translation mixture of a small amount of cytoplasmic extract from HeLa cells. For this reason, we usually translate poliovirus RNA in a reticulocyte lysate supplemented with HeLa cell extract, and the experiment for which results are shown in Fig. 1 was performed with a supplemented lysate. The degree and specificity of restriction, however, were the same in nonsupplemented and supplemented reticulocyte lysates, as was the profile of protein products translated from VSV mRNA. Therefore, all VSV translations described below were performed in standard reticulocyte lysates, containing no added HeLa cell cytoplasm.

Correlation of host cell shutoff with appearance of restriction activity. As indicated above, specific restriction activity was found only in material derived from polio-virus-infected cells; uninfected HeLa cells contained no restriction activity. The kinetics of appearance of this activity in cells as a function of time postinfection is shown in Fig. 2. Extracts of cells were prepared at different times after infection and assayed for their ability to restrict translation of VSV mRNA in a rabbit reticulocyte lysate. Restriction activity appeared between 1 and 2 h postinfection and continued to accumulate up to 4 h postinfection. These kinetics correlate quite closely with the rates of host cell protein synthesis occurring in vivo, determined by pulse-labeling with [^{35}S]methionine (Fig. 2).

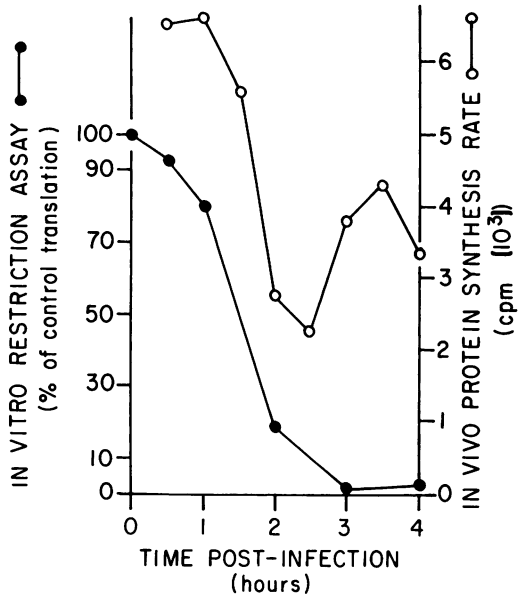

FIG. 2. Kinetic analysis of host cell shutoff and appearance of translation restriction activity. The protein synthesis rate in infected HeLa cells was determined by pulse-labeling (10 min) cells with ^{14}C-amino acids (0.5 μCi/2.5 × 10^6 cells per ml) at the indicated times postinfection. Translation restriction activity was determined by in vitro restriction assays of RSW from cells harvested at the indicated times postinfection.

The secondary burst of protein synthesis was due to translation of poliovirus mRNA. Thus, virus-infected cells demonstrating specific inhibition of cellular protein synthesis simultaneously accumulate an activity that restricts protein synthesis in vitro.

It has long been known that cells infected at a high multiplicity in the presence of 1 mM guanidine-HCl manifest virus-induced inhibition of protein synthesis, despite the absence of detectable virus replication (17, 29). Extracts prepared from such guanidine-treated, infected cells also contained restriction activity (Fig. 3, lane e). The band seen in lanes a, d, and e represents an unidentified background in this translation but does not indicate VSV-directed protein synthesis, since it was present in reaction mixtures containing no mRNA (lane a). The effect of guanidine on virus replication was monitored by the failure of these cells to incorporate any detectable [^{3}H]uridine into viral RNA or [^{35}S]methionine into viral protein. The addition of cycloheximide to the guanidine-treated cells at the time of infection, however, completely prevented the appearance of restriction activity (Fig. 3, lane f). The latter condition prevents viral gene expression from infecting virus and was previously used to demonstrate the requirement for some viral protein synthesis to effect host cell shutoff (1, 29). Thus, the appearance of restriction activity in infected cells occurred only under those conditions that also resulted in virus-induced inhibition of host cell protein synthesis.

