and influence the regioselectivity of attack on a given substrate.

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Registry No. **1**, 108-86-1; **1**, *o*-OH, 95-56-7; **1**, *m*-OH, 591-20-8; **1**, *p*-OH, 106-41-2; **2**, 98-95-3; **2**, *o*-OH, 88-75-5; **2**, *m*-OH, 554-84-7; **2**, *p*-OH, 100-02-7; **3**, 100-47-0; **3**, *o*-OH, 611-20-1; **3**, *m*-OH, 873-62-1; **3**, *p*-OH, 767-00-0; **4**, 100-66-3; **4**, *o*-OH, 90-05-1; **4**, *m*-OH, 150-19-6; **4**, *p*-OH, 150-76-5; **5**, 108-88-3; **5**, *o*-OH, 95-48-7; **5**, *m*-OH, 108-39-4; **5**, *p*-OH, 106-44-5; **6**, 92-52-4; **6**, *o*-OH, 90-43-7; **6**, *m*-OH, 580-51-8; **6**, *p*-OH, 92-69-3; phenol, 108-95-2; benzyl alcohol, 100-51-6; 3,5-$D_2C_6H_3Br$, 7291-07-8; 2,4,6-$D_3C_6H_2Br$, 13122-41-3; 4-DC_6H_4Br, 13122-33-3; 3,5-$D_2C_6H_3NO_2$, 89908-09-8; 2,4,6-$D_3C_6H_2NO_2$, 28147-77-5; 3,5-$D_2C_6H_3CN$, 37960-86-4; 2,4,6-$D_3C_6H_2CN$, 13137-55-8; 3,5-$D_2C_6H_3OCH_3$, 55415-90-2; 2,4,6-$D_3C_6H_2OCH_3$, 2567-25-1; 3,5-$D_2C_6H_3CH_3$, 16954-38-4; 2,4,6-$D_3C_6H_2CH_3$, 13122-37-7; 3,5-$D_2C_6H_3C_6H_3D_2$-3,5, 6120-98-5; 2,4,6-$D_3C_6H_2C_6H_2D_3$-2,4,6, 6081-66-9.

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Monoclonal Antibody Specific for Yeast Elongation Factor 3†

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ABSTRACT: Hybridomas have been prepared by fusing mouse myeloma (P3 × 63 Ag8) cells with spleen cells of mice immunized with a yeast fraction enriched with respect to nonribosomal translational components. Cloned hybridoma lines were grown in the form of ascites tumors, and the monoclonal antibodies produced were purified from the ascites fluid by chromatography on DEAE-Affi-Gel Blue. One of the antibodies, from a hybridoma cell line designated as PSH-1, inhibited the translation of natural mRNA and poly(U) and polysomal chain elongation in a cell-free protein-synthesizing system from yeast. Resolution and partial purification of the elongation factors indicated that the monoclonal antibody from PSH-1 did not interact with EF-1 or EF-2 but reacted with and inactivated EF-3, the 125 000 molecular weight additional elongation factor specifically required with yeast ribosomes. The EF-3 purified from the cytosol by immunoaffinity chromatography was comparable to that prepared by ion-exchange chromatography. Evidence was obtained which indicated that EF-3 was essential for the translation of natural mRNA as well as poly(U), was associated with polysomes but not ribosomal subunits, and was required for every cycle in the elongation phase of protein synthesis.

Immunological techniques have provided important tools for the biological characterization of components involved in protein synthesis and the reactions that they carry out. Specific polyclonal antisera to prokaryotic and eukaryotic protein synthesis components, such as ribosomes and translational factors, have been prepared (Howe et al., 1978; Petryshyn et al., 1979; Khanh et al., 1979; Van Duin et al., 1979; Lelong et al., 1979; Zinker, 1980; Politz & Glitz, 1980; Ghosh-Dastidar et al., 1980; Tanaka et al., 1980; Stoffler et al., 1980; Kahan et al., 1981; Fahnestock et al., 1981; Van Duin & Wijnands, 1981; Kastner et al., 1981; Brown-Luedi et al., 1982; Meyer et al., 1982; Howe & Hershey, 1982; Olson et al., 1982). One of the problems inherent in the use of polyclonal antibodies has been the requirement for pure specific proteins for immunization, which in many cases are available only in

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limited quantities, require laborious purification procedures, and may exhibit a low degree of immunogenicity. One of the advantages of the monoclonal antibody techniques (Kohler & Milstein, 1975) is the ability to immunize with only partially purified proteins, or with a mixture of proteins, and then to select for the specific antibody of interest by cell cloning. The development of the monoclonal antibody procedure has made it possible to produce highly specific antibodies that can be used as immunochemical probes in protein synthesis. For example, monoclonal antibodies have been used for the characterization of prokaryotic and eukaryotic ribosomal proteins (Shen et al., 1980; Kalthoff et al., 1982; Towbin et al., 1982) and in the analysis of the eukaryotic cap-binding protein (Sonenberg et al., 1981). Recently, a number of monoclonal antibodies to various nonribosomal translational components from yeast have been obtained in this laboratory; this report describes one of these monoclonal antibodies, which reacts with the yeast-specific elongation factor EF-3, and its effect on in vitro protein synthesis.

Experimental Procedures

Immunization and Production of Hybridomas. Balb/c female mice were immunized with four intraperitoneal injections (10 mg of protein for each) of a ribosomal high-salt extract over a 3-month period. The extract was prepared by centrifuging yeast postpolysomal fraction (Gasior et al., 1979; Feinberg et al., 1982) at 250000*g* for 4 h, resuspending the sedimented ribosomes and ribosomal subunits in a solution containing 0.5 M KCl, 20 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (Hepes) (pH 7.4), 2 mM $Mg(OAc)_2$, 2 mM dithiothreitol, and 20% glycerol, and, after 30 min at 2 °C, centrifuging at 250000*g* for 4 h to remove the particles; the extract was dialyzed against 50 mM sodium phosphate buffer (pH 7.4)–0.15 M NaCl for immunization. Single cell suspensions prepared from spleens of immunized mice by repeated drawing and ejection in 5 mL of Eagle's minimal essential medium/α modified (MEM/α; KC Biological, Inc.) through a 25-gauge needle were washed 3 times in Eagle's MEM/α by low-speed centrifugation (200*g*, 5 min), and 10^8 cells were fused to 10^7 P3 × 63 Ag8 myeloma cells (Kohler & Milstein, 1975) by using poly(ethylene glycol). The mixed cell suspension was centrifuged at 600*g* for 7 min, 0.8 mL of warm (37 °C) 30% poly(ethylene glycol) (BDH, M_r 1000) was added to the pellet dropwise over a 1-min period, and the resuspended cells were incubated at 37 °C for an additional minute. The poly(ethylene glycol) was diluted by the addition of 10 mL of Eagle's MEM/α, over a 2-min period; the suspension was centrifuged at 600*g* for 3 min and maintained at 37 °C for 3 min prior to removal of the supernatant by decantation. The cells were gently resuspended in 20 mL of Eagle's MEM/α containing 10% fetal calf serum and 8% horse serum, and 0.2-mL aliquots were incubated in individual wells in a multiwell microculture plate. The cells were maintained for 2 weeks in HAT medium (100 μM hypoxanthine, 10 μM aminopterin, and 30 μM thymidine in Eagle's MEM/α–10% fetal calf serum–8% horse serum), with frequent changes, and then for 1 week in the same media but without aminopterin.

