Yeast Cells Are Incapable of Translating RNAs Containing the Poliovirus 5' Untranslated Region: Evidence for a Translational Inhibitor

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We have expressed in the yeast *Saccharomyces cerevisiae* a full-length poliovirus cDNA clone under the control of the *GAL10* promoter to better characterize the effect of poliovirus on host cell metabolism. We find that yeast cells are unable to translate poliovirus RNA in vivo and that this inhibition is mediated through the 5' untranslated region of the viral RNA. The in vivo inhibition of translation of poliovirus RNA and P2CAT RNA (which contains the 5' untranslated region fused upstream of the bacterial chloramphenicol transferase gene) can be mimicked in vitro in yeast translation lysates. In fact, a *trans*-acting inhibitor present in yeast lysates can inhibit translation of either poliovirus or P2CAT RNA in HeLa cell translation lysates. In contrast, when the inhibitor is added to translations programmed with chloramphenicol acetyltransferase RNA, yeast prepro- α -factor RNA, or an RNA containing the internal ribosome entry site of encephalomyocarditis virus, no inhibition is seen. The inhibitory activity has been partially purified by DEAE-Sephacel chromatography. The partially purified inhibitor is heat stable, escapes phenol extraction, is resistant to proteinase K and DNase I treatment, and is sensitive to RNase A digestion, suggesting that the inhibitor is an RNA. In an in vitro translation assay, the inhibitory activity can be overcome by increasing the concentration of HeLa cell lysate but not P2CAT RNA, suggesting that the inhibitor interacts (directly or indirectly) with one or more components of the HeLa cell translational machinery rather than with the viral RNA.

Poliovirus is a member of the *Picornaviridae* family. It contains a single-stranded, positive-polarity RNA which is 7.4 kb in length (19, 31). The RNA is not capped, but instead has a small virus-encoded protein, VPg, linked to its 5' end. VPg is rapidly removed upon infection or introduction of the RNA into cell-free translation lysates, leaving pUp at the 5' end of the RNA. Type 1 poliovirus contains a long untranslated region (UTR; 742 nucleotides) with eight short open reading frames (ORFs; some overlapping) upstream of the initiator methionine codon. Disruption of these ORFs by linker insertion or by a point mutation of each AUG shows that they do not play a role in viral replication (26, 37). Recent evidence indicates that ribosomes do not scan poliovirus RNA from the 5' end, as is the case for most eukaryotic mRNAs (21). Instead, sequences between nucleotides 320 and 631 appear to mediate cap-independent, internal initiation of translation (5, 27, 29, 38). The sequences responsible for internal initiation are referred to as the internal ribosome entry site (IRES) (15, 16; reviewed in 14). A similar internal entry of ribosomes also has been seen with two other picornaviruses, encephalomyocarditis virus (ECMV) (16) and foot-and-mouth disease virus (4). Recently, the cellular mRNA encoding the immunoglobulin heavy-chain-binding protein was also shown to be capable of internal initiation (22).

Poliovirus RNA is translated as a polypeptide which is self-cleaved by virus-encoded proteases, releasing structural and nonstructural proteins. The presence of viral proteins leads to a rapid shutoff of host cell transcription and translation caused by modification (e.g., proteolysis or dephos-

phorylation) of specific cellular factors (9, 10, 18, 20, 35). Complete characterization of these altered factors has been hampered in part by the logistical problems of obtaining a large amount of starting material for cellular protein purification and the inability to genetically manipulate HeLa cells. However, these limitations can be overcome by studying gene regulation in yeast cells. The mechanisms of transcription and translation in yeast cells are functionally similar to those of the mammalian system. In fact, yeast transcription factor IID (TFIID, the TATA-binding factor) can substitute for human TFIID in reconstituted transcription assays (7, 8), and mammalian translation initiation factor eIF-4E can substitute for yeast eIF-4E in vivo (2). Thus, the presence of poliovirus proteins in yeast cells would be expected to result in effects similar to those seen in infected HeLa cells.

In this study we have expressed a poliovirus cDNA clone in *Saccharomyces cerevisiae* with the hope of better characterizing the effect of poliovirus on cellular processes. Interestingly, we find that yeast cells are unable to translate poliovirus RNA in vivo and that this effect is a consequence of the 5' UTR of the viral RNA. The inability of yeast cells to translate RNAs containing the 5' UTR can also be demonstrated in vitro with cell-free translation assays. This effect appears to be due to a *trans*-acting factor, since an activity present in yeast cell lysates inhibits the ability of HeLa cell extracts to translate these same RNAs. The activity binds strongly to DEAE-Sephacel, eluting at between 0.6 and 1.0 M potassium acetate. Initial characterization of the inhibitory activity suggests that it is an RNA rather than a protein and that it interacts (directly or indirectly) with a component of the HeLa cell translational machinery.

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MATERIALS AND METHODS

Cells and viruses. HeLa cells were grown as Spinner cultures in minimal essential medium (GIBCO Laboratories) supplemented with 1 g of glucose per liter and 6% newborn calf serum. Poliovirus RNA (type 1 Mahoney) was prepared as described previously (36). The *S. cerevisiae* strain used was YM259 (a *his3Δ200 tyr1 ade2-101^{och} ura3-52 GAL SUC2*), provided by M. Grunstein.

