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Characterization of a 40S Ribosomal Subunit Complex in Polyribosomes of *Saccharomyces cerevisiae* Treated with Cycloheximide

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Under specific conditions cycloheximide treatment of *Saccharomyces cerevisiae* caused the accumulation of a type of polyribosome called "halfmer." Limited ribonuclease digestion of halfmers released particles from the polyribosomes identified as 40S ribosomal subunits. The data demonstrated that halfmers are polyribosomes containing an additional 40S ribosomal subunit attached to the messenger ribonucleic acid. Protein gel electrophoretic analysis of halfmers revealed numerous nonribosomal proteins. Two of these proteins comigrate with subunits of yeast initiation factor eIF_2.

In current models of the initiation of eucaryotic protein synthesis, the 40S ribosomal subunit binds messenger ribonucleic acid (RNA) before the attachment of the 60S ribosomal subunit. Progress in the study of this process in yeast has been limited by the absence of an in vitro translation system, which was developed only very recently (7). As an alternative approach to in vitro investigation of initiation factors, we have isolated and characterized complexes formed in vivo in the presence of the antibiotic cycloheximide.

Cycloheximide, an inhibitor of eucaryotic protein synthesis (22), is known to inhibit elongation over a wide range of concentrations (2, 25, 27, 32). At lower concentrations there is also evidence for inhibition of initiation (2, 4, 9, 24, 29). Using a low concentration of cyloheximide, we have detected and characterized a type of polyribosome, termed "halfmer" (9, 14, 19, 20), which has an extra 40S ribosomal subunit. The halfmer is an apparent product of a cycloheximide-induced inhibition of binding of the 60S ribosomal subunit to the 40S ribosomal subunit initiation complex on the polyribosome. Here we report that nonribosomal proteins are associated with the halfmer, and two comigrate with subunits of initiation factor eIF_2 in polyacrylamide gels.

MATERIALS AND METHODS

Strains and media. *Saccharomyces cerevisiae* haploid strain A364A (ATCC 22244) was grown on "modified synthetic complete" (SCY) medium (13) and YM-1 and YM-5 media (11). Spheroplasts were incubated in YM-5 medium containing 0.5 M $MgSO_4$. Low-phosphate medium was as described by Rubin (30).

Buffers. The following buffers were used: (i) lysing (L) buffer, 0.01 M tris(hydroxymethyl)aminomethane hydrochloride–0.1 M KCl–0.03 M $MgSO_4$–0.006 M 2-mercaptoethanol, pH 7.4 at 25°C; (ii) high-salt (H) buffer, L buffer containing 0.5 M KCl; (iii) TKMD buffer, 0.02 M tris(hydroxymethyl)aminomethane hydrochloride–0.05 M KCl–0.005 M $MgCl_2$–0.001 M dithiothreitol , pH 7.4 at 25°C; and (iv) TKM buffer, 0.025 M tris(hydroxymethyl)aminomethane acetate–0.06 M potassium acetate–0.01 M magnesium acetate, pH 8.0 at 25°C.

Cell growth and spheroplast preparation. Cells were grown overnight to a density of approximately 10^7 cells per ml, collected by centrifugation, and converted to spheroplasts with glusulase (Endo Laboratories, Garden City, N.Y.) (16). The cells recovered for 2.5 h in YM-5 medium containing 0.5 M $MgSO_4$ before cycloheximide (Sigma Chemical Co., St. Louis, Mo.) addition and collection by centrifugation. The cell pellets were either used immediately or rapidly frozen to −70°C.

Radioactive labeling of ribosomal proteins. Cells grown as above in low-phosphate medium were converted to spheroplasts and diluted into low-phosphate YM-5 medium containing 0.5 M $MgSO_4$ (30). Fifty microcuries of carrier-free [^{32}P]phosphoric acid (New England Nuclear Corp., Boston, Mass.) was added per milliliter of culture, and spheroplasts were allowed to recover for 2.5 h before cycloheximide (10 μg/ml of culture) was added. Cells were collected after 10 min at room temperature, and the cell pellets were frozen.

Cells were also grown in SCY medium, containing 2.5 μCi of a ^{14}C-labeled L-amino acid mixture (New England Nuclear Corp.) per ml, to early stationary growth phase (5×10^7 cells per ml). This culture was diluted with 10 volumes of YM-1 medium and grown for one generation before collection, spheroplasting, and recovery as described above.