Hybridoma culture fluids were assayed for antibody activity to the high-salt ribosomal extract with a solid-phase radioimmunoassay as described below, and those cultures which showed a positive reaction were cloned by limited dilution, without feeder cells, in microculture plates that had been preincubated for 24–48 h with serum-containing media in order to condition the surface. The cloned cultures were tested for antibody activity, the positive ones were expanded, and cell stocks were stored frozen in Eagle's basal medium–Hank's balanced salt solution–15% dimethyl sulfoxide in liquid nitrogen.

Purification of Monoclonal Antibodies. Approximately 10^7 cloned hybridoma cells were injected intraperitoneally into Balb/c mice to produce ascites tumors, and after 12–21 days, the tumors were removed and centrifuged at 10000*g* for 10 min, and the supernatant fluids were dialyzed overnight at 2 °C against 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 8.0 at 20 °C)–28 mM NaCl. All subsequent steps were carried out at 2 °C. One milliliter of ascites fluid was applied to the top of the 0.8 × 15 cm (7.5-mL bed volume) column of DEAE-Affi-Gel Blue (Cibacron Blue F3GA–diethylaminoethylagarose, Bio-Rad Labs), washed with 1 bed volume of the 20 mM Tris–28 mM NaCl solution, and eluted with 20 mM Tris-HCl (pH 8.0) containing 84 mM NaCl. Two-milliliter fractions were collected and tested for UV-adsorbing material (at 280 nm) and for antibody activity against the high-salt ribosomal extract as measured with the solid-phase radioimmunoassay. With several hybridoma products, a sharp protein peak containing the antibody was recovered from the columns between 60 and 80 mM NaCl. Analyses for protease activity in the eluates, using a protease substrate gel (Bio-Rad Labs), were negative.

Immunological Assay Procedures. Solid-phase radioimmunoassays (Tsu & Herzenberg, 1980) were carried out by coating individual wells in a poly(vinyl chloride) microtiter plate with 50 μL of high-salt ribosomal extract containing 0.1 mg of protein/mL in PBS (50 mM sodium phosphate buffer, pH 7.4, and 0.15 M NaCl) or various fractions obtained in the course of purification of translational factors. After 1 h at ambient temperature, the wells were washed 3 times with PBS containing 0.1% bovine serum albumin (BSA), 50 μL of expended hybridoma culture media (hybridoma supernatant) or DEAE-Affi-Gel Blue purified monoclonal antibody was added, and the plates were incubated for 1 h at ambient temperature. The wells were than washed 3 times with PBS–BSA and incubated for another hour with 20 μL of a 1:10 dilution of ^{125}I-labeled goat anti-mouse Ig's (8–10 μCi/mg, 100 μCi/mL; New England Nuclear). The solution was then carefully removed by aspiration, the wells were washed 3 times (by aspiration) with 250 μL of PBS–BSA, and the individual wells were cut and analyzed for radioactivity in a scintillation counter.

An enzyme-linked immunosorbant assay was used for antibody typing (Engvall et al., 1971). Wells of poly(vinyl chloride) microtiter plates coated with the high-salt ribosomal extract were incubated with 250 μL of PBS–BSA solution for 3 h at ambient temperature and then with 50 μL of supernatant from individual hybridomas or DEAE-Affi-Gel Blue purified monoclonal antibody for 2 h and washed 3 times with PBS–BSA. Individual wells received 50 μL of class-specific (rabbit, anti-mouse, λ, γ_1, γ_{2a}, γ_{2b}, γ_3, or μ) antibodies (Boehringer Mannheim), and after 2 h at ambient temperature, the plates were washed with PBS–BSA, incubated for 2 h with 50 μL of peroxidase-coupled goat anti-rabbit IgG, washed, and incubated with 50 μL of a solution containing 1 mM 2,2'-azinobis(3-ethylbenzothiazolidinesulfonate) and 0.03% H_2O_2. Positive wells were indicated by the development of green color between 20 and 40 min.

Immunoaffinity Chromatography. Monoclonal antibody purified on DEAE-Affi-Gel Blue columns was coupled to Affi-Gel 10 (Bio-Rad Labs). One milliliter of the gel, containing 1 mg of coupled IgG, was equilibrated at 4 °C with a 10 mM sodium phosphate (pH 7.4)–0.15 M NaCl solution

and used to prepare a 0.7 × 1.5 cm column in a 0.7 × 10 cm glass tube. All subsequent steps were also carried out at 4 °C. About 2.5 mg of protein of a high-salt (0.5 M KCl) cytosol (HS-cytosol) extract (Feinberg et al., 1982), dialyzed against the sodium phosphate–saline solution described above, was passed through the IgG–Affi-Gel 10 column 5 times; the gel was resuspended gently in the cytosol solution each time before the column was allowed to drain. The gel was then washed with 25 mL of the phosphate–saline solution containing 0.1% Triton X-100 followed by 25 mL of the phosphate–saline solution without Triton X-100. The bound protein was eluted with 0.2 M glycine (pH 2.3)–0.15 M NaCl and immediately adjusted to pH 7.0 with KOH.

In Vitro Protein Synthesis. The translation of exogenous natural mRNA and poly(U), in nuclease-treated yeast postpolysomal extracts, was carried out as described (Gasior et al., 1979; Feinberg et al., 1982). When the effects of antibodies on protein synthesis were tested, the monoclonal preparations obtained from the DEAE-Affi-Gel Blue columns were dialyzed against a solution containing 20 mM Hepes–KOH buffer (pH 7.4), 100 mM KOAc, 2 mM $Mg(OAc)_2$, 2 mM dithiothreitol, and 20% glycerol and then preincubated with the yeast postpolysomal extract for 15–20 min at 20 °C prior to the assay for protein synthesis. The mRNA-dependent incorporation of [^{3}H]leucine and the poly(U)-dependent incorporation of [^{3}H]phenylalanine into protein were used to measure protein synthesis. In some of the experiments on poly(U) translation, [^{3}H]Phe-tRNA was incubated with yeast cytosol and yeast or rat liver ribosomes, instead of yeast postpolysomal extract.

Phenylalanyl-tRNA synthetase activity was determined by incubating yeast cytosol, [^{3}H]phenylalanine, and ATP in buffered salts–dithiothreitol as described (Feinberg et al., 1982). When the effect of monoclonal antibody on this activity was examined, the cytosol was preincubated with the antibody for 20 min at 20 °C prior to the assay for Phe-tRNA synthesis.