Properties of the restriction activity. Although the direct addition of infected HeLa cell material to the translation reaction mixture resulted in a manifestation of restriction activity, it was found that preincubation of the reticulocyte lysate at 30 or 37°C with the restricting activity provided a considerable enhancement in the sensitivity of the assay. The degree of restriction of VSV translation was enhanced with increasing time of preincubation of the lysate with RSW from polio-virus-infected HeLa cells (Fig. 4A). Preincubation for 30 min resulted in a greater than 50-fold increase in measurable restriction activity while causing only minimal losses in the translational activity of the lysate. These data suggested that some time-dependent reaction was occurring between a component(s) of the reticulocyte lysate and a component(s) in the infected HeLa cell extract. This reaction was temperature dependent, since preincubation at 0°C resulted in no increased restriction activity (data not shown). Similarly, the degree of restriction produced was directly proportional to the amount of infected HeLa cell material present (Fig. 4B). Taken together, these results suggest that restriction activity represents a catalytic rather than a stoichiometric event.

Relationship of restriction activity to virus-induced p220 cleavage activity. Previous studies in this laboratory have been directed at the identification and characterization of the activity in poliovirus-infected cells that catalyzes the cleavage of the 220-kDa polypeptide component of the CBP complex IF (25, 26). Since a great deal of circumstantial evidence has been accumulated and presented to support the hypothesis that cleavage of p220 is responsible for the failure of infected cells to translate capped mRNAs (12, 13, 34), it seemed reasonable to explore a possible relationship between p220 cleavage activity and the restriction activity described here. To this end, we attempted to purify the restriction activity from infected-cell extracts and determine whether it copurified with p220 cleavage activity. Upon initial fractionation of infected cytoplasmic extracts, the

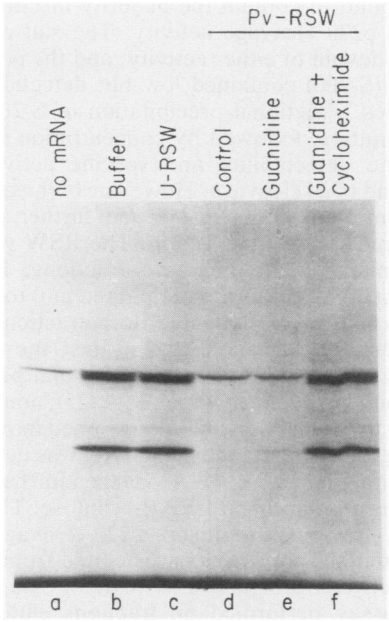

FIG. 3. Appearance of restriction activity in HeLa cells infected with poliovirus in the presence of 1 mM guanidine-HCl. HeLa cells were infected with poliovirus at a multiplicity of 300 PFU per cell and divided into three aliquots. One aliquot was adjusted to 1 mM guanidine-HCl, and another received 100 μg of cycloheximide per ml in addition to 1 mM guanidine. Cultures were incubated at 37°C for 4 h, harvested, and fractionated, and RSW was tested for restriction activity. Translation reaction mixtures in lanes b to f contained VSV mRNA. Lysates were preincubated for 30 min with 2 μl of uninfected-cell RSW (U-RSW) or infected-cell RSW (Pv-RSW) prepared from control cells or cells infected in the presence of guanidine or guanidine plus cycloheximide, as indicated.

