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# Characterization of Initiation Factor 3 from Wheat Germ. 2. Effects of Polyclonal and Monoclonal Antibodies on Activity†

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ABSTRACT: Rabbit polyclonal antibodies to wheat germ initiation factor 3 (eIF-3) were obtained and were shown to react strongly with 4 of the 10 subunits of eIF-3 (pp116, pp87, pp56, and pp36). Two mouse monoclonal antibodies were obtained, one of which reacts specifically with pp87 and one of which reacts specifically with pp36. Highly purified anti-pp87 has no effect on the activity of eIF-3. Highly purified polyclonal antibodies and anti-pp36 inhibit the ability of eIF-3 to support polypeptide synthesis in vitro and the ability of eIF-3 to support mRNA binding to 40S ribosomal subunits. These results provide additional evidence that pp116, pp87, and pp36 are in exposed positions in the eIF-3 particle and that pp36 is essential for activity.

In the preceding paper (Lauer et al., 1985), it was shown that four of the subunits of wheat germ initiation factor 3 (eIF-3)[1] (pp116, pp107, pp87, and pp36) are susceptible to proteolytic degradation, strongly suggesting that these polypeptides are in exposed positions in the eIF-3 particle. The data also indicated that pp116 and/or pp36 are essential for activity. Here we report the effects of polyclonal and monoclonal antibodies on the activity of eIF-3. The results obtained provide additional evidence that pp116, pp87, and pp36 are in exposed positions in the eIF-3 particle and that pp36 is essential for activity.

## Materials and Methods

*Materials.* eIF-3 was prepared as described in the preceding paper (Lauer et al., 1985). Goat anti-rabbit IgG (H + L) conjugated to horseradish peroxidase and rabbit anti-mouse IgM + IgA + IgG conjugated to horseradish peroxidase, the MonoAb Id EIA kit, and ABTS were obtained from Zymed. Nitrocellulose paper was from Schleicher & Schuell, and the HRP color development reagent was from Bio-Rad Laboratories.

*Assays.* The abilities of eIF-3 to support polypeptide synthesis (assay A), to bind to 40S ribosomal subunits (assay B), and to support the binding of $^{125}$I-labeled STNV RNA to 40S ribosomal subunits (assay C) were determined as described in the preceding paper (Lauer et al., 1985).

*Production of Polyclonal Antibodies to eIF-3.* A 5-lb, male New Zealand rabbit was injected intramuscularly with 0.2 mg of eIF-3 (CM-150 fraction) in 0.7 mL of Freund's complete adjuvant. Two booster injections, each containing 0.5 mg of eIF-3 in 0.75 mL of Freund's incomplete adjuvant, were given 4 and 8 weeks after the initial injection. Twelve days after the second boost, the rabbit was sacrificed, and the serum fraction of the blood was collected.

*Production of Monoclonal Antibodies to eIF-3.* Monoclonal antibodies were produced in the Central Hybridoma Facility at The University of Texas at Austin (director: Dr. Paul Gottlieb). A female Balb/cAM mouse was injected intraperitoneally with 0.13 mg of eIF-3 (CM-150 fraction) in 0.2 mL of Freund's complete adjuvant. A second injection in Freund's incomplete adjuvant was given 2 weeks later, and 2 weeks after the second injection, 0.2 mg of eIF-3 in buffer was injected intravenously. Three days later, the mouse was sacrificed, and the spleen was removed. Hybridomas were produced by fusion of the spleen cells with P3×63A$_G$8 myeloma cells by the procedure of Kennett et al. (1978). Twenty hybridomas producing antibodies that reacted strongly with eIF-3 in the ELISA (described below) were obtained. Ascites fluid was produced in pristane-primed Balb/c mice and used as a source of monoclonal antibodies. Monoclonal antibodies in ascites fluid produced in response to hybridomas, A8.1 and 5C-12, as well as to the parent cell, P-3, were purified as described below.