Protein Transfer and Immunoblotting. Polypeptides were resolved by one-dimensional sodium dodecyl sulfate (SDS) gel electrophoresis (Laemmli, 1970) using 1.5-mm slab gels consisting of a 10% acrylamide–2.7% bis(acrylamide) resolving gel and a 4% acrylamide–2.7% bis(acrylamide) stacking gel. Proteins were transferred from polyacrylamide gels to hybridization membranes (Gene Screen, New England Nuclear) essentially as described by Towbin et al. (1979), but with 0.1% SDS in the transfer buffer and electrophoresis (3 V/cm for 12 h) at 30 °C. After electrophoretic transfer, the blots were incubated as follows, at ambient temperature with constant agitation: (a) 1 h with 10 mM Tris-HCl buffer (pH 7.4)–0.15 M NaCl–5% BSA; (b) 2 h with the Tris–saline–BSA buffer containing 1 μg/mL DEAE-Affi-Gel Blue purified monoclonal antibody; (c) 10 min with Tris–saline–BSA; (d) twice for 10 min with Tris–saline–BSA containing 0.05% Nonidet P-40; and (e) 10 min with Tris–saline–BSA. To detect the transfer membrane bound antibody, the blot was incubated for 45 min with a 1:200 dilution of ^{125}I-labeled goat anti-mouse IgG's in Tris–saline–BSA, washed once with Tris–saline–BSA, twice with Tris–saline–BSA–Nonidet, and once with Tris–saline–BSA as described above, and then subjected to autoradiography with Kodak X-Omat film.

Results

The cell-free protein-synthesizing system from yeast cells was markedly inhibited by serum from control and immunized mice, and by supernatant from cultures of P3 × 63 myeloma cells or from hybridomas prepared from control or immunized mice. Therefore, in order to test the effects of the antibodies on in vitro protein synthesis, it was necessary to purify them prior to use in the protein-synthesis assays. Antibodies were purified, on DEAE-Affi-Gel Blue columns, from the fluid of hybridomas grown in the form of ascites tumors since the concentration of monoclonal antibodies in ascites fluid was typically 3–15 mg/mL, compared to 10–100 μg/mL in cell culture media. The effects of several purified IgG preparations, obtained from various cell lines, on the in vitro translation of mRNA were examined by incubation yeast postpolysomal extracts with [^{3}H]leucine, yeast polysomal RNA, other components required for protein synthesis, and varying concentrations of monoclonal antibodies. As seen in Figure 1, the addition of relatively small amounts of IgG from two hybridomas, designated as PSH-1 (circles) and PSH-2 (triangles), inhibited the translation of mRNA. Inhibition was dependent on the concentration of antibody, and over 80% inhibition was obtained with about 10 μg of PSH-1 IgG per incubation. A similar preparation obtained from a control myeloma cell line, P3 × 63 (squares), used to construct these hybrids, had no effect on protein synthesis.

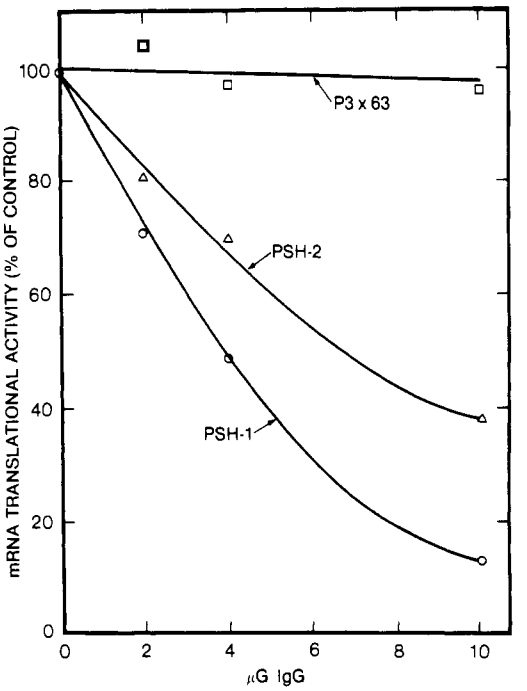

FIGURE 1: Effect of various antibodies on the translation of natural mRNA in nuclease-treated postpolysomal yeast extracts. Approximately 0.15 mg of extract protein was incubated for 20 min at 20 °C alone (100% value) or in the presence of varying concentrations of purified antibody from control cell line P3 × 63 (□), hybridoma cell line PSH-1 (○), or hybridoma PSH-2 (△). The rest of the components required for protein synthesis, including [^{3}H]leucine (2.0 μM final concentration, 59.8 Ci/mmol) and 20 μg of yeast polysomal RNA, were added, and the incubations, in a total volume of 0.05 mL, were continued for an additional 90 min at 20 °C. At the end of the incubation, the hot (90 °C) 5% trichloroacetic acid insoluble fractions were prepared, collected on glass-fiber filters, washed, dried, and counted. The control, 100% value, was 325 000 cpm incorporated into protein.

Translation of natural mRNA requires the initiation, elongation, and termination sequence of reactions. To determine whether the inhibition obtained with these antibodies was due to an effect on chain elongation, the IgG preparations were added to incubations containing yeast extract, [^{3}H]-phenylalanine, poly(U), and the other protein-synthesis components. Figure 2A shows that the addition of IgG from the control cell line P3 × 63 (squares) or from the hybridoma line PSH-2 (triangles) had no effect on the poly(U)-dependent incorporation of radioactive phenylalanine into polyphenyl-

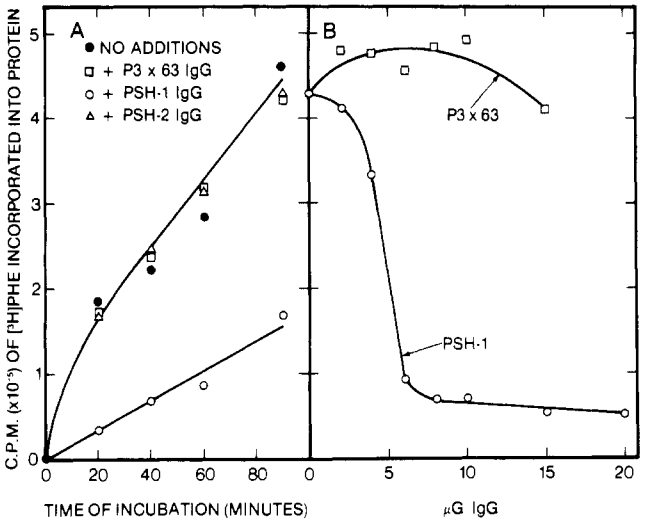

FIGURE 2: Effect of various antibodies on the time-dependent translation of poly(U). The yeast postpolysomal extract (0.43 mg of protein) was incubated for 20 min at 20 °C alone (●) or in the presence of 30 μg of purified antibody from cell lines P3 × 63 (□), PSH-1 (○), or PSH-2 (△). The rest of the components required for poly(U) translation, including [^{3}H]phenylalanine (2.0 μM final concentration, 5 Ci/mmol) and 150 μg of poly(uridylic acid), were added, and the incubations, in a total volume of 0.15 mL (30-μL aliquots), were removed at varying periods of time up to 90 min and analyzed for radioactivity. (B) Effect of antibody concentration on the translation of poly(U). Yeast extract was preincubated alone or in the presence of varying concentrations of antibody from P3 × 63 (□) or PSH-1 (○) cell lines; then all the other components required for poly(U) translation were added, including [^{3}H]Phe and poly(U), and the incubations were continued for 60 min.

alanine; the rates of incorporation with these two antibodies were similar to those in control incubations that did not contain antibody (closed circles). However, the translation of poly(U) was markedly depressed in the presence of IgG from cell line PSH-1 (open circles). Inhibition was dependent on the concentration of IgG from PSH-1 (B, open circles), and maximum inhibition was obtained with 5–10 μg per incubation. A more detailed characterization of the monoclonal antibody obtained from hybridoma cell line PSH-1, which inhibited polypeptide chain elongation, is described below.