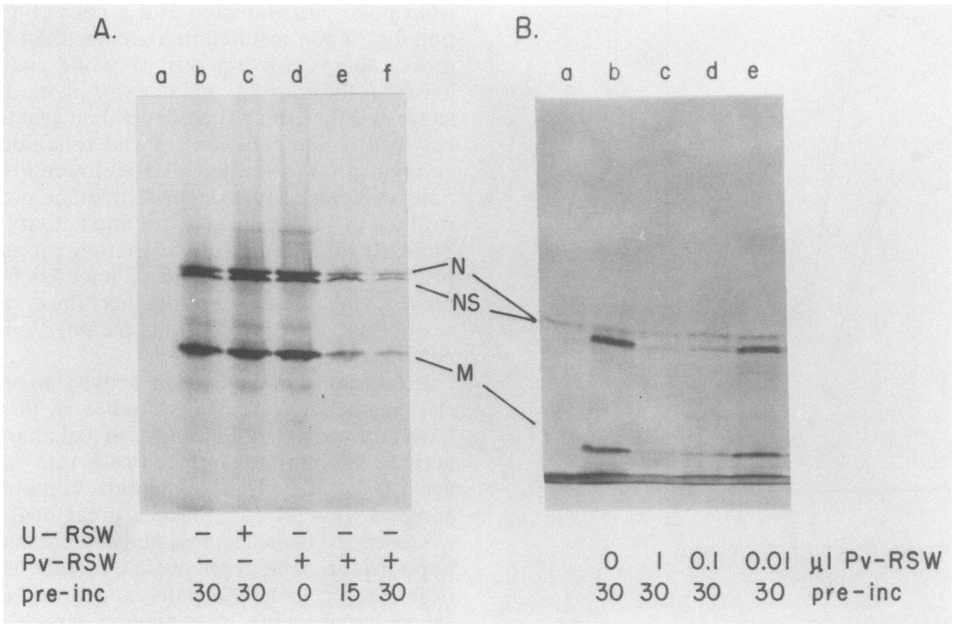

FIG. 4. Effect of time of preincubation and concentration on in vitro translation restriction activity. (A) Lysates (20 µl) were preincubated (pre-inc) at 30°C for 0, 15, or 30 min, as indicated, with 3 µl of buffer A (lanes a and b), RSW from uninfected cells (U-RSW) (lane c), or RSW from poliovirus-infected cells (Pv-RSW) (lanes d to f) before addition of VSV mRNA to all reaction mixtures except that in lane a. (B) Lysates were preincubated for 30 min at 30°C with dilutions of Pv-RSW (lanes c to e) or 1 µl of buffer A (lanes a and b). Translation reaction mixtures in lanes b to e contained 1 µg of VSV mRNA. Samples from each reaction mixture were analyzed by SDS-PAGE and autoradiography. N, NS, and M, VSV proteins.

RSW was found to contain the majority of both restriction activity and p220 cleavage activity. The salt-washed ribosomes were devoid of either activity, and the postribosomal supernatant (S-200) contained low but detectable levels of both activities. Fractional precipitation of S-200 with 40% ammonium sulfate, followed by concentration and dialysis, increased the detectability and specific activity of both restriction and p220 cleavage. However, both activities were more concentrated in the RSW, and further purifications were performed with this fraction. The RSW was resolved with ammonium sulfate into two fractions, RSW-A and RSW-B, containing proteins precipitated at 0 to 40% and 40 to 70% saturation, respectively. Both fractions were suspended in buffer A and dialyzed against the same buffer before assay. We had previously shown that p220 cleavage activity is found exclusively in RSW-A (25); none is found in RSW-B. Restriction activity also partitioned into the RSW-A fraction (Fig. 5A, lane d), and no activity was detected in the RSW-B fraction (lane f). RSW-A was then further purified by column chromatography on DEAE-cellulose. This resin had been shown previously to adsorb p220 cleavage activity at low ionic strength, but activity was eluted from the column with KCl between 0.14 and 0.20 M (25). The results of a restriction assay performed on fractions eluted from the same DEAE-cellulose column with a KCl concentration gradient of 0.1 to 0.5 M are shown in Fig. 5B. Restriction activity eluted as a single peak, with maximum activity in fraction 9, corresponding to 0.16 M KCl. p220 cleavage activity assays performed on the same column fractions from numerous preparations showed that the same fractions always contained both activities (25).

Fractions eluted from DEAE-cellulose that contained both p220 cleavage activity and restriction activity were then dialyzed into buffer B and further fractionated by phosphocellulose chromatography. We have previously shown that p220 cleavage activity is not adsorbed to this column resin (25). Restriction activity behaved similarly on this column (Fig. 5C). All the activity was recovered in the unadsorbed (flowthrough) fraction (lane d); none was detected in the fractions containing proteins bound to the column and eluted with 0.1 or 0.5 M KCl (lanes e and f). Unfortunately, even though p220 cleavage or restriction activity was very stable in crude fractions stored at −70°C, efforts to further purify these activities after elution from DEAE-cellulose and phosphocellulose resulted in a marked loss of both activities, especially upon storage of purified fractions at either −70 or 4°C. Purication through phosphocellulose usually was approximately 1,500-fold. Examination of SDS-polyacrylamide gels stained with silver showed that more than 50 polypeptides remained in this fraction (data not shown). The sequential purification steps and the coordinate purification of the two activities are shown in Table 1. These data suggest that both activities may reside in the same protein(s).