Lysate preparation and analysis. Spheroplast pellets containing about 3×10^7 cells were lysed at 0°C

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in L buffer (12), the cell debris was removed by centrifugation (17,000 × *g*, 5 min), and the supernatant was layered on appropriate sucrose gradients.

Lysates were fractionated on 37-ml linear gradients of 10 to 45% (wt/vol) sucrose containing either L or H buffer by centrifugation at 27,000 rpm at 3°C for 6.5 h in a Beckman SW27 rotor. The gradients were collected, and the ribonucleoprotein particles from appropriate fractions were concentrated by ethanol precipitation (21) and suspended in TKMD buffer.

Sedimentation analysis of subunits. Subunits derived from polyribosomes were prepared by incubation for 15 min at 30°C with 1 mM puromycin (34) and sedimentation through 10 to 25% (wt/vol) sucrose gradients containing TKMD buffer made 0.5 M in KCl. Gradients used for the preparation of subunits for the analysis presented in Fig. 5 contained 37.5 ml and were centrifuged at 22,000 rpm for 12 h at 15°C in a Beckman SW27 rotor.

To analyze the subunits derived from halfmer polyribosomes, we found it necessary to subject these structures to partial ribonuclease (RNase) digestion. One microliter of a 0.1-mg/ml solution of pancreatic RNase A was added to 0.2 ml of L buffer at 0°C for each unit equivalent of absorbance at 260 nm of halfmer or disomes and trisomes to be analyzed. This mixture was immediately layered on a 12-ml analytical gradient containing L buffer and centrifuged at 41,000 rpm at 10°C for 3.5 h in a Beckman SW41 rotor.

Gel electrophoresis. Ribonucleoprotein particles were electrophoresed into slab gels containing 2.5% acrylamide and 0.5% agarose, using a vertical gel apparatus (E-C Apparatus Corp., St. Petersburg, Fla.) as described previously (5, 6) except that acetate was substituted for chloride in the TKM buffer used, and electrophoresis was at 100 V and 10°C for 6 to 8 h with buffer changes at 2- to 3-h intervals. The samples were applied to the empty slots after mixing them with equal volumes of 0.5% agarose containing TKM buffer at 50°C. The gel was stained with Stains-All (Eastman Organic Chemicals, Rochester, N.Y.) as described previously (5).

Ribosomal protein extraction. Proteins were extracted from subunits and polyribosomes in 67% acetic acid–0.2 M $MgCl_2$ (10, 18) and precipitated by 5 volumes of acetone at −20°C (3). Insoluble material was resuspended in 7 M urea–0.01 M bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane acetate (pH 4.0)–1 mM dithiothreitol and clarified by centrifugation before analysis by two-dimensional gel electrophoresis. Protein precipitates to be analyzed by one-dimensional sodium dodecyl sulfate-gel electrophoresis were prepared as described (23).

Electrophoresis of ribosomal proteins. Protein samples were analyzed by one-dimensional sodium dodecyl sulfate-gel electrophoresis as described by Laemmli (23) or by two-dimensional gel electrophoresis with a technique developed by Tokimatsu (H. Tokimatsu and A. E. Dahlberg, manuscript in preparation) which combines two currently used methods: the first-dimension procedure of Mets and Bogorad (28) and the second-dimension slab gel of Kaltschmidt and Wittmann (17, 18). The first-dimension procedure (28) was modified as follows. The cylinder gels (2 mm by 11 cm) were overlaid during polymerization with 6 M urea–0.01 M bis(2-hydroxyethyl)imino-tris-(hydroxymethyl)methane acetate, pH 4.0. After 45 min at room temperature this overlay was replaced with the buffer above containing 1% 2-mercaptoethanol, and polymerization was allowed to continue for 1 h. Gels were electrophoretically prerun for 45 min before sample application and electrophoresed at no more than 2 mA per gel. These modifications facilitated the reproducible entry of certain acidic proteins into the gel. Modifications of the second-dimension slab gel (15, 17) included (i) forming a slot for the first-dimension gel in the second-dimension slab in a vertical gel apparatus (E-C Apparatus Corp.); (ii) holding the first-dimension gel in place in the slot [filled with 6 M urea–0.057 M bis(2-hydroxyethyl)imino-tris-(hydroxymethyl)methane acetate, pH 5.0] with fiber glass screening, eliminating the need for a slot gel solution; and (iii) electrophoresing at 19°C and 35 mA per gel with a constant-current power supply. *N,N'*-Diallyltartardiamide, 3.7 g/liter, was substituted for 2.5 g of *N,N'*-methylenebisacrylamide per liter in the second-dimension gel when the gel was to be dried for autoradiography or cut and solubilized for scintillation counting (1).