Plasmid construction. pUC19CAT was provided by R. Gaynor. The *HindIII*-*Bam*HI fragment containing the chloramphenicol acetyltransferase (CAT) gene was subcloned into pGEM3, creating pG3CAT. pP2CAT was created by digesting vector pP25' (provided by V. Racaniello [27]) with *Eco*RV and *Bam*HI and ligating it to the CAT gene from pG3CAT, which had been digested with *HindIII*, end filled with the Klenow fragment, digested with *Bam*HI, and gel purified. pBMCAT was constructed by cloning an end-filled *HindIII*-*Bam*HI CAT gene fragment from pG3CAT into the *Eco*RI site (also end filled) of vector pBM258 (provided by M. Grunstein [referred to as pBM150 in reference 17]). pBMP2CAT was constructed by digesting pP2CAT with *HindIII*, end filling, adding *Eco*RI linkers, and removing P2CAT as an *Eco*RI fragment and ligating it into the *Eco*RI site in pBM258. A full-length poliovirus cDNA was constructed by cloning an *Aat*II-*Eco*RI fragment of pT7XL (provided by E. Wimmer and representing the 3' 6.3 kb of the viral cDNA) and a *Sal*I-*Aat*II fragment of p5'Mut0 (provided by V. Racaniello and representing the 5'-most 1.1 kb of the viral cDNA [a *Sal*I linker had been inserted into the *HindIII* site at the 5' end of the clone]) into pBluescript KS (Stratagene Corp.), which had been digested with *Sal*I and *Eco*RI. An *Eco*RI linker was added at the *Sal*I site, and the resultant full-length cDNA was excised from the vector by *Eco*RI digestion and cloned into the *Eco*RI site in pBM258.

α 36 (provided by D. Meyer) contains the yeast prepro- α -factor gene downstream of the Sp6 RNA polymerase promoter. This gene encodes an 18-kDa protein. pCITE-1 was purchased from Novagen. This plasmid contains 586 nucleotides of the ECMV noncoding region, including sequences necessary for cap-independent translation (the IRES) upstream of an insert coding for a 390-amino acid-peptide and downstream of the T7 RNA polymerase promoter.

HeLa cell translation extracts. HeLa cell extracts were prepared as previously described (32) with the following modifications. A starting cell concentration of 8×10^5 cells per ml was used. Micrococcal nuclease treatment was performed by adjusting the lysate to 2 mM CaCl_2 , adding 600 U of S7 nuclease (Boehringer Mannheim Biochemicals) per ml in 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4), and incubating the mixture for 25 min at room temperature. The nuclease was inactivated by adjusting the lysate to 4 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA). After dialysis, 25- μ l aliquots of the lysate were frozen at -70°C and discarded after one use.

Yeast cell-free translation. Yeast cell extracts were prepared as described previously (33) with the following modifications. Micrococcal nuclease treatment (described above) was performed on the supernatant following the Sorvall centrifugation step. Also, the Sephadex G-25 column was omitted in favor of dialysis against at least 100 volumes of lysis buffer (100 mM KOAc [OAc is acetate], 2 mM $\text{Mg}(\text{OAc})_2$, 2 mM dithiothreitol, 20% glycerol, 20 mM HEPES [pH 7.4]) plus 1 mM phenylmethylsulfonyl fluoride. Lysates were divided into aliquots and stored at -70°C .

These lysates could be used multiple times without loss of activity.

In vitro transcriptions and translations. RNA transcripts were prepared as follows. pG3CAT DNA was linearized with either *Bam*HI (for sense transcripts) or *HindIII* (for antisense "riboprobe" transcripts). pP2CAT was linearized with *Bam*HI for sense transcripts. α 36 and pCITE-1 were linearized with *Pvu*II and *Xba*I, respectively. Uncapped RNA was transcribed by the Promega Biotec protocol with either Sp6 or T7 RNA polymerase. Capped RNA was transcribed as described above but in the presence of 0.04 A_{260} unit of $m^7\text{G}(5')\text{ppp}(5')\text{G}$ (Pharmacia) per ml and 60 μM GTP for 1 h and then for 10 to 15 min with an additional 60 μM GTP. Unlabeled RNAs were phenol-chloroform extracted, ethanol precipitated, and resuspended in DEPC water at 1 $\mu\text{g}/\mu\text{l}$ prior to use in translation. Labeled RNA was made with 50 μM unlabeled UTP and 100 μCi of [^{32}P]UTP (3,000 Ci/mmol; Amersham). Reaction mixtures were incubated for 1 h at 37°C . Poliovirus-specific riboprobe was made from plasmid pProPol (24), which had been digested with *Bgl*II and transcribed with Sp6 RNA polymerase. This probe is complementary to poliovirus nucleotides 5601 to 7400. The probe was added directly to prewarmed (65°C) Northern (RNA) blot hybridization solutions.

HeLa cell-free translation conditions were as described previously (32), except that only 30 μCi of [^{35}S]methionine (>800 Ci/mmol; Amersham) was used per reaction and 40 U of RNasin (Promega Biotec) was used per reaction. Yeast cell-free translation conditions were identical to those described above, except that reaction mixtures were incubated at room temperature. The CAT RNA used in all translation reactions was capped, while the P2CAT RNA was not. Experiments requiring mixing of extracts were performed at 37°C . After translation, all reaction mixtures were treated for 5 min with 1 μl of RNase A (10 mg/ml; Boehringer) at 37°C , precipitated with 3 volumes of ice-cold acetone, resuspended in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer, heated, and subjected to electrophoresis on 12.5% SDS-polyacrylamide gels. Gels were fixed in 10% methanol-10% acetic acid, washed in water, incubated in 1 M salicylic acid for 20 min, dried, and exposed to Kodak XAR film overnight at -70°C .