*Enzyme-Linked Immunoadsorbent Assay (ELISA).* The ELISA was performed by a modification of the procedure of Bahr et al. (1980). The microtiter plate wells were coated with 0.25 μg of eIF-3 in 50 μL of buffer B-100. The conjugated second antibody was either goat anti-rabbit IgG conjugated

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[1] Abbreviations: eIF-3, initiation factor 3; ABTS, 2,2′-azinobis(3-ethyl-6-benzothiazolinesulfonic acid); GMP-PNP, 5′-guanylyl imidodiphosphate; STNV, satellite tobacco necrosis virus; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunoadsorbent assay; EITB, enzyme-linked immunoelectrotransfer blot; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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to horseradish peroxidase or rabbit anti-mouse IgM + IgA + IgG conjugated to horseradish peroxidase. The substrate solution (0.1 mL containing 0.7 mg/mL ABTS, 0.03% hydrogen peroxide, and 0.1 M sodium citrate, pH 4.2) was added, and the absorbance at 414 nm was measured.

*Enzyme-Linked Immunoelectrotransfer Blot (EITB) Technique.* EITB was carried out by a modification of the procedure described by Tsang et al. (1983). eIF-3 was first separated into its subunits by electrophoresis in a SDS–12.5% polyacrylamide gel as described in the preceding paper (Lauer et al., 1985). The polypeptides were then transferred to nitrocellulose paper by electrophoresis at 200 mA for 5 h. HRP color development reagent was used as a substrate for the peroxidase-conjugated second antibody.

*Purification of Polyclonal Antibodies.* Solid ammonium sulfate was added to rabbit serum (60–70 mL) to bring the percent of saturation to 50%. The precipitated proteins were suspended in buffer TK (10 mM Tris-HCl, pH 8.0) containing 30 mM KCl (buffer TK-30), dialyzed against the same buffer, and chromatographed on DEAE Affi-Gel Blue by the procedure recommended by Bio-Rad Laboratories, Inc. A sample of the 0–50% ammonium sulfate fraction containing 125–150 mg of protein was applied to a 30-mL DEAE Affi-Gel Blue column equilibrated in buffer TK-30. The column was washed with buffer TK-30, followed by buffer TK-100. The 30 mM KCl and the 100 mM KCl fractions containing high amounts of protein were pooled separately and tested for the presence of eIF-3-specific antibodies in the ELISA; the majority of the anti-eIF-3 activity was found in the 30 mM KCl fraction. The proteins in TK-30 fractions were precipitated by the addition of solid ammonium sulfate (50%), suspended in and dialyzed against buffer HK-100 (10 mM Hepes/KOH, pH 7.6, and 100 mM KOAc), and used as a source of polyclonal anti-eIF-3 antibodies. The remaining proteins were removed from the column by washing with 2 M guanidine hydrochloride. The column was reequilibrated by extensive washing with buffer TK-30 prior to reuse.

*Purification of Monoclonal Antibodies.* Solid ammonium sulfate was added to 10 mL of ascites fluid to bring the percent of saturation to 50%. The precipitated proteins were suspended in buffer TK-0, dialyzed against the same buffer, and then purified by chromatography on DEAE Affi-Gel Blue according to the procedure of Bruck et al. (1982). A sample of the 0–50% ammonium sulfate fraction of ascites fluid containing 100–125 mg of protein was applied to a 25-mL DEAE Affi-Gel Blue column equilibrated in buffer TK-0. After being washed with 30–40 mL of buffer TK-0, a 125-mL linear gradient from 0 to 100 mM KCl in buffer TK-0 was applied to the column, and 2.5-mL fractions were collected. Aliquots of the fractions were assayed in the ELISA and analyzed by SDS–polyacrylamide gel electrophoresis. Those fractions which gave a strong positive response in the ELISA and contained few, if any, polypeptides other than immunoglobulin polypeptides ($M_r$ 52 000 and 30 000) were pooled and treated as described above for the polyclonal antibodies. The same procedure was followed for the P3 antibodies, but these fractions were pooled on the basis of polypeptide composition alone. The majority of the immunoglobulin polypeptides eluted from the DEAE Affi-Gel Blue column between 60 and 80 mM KCl in each case. The subclass of each monoclonal antibody was determined with the MonoAb Id EIA kit (Zymed). Both hybridomas, A8.1 and 5G-12, produced antibodies of the $IgG_{2b}$ subclass.

*Determination of Protein Concentration.* The protein concentrations of serum or ascites fluid samples were determined by the method of Warburg & Christian (1942). The concentration of purified polyclonal antibodies was determined by assuming $E^{1\%}_{280nm} = 13.5$ (Stevenson & Dorrington, 1970), and the concentration of purified monoclonal antibodies was determined by assuming $E^{1\%}_{280nm} = 14$ (Ey et al., 1978).