RESULTS

Cycloheximide effects on polyribosomes. The effects of the inhibition of protein synthesis by cycloheximide on polyribosome structure were examined. Spheroplasts of *S. cerevisiae* were incubated with different concentrations of the antibiotic before lysis and analysis by sucrose gradient centrifugation. Polyribosomes were analyzed in gradients containing 0.5 M KCl, which dissociated "runoff" ribosomes into ribosomal subunits, allowing one to detect monosomes actually engaged in protein synthesis (26). Spheroplasts not treated with cycloheximide (Fig. 1A) had large ribosomal subunit peaks and only small peaks of polyribosomes. Spheroplasts incubated with a high concentration of cycloheximide (100 μg/ml) (Fig. 1C) had fewer runoff ribosomes and a substantial increase in larger-size polyribosomes, consistent with the reported inhibition of elongation at this concentration (8, 32). Spheroplasts incubated with a low concentration of cycloheximide (10 μg/ml) had discrete peaks sedimenting between each pair of polyribosome peaks (Fig. 1B, arrows; also present, but less apparent, in Fig. 1C). These intermediate peaks, called halfmers (14, 19, 20), were detectable in some gradients through the pentasome region. Optimum conditions for production of halfmers were incubation of spheroplasts in cycloheximide at 10 μg/ml for 10 min or longer at 22°C. Lower (5-μg/ml) and higher (25- to 100-μg/ml) concentrations (Fig. 1C) reduced the yield of halfmers. Growth in a rich medium with slow shaking was also necessary.

The composition of the halfmers was sug-

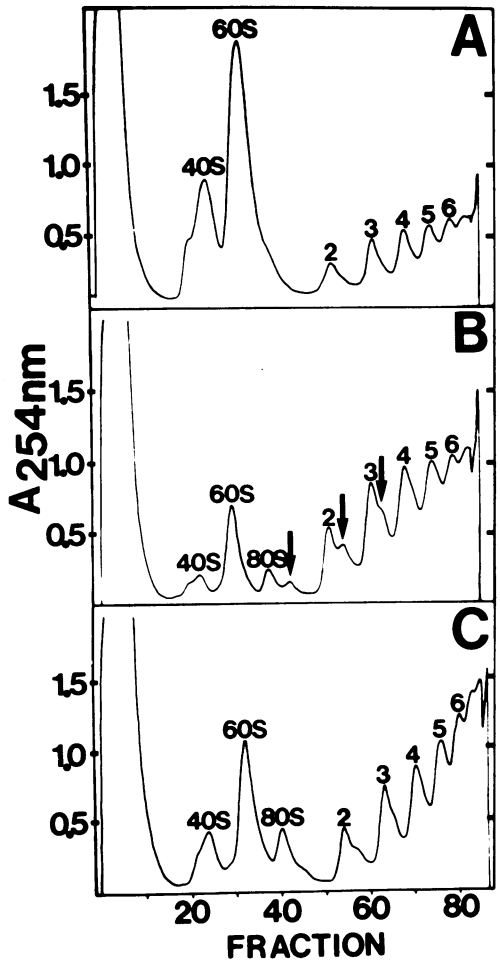

FIG. 1. *Accumulation of halfmer polyribosomes. Spheroplast cultures, prepared as described in the text, were collected at room temperature; these were either incubated without cycloheximide (A) or incubated for 10 min at room temperature with 10 (B) or 100 (C) μg of cycloheximide per ml before collection and lysis. The lysates were analyzed on 10 to 45% sucrose gradients containing H buffer as described in the text. Halfmer polyribosomes are indicated by arrows in (B). A_{254nm}, Absorbance at 254 nm.*