Induction of yeast cells for RNA and protein. Yeast cultures were grown, made competent by the lithium acetate method, and transformed with plasmid DNA as described previously (3). Colonies which grew on plates lacking uracil were grown in liquid medium on a shaker at 30°C . For induction, cultures which had reached an optical density at 600 nm of 0.5 were washed twice with medium containing galactose, resuspended in this medium, and grown overnight. Protein labeling was performed with medium containing galactose, lacking methionine, and supplemented with 20 μCi of [^{35}S]methionine ($>1,000$ Ci/mmol; ICN) per ml of culture.

Preparation of total yeast RNA, formaldehyde gel electrophoresis, and Northern transfer were done as described previously (3). Northern blots were prehybridized in solution X (containing 10% dextran sulfate, 40% deionized formamide, $4 \times \text{SSC}$ [$1 \times \text{SSC}$ is 0.15 M NaCl plus 0.015 M sodium citrate], 20 mM Tris [pH 7.4], $1 \times$ Denhardt's solution [containing 0.02% each Ficoll 400, polyvinylpyrrolidone, and bovine serum albumin {BSA}], 20 μg of salmon sperm DNA per ml, and 200 μg of tRNA per ml) for at least 1 h at 65°C . Hybridization was carried out overnight at 65°C in fresh solution X (prewarmed to 65°C) with the ^{32}P -labeled RNA probe. After hybridization, blots were washed three times in approximately 150 ml of 0.1X SSPE (15 mM NaCl,

1 mM NaH_2PO_4 , 0.1 mM EDTA)–0.1% SDS at 65°C for 30 to 45 min. Blots were exposed to Kodak XAR film at –70°C for 4 h or overnight.

Immunoprecipitations. Yeast cell extracts for immunoprecipitations were prepared as described previously (13) with 5 ml of yeast cultures. In brief, cultures were pelleted, washed once in 1 ml of phosphate-buffered saline, and resuspended in 200 μl of RIPA buffer (150 mM NaCl, 1% Nonidet P-40 [Sigma Chemical Co.], 0.5% deoxycholate, 0.1% SDS, 50 mM Tris [pH 8.0]) in a Microfuge tube. An equal volume of glass beads was added, the tubes were vortexed for 2 min at 4°C and centrifuged in a Microfuge for 5 min, and the supernatant was recovered for immunoprecipitations. Poliovirus-infected, [^{35}S]methionine-labeled HeLa cell extracts were prepared as described previously (34). Anticapsid antibodies were purchased from the American Type Culture Collection. Protein A-Sepharose (75 μl of a 20% solution in RIPA buffer plus 0.2% BSA) was used to precipitate the immune complexes. Sepharose-coupled anti-CAT antibodies were purchased from 5'-3' Corp. Immunoprecipitations were done on ice overnight at 4°C with either 30 μl of anticapsid antibody or 5 μl of anti-CAT antibody in a 500- μl total volume of RIPA buffer. Approximately 3.5×10^6 cpm of labeled protein was used in the immunoprecipitations. Immunoprecipitates were washed three times with 500 μl of RIPA buffer, transferred to a fresh Microfuge tube, and washed once more. Pellets were resuspended in 30 μl of SDS-PAGE sample buffer, heated for 5 min at 95°C, spun in a Microfuge to pellet the Sepharose beads, and electrophoresed on 12.5% SDS-polyacrylamide gels. Approximately 7×10^5 cpm of labeled protein was analyzed in experiments without immunoprecipitation. Gels were treated as described above.

Partial purification and characterization of the inhibitor. Yeast cell lysates were made as described above, except that they were not treated with micrococcal nuclease. Lysates were batch loaded onto DEAE-Sepharose (Pharmacia) which had been equilibrated with lysis buffer (100 mM KOAc). Fractions were step eluted at 0.3, 0.6, and 1.0 M KOAc, dialyzed back to 100 mM KOAc, divided into aliquots, and frozen at –70°C. The 1.0 M DEAE fraction was heat treated for 10 min at 95°C and centrifuged in a Microfuge briefly to pellet debris, and the supernatant was divided into aliquots and frozen. Phenol extraction involved extraction with phenol-chloroform equilibrated with RNA buffer (0.5 M NaCl, 200 mM Tris [pH 7.5], 10 mM EDTA [4]) and extraction with chloroform only. Approximately one-half of the aqueous volume was collected from each extraction to eliminate any possibility of disturbing the interface.

RESULTS

Poliovirus RNA is not translated in yeast cells. A full-length copy of the poliovirus cDNA was cloned into pBM258 (a vector which contains sequences for replication in both *E. coli* and yeast cells) under the control of the *GAL10* promoter. *S. cerevisiae* cells transformed with the resultant plasmid, pBMPOLIO (Fig. 1), were grown on galactose to induce the expression of the poliovirus cDNA, and the growth characteristics were compared with those of yeast cells transformed with pBM258. We had expected that the presence of viral proteins would be deleterious to yeast cells but were surprised to find that the growth of yeast cells transformed with pBMPOLIO was not different from the growth of yeast cells transformed with pBM258 (data not shown). To determine whether the poliovirus cDNA was