To further explore the relationship between restriction activity and p220 cleavage activity, the kinetics of appearance of p220 cleavage activity was determined as a function of time postinfection, as had been done for restriction activity (see above; Fig. 2). Again, cleavage activity was demonstrable in infected-cell RSW by 2 h postinfection (Fig. 6, lane f). As expected, the endogenous p220 that was in the infected cells was cleaved to generate the characteristic p100 to 130 bands concomitantly with the appearance of p220 cleavage activity (lane e). Thus, the appearance of both restriction activity and p220 cleavage activity occurs coordinately in infected cells, and the kinetics correlate well with p220 cleavage and shutoff of host protein synthesis in vivo. A similar experiment was performed with cells infected in the presence of guanidine. As indicated above (Fig. 3), restriction activity was produced in guanidine-treated infected cells; the immunoblot shown in Fig. 7 demonstrates

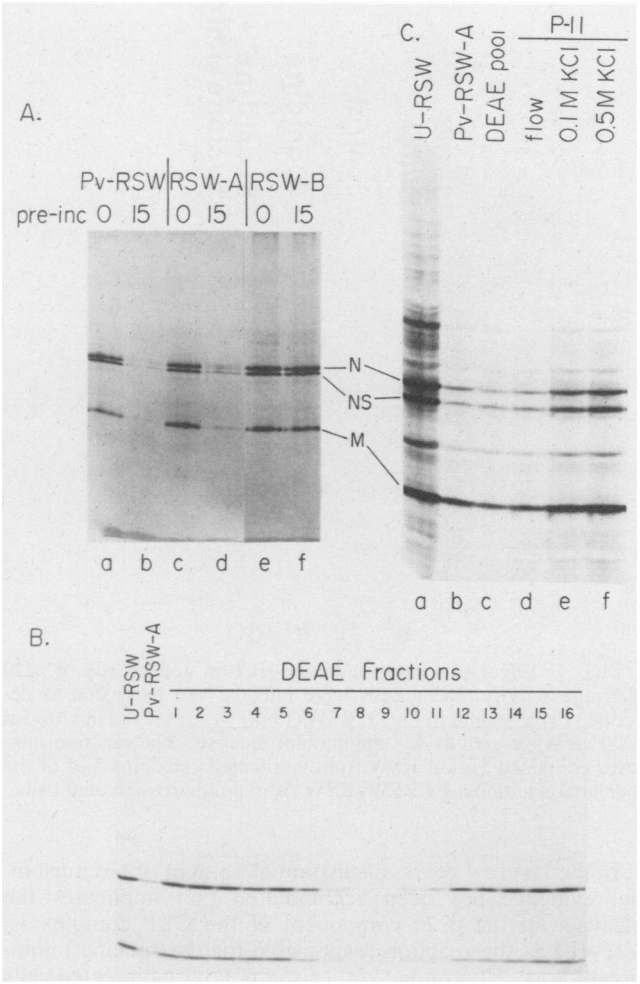

FIG. 5. Partial purification of translation restriction activity from poliovirus-infected HeLa cell extracts. (A) Ammonium sulfate fractionation of restriction activity. RSW from poliovirus-infected cells (Pv-RSW) (lanes a and b) or ammonium sulfate fractions RSW-A (0 to 40% saturation precipitate; lanes c and d) and RSW-B (40 to 70% saturation precipitate; lanes e and f) were tested in translation restriction assays. RSW samples (1 μl) were preincubated with 20 μl of reticulocyte lysate for 0 min (lanes a, c, and e) or 15 min (lanes b, d, and f) before VSV mRNA was added and translation reactions were begun. After 90 min of translation, samples were analyzed by SDS-PAGE and autoradiography. (B) DEAE column chromatography of restriction activity. RSW-A from poliovirus-infected cells was applied to a DEAE-cellulose column and eluted with a KCl gradient from 0.1 to 0.5 M. Samples (1 μl) of uninfected-cell RSW, RSW-A from infected cells, or DEAE column fractions were preincubated with 10 μl of reticulocyte lysate for 30 min at 30°C before translation of VSV mRNA was initiated. After translation, samples were analyzed by SDS-PAGE and autoradiography. (C) Phosphocellulose column chromatography of restriction activity. Fractions from DEAE column chromatography containing restriction activity were pooled, dialyzed, and fractionated on phosphocellulose as described in the text. Samples (1 μl) of an uninfected-cell RSW (lane a), the Pv-RSW-A from infected cells (lane b), the DEAE pool (lane c), the phosphocellulose flowthrough (lane d), the 0.1 M KCl eluate (lane e), and the 0.5 M KCl eluate (lane f) were preincubated with lysate, assayed for restriction activity, and analyzed as in panel A.