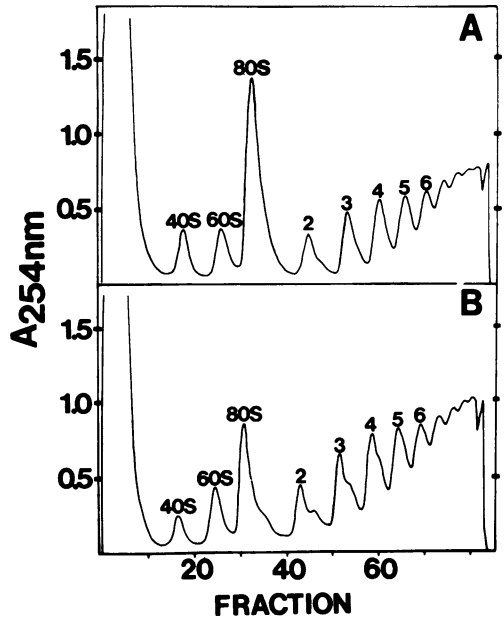

FIG. 2. *Decrease in free 40S ribosomal subunit pool associated with halfmer accumulation. Spheroplast cultures were prepared as described in the text and either collected by pouring directly onto 1 M sorbitol ice containing cycloheximide at a final concentration of 10 μg/ml before centrifugation (A) or incubated with cycloheximide (10 μg/ml) at 22°C for 10 min before collection (B). Lysates were analyzed on 10 to 45% sucrose gradients in L buffer centrifuged for 5 h at 27,000 rpm as described in the text. A_{254nm}, Absorbance at 254 nm.*

gested by the difference in free 40S ribosomal subunit pools in cycloheximide-treated and untreated spheroplasts. Polyribosomes were analyzed in sucrose gradients containing only 0.1 M KCl to prevent dissociation of runoff ribosomes. Lysates were prepared from spheroplasts collected at 0°C in the presence of cycloheximide (10 μg/ml) (Fig. 2A) or incubated in cycloheximide (10 μg/ml) for 10 min at 22°C (Fig. 2B) before collection. The results showed that the appearance of halfmers coincides with a reduction in the pool of free 40S subunits, suggesting that 40S subunits became a part of the halfmers.

Halfmers contain an extra 40S subunit. Halfmers were treated by limited RNase digestion, and the products were analyzed on a sucrose gradient. The results showed the release of a particle (Fig. 3A, arrow) sedimenting at a rate comparable to that for 40S ribosomal subunits derived from runoff ribosomes analyzed in a parallel gradient (Fig. 3C). RNase treatment of disome and trisome samples did not produce this particle (Fig. 3B).

In a similar experiment we took advantage of the excellent separation of halfmers from regular polyribosomes by electrophoresis in agarose-acrylamide composite gels as described previously for bacterial polyribosomes (5, 6). A halfmer sample was treated briefly with RNase and electrophoresed along with an untreated sample (control) into a composite gel. The untreated sample (Fig. 4, slot 1) contained monosomes (80S), disomes (2x), and trisomes (2x), as well as halfmers of sizes "one-and-one-halfmer" (1H) and "two-and-one-halfmer" (2H). Limited diges-

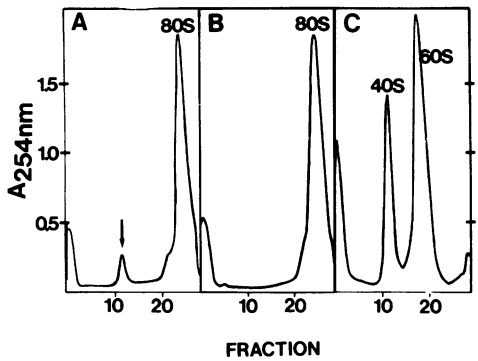

FIG. 3. *Sedimentation analysis of RNase-cleaved halfmer. Two-and-one-halfmer and three-and-one-halfmer polyribosome sample (A) and a disome and trisome sample (B) were treated with RNase, and products were analyzed by sucrose gradient sedimentation as described in the text. The 40S-size particle released by this treatment only from the halfmer sample is indicated by the arrow. Runoff ribosomes were dissociated into ribosomal subunits in a parallel gradient (C) as described in the text.* A_{254nm}, *Absorbance at 254 nm.*