FIG. 1. Plasmids used in these experiments. Sp6, Sp6 RNA polymerase promoter; T7, T7 RNA polymerase promoter; GAL10, yeast *GAL10* promoter; UTR, poliovirus 5' UTR; CAT, bacterial CAT gene; POLIO, full-length poliovirus type 1 (Mahoney) cDNA. The arrows represent the direction of transcription. Not shown is pBM258, the parental yeast-*E. coli* shuttle vector.

actually expressed, we performed Northern blot analyses. Total RNA was isolated from yeast cells transformed with either pBM258 or pBMPOLIO, run on a denaturing agarose gel, transferred to a nitrocellulose membrane, and probed with a poliovirus-specific riboprobe. Poliovirus-specific sequences were not detected in yeast cells transformed with pBM258 or pBMPOLIO and grown on glucose as the carbon source (Fig. 2A, lanes 2 and 3) or from yeast cells transformed with pBM258 and grown on galactose (lane 4). However, a poliovirus-specific RNA was detected in yeast cells transformed with pBMPOLIO and grown on galactose (lane 5). This RNA species had a mobility similar to that of poliovirus RNA which was purified from infected HeLa cells and which also hybridized to the riboprobe (lane 1). Thus, it appeared that the poliovirus cDNA was transcribed in yeast cells to yield a stable, poliovirus-specific RNA of the appropriate size.

To determine whether the poliovirus-specific RNA was translated to make viral proteins, we grew yeast cells transformed with either pBM258 or pBMPOLIO on galactose in the presence of [^{35}S]methionine to label proteins. Extracts made from these yeast cells were incubated with antisera specific for poliovirus capsid proteins. Immunoprecipitation of infected HeLa cell extracts also labeled with [^{35}S]methionine served as the positive control (Fig. 2B, lane 4). The antibody was unable to precipitate detectable amounts of

FIG. 2. Northern analysis and immunoprecipitation of yeast cells transformed with pBM258 and pBMPOLIO. (A) Northern analysis was performed with total yeast RNA (10 μ g per lane) and poliovirus RNA (0.5 ng per lane). The blot was probed with a 32 P-labeled, poliovirus-specific riboprobe as described in Materials and Methods. RNA was isolated from yeast cells transformed with pBM258 (lanes 2 and 4) or pBMPOLIO (lanes 3 and 5) and grown on glucose (DEX) or galactose (GAL) as the carbon source. Poliovirus (PV) RNA was run in lane 1. The poliovirus-specific RNA is indicated by the arrow. (B) Immunoprecipitation of [35 S]methionine-labeled yeast cells transformed with pBM258 and pBMPOLIO and poliovirus-infected HeLa cell extracts. Labeled extracts were either untreated (lanes 1 to 3) or incubated with anticapsid antibody (Ab) on ice overnight (lanes 4 to 6). Samples were processed as described in Materials and Methods. Size markers (in kilodaltons) are shown on the left. The arrows indicate the migration of immunoprecipitated poliovirus capsid proteins from the infected HeLa cell extracts.

labeled viral proteins from yeast cells transformed with pBM258 (lane 5) or pBMPOLIO (lane 6), even when the film was overexposed (data not shown). Extracts which were not incubated with antibody showed many labeled bands, indicating that the [35 S]methionine was incorporated into proteins (lanes 1 to 3). Thus, although the poliovirus cDNA was expressed in yeast cells transformed with pBMPOLIO and grown on galactose, these yeast cells were unable to translate the poliovirus-specific RNA.

Translational inhibition is mediated through the 5' UTR. Because poliovirus RNA contains a long 5' UTR and translation initiates internally in HeLa cells, yeast cells transformed with pBMPOLIO may have been unable to translate poliovirus RNA because of the 5' UTR. To determine whether this was the case, we constructed two new plasmids: pBMCAT, which contains the bacterial CAT gene cloned into pBM258, and pBMP2CAT, in which the 5' UTR is first fused to the CAT gene and then inserted into pBM258 (Fig. 1). Yeast cells transformed with pBMCAT and pBMP2CAT were induced to express the CAT genes with galactose, and total RNA was extracted and subjected to Northern analysis. When hybridized to a 32 P-labeled CAT antisense riboprobe, these blots showed the presence of intact RNAs of the appropriate sizes for CAT and P2CAT mRNAs (Fig. 3A, lanes 2 and 3). RNA from yeast cells transformed with parental vector pBM258 did not hybridize to the labeled probe (lane 1).

To determine whether these RNAs were translated, we prepared extracts from yeast cells grown in the presence of galactose and [35 S]methionine. Immunoprecipitations with anti-CAT antibodies showed that only yeast cells transformed with pBMCAT made the CAT protein (Fig. 3B, lane 2). Yeast cells transformed with pBMP2CAT did not make any CAT protein (Fig. 3B, lane 3), despite the presence of CAT-specific RNA in yeast cells transformed with this construct (Fig. 3A, lane 3). Longer exposures of the film also failed to detect CAT protein in yeast cells transformed with pBMP2CAT (data not shown). Thus, the RNA containing the 5' UTR was not translated *in vivo*. To investigate the mechanism of this translational inhibition, we used cell-free translation systems to translate CAT, P2CAT, and poliovirus RNAs.