that p220 cleavage activity was also present in these cells. When cycloheximide was added to such cells to prevent expression of the parental viral genome, no p220 cleavage activity appeared (Fig. 7, lane d). Thus, the appearance of both restriction activity and p220 cleavage activity required viral gene expression but not virus replication and both of these activities demonstrated properties expected of the mediator of host cell shutoff.

Cleavage of reticulocyte p220 during restriction. The results described above strongly suggested that restriction of translation of capped mRNAs in reticulocyte lysates was an accurate model of host cell shutoff and that the mediator of both restriction in vitro and shutoff in vivo was likely to be p220 cleavage activity. If this was the case, then it would be predicted that during restriction of reticulocyte lysates in vitro, endogenous reticulocyte p220 should be cleaved by the virus-induced p220 cleavage activity that was shown to cleave HeLa cell p220. Testing this prediction in reticulocyte lysates initially proved difficult because neither our monoclonal nor polyclonal antibodies that were prepared against HeLa cell p220 sequences reacted well with rabbit p220. However, if sufficient antigen was concentrated by preparation of reticulocyte RSW or ammonium sulfate precipitations (50% saturation) of whole lysates, a detectable immunoblot could be obtained. Therefore, reticulocyte lysates were restricted or mock restricted by incubation for 60 min at 37°C with either poliovirus-infected-cell restricting activity or buffer, respectively, and then RWSs were prepared from the two lysates. The proteins in these RSWs were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-p220 antibody. The bulk of the p220 antigen in the RSW of the restricted lysate remained intact (Fig. 8, lane c), although a small amount of cleavage (not more than approximately 20% in several different experiments) did occur. It should be noted that the cleavage products present in the infected-HeLa-cell restricting material did not sediment with the reticulocyte ribosomes and thus did not appear in the concentrated RSW fractions shown (lanes b and c). The amount of p220 cleavage activity present in the restriction incubation mixture was sufficient to cleave all of the HeLa cell p220 added in a control incubation (lane f) but was sufficient to cleave only a fraction of the rabbit p220 in a reticulocyte RSW preparation (lane j). When other lysates were restricted by the same protocol and concentrated by

TABLE 1. Copurification of restriction activity and p220 cleavage activity

Procedure	Fraction	Restriction activity	p220 cleavage activity
Cell fractionation	S-200	+/−	+/−
	RSW	+	+
	RSW-A	+	+
	RSW-B	−	−
DEAE chromatography[a]	Unadsorbed	−	−
	0.01–0.14 M KCl	−	−
	0.14–0.2 M KCl	+	+
	0.2–0.5 M KCl	−	−
Phosphocellulose chromatography[b]	Unadsorbed	+	+
	0.1 M KCl	−	−
	0.5 M KCl	−	−

[a] RSW-A was applied to DEAE-cellulose.
[b] DEAE 0.14 to 0.2 M KCl fraction was applied to phosphocellulose.

ammonium sulfate precipitation of the whole reaction mixture, immunoblotting again revealed only a small amount of p220 cleavage (approximately 25%) (data not shown). All p220 antigens were analyzed by this procedure. In each instance, samples from the incubated lysates were removed and analyzed for their ability to translate VSV mRNA. Translation was always reduced by greater than 90% in the restricted lysate compared with that in the mock-restricted lysate. It was not possible to quantitate either the actual amount of reticulocyte p220 cleaved or the amount of p100 to p130 products formed due to the differences in antibody reactivity between the rabbit and human antigens; however, it was apparent that essentially complete restriction could be obtained, even though a major portion of the p220 antigen in the lysate remained uncleaved.