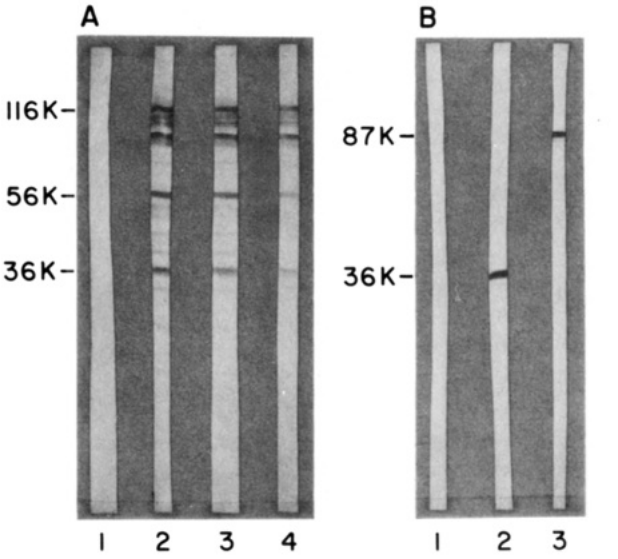


FIGURE 1: Specificity of polyclonal and monoclonal antibodies to eIF-3. The EITB assay was performed as described under Materials and Methods. The first antibody was as indicated. (A) Lane 1, 9 μg/mL preimmune rabbit antibodies; lanes 2, 3, and 4, 3, 1, and 0.3 μg/mL, respectively, of polyclonal anti-eIF-3. (B) Lane 1, 5 μg/mL P3 antibody; lane 2, 0.2 μg/mL anti-pp36; lane 3, 0.2 μg/mL anti-pp87.

## RESULTS

The interaction of purified polyclonal antibodies with the various subunits of eIF-3 was determined by the EITB technique, and the results are shown in Figure 1, panel A. At a high concentration of the polyclonal antibodies (lane 2, 3 μg/mL), interactions with most of the subunits of eIF-3 were observed. At lower concentrations (lanes 3 and 4, 1 and 0.3 μg/mL, respectively), interactions were seen primarily with four of the subunits, pp116, pp87, pp56, and pp36. Antibodies purified from preimmune serum (lane 1) showed no detectable interaction with any of the subunits of eIF-3. In separate experiments, the ability of purified eIF-3 polyclonal antibodies to interact with other wheat germ initiation factors (eIF-2, eIF-4A, and eIF-4B) and elongation factors (EF-1α and EF-2) was determined by the EITB technique, and no detectable interaction was observed.

The specificities of the three purified monoclonal antibodies (P3, A8.1, and 5G-12) were also determined by the EITB technique, and the results are shown in Figure 1, panel B. Purified antibodies from the parent P3 myeloma cell line showed no detectable interaction with any of the subunits of eIF-3 when tested at a high concentration (lane 1, 5 μg/mL). Purified antibodies from hybridoma A8.1 reacted only with pp36 (lane 2) while purified antibodies from hybridoma 5G-12 reacted only with pp87 (lane 3). When the concentrations of these antibodies were raised to 5 μg/mL, no binding to the other subunits of eIF-3 was observed. These data indicate that the other subunits of eIF-3 are probably not either precursors or degradation products of pp87 or pp36. In all subsequent experiments, purified antibodies from A8.1 and 5G-12 were used and designated anti-pp36 and anti-pp87, respectively.

The ability of the various antibody preparations to inhibit in vitro polypeptide synthesis was studied, and the results are shown in Figure 2. In the presence of a limiting amount of

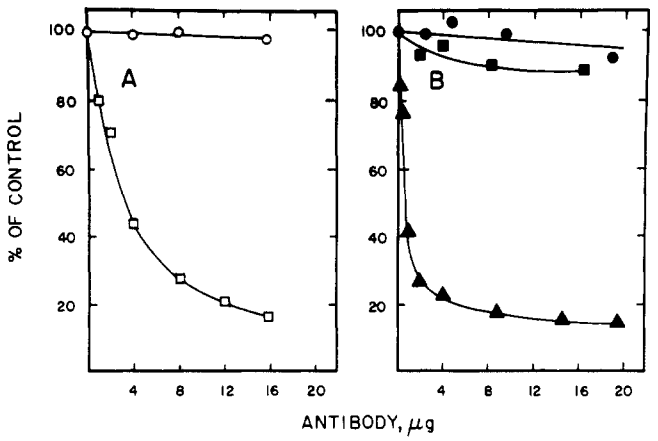


FIGURE 2: Effect of antibodies to eIF-3 on in vitro polypeptide synthesis. In vitro polypeptide synthesis was measured as previously described [see assay A in Lauer et al. (1985)] in the presence of 0.7 μg of eIF-3 (fraction CM-150) and antibody as indicated. (A) (○) Preimmune rabbit antibodies; (□) polyclonal anti-eIF-3. (B) (●) P3 antibody; (■) anti-pp87; (▲) anti-pp36.