Inhibition of translation *in vitro*: evidence for a translational inhibitor in yeast cells. HeLa and yeast cell lysates were used to translate CAT, P2CAT, and poliovirus RNAs *in vitro*. HeLa cell lysates translated poliovirus RNA (Fig. 4A, lanes 2 and 3), CAT RNA (Fig. 4B, lane 1), and P2CAT RNA (Fig. 4B, lane 4) quite efficiently. Consistent with the *in vivo* results (Fig. 2 and 3), yeast cell lysates were able to translate CAT RNA (Fig. 4B, lane 3) but not P2CAT RNA (Fig. 4B, lane 6) or poliovirus RNA (Fig. 4A, lane 5). Interestingly, the addition of a small amount of yeast cell lysate to the HeLa cell translation reactions prevented translation of RNAs containing the 5' UTR (Fig. 4A, lane 4, and Fig. 4B,

FIG. 3. Northern analysis and immunoprecipitation of yeast cells transformed with pBM258, pBMCAT, or pBMP2CAT. (A) Northern analysis was performed on total yeast RNA (10 μ g per lane) isolated from yeast cells transformed with the plasmid indicated above each lane. The blots were probed with a ³²P-labeled CAT antisense riboprobe. RNA size markers (in kilobases) are shown on the left. The upper and lower arrows on the right indicate the migration of P2CAT and CAT RNAs, respectively. (B) Immunoprecipitation was performed by incubating [³⁵S]methionine-labeled proteins from yeast cells transformed with the plasmid indicated above each lane with anti-CAT antibody. Samples were treated as described in Materials and Methods. Protein size markers (in kilodaltons) are shown on the left, and the location of the CAT protein product is indicated on the right.

FIG. 5. Fractionation of the inhibitory activity over DEAE-Sephacel. Yeast cell extract was loaded onto DEAE at 0.1 M KOAc and step eluted at 0.3, 0.6, and 1.0 M KOAc. Fractions were dialyzed to 0.1 M KOAc and assayed for their ability to inhibit the translation of P2CAT (lanes 1 to 6) and CAT (lanes 7 to 12) RNAs in HeLa cell translation lysates. A total of 3.6 μ g of each fraction was added in a final volume of 4 μ l (fractions were diluted in lysis buffer when necessary). The amount of HeLa cell lysate used in each lane was 72 μ g. B, buffer; FT, flowthrough. 0.3, 0.6, and 1.0 indicate the molarity of KOAc used to elute each fraction from the DEAE column.

lane 5). Mixing of extracts had little effect on translation of CAT RNA (Fig. 4B, lane 2). The addition of lysis buffer did not affect translation of P2CAT RNA (Fig. 5 and 6 and data not shown), implying that the inhibition was not simply a consequence of buffer conditions. These results suggest that yeast cells contain a translational inhibitor whose effects are mediated through the UTR and which functions in *trans*.

It was possible that one of the short ORFs found in the

FIG. 4. In vitro translation assays. (A) HeLa or yeast cell-free translation lysates were used to translate poliovirus RNA. HeLa (H) cell lysates were used at 34 μ g (lanes 1, 2, and 4) or 68 μ g (lane 3) of total protein. Yeast (Y) cell lysates were used at 25 μ g (lane 4) or 75 μ g (lanes 5 and 6) of total protein. One microgram of poliovirus (PV) RNA was added to lanes 2 to 5. —, no additional components added. (B) HeLa or yeast cell-free translation lysates were used to translate CAT or P2CAT RNA. Fifty two micrograms of HeLa cell lysate was used in lanes 1, 2, 4, and 5; 30 μ g of yeast cell lysate was used in lanes 2 and 5, and 90 μ g was used in lanes 3 and 6. Approximately 1 μ g of CAT (C) or P2CAT (P) RNA was added per reaction. The location of the CAT gene product is indicated on the right.

FIG. 6. Initial characterization of the inhibitory activity by an in vitro translation assay. (A) Translation of P2CAT RNA by HeLa cell lysates. Translations were performed in the presence of no additional components (lane 1), yeast lysis buffer (lane 2), inhibitor (1.0 M DEAE column fraction) (lane 3), or heat-treated inhibitor (lane 4). Lysis buffer or heat-treated inhibitor was extracted with phenol-chloroform (see Materials and Methods), and the aqueous phases were added to the translation reactions (lanes 5 and 6). Lysis buffer or heat-treated inhibitor was incubated with 10 μ g of proteinase K (lanes 7 and 8) or 100 μ g of RNase A (lanes 9 and 10) per ml for 15 min at 37°C before phenol-chloroform extraction and addition to the translation reactions. In all lanes, 3 μ l of buffer or inhibitor was added. (B) Translation of CAT RNA by HeLa cell lysates in the presence of 3 μ l of buffer (lane 1), inhibitor (lane 2), or heat-treated inhibitor (lane 3). B, buffer; I, inhibitor (1.0 M DEAE fraction); Δ I, heat-treated (95°C for 10 min) inhibitor; -, no additional components added. The amount of HeLa cell lysate used in each lane was 72 μ g.

poliovirus 5' UTR was translated in the presence of yeast cell lysates, resulting in a protein product which inhibited translation. To address this possibility, we preincubated yeast cell translation lysates for 10 min with either P2CAT RNA or an RNA encoding only the 5' UTR, added CAT RNA, and continued incubation for another 60 min. Under these conditions, CAT RNA was still translated with the same efficiency as when there was no RNA present during the preincubation step, indicating that the ORFs present in the poliovirus 5' UTR do not code for a translational repressor (data not shown). While it cannot be ruled out that a leader peptide which specifically inhibits internal initiation is translated, we feel that this possibility is unlikely since (i) it appears that these ORFs are not translated either in poliovirus-infected HeLa cells or in cell-free translation systems (26, 37) and (ii) the inhibitory activity has been partially purified and appears not to be a protein, but an RNA (see below).