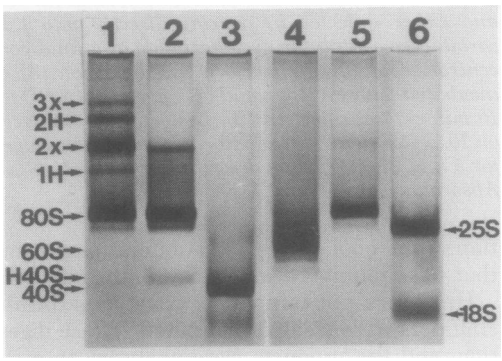

FIG. 4. *Composite gel electrophoretic analysis of halfmers and RNase-treated halfmers. A sample from the two-and-one-halfmer region of a sucrose gradient was electrophoresed into a composite gel along with a similar sample treated with RNase and marker ribosomes and ribosomal RNA as described in the text. Slots: 1, 0.5 unit of absorbance at 260 nm of the untreated halfmer; 2, the same sample to which 0.05 μg of pancreatic RNase A was added at 0°C immediately before application; 3 and 4, 0.2 unit of absorbance at 260 nm of derived 40S and 60S ribosomal subunits, respectively; 5, 0.2 unit of absorbance at 260 nm of monosome; 6, the same sample to which sodium dodecyl sulfate (2%) had been added to release 25S and 18S ribosomal RNA. Stained bands are designated 80S (monosome), 2x (disome), 3x (trisome), 1H (one-and-one-halfmer), 2H (two-and-one-halfmer), 40S and 60S (ribosomal subunits), H40S (the particle released from halfmer by RNase), and 25S and 18S (yeast ribosomal RNAs).*

tion by RNase converted all halfmers and all but a few disomes into monosomes (Fig. 4, slot 2). An additional new band (H40S) was also noted migrating only slightly slower than marker 40S subunits of slot 3. The mobility of this new band differed from those of 60S subunits (slot 4) and 25S and 18S ribosomal RNAs (slot 6). The results indicated that a 40S subunit attached to the halfmer's messenger RNA was released by RNase cleavage. Its slightly slower mobility than that of the derived 40S ribosomal subunits could indicate a more unfolded structure, possibly due to RNase cleavage of the ribosomal RNA or differences in protein composition.

Protein composition of halfmers. The protein composition of the halfmer-associated 40S ribosomal subunit was examined. Cells were labeled with ^{14}C-amino acids as described in Materials and Methods. The 40S ribosomal subunits released from RNase-treated halfmers were isolated as described in the legend to Fig. 3. Proteins were extracted along with carrier proteins from polyribosome-derived 40S ribosomal subunits and electrophoresed in two-dimensional acrylamide gels. The stained gel (Fig. 5, left) shows the ribosomal protein pattern obtained for the carrier 40S ribosomal subunit proteins. An autoradiogram of this dried gel (Fig. 5, right panel) shows the pattern of ^{14}C proteins from the halfmer-associated 40S subunit. The latter pattern is consistent with proteins of 40S ribosomal subunits. The same two ribosomal proteins (Fig. 5, A and B) were phosphorylated in the halfmer-associated 40S subunit and in 40S ribosomal subunits derived from polyribosomes of cells labeled with [^{32}P]orthophosphate as described in Materials and Methods (data not shown).

Several proteins were partially or completely dissociated from both the halfmer and its associated 40S subunit during RNase treatment. They were recovered by acetone precipitation of fractions from the top of a sucrose gradient similar to that shown in Fig. 3A. The locations of these proteins in an autoradiogram of a two-dimensional gel are indicated by letters C, D, and E in Fig. 5 (right) rather than showing the actual autoradiogram. Protein D was the only ribosomal protein. It was partially released from the ribosomal subunit, as evidenced by its appearance in the autoradiogram of Fig. 5 (right). The proteins indicated by C and E were also found on nascent 40S ribosomal subunits as well as on the halfmer, but not on polyribosomes (data not shown).

Halfmer-associated proteins were further analyzed by sodium dodecyl sulfate-gel electrophoresis of a one-and-one-halfmer sample (not