The immunoglobulin chains of the monoclonal antibody obtained from hybridoma line PSH-1 were typed by using an enzyme-linked immunosorbant assay, as described under Experimental Procedures. The monoclonal antibody from cell line PSH-1 was found to belong to the IgG class consisting of light chains and γ_{2b} heavy chains.

Since the utilization of phenylalanine in protein synthesis requires its activation and esterification to tRNA^{Phe}, as well as ribosomes and elongation factors, the effect of these antibody preparations on phenylalanyl-tRNA synthetase was examined (Table I). The addition of IgG from myeloma cell line P3 × 63 or from PSH-1 (rows 2 and 3) had no effect on the formation of Phe-tRNA from phenylalanine, as compared to incubations in the absence of antibody (row 1). The results presented in Figure 3 are also consistent with the finding that the PSH-1 antibody did not react with Phe-tRNA synthetase; when preformed radioactive Phe-tRNA was used as the substrate for poly(U) translation, thus bypassing the aminoacyl-tRNA synthetase, inhibition of the chain elongation reactions was obtained with the antibody from PSH-1 (circles); the antibody preparation from cell line P3 × 63 (squares) had no effect. It should be noted that this assay system with Phe-tRNA, which required different ionic conditions and buffers, as well as lower concentrations of yeast extract, was

Table I: Effect of Antibodies from Myeloma and Hybridoma Cells on Phe-tRNA Synthetase[a]

additions	cpm of [^{3}H]Phe-tRNA synthesized
none	53 900
P3 × 63 IgG	54 600
PSH-1 IgG	55 400

[a] Approximately 22 μg of high-salt cytosol protein, in buffered salts–dithiothreitol, 120 μg of yeast deacylated tRNA, and 100 μM [^{3}H]phenylalanine (1 Ci/mmol) were incubated in the presence and absence of 1 mM ATP. Where noted, 10 μg of purified antibody from P3 × 63 or from PSH-1 was incubated with the cytosol for 20 min at 20 °C prior to the addition of tRNA, [^{3}H]Phe, and ATP. After 30 min at 20 °C, the cold (2 °C) 5% trichloroacetic acid insoluble fractions were prepared, collected on glass-fiber filters, washed with cold trichloroacetic acid, dried, and counted. The values for Phe-tRNA formed were corrected by subtracting the values obtained without ATP.

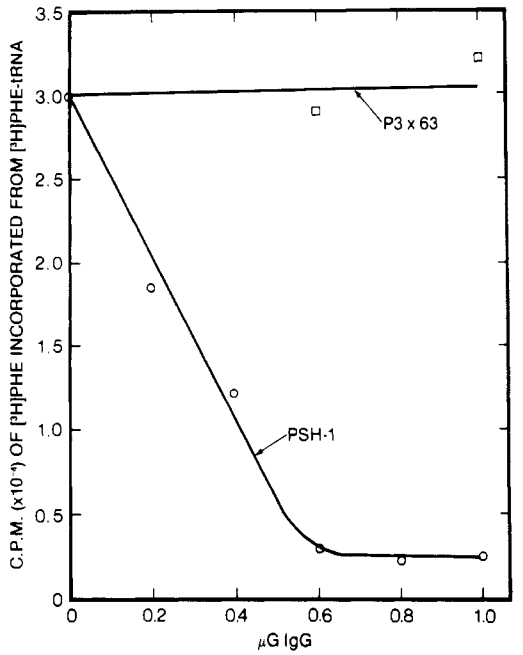

FIGURE 3: Effect of varying antibody concentrations on the poly(U)-dependent incorporation of phenylalanine from [^{3}H]Phe-tRNA into polyphenylalanine. Approximately 5.4 μg of yeast HS-cytosol and 9 μg of salt-extracted yeast 80S ribosomes were incubated for 15 min at 20 °C alone or in the presence of varying concentrations of purified antibody from P3 × 63 (□) or PSH-1 (○); all of the other components required for poly(U) translation, including 67 μg of [^{3}H]Phe-tRNA (2100 cpm/μg) and 100 μg of poly(uridylic acid), were added and the incubations continued for an additional 15 min.

inhibited by much lower levels of PSH-1 antibody than the assays with free phenylalanine described in Figure 2.

Evidence that the monoclonal antibody from PSH-1 reacted with an elongation factor rather than ribosomes is presented in Figure 4. In this experiment, a series of incubations containing all of the components necessary to translate poly(U) with [^{3}H]Phe-tRNA received sufficient PSH-1 antibody to inhibit protein synthesis about 75%; the additon of a dialyzed high-salt (0.5 M KCl)–250000g supernatant fraction (HS-cytosol) from yeast to these IgG-containing incubations (open circles) led to the restoration of poly(U) translation almost to the uninhibited level. The HS-cytosol contained the translational factors required for protein synthesis but was devoid of ribosomes and ribosomal subunits. The levels of HS-cytosol which were capable of rescuing the activity inhibited by PSH-1 antibody did not affect the normal level of poly(U) translation in the absence of antibodies (closed circles) or in the presence of IgG from cell line P3 × 63 (squares).

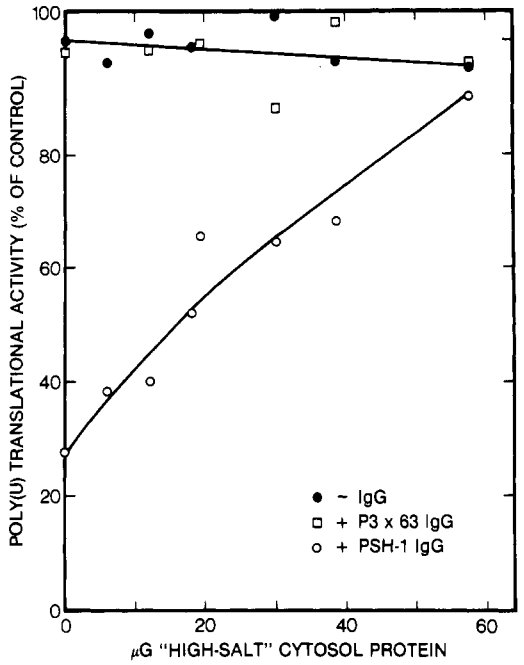

FIGURE 4: Effect of cytosol on the PSH-1 antibody-inhibited translation of poly(U). Approximately 0.15 mg of yeast postpolysomal extract protein was incubated for 20 min at 20 °C alone (●) or in the presence of 10 μg of purified antibody from the P3 × 63 (□) cell line or 5 μg of antibody from the PSH-1 (○) cell line. The rest of the components required for poly(U) translation, including [^{3}H]-phenylalanine (4.76 μM final concentration; 21 Ci/mmol), poly-(uridylic acid) (50 μg), and varying concentrations of HS-cytosol, were added and the incubations continued for an additional 60 min. The 100% value, obtained from samples that were not preincubated for 20 min and did not contain antibody, was 468 500 cpm of [^{3}H]Phe incorporated into protein.