Partial purification and initial characterization of the inhibitory activity. Yeast cell lysates were fractionated on a DEAE-Sephacel column to partially purify the inhibitory activity. Extracts were prepared by the same protocol as that used for translation extracts but were not treated with micrococcal nuclease. Extracts were loaded onto DEAE at 0.1 M KOAc and step eluted at 0.3, 0.6, and 1.0 M KOAc. The fractions were dialyzed back to 0.1 M salt and assayed for the ability to inhibit translation of P2CAT RNA by HeLa cell translation lysates. As a control for nonspecific effects, the fractions were also tested for their effect on the translation of CAT RNA by HeLa cell lysates. Both the flowthrough and the 0.3 M wash inhibited translation of P2CAT RNA slightly (Fig. 5, lanes 3 and 4), but this effect

was also seen with CAT RNA (lanes 9 and 10) and thus was probably nonspecific. The 0.6 M fraction stimulated translation of both P2CAT and CAT RNAs (compare lane 5 with lane 2 and lane 11 with lane 8) and most likely contained a limiting component of the translation lysates. Most dramatic, however, was the effect of the 1.0 M fraction. While the addition of this fraction to HeLa cell lysates translating CAT RNA did not result in a significant change in translation efficiency (compare lanes 8 and 12), translation of P2CAT RNA was drastically inhibited (compare lanes 2 and 6). The addition of lysis buffer to the translation reactions had no effect on translation of P2CAT RNA, although translation of CAT RNA was reduced somewhat (compare lane 1 with lane 2 and lane 7 with lane 8). Thus, the activity which specifically inhibits translation of P2CAT RNA can be fractionated over DEAE-Sephacel and elutes between 0.6 and 1.0 M KOAc.

To better characterize the inhibitory activity, we heat treated the 1.0 M column fraction to determine whether the activity was heat stable or labile. The partially purified inhibitor was heated at 95°C for 10 min and spun briefly in a Microfuge to pellet debris, and the supernatant was added to HeLa cell translation lysates containing P2CAT RNA. The inhibitor still retained its activity after this treatment (Fig. 6A, compare lane 4 with lanes 1 and 2). In fact, heat treatment of the 1.0 M DEAE column fraction resulted in a severalfold purification of the activity, as the heat-treated inhibitor was more active than the same volume of untreated inhibitor (compare lanes 3 and 4). The addition of heat-treated lysis buffer had no effect on translation of P2CAT RNA (data not shown). Neither the untreated nor the heat-treated inhibitor prevented translation of CAT RNA

FIG. 7. Effects of increasing amounts of HeLa cell lysate or RNA on translation in the presence of the inhibitor. (A) Translation of P2CAT RNA by different concentrations of HeLa cell lysates. Translations were performed in the presence of 3 μ l of lysis buffer (lanes 1 to 3) or heat-treated inhibitor (lanes 4 to 6). The volume of HeLa cell lysate added (in microliters) is shown above each lane. The HeLa cell lysate protein concentration was 30 μ g/ μ l. (B) Translation of different amounts of P2CAT RNA by HeLa cell lysates. Translations were performed in the presence of 3 μ l of lysis buffer (lanes 1 to 4) or heat-treated inhibitor (lanes 5 to 8). Sixty micrograms (2 μ l) of HeLa cell lysate was used in each lane. The amount of RNA added (in micrograms) is shown above each lane. H, HeLa cell lysate; B, lysis buffer; Δ I, heat-treated inhibitor.

(Fig. 6B, lanes 1 to 3). These data suggested that the inhibitor was either a very heat-stable protein or perhaps a nucleic acid or nucleoprotein.

Because the lysates had not been treated with micrococcal nuclease prior to fractionation, it was possible that the activity would be sensitive to this nuclease. However, treatment of the 1.0 M DEAE column fraction with micrococcal nuclease and inactivation of the nuclease with EGTA had no effect on the ability of the inhibitor to inhibit translation of P2CAT RNA by HeLa cell lysates (data not shown). The nuclease was active, however, since it was able to digest 10 μ g of total yeast RNA under identical reaction conditions.

Phenol-chloroform extraction was used to determine whether the inhibitory activity had a protein component. Lysis buffer which had been extracted with phenol-chloroform slightly inhibited translation of P2CAT RNA and served as a control for the toxicity of the extraction process (Fig. 6A, lane 5). Extraction of the heat-treated inhibitor with phenol-chloroform did not remove the inhibitory activity (compare lanes 5 and 6). This observation allowed for digestion of the heat-treated inhibitor with RNase A and proteinase K, two enzymes which could not be adequately inhibited by chemical means but which could be removed by phenol extraction. Heat-treated inhibitor was incubated with 100 μ g of RNase A or 10 μ g of proteinase K per ml at 37°C for 15 min, phenol-chloroform extracted, and added back to HeLa cell translation lysates containing P2CAT RNA. Since any residual RNase A or proteinase K activity would have inhibited the translation reactions, buffer alone was also treated with these enzymes, extracted with phenol-chloroform, and added to the translation reaction mixtures. Buffer thus treated did not affect translation of P2CAT RNA, indicating that the enzymes were completely removed by phenol extraction (lanes 7 and 9). Incubation of the heat-treated fraction with proteinase K did not remove the inhibitor (lane 8), while RNase A treatment effectively

removed all inhibitory activity, allowing translation of P2CAT RNA (lane 10). Thus, the inhibitor appears to be an RNA molecule. Precisely why it is resistant to micrococcal nuclease treatment is not known, but this resistance could be due to the presence of an extensive secondary structure.