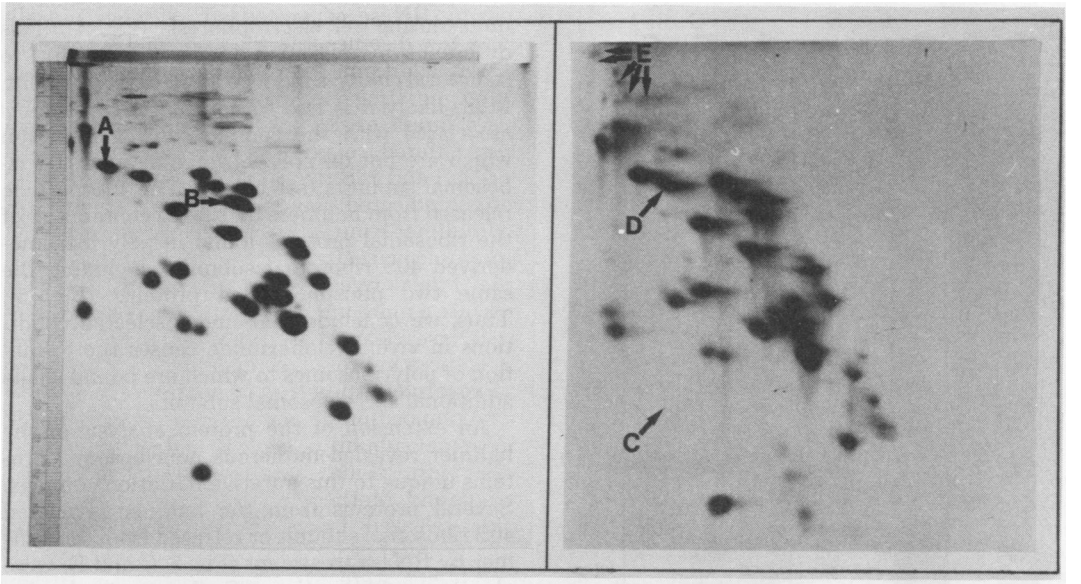

FIG. 5. *Protein composition of the 40S particle derived from halfmers. RNase-released 40S particles were prepared as described in the legend to Fig. 3A, using halfmers labeled in vivo with ^{14}C-amino acids. Nonradioactive 40S subunits were added as carrier, and the proteins were extracted and electrophoresed in a two-dimensional acrylamide gel as described in the text. Protein migration was from left to right (first dimension) and top to bottom (second dimension). The stained gel (left) shows carrier proteins of the polyribosome-derived 40S particle. An autoradiogram of the gel (right) shows the protein composition of the halfmer-associated 40S subunit derived by RNase treatment. For a discussion of proteins indicated by letters, see text.*

RNase treated). Figure 6 shows the separation of multiple stained bands on high-molecular-weight proteins from the halfmer sample (arrows, slot 3) which were not present in polyribosome samples (slots 1 and 6) or on 80S monosomes (slot 5). Four of the halfmer-associated proteins did correspond in mobility to proteins on native 40S ribosomal subunits (arrows, slot 4), and two halfmer-associated proteins (labeled I in slot 3) were also present on polyribosomes (slots 1 and 6), but not detectable on runoff 80S monosomes (slot 5). Finally, two halfmer-associated proteins (labeled II in slot 3) had the same mobilities as two of the three subunits of initiation factor eIF_2 in yeast (slot 2) (R. A. Baan, P. Keller, and A. E. Dahlberg, J. Biol. Chem., in press).

DISCUSSION

Here we show that under rather specific conditions of cell growth and cycloheximide treatment in vivo, a unique type of polyribosome is produced in yeast. This structure, termed halfmer, has also been observed in vitro in the reticulocyte translation system with pactamycin (9, 19, 20) or sodium fluoride (14) treatment and in vivo in Ehrlich ascites cells treated with anisomycin (33). The halfmer is a polyribosome (or monosome) to which is attached an additional 40S ribosomal subunit, presumably in the form of an initiation complex. In this paper we have extended the studies on the halfmer by isolating the 40S ribosomal subunit and characterizing the associated nonribosomal protein in an attempt to identify translation factors bound to the complex.

Our evidence that cycloheximide causes the production of halfmers in yeast includes the separation of halfmers from normal polyribosomes in sucrose density gradients (Fig. 1 and 2) and even more distinctly in agarose-acrylamide gels (Fig. 4). Halfmers are produced at low concentrations of cycloheximide, which reportedly inhibit initiation (2, 4, 9, 24, 29). The inhibition is probably in the "joining reaction" of 60S to 40S ribosomal subunits on messenger RNA, as reported for pactamycin (19, 20) and anisomycin (33). Higher concentrations of cycloheximide (100 μg/ml), which primarily block translocation and produce fewer halfmers (Fig. 1C), have actually been used to prevent formation of halfmers by anisomycin (33). The cycloheximide-dependent formation of halfmers coincides with an expected decrease in free 40S ribosomal sub-