DISCUSSION

This report describes the development of a sensitive in vitro assay for an activity, present in polio-virus-infected cells, that specifically restricts the translation of capped mRNAs in cell-free translation systems derived from rabbit reticulocytes or HeLa cells. In these lysates, the translation of poliovirus RNA, which is not capped, is unaffected. This specific restriction of translation in vitro appears to constitute a good model for the study of poliovirus-induced inhibition of host cell protein synthesis that occurs in infected cells by several criteria. (i) Restriction activity was absent in uninfected HeLa cells; it appeared as a function of time postinfection with kinetics that parallel the inhibition of host cell protein synthesis (Fig. 2). (ii) Restriction activity appeared in cells infected in the presence of 1 mM guanidine but not in cells infected in the presence of guanidine and cycloheximide together (Fig. 3), demonstrating that viral gene expression but not replication is required for its formation. The same is true for the induction of host cell shutoff (1, 29). (iii) It demonstrated the stringent specificity of restricting translation of capped mRNAs but not poliovirus RNA (Fig. 1; 3, 4). (iv) It acted at the precise step of binding of mRNA to the ribosome (3) that is inhibited during inhibition of host protein synthesis in vivo (10). Thus, by all criteria tested, the restriction measured in vitro has all of the properties of the inhibition of host cell protein synthesis in vivo, and it is likely that identification of the restricting activity will also identify the mediator of host cell shutoff.

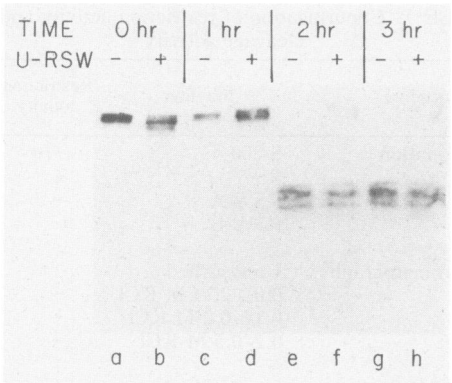

FIG. 6. Appearance of p220 cleavage activity in infected cells. Cytoplasmic extracts were made from infected HeLa cells harvested at the indicated times postinfection. Portions (10 μl) of extract were analyzed directly by immunoblot analysis (lanes a, c, e, and g) or incubated with 10 μl of uninfected-cell RSW (U-RSW) at 37°C for 60 min before immunoblot analysis (lanes b, d, f, and h).

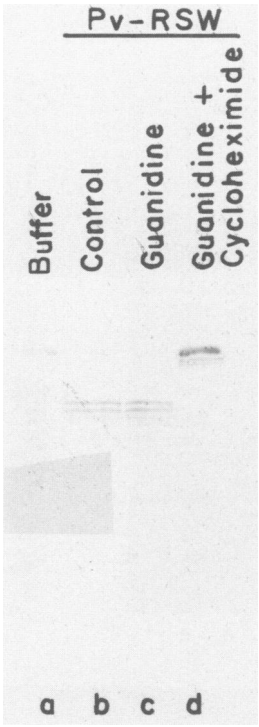

FIG. 7. Effect of 1 mM guanidine-HCl on appearance of p220 cleavage activity. HeLa cells were infected and harvested as described in the legend to Fig. 3. RSWs (5 μl) were assayed in vitro for p220 cleavage activity by immunoblot analysis. The reaction mixtures contained 5 μl of RSW from uninfected cells plus 5 μl of the indicated additions. Pv-RSW, RSW from poliovirus-infected cells.