The next experiments were aimed at determining whether the inhibitor mediated its effect directly through the poliovirus 5' UTR or through an interaction with a HeLa cell factor needed specifically for translation of P2CAT RNA. To differentiate between these two possibilities, we added an excess of either P2CAT RNA or HeLa cell lysate to the reaction mixtures to saturate the inhibitor and restore translation. Unfortunately, HeLa cell translation lysates were sensitive to high levels of protein and RNA, so that the addition of a very large excess of either was not possible. However, it appears that a small increase in the amount of HeLa cell lysate used for translation could overcome the inhibition seen with the heat-treated inhibitor, while an increase in the RNA amount could not. Translational inhibition (Fig. 7A, compare lanes 1 and 4) could be overcome by increasing the amount of HeLa cell lysate used in the translation reaction (lanes 5 and 6). The same titration of HeLa cell lysate in the absence of the inhibitor did not result in a significant change in the amount of CAT protein produced (lanes 1 to 3). In a second experiment, the HeLa cell lysate concentration was kept constant while P2CAT RNA was titrated over a sixfold range (Fig. 7B). In the absence of the inhibitor, the level of translation remained essentially constant with two-, four-, and sixfold increases in P2CAT RNA levels (compare lane 1 with lanes 2, 3, and 4). Over this same range of RNA concentrations, the inhibitor remained active (lanes 5 to 8), indicating that the RNA was not the direct target of the inhibitor. These results suggest that the yeast inhibitor interacts with a component of the HeLa cell translation lysate to prevent efficient translation of P2CAT RNA.

Effect of the inhibitory activity on other RNAs. We next

FIG. 8. Effect of the inhibitor on the translation of other RNAs. HeLa cell lysates were used to translate P2CAT, CAT, α 36, and pCITE-1 RNAs. α 36 RNA encodes the 18-kDa yeast prepro- α -factor protein, and pCITE-1 RNA contains the EMCV IRES fused to an RNA encoding a 390-amino-acid peptide. Translations were performed in the presence of RNA made from the plasmid indicated above each lane and in the presence of 1 μ l of lysis buffer (B) or inhibitor (I), as indicated above each lane. The inhibitor was made by treating a yeast S-10 lysate with micrococcal nuclease (600 U/ml) and DNase I (1 μ g/ml) for 25 min at 22°C and with proteinase K (50 μ g/ml) for 30 min at 37°C, phenol-chloroform extracting the lysate twice, and collecting the inhibitory RNA by ethanol precipitation. The pellet was resuspended in lysis buffer. The nucleic acid concentration was 4 μ g/ μ l. The amount of HeLa cell lysate used in each lane was 50 μ g. The arrows indicate the locations of the translated proteins. Samples were treated as described in Materials and Methods, except that the samples loaded in lanes 7 and 8 were electrophoresed on a 15% gel.

tested whether the inhibitor had any effect on the translation of two other RNAs, the yeast prepro- α -factor RNA (α 36) and an RNA containing the IRES of EMCV. EMCV and poliovirus are both members of the *Picornaviridae* family, with EMCV classified in the cardiovirus subgroup and poliovirus classified in the enterovirus subgroup. The EMCV IRES directs internal initiation of translation, but the cellular factors involved must be somewhat different from those used by poliovirus, since EMCV RNA is translated by both HeLa cell and rabbit reticulocyte lysates (RRL) while poliovirus RNA is translated accurately and efficiently by HeLa cell lysates but not by RRL (6, 12). The yeast inhibitor used in these experiments was purified in a slightly different manner from that used before, as described in the legend to Fig. 8. The inhibitor still maintained its specificity for P2CAT RNA, as the addition of the inhibitor prevented translation of P2CAT RNA but not CAT RNA (Fig. 8, compare lane 1 with 2 and lane 3 with lane 4). The addition of the inhibitor to HeLa cell translation lysates containing α 36 RNA did not result in translational inhibition; in fact, some stimulation was routinely seen (Fig. 8, compare lanes 7 and 8). The reason for this translational stimulation is not known at present. Translation of pCITE-1 RNA, which contains the EMCV IRES fused to an RNA coding for a 390-amino-acid protein, was also not decreased by the presence of the inhibitor (lanes 5 and 6). Thus, the inhibitor appears to be very specific for the poliovirus 5' UTR.

DISCUSSION

We have shown here that a full-length poliovirus cDNA clone under the control of the *GAL10* promoter is not efficiently expressed in the yeast *S. cerevisiae*. Although yeast cells induced with galactose make a poliovirus-specific mRNA, the RNA is apparently not translated. P2CAT RNA, which contains the poliovirus 5' UTR fused upstream of the CAT gene, is also not translated *in vivo*, indicating that the inhibition is due to the presence of the 5' UTR. The inability of yeast cells to efficiently translate poliovirus and P2CAT RNAs *in vivo* can be recapitulated *in vitro* and appears to be due to a translational inhibitor present in yeast cells. The inhibitor is capable of inhibiting translation of these RNAs *in trans* in HeLa cell lysates. The inhibitory activity from yeast cells binds strongly to DEAE-Sephacel and elutes between 0.6 and 1.0 M potassium acetate. The activity is heat stable (95°C for 10 min), proteinase K resistant, DNase I resistant (Fig. 8 and data not shown), not extractable by phenol, and sensitive to RNase A treatment. All of these properties and its ability to bind DEAE relatively strongly suggest an RNA rather than a protein nature for the inhibitor. The inhibitor is very specific for the poliovirus 5' UTR, since it does not prevent translation of CAT, α 36, or pCITE-1 (EMCV) RNA.