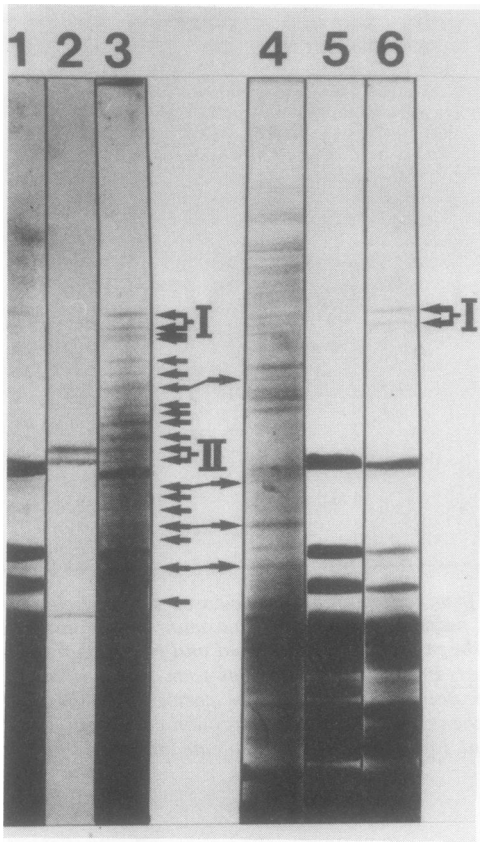

FIG. 6. *Nonribosomal proteins associated with halfmers. Protein samples from native 40S subunits, 80S monoribosomes, one-and-one-halfmers, and disomes isolated from preparative sucrose gradients containing L buffer were electrophoresed into a sodium dodecyl sulfate-protein gel to separate and identify large-molecular-weight proteins associated with each fraction. Protein bands were visualized by staining as described in the text. Slots: 1 and 6, 60 mg of polyribosomes; 2, 2 mg of yeast initiation factor eIF_2; 3, 30 mg of one-and-one-halfmer; 4, 30 mg of nascent 40S ribosomal subunits; 5, 50 mg of 80S monosomes. Arrows indicate nonribosomal proteins associated with halfmers. For a discussion of proteins indicated by roman numerals and arrows, see text.*

units (Fig. 2). More direct evidence that an additional 40S ribosomal subunit is associated with halfmers is seen in the experiments involving limited RNase treatment. Conditions which cleave messenger RNA linking separate 80S ribosomes in polyribosomes release ribonucleoprotein particles not found in normal disomes and trisomes (Fig. 3) which cosediment with derived 40S ribosomal subunits. The RNase-released particles also migrate just slightly slower than polyribosome-derived 40S ribosomal subunits during gel electrophoresis (Fig. 4). This difference in migration rate could be due to structural changes induced by RNase treatment. More likely it is due to nonribosomal proteins associated with the complex during initiation which are not on polyribosome-derived 40S ribosomal subunits (see below). The 40S particle released from halfmers by RNase contains all of the ribosomal proteins found in polyribosome-derived 40S ribosomal subunits, including the same two phosphorylated proteins (Fig. 5). Thus, we conclude that under selected conditions in vivo, cycloheximide causes the formation of polyribosomes to which are bound single additional 40S ribosomal subunits.

An extension of the protein analysis of the halfmer revealed numerous nonribosomal proteins unique to this putative initiation complex. Several proteins from the halfmer-associated 40S ribosomal subunit or released from the halfmer by RNase treatment (Fig. 5, C and E) were also present on native 40S ribosomal subunits, but were not found on polyribosomes. A more direct comparison of proteins, achieved by sodium dodecyl sulfate-gel electrophoresis, showed that the intact halfmer contained a large number of high-molecular-weight proteins not present on normal polyribosomes or 80S monosomes (Fig. 6). Again, a few of these same proteins were associated with native 40S ribosomal subunits, but they have not been characterized further. Two of the proteins (Fig. 6, I) were also on polyribosomes, but not on runoff 80S monosomes, and may be involved in the elongation step of translation. Finally, two other proteins associated with the halfmer (Fig. 6, II) correspond in mobility to two of the three subunits of eIF_2 recently isolated in our laboratory (Baan et al., in press). This observation is consistent with the established sequence of events for initiation, which involves the binding of eIF_2 and eIF_3 to the 40S ribosomal subunit and messenger RNA before the 60S ribosomal subunit joining reaction (for review, see reference 31). Further isolation and characterization of these translation factors and their identification on halfmers is now in progress.

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