The findings presented above indicated that the inhibitory effect of PSH-1 antibody was localized to the chain elongation phase of protein synthesis, that it was not due to an effect on Phe-tRNA synthetase or ribosomes, and that the activity could be restored by the addition of a soluble component. These observations suggested that the antibodies were probably reacting with an elongation factor; therefore, resolution and purification of the elongation factors were carried out in order to identify the critical component. It should be noted that although most mammalian systems require only two elongation factors (EF-1 and EF-2), evidence for an additional factor (EF-3) in yeast has been reported (Skogerson, 1979; Dasmahaparta & Chakraburtty, 1981). The proteins in the HS-cytosol fraction were precipitated with ammonium sulfate, dialyzed, and chromatographed on a column of DEAE-Sephadex A-50 (Figure 5). Two major protein peaks (A, closed circles) were recovered from the column, one in fractions 6–20 and the other in fractions 36–64. Column fractions from the middle of each of the two peaks, numbered 11 and 48, were tested individually and together for their ability to catalyze the poly(U)-dependent synthesis of polyphenylalanine from radioactive Phe-tRNA, in the presence of the other essential components. Efficient translation of poly(U) was obtained only when the two fractions were incubated together, indicating that they contained the complementary factors required for chain elongation. Fractions 8–30 were than assayed for polyphenylalanine synthesis in the presence of fraction 48, and fractions 42–70 were assayed in the presence of fraction 11.

The results presented in Figure 5A show the distribution of elongation factor EF-1 (squares), and of elongation factors EF-2 and EF-3 (triangles) which were not resolved by this procedure (Dasmahaparta & Chakraburtty, 1981); the EF-1

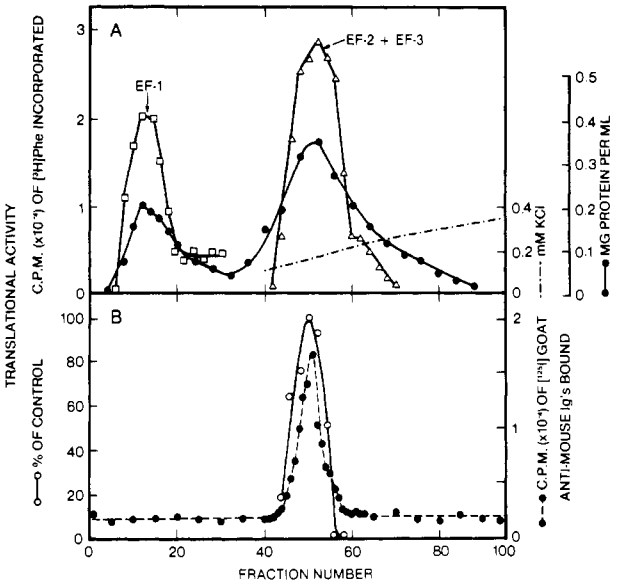

FIGURE 5: Chromatography of cytosol proteins on DEAE-Sephadex. Approximately 400 mg of protein precipitated from the HS-cytosol fraction with 70% saturated ammonium sulfate was chromatographed on a column of DEAE-Sephadex A-50 and eluted with a KCl gradient as described (Dasmahaparta & Chakraburtty, 1981). Twenty-milliliter fractions were collected. (A) Elution pattern obtained from the column for protein (●), EF-1 (□), and EF-2 plus EF-3 (△), as described in the text. (B) Elution pattern for material that restored the poly(U) translational activity inhibited by monoclonal antibody from hybridoma cell line PSH-1 (○) and for material that reacted with PSH-1 IgG as determined by the solid-phase radioimmunoassay (●).

was recovered in the effluent volume, and the EF-2 plus EF-3 was eluted with KCl concentrations of about 0.125 M. Various fractions from the column were also tested individually for their ability to react with IgG from PSH-1, by using the solid-phase radioimmunoassay. Aliquots of the column fractions were adsorbed to individual wells in plastic plates and incubated first with the hybridoma (PSH-1) antibody and then with ^{125}I-labeled goat anti-mouse antibodies, and the wells were counted as described under Experimental Procedures. The results indicated that the PSH-1 IgG-reacting material from the column was eluted as a sharp peak in fractions 45–57 (Figure 5B, closed circles) in the same region as the combined EF-2 and EF-3. In addition, various fractions from the column were tested individually for their ability to overcome the inhibitory effect of limiting amounts of the monoclonal antibody from hybridoma line PSH-1 (B, open circles). Fractions between numbers 40 and 60 contained an activity that restored poly(U) translation in the presence of the antibody; fractions corresponding to the EF-1 activity, such as fraction 12, or other fractions from the column did not rescue the system from inhibition.

The elution profiles shown in Figure 5 for translational factors EF-2 and EF-3 (A), for the material that reacted with PSH-1 antibody (B), and for the activity that restored antibody-inhibited translation (B) coincided. These results ruled out EF-1 as the elongation component that reacted with the antibody. It was important, therefore, to determine whether PSH-1 IgG was directed against EF-2 or EF-3. Indirect evidence that EF-3 was the reactive component was obtained by comparing the effect of PSH-1 IgG on poly(U) translation in incubations containing yeast cytosol as a source of elongation factors and purified rat liver or yeast ribosomes (Table II). Yeast cytosol contains all three elongation factors (EF-1, EF-2, and EF-3), but rat liver ribosomes require only EF-1 and EF-2 while yeast ribosomes also require the third factor (EF-3) for chain elongation. The addition of IgG from control myeloma

Table II: Effect of Antibodies from Myeloma and Hybridoma Cells on Poly(U) Translation with Yeast Cytosol and Rat Liver or Yeast Ribosomes[a]

additions	poly(U) translational activity (% of control)	
	rat liver ribosomes	yeast ribosomes
P3 × 63 IgG	94	97
PSH-1 IgG	89	37

[a] Approximately 11 μg of yeast HS-cytosol protein, in buffered salts–dithiothreitol, was incubated as such or in the presence of 3 μg of purified antibody from P3 × 63 or PSH-1, for 15 min at 20 °C. Purified rat liver (300 μg) or yeast (17.5 μg) 80S ribosomes, 50 μg of [^{3}H]Phe-tRNA (3300 cpm/μg), 200 μg of poly(U), and the other components required for protein synthesis were added, and the incubations, in a total volume of 0.2 mL, were continued for an additional 15 min. At the end of the incubation, the hot acid-insoluble fractions were prepared and counted. The results are expressed as the percent of the respective controls, without antibody; the control values were 21 700 cpm incorporated with rat liver ribosomes and 49 500 cpm incorporated with yeast ribosomes.