Our results indicate that the inhibition of translation of poliovirus RNA in yeast cells is mediated through the 5' UTR of the viral RNA. However, the exact details of how the inhibitor works are not known at present. The inability of yeast cells to translate virion or P2CAT RNA is not due to the absence of a 5' methyl cap structure at the end of the RNA because P2CAT RNA transcribed *in vitro* in the presence of m⁷GpppG is also not translated (data not shown). Furthermore, the RNA made *in vivo*, either from pBMPOLIO or pBMP2CAT, is presumably capped and is not translated.

The inhibitor appears to interact with a component of the translational machinery rather than to directly bind to the input RNA, since the addition of excess HeLa cell lysate relieves the inhibition, whereas the addition of increasing amounts of RNA to the translation reaction mixture does not (Fig. 7A and B). It is possible that a cellular protein which binds specifically to the 5' UTR is a target for the inhibitor. Several proteins which bind to specific regions of the viral 5' UTR have been identified. A membrane-associated, 50-kDa protein has been shown to bind to an RNA hairpin structure between nucleotides 178 and 224 (25). Another protein, p52, binds to an RNA fragment spanning nucleotides 559 to 624 of the UTR and is believed to play a role in internal initiation of translation (23). Del Angel et al. have shown the binding of cellular proteins to two regions, nucleotides 97 to 182 and nucleotides 510 to 629 (whether this complex contains p52 is not known) (11). If any of these proteins are involved in translation, they may facilitate ribosome or initiation factor binding to the IRES located within the 5' UTR. In fact, the two complexes described by del Angel et al. both contain eIF2 α , suggesting the need for cellular proteins to position the translational machinery on the viral RNA (11). We suspect that it would be this function of the protein, rather than the RNA-binding property itself, which would be altered in the presence of the inhibitor, since increasing the amount of template mRNA does not relieve inhibition. It is also possible that the inhibitor can irreversibly saturate all the RNA-binding sites in the protein so that increasing the mRNA concentration still has no effect. In either case, it will be interesting to determine whether the addition of an excess

of one of the RNA-binding proteins described above can relieve translational inhibition.

The fact that translation of pCITE-1 RNA (containing the EMCV IRES) is not decreased by the addition of inhibitor argues that the inhibitor interacts with a factor needed specifically for poliovirus RNA to direct internal initiation. Although both poliovirus and EMCV use internal initiation of translation, their respective IRES elements are not conserved, and they show differences in the cell types in which they are functional (reviewed in reference 14). For example, the EMCV IRES is functional (in terms of initiating translation accurately and efficiently) in RRL and HeLa cell lysates, whereas the poliovirus IRES is functional in HeLa cell lysates but not in RRL (28). The inability of poliovirus RNA to translate accurately and efficiently in RRL appears to be due to the absence of a *trans*-acting factor(s) which can be supplied by HeLa cell extracts (6, 12, 30). A candidate for this factor is p52, since it binds to the poliovirus 5' UTR within the IRES and is much more abundant in HeLa cell lysates than in RRL (23). The inhibitor described here could interfere with this protein, which could explain why the translation of P2CAT but not pCITE-1 RNA is inhibited. Future studies with 5' UTR mutants should determine the exact RNA sequence through which the inhibitor acts and consequently which RNA-binding protein(s) can be considered a potential target for the inhibitor.

The specificity of the inhibitory activity for the poliovirus 5' UTR should make it a valuable tool for studying internal initiation on this IRES. Our preliminary results suggest that a small (~30-base) RNA contains the inhibitory activity described here. If the inhibitor functions by binding to IRES-binding proteins, it should be possible to identify (and purify) such proteins by using the sequence of the inhibitor in RNA gel shifts and RNA affinity chromatography. If a protein which can relieve the inhibition *in vitro* is found, overexpression of such a factor *in vivo* should allow translation of full-length poliovirus RNA in yeast cells. Altman et al. have reported a strain of *S. cerevisiae* which can translate (albeit inefficiently) UTR-CAT gene fusions present as the second gene in bicistronic RNAs (1). Perhaps in this strain the inhibitor is present in lower amounts or the putative target factor is present in higher amounts. We have been unable to detect any translation of P2CAT RNA in the two strains that we have used (Fig. 2 and 3 and data not shown).

Finally, what is the function of the inhibitor in yeast cells? Macejak and Sarnow have recently published a report showing that internal initiation can take place on the cellular mRNA encoding the immunoglobulin heavy-chain-binding protein (22). The authors speculate that this mRNA may be translated by both cap-dependent and cap-independent mechanisms, possibly in a cell cycle-dependent manner (correlating with the phosphorylation state of eIF-4F) (22). An intriguing possibility is that yeast cells contain genes which initiate translation internally and that the inhibitory RNA described here can be used to regulate the expression of these genes posttranscriptionally. Purifying and sequencing of the inhibitor and determining its mode of action will help us define any role it may have in translation in yeast cells.

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