In the last few years, a substantial amount of circumstantial evidence has been accumulated that implicates the cleavage of the p220 component of the CBP complex IF (eIF-4F) as the reaction responsible for the specific inhibition of host cell protein synthesis in poliovirus-infected cells (12, 22, 34). The biochemical mediator of this protein cleavage is not known, although both poliovirus proteases, 3C and 2A, appear to have been excluded as direct catalysts of p220 cleavage (21, 25, 26). These results provided sufficient reason for us to examine in this study the possible relationship between restriction activity and p220 cleavage activity. Again, the kinetics of appearance of the two activities, as well as the effects of guanidine and cycloheximide on their formation, correlated well. In addition, both activities copurified precisely through several cell fractionation, salt precipitation, and ion-exchange chromatography procedures, resulting in preparations purified approximately 1,500-fold for both activities. The results suggest that the two activities may reside in the same protein, but further purification will be required for identification of the active polypeptides(s).

The surprising result in this study was the finding that virtually complete restriction of translation could be induced in reticulocyte lysates, but only a partial cleavage of the reticulocyte p220 occurred. These experiments presented technical difficulties because of the poor reactivity of antibodies raised aginst human (HeLa cell) p220 with rabbit antigens, and thus it was not possible to quantitate the absolute amounts of p220 that remained intact or were cleaved. Nevertheless, it is clear that most of the p220, even in a completely restricted lysate, was not cleaved. It is, of

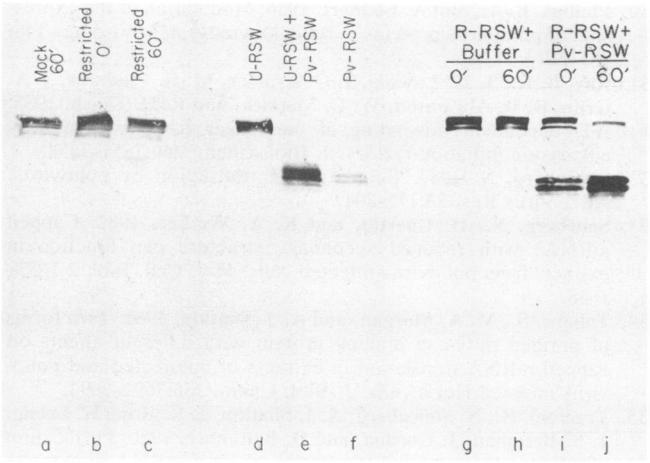

FIG. 8. Cleavage of reticulocyte p220 in restricted lysates. Shown is an immunoblot analysis of various reticulocyte RSW preparations. Lysate (3 ml) was incubated for 60 min at 37°C with 0.6 ml of buffer A (lane a) or RSW from poliovirus-infected cells (Pv-RSW) (lane c) before isolation of reticulocyte RSW as described in the text. Lane b, RSW from 3 ml of lysate incubated with Pv-RSW at 0°C for 60 min. One-third of each RSW preparation was analyzed in this figure. Lanes d to f show p220 cleavage activity on human p220. Uninfected-HeLa-cell RSW (U-RSW) (10 μl) and Pv-RSW (2 μl) were incubated for 60 min, either alone (lanes d and f) or together (lane e). Lanes g to j show p220 cleavage activity in reticulocyte RSW substrate. RSW (5 μ) was incubated with buffer A (5 μl) for 0 (lane g) or 60 (lane h) min or with 5 μl of Pv-RSW for 0 (lane i) or 60 (lane j) min.

course, possible that a small proportion of p220 cleavage is sufficient to produce quite large effects on the overall translation rates or that the process of formation of some amount of p220 degradation products is the cause of translational restriction. However, these arguments require the assumption of undocumented kinetic models and must be subjected to further testing and evaluation before firm conclusions can be drawn. Alternatively, the infected-cell p220 cleavage activity could bind and inactivate the reticulocyte CBP complex without proteolytic cleavage. It is still possible that despite the similarity in the behavior of restricted lysates and extracts prepared from polio-virus-infected cells, the restriction assay described here is not a suitable model for shutoff and that cleavage of p220 is unrelated to restriction, although it may be the mechanism responsible for shutoff. Lastly, the evidence linking p220 cleavage to host cell shutoff is still correlative and indirect, and the possibility that p220 cleavage is not the cause of virus-induced inhibition of host protein synthesis, although unlikely, remains.

ACKNOWLEDGMENTS

We are indebted to Diane Etchison for monoclonal antibody to p220.

This work was supported by Public Health Service grant AI-12387 (to E.E.) and by fellowship AI-07085 (to R.E.L.) from the National Institutes of Health.

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