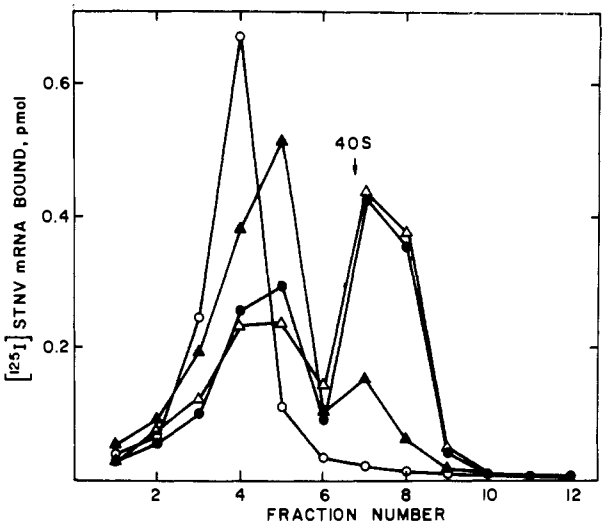


FIGURE 3: Effects of anti-pp87 and anti-pp36 on the ability of eIF-3 to support the binding of STNV RNA to 40S ribosomal subunits. The assay conditions and sucrose density gradient analysis were the same as those described in the preceding paper [see assay C in Lauer et al. (1985)]. (○) No eIF-3; (●) 5.5 μg of eIF-3; (▲) 5.5 μg of eIF-3 and 30 μg of anti-pp87; (▲) 5.5 μg of eIF-3 and 30 μg of anti-pp36.

eIF-3 (0.7 μg), neither the preimmune polyclonal antibody (panel A) nor the P3 antibody (panel B) inhibited polypeptide synthesis to a significant extent at concentrations up to 20 μg. Addition of 3.5 μg of the polyclonal antibodies or of 0.65 μg of anti-pp36 resulted in a 50% inhibition of polypeptide synthesis, and greater than 80% inhibition was observed with 12 μg of the polyclonal antibodies or 2 μg of anti-pp36. The inhibitory effects of these antibodies were completely overcome by the addition of 1 μg of eIF-3. In contrast, anti-pp87 had very little inhibitory effect; no greater than 15% inhibition was observed. In separate experiments, the ability of anti-pp36 to inhibit polypeptide synthesis directed by globin mRNA or STNV RNA was determined, and the degree of inhibition was the same as that observed with yeast polysomal RNA.

In separate experiments, attempts were made to determine the effects of the antibodies on the ability of eIF-3 to bind to 40S ribosomal subunits [see assay B in Lauer et al. (1985)]. In the presence of anti-pp36, no decrease in the binding of eIF-3 to 40S ribosomal suubunits was observed (data not shown). In the presence of polyclonal anti-eIF-3 or monoclonal anti-pp87, precipitation of both eIF-3 and the ribosomal subunits occurred when the reaction mixtures were fixed with glutaraldehyde. It was not possible, therefore, to determine the effects of these antibodies on the binding of eIF-3 to 40S ribosomal subunits. The reason that precipitation occurred in the presence of glutaraldehyde with polyclonal anti-eIF-3 or monoclonal anti-pp87, but not with monoclonal anti-pp36, is not known at this time.