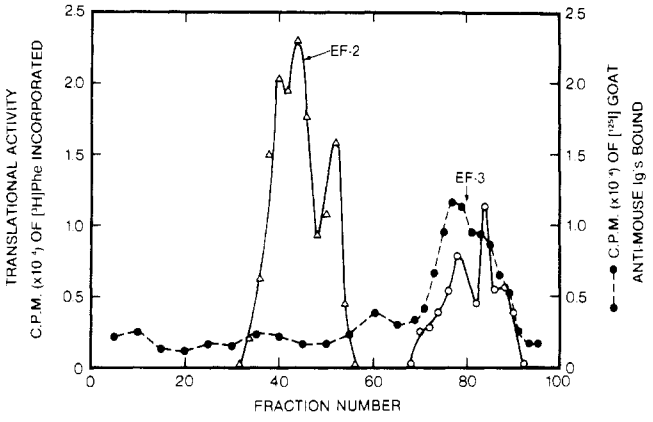

FIGURE 6: Chromatography of combined EF-2 and EF-3 on CM-Sephadex. Approximately 50 mg of protein obtained from fractions 40–60 from the DEAE-Sephadex column was chromatographed on a CM-Sephadex column and eluted with an NH_4Cl gradient, essentially as described with CM-Sepharose. The elution pattern for EF-2 (△) and for EF-3 (○) was determined by using the poly(U) translation assay, which requires the three complementary factors with yeast ribosomes; the elution pattern for material that reacted with the monoclonal antibody from hybridoma cell line PSH-1 (●) was determined by using the solid-phase radioimmunoassay.

cell line P3 × 63 had no effect on protein synthesis with either rat liver or yeast ribosomes. The addition of the monoclonal antibody from hybridoma line PSH-1, however, markedly inhibited poly(U) translation in incubations containing yeast ribosomes, which require EF-3, whereas incubations containing yeast cytosol and rat liver ribosomes, which require only EF-1 and EF-2, were unaffected by the antibody.

More definitive evidence that EF-3 was the PSH-1 monoclonal antibody reactive component was obtained by chromatographing the fractions eluted from DEAE-Sephadex, which contained combined EF-2 and EF-3, on CM-Sephadex, which resolves these two elongation factors. Solid-phase radioimmunoassay with individual fractions from this column (Figure 6) revealed that fractions 70–90 contained material that reacted with the antibody from PSH-1 (closed circles). Pooled material from fractions 70–90 and EF-1 obtained by chromatography on DEAE-Sephadex as described in Figure 5 were used to assay the column fractions for the third factor by their ability to stimulate poly(U) translation with yeast ribosomes. The results revealed that the complementary elongation factor, EF-2, was eluted from the CM-Sephadex in fractions 34–54 (triangles). Column fractions were then

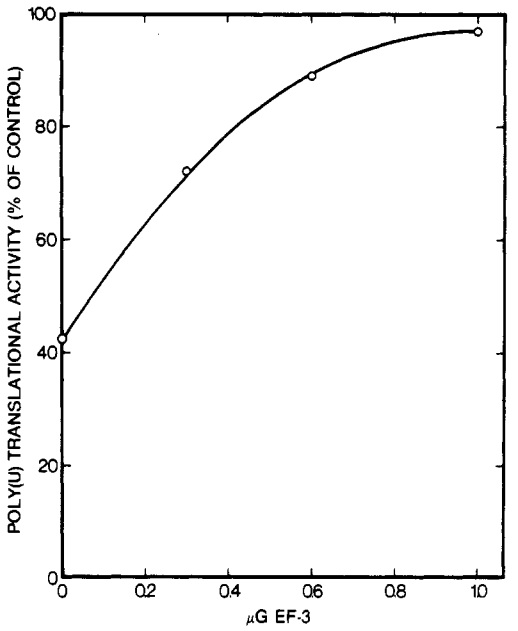

FIGURE 7: Effect of varying concentrations of purified EF-3 on the PSH-1 antibody-inhibited translation of poly(U). Approximately 5.4 μg of yeast HS-cytosol protein and 9 μg of purified yeast 80S ribosomes were incubated alone or with 0.6 μg of purified monoclonal antibody from hybridoma cell line PSH-1 for 20 min at 20 °C. The rest of the components required for poly(U) translation, including [^{3}H]-Phe-tRNA (50 μg; 3300 cpm/μg) and poly(uridylic acid), were then added; incubations containing PSH-1 IgG also received varying concentrations of EF-3 (○), purified by chromatography on DEAE- and CM-Sephadex. Incubations were continued for an additional 20 min. The 100% value, obtained in the absence of PSH-1 antibody, was 34 400 cpm of [^{3}H]Phe polymerized.

analyzed for EF-3 activity by using the resolved EF-2 and EF-1 in similar incubations. The analyses indicated that EF-3 (open circles) was eluted from the column in the same fractions (70–90) that contained PSH-1 antibody-reactive material.

The EF-3 recovered from the CM-Sephadex column in fractions 70–90 was also tested for its ability to restore the activity of PSH-1 antibody-inhibited poly(U) translation (Figure 7). A series of tubes containing the components required to translate poly(U) with [^{3}H]Phe-tRNA received sufficient PSH-1 antibody to inhibit protein synthesis about 60%. The addition of varying concentrations of resolved, partially purified EF-3 to these IgG-containing incubations led to the restoration of poly(U) translation; approximately 1 μg of the EF-3 preparation stimulated protein synthesis to the levels obtained in the control incubations in the absence of antibody.

Polyacrylamide gel electrophoresis (in SDS), protein transfer, and immunoblot analyses of EF-3 preparations are shown in Figure 8. For reference, the Coomassie Blue stained pattern of polypeptides in yeast cytosol is shown in track A; about 100 polypeptide bands are visible. The material purified from the yeast HS-cytosol by using one-step immunoaffinity chromatography on Affi-Gel 10 as described under Experimental Procedures revealed a major component with a molecular weight of about 125 000 (125K) and several lower molecular weight polypeptides on gel electrophoresis (B). The molecular weight of 125 000 is in agreement with that reported by Dasmahaparta & Chakraburtty (1981). Protein blot analysis (Towbin et al., 1979) of this material was carried out by transferring the proteins on the polyacrylamide gel to nitrocellulose membranes electrophoretically and treating them first with PSH-1 IgG and then with radioactive goat anti-mouse IgG. The radioactive EF-3–PSH-1 IgG–goat anti-

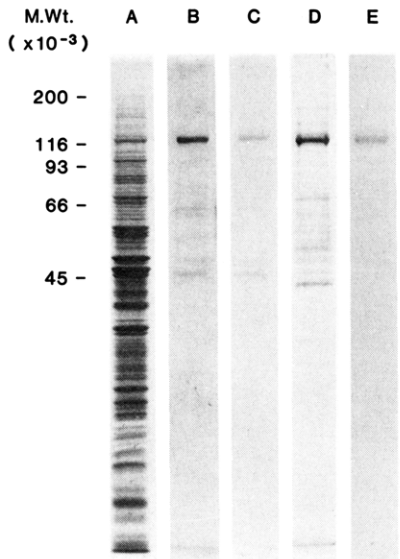

FIGURE 8: Electrophoretic and immunoblot analysis of preparations containing EF-3. (A) Yeast cytosol, stained gel; (B) purified EF-3 preparation obtained by immunoaffinity chromatography, stained gel; (C) autoradiography of gel shown in (B, affinity chromatography purified EF-3), after protein transfer and immunoblotting; (D) purified EF-3 obtained by ion-exchange chromatography, stained gel; (E) autoradiography of gel shown in (D, ion-exchange chromatography purified EF-3), after protein transfer and immunoblotting.

mouse antibody complex was detected by exposure of X-ray film to the treated transfer membrane. The results (C) revealed that the 125K material reacted very strongly with the PSH-1 monoclonal antibody; the lower molecular weight polypeptides also gave a positive reaction in this assay. The possibility exists that the smaller components may be breakdown products of EF-3 that have retained their ability to interact with the antibody. Indeed, similar products were observed on prolonged storage of EF-3, or on freezing and thawing, particularly in the absence of protease inhibitors. The polypeptide pattern obtained on gel electrophoresis of the EF-3 purified by chromatography on DEAE- and CM-Sephadex chromatography, as described above, is shown in track D. The major component, accounting for over 90% of the total protein in the gel, also had a molecular weight of about 125 000; some 12 polypeptide bands, present in much lower concentrations, were also detected. Protein immunoblot analysis of this preparation (E) revealed that only the 125K band reacted positively with the monoclonal antibody from PSH-1, suggesting that the lower molecular weight polypeptides recovered with EF-3 on chromatography are immunologically unrelated contaminants or nonreactive breakdown products.