The effects of anti-pp87 and anti-pp36 on the ability of eIF-3 to support the binding of mRNA to 40S ribosomal subunits are shown in Figure 3. The 40S ribosomal subunits were incubated with $^{125}$I-labeled STNV RNA (an uncapped mRNA), eIF-2, Met-tRNA$_i$, GMP-PNP, eIF-4A, eIF-4B, and ATP in the absence and presence of eIF-3. The reaction mixtures were centrifuged through sucrose density gradients, and the gradient fractions were passed through nitrocellulose filters as described in the preceding paper (Lauer et al., 1985). In the absence of eIF-3, STNV RNA was found primarily in fractions 3 and 4. Very little (<0.05 pmol) was found in the 40S region of the gradient (fractions 7 and 8). The peak of STNV RNA observed in fractions 3 and 4 was probably due to the formation of a complex between STNV RNA and one or more of the initiation factors present in the reaction mixture, because free STNV RNA is not retained on a nitrocellulose filter. In the presence of the initiation factors and a limiting amount of eIF-3 (approximately 1 pmol of eIF-3 per picomole of 40S ribosomal subunits), there was a decrease in the amount of STNV RNA found in fractions 3 and 4 and an increase in the amount of STNV RNA found in the 40S region of the gradient (fractions 7 and 8). Approximately 0.8 pmol of STNV RNA was present in the 40S region. Addition of anti-pp36 decreased the amount of mRNA found in the 40S region from 0.8 to 0.2 pmol. In contrast, anti-pp87 did not decrease the binding of STNV RNA to 40S ribosomal subunits. In separate experiments, polyclonal antibodies to eIF-3 were found to reduce the amount of STNV RNA bound to 40S ribosomal subunits to the same extent as anti-pp36. These data are in agreement with the data presented in Figure 2 showing that polyclonal antibodies and anti-pp36 inhibit polypeptide synthesis and anti-pp87 does not. Furthermore, these data indicate that polyclonal antibodies and anti-pp36 inhibit polypeptide synthesis by inhibiting the ability of eIF-3 to support mRNA binding to 40S ribosomal subunits.

## Discussion

Rabbit polyclonal antibodies to wheat germ eIF-3 were obtained and shown to react strongly with four of the subunits of eIF-3 (pp116, pp87, pp56, and pp36). Two mouse monoclonal antibodies were also obtained, one of which reacts specifically with pp87 (anti-pp87) and one of which reacts specifically with pp36 (anti-pp36). Purified polyclonal antibodies and anti-pp36 inhibit the ability of eIF-3 to support polypeptide synthesis in vitro and the ability of eIF-3 to support the binding of mRNA to 40S ribosomal subunits. Anti-pp87, however, does not interefere with the functioning of eIF-3 either because pp87 is not required or because binding of the antibody does not interfere with the function of pp87. The lack of interaction of anti-pp36 or anti-pp87 with the other subunits of eIF-3 indicates that the polypeptides do not contain common amino acid sequences and therefore do not have a precursor–product relationship.

In summary, these data provide additional evidence that pp116, pp87, and pp36 are in exposed positions in the eIF-3 particle and that pp36 is essential for activity. Further work will be necessary to determine whether pp116 is also essential for activity.

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# Human Serum Amyloid A (SAA): Biosynthesis and Postsynthetic Processing of PreSAA and Structural Variants Defined by Complementary DNA†

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ABSTRACT: To study structural variants of human serum amyloid A (SAA), an apoprotein of high-density lipoprotein, complementary DNA clones were isolated from a human liver library with the use of two synthetic oligonucleotide mixtures containing sequences that could code for residues 33–38 and 90–95 of the protein sequence. The SAA-specific cDNA clone (pA1) contains the nucleotide sequence coding for the mature SAA and 10 amino acids of the 18-residue signal peptide. It also includes a 70 nucleotide long 3′-untranslated region and approximately 120 bases of the poly(A) tail. The derived amino acid sequence of pA1 is identical with the $\alpha$ form of apoSAA1. A fragment of pA1 containing the conserved (residues 33–38) region of SAA also hybridized with RNA from human acute phase liver and acute phase stimulated, but not unstimulated, mouse and rabbit liver. In contrast, a fragment corresponding to the variable region hybridized to a much greater extent with human than with rabbit or murine RNA. Human acute phase liver SAA mRNA ($\sim$600 nucleotides in length) directs synthesis of preSAA ($M_r$ 14000) in a cell-free translating system. In a *Xenopus* oocyte translation system preSAA is synthesized and processed to the mature $M_r$ 12000 product. The complete 18 amino acid signal peptide sequence of preSAA was derived from sequencing cDNA synthesized by "primer extension" from the region of SAA mRNA corresponding to the amino terminus of the mature product. Two other SAA-specific cDNA clones (pA6 and pA10) differed from pA1 in that they lack the internal *Pst*I restriction enzyme site spanning residues 54–56 of pA1. Thus, there are