The effect of inactivation of EF-3 on the chain elongation phase of protein synthesis with natural mRNA was examined by preincubating the complete system for a short period of time, in order to allow initiation reactions to occur, prior to the addition of the monoclonal antibody from PSH-1. Several reaction vessels containing yeast postpolysomal extract, [^{3}H]leucine, yeast mRNA, and the other components required for protein synthesis were incubated at 20 °C as described (Gasior et al., 1979; Feinberg et al., 1982). After 10 min, as shown in Figure 9, one incubation received antibody from cell line P3 × 63, another received 7-methylguanosine 5′-monophosphate (m^{7}GMP), the third received monoclonal antibody from hybridoma line PSH-1, and cycloheximide was added to the fourth incubation; one incubation mixture, a control, did not receive any additions. The incubations were continued at 20 °C; aliquots were removed at varying time periods and analyzed for radioactive protein. As shown in this figure,

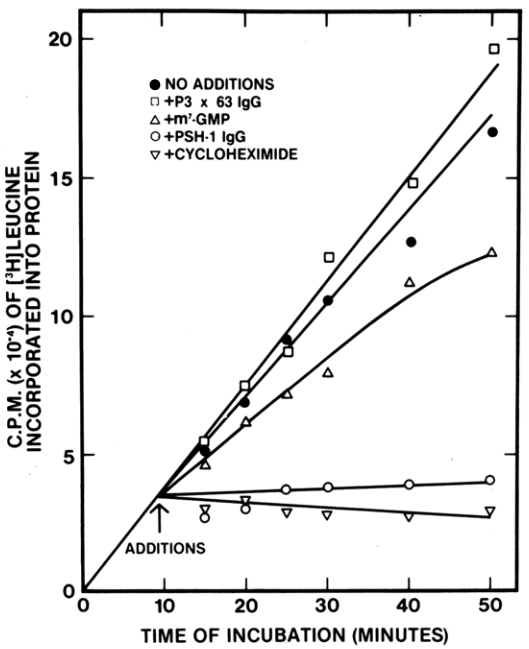

FIGURE 9: Effect of various antibodies and protein-synthesis inhibitors on the translation of mRNA. A set of reaction mixtures, each the equivalent of several incubation volumes (0.3 mL), containing yeast postpolysomal extract, [^{3}H]leucine, yeast mRNA, and all the other components required for protein synthesis were incubated for 10 min at 20 °C. Individual reaction mixtures then received one of the following, and the incubations were continued for varying periods of time: 70 μg of antibody from control cell line P3 × 63 (□); 1.2 mM (final concentration) 7-methylguanosine 5′-monophosphate (△); 70 μg of monoclonal antibody from hybridoma cell line PSH-1 (○); or 1 mM (final concentration) cycloheximide (△); one of the reaction mixtures (●) did not receive any additions. After varying periods of time, 20-μL aliquots were withdrawn and assayed for radioactive protein.

protein synthesis in the control incubation (closed circles) was essentially linear for up to 50 min, and the addition of IgG from P3 × 63 (squares) had no effect. In contrast to the initiation inhibitor m^{7}GMP (upward triangles), whose inhibitory effects became gradually apparent with time, the addition of the elongation inhibitor cycloheximide (inverted triangles) and the monoclonal antibody from PSH-1 (open circles) resulted in the immediate and complete cessation of protein synthesis.

Ribosomal native 40S and 60S subunits, ribosomes and monosomes, polysomes, and cytosol were examined for reactivity with the monoclonal antibody from PSH-1; samples were incubated with PSH-1 IgG and radioactive goat anti-mouse antibody by using the solid-phase radioimmunoassay described above. Antibody-reacting material was detected in the cytosol, polysomes, and 80S particles, but the 40S and 60S subunit preparations did not react with the monoclonal PSH-1 antibody. Using a quantitative competitive radioimmunoassay, to be described elsewhere, it was estimated that approximately 50% of the polysomal ribosomes and 2% of the 80S ribosomes (monosomes) contained a molecule of EF-3. The 40S and 60S subunit preparations remained unreactive to PSH-1 IgG even when the samples were made 0.5 M with respect to KCl, in order to dissociate nonribosomal proteins from the particles prior to the solid-phase assay. In addition, antibody-reacting material was not detected in the ribonucleoprotein particles obtained from ribosomal–polysomal preparations after extraction with 0.5 M KCl.

Discussion

A monoclonal antibody purified from one of the cloned hybridomas (PSH-1) prepared in this laboratory inhibits the

translation of mRNA by blocking chain elongation. The antibody from cell line PSH-1 reacts with and inactivates EF-3, the 125 000 molecular weight polypeptide which is required for protein synthesis only with yeast ribosomes. Previous studies on the role of EF-3 were carried out by using the poly(U)-dependent incorporation of phenylalanine into polyphenylalanine (Skogerson, 1977, 1979; Dasmahaparta & Chakraburtty, 1981). The data presented here, that the antibody-dependent inactivation of EF-3 inhibits the translation of natural mRNA, indicate that this elongation factor is a normal requirement for protein synthesis in yeast and is not an artifact resulting from the use of an artificial template.

The requirement for EF-3, in addition of EF-1 and EF-2, for the translation of poly(U) with yeast ribosomes implies a role for this factor in polypeptide chain elongation. Although EF-3 exhibits strong GTPase and ATPase activities (Skogerson, 1979; Dasmahaparta & Chakraburtty, 1981), the exact site and mechanism of action within the elongation cycle of reactions remain to be established; for example, EF-3 does not appear to be involved in the EF-1- and GTP-dependent binding of aminoacyl-tRNA to ribosomes, the translocation-dependent formation of *N*-acetylphenylalanylpuromycin, or the formation of the ribosome–EF-2–GDP–fusidic acid complex (Skogerson, 1977). It has been suggested that EF-3 may be a loosely bound ribosomal protein which is not required for a specific step in the elongation cycle but may be involved in the coordination of the individual steps (Skogerson, 1977). As described above (Figure 9), inactivation of EF-3 by the monoclonal antibody, after polypeptide chain initiation reactions have been allowed to occur, results in an immediate block in protein synthesis; this observation suggests that EF-3 is required for every cycle in elongation. The known individual steps in chain elongation are the following: (1) EF-1- and GTP-dependent binding of aminoacyl-tRNA; (2) ribosome-catalyzed peptidyltransferase reaction; and (3) EF-2- and GTP-dependent translocation. These reactions are repeated sequentially as every internal codon is translated. There are some minor variations in the case of the first internal codon (following the initiation codon AUG) and the termination codon; in the former, binding of aminoacyl-tRNA to the ribosomal A site occurs when the P site is occupied by an aminoacyl-tRNA rather than a peptidyl-tRNA, and in the latter, a protein (termination factor) rather than an aminoacyl-tRNA is bound to the A site prior to the final peptidyltransferase reaction. The data presented in Figure 9 not only demonstrated that EF-3 is required for every cycle in chain elongation but also rule out the first cycle involving the 80S initiation complex as well as the termination reactions as specific sites of action; in both of these cases, inhibition with PSH-1 IgG would be expected to be similar to that with m^7GMP rather than cycloheximide.

Since EF-3 is involved in chain elongation, it would be expected to be found in association with polysomes and monosomes. If it is indeed a translational factor, it would be expected to cycle between the particles and the soluble fraction of the cytoplasm and to be found both on ribosomes engaged in protein synthesis and in the cytosol, but not necessarily on ribosomal subunits. The observations reported here are consistent with this interpretation. Failure to detect EF-3 in ribosomal subunits would argue against its being a ribosomal protein, although it could be a loosely bound ribosomal protein, as suggested (Skogerson, 1977), which is removed on centrifugation. The lack of stoichiometry between EF-3 and polysomal ribosomes could be a consequence of the cycling process during elongation; it could indicate that the factor is loosely bound and removed on centrifugation or that its antigenic domain is masked when it is associated with the particle. The factor does not appear to be tightly bound to polysomes; it is extensively removed by centrifugation through a low-salt discontinuous sucrose gradient. The observation that the antibody inactivates EF-3 in the middle of the elongation phase suggests that it must be sensitive to the antibody when it is bound to the particle or, more likely, that the factor dissociates from the particle at every cycle in chain elongation.

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Registry No. Poly(U), 27416-86-0.

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Synthesis and Biological Activity of Tubercidin Analogues of ppp5′A2′p(5′A2′p)$_n$5′A†

Jean-Claude Jamoulle, Jiro Imai, Krystyna Lesiak, and Paul F. Torrence*

ABSTRACT: A series of tubercidin (7-deazaadenosine) analogues of 2–5A of the general formula p5′(c^{7}A)2′p[5′(c^{7}A)-2′p]$_n$5′(c^{7}A) (n = 0–5) were prepared by lead ion catalyzed polymerization of the 5′-phosphoroimidazolidate of tubercidin. Through the corresponding imidazolidates, these oligonucleotide 5′-monophosphates were converted to the 5′-triphosphates. All reported structures were corroborated by enzyme digestion and ^{1}H or ^{31}P nuclear magnetic resonance. When evaluated for its ability to bind to the 2–5A-dependent endonuclease of mouse L cells, the tubercidin analogue of trimeric 2–5A, namely, ppp5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A), and the corresponding tetramer were bound as effectively as 2–5A itself; nonetheless, it and the corresponding tetramer, ppp5′-(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A), failed to stimulate the 2–5A-dependent endonuclease as judged by its inability to inhibit translation in extracts of mouse L cells programmed with encephalomyocarditis virus RNA and to give rise to ribosomal RNA cleavage in the same cell system under conditions where 2–5A showed activity at 10^{-9} M. The trimer, ppp5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A), was an antagonist of 2–5A action in the L cell extract. In the lysed rabbit reticulocyte system, both the trimeric and tetrameric tubercidin 2–5A analogues were bound to the 2–5A-dependent endonuclease as well as 2–5A, but in this case, the tetramer triphosphate, ppp5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A)2′p5′(c^{7}A), was just as potent an inhibitor of translation as 2–5A tetramer triphosphate. Moreover, this inhibition was prevented by the established 2–5A antagonist p5′A2′p5′A2′p5′A. The tubercidin analogues of 2–5A also were bound to the endonuclease of Daudi lymphoblastoid cells albeit slightly less effectively than 2–5A. Thus, it appears that the purine N(7) moieties of 2–5A are not involved in binding to the 2–5A-dependent endonuclease but one or more are required for activation of the 2–5A-dependent endonuclease of mouse L cells. These results also serve to underscore the inherent differences between the mouse L cell enzyme and the rabbit reticulocyte RNase L.

The probable role of the unique oligoribonucleotide 2–5A[1] (Kerr & Brown, 1978) and its associated enzymes, 2–5A synthetase,[1] RNase L, and a 2′,5′-phosphodiesterase, in the antiviral action of interferon [reviewed by Revel (1979), Lengyel (1982), Sen (1982) and Torrence (1982)] and the possible role of the above elements in the antiproliferative effects of interferon (Kimchi et al., 1981), as well as in cellular regulation of growth or differentiation (Stark et al., 1979; Etienne-Smekens et al., 1983), have stimulated considerable interest in the synthesis and biological activities of 2–5A analogues [reviewed by Imai & Torrence (1983) and Johnston & Torrence, 1984)]. Although a number of modifications have been executed at the 5′- and/or 2′-terminus (Imai et al., 1982; Silverman et al., 1981; Haugh et al., 1983; Torrence et al., 1982, 1984; Imai & Torrence, 1984) or in the sugar–phosphate backbone (Baglioni et al., 1981; Lesiak et al., 1983; Sawai et al., 1983; Eppstein et al., 1982), relatively little is understood regarding the influence of base modifications on binding to and activation of RNase L.

One base modification of interest is the substitution of a CH moiety for the adenine N(7), resulting in the replacement of adenosine with tubercidin (7-deazaadenosine, 4-amino-7-β-D-ribofuranosyl-7*H*-pyrrolo[2,3-*d*]pyrimidine). In some instances, the adenine N(7) may be a recognition element in enzymic transformation; for example, tubercidin 5′-triphosphate was not a substrate of adenosine deaminase (Ikehara & Fukui, 1974) or nucleoside phosphorylase (Bloch, 1975). Incorporation of tubercidin or its inosine counterpart, 7-deazainosine, into polynucleotides results in significant changes in interferon-inducing ability (DeClercq et al., 1974; Torrence et al., 1974) and nucleic acid conformation (Ikehara & Fukui, 1968; Bobst et al., 1976; Miles et al., 1979). Finally, Wreschner et al. (1981b) and Floyd-Smith et al. (1981) have noted that the preferred cleavage sites of RNase L all contain uridylate residues, which are complementary to the adenosines of 2–5A. Replacement of adenosine with tubercidin allows a test of the possibility that Hoogsteen hydrogen bonding could be involved in activation of RNase L.

Materials and Methods

Chromatography. Thin-layer chromatography (TLC) was carried out on PEI–cellulose F in 0.25 M NH_4HCO_3 (system a) or on cellulose F in isobutyric acid–concentrated NH_4OH–0.2 M EDTA (100:60:0.8) (system b) or on silica gel Merck 60F254 in 2-propanol–concentrated NH_4OH–H_2O (7:1:2) (system c) or PEI–cellulose F in 0.1 M ammonium bicarbonate (system d). High-performance chromatography (HPLC) was

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[1] Abbreviations: 2–5A, ppp5′A2′p(5′A2′p)$_n$5′A, where n = 1 to about 10; RNase L, 2–5A-dependent endoribonuclease; 2–5A synthetase, enzyme that, after activation by double-stranded RNA, effects the conversion nATP → pppA(pA)$_{n-1}$ + (n–1)PP$_i$; PEI, poly(ethylenimine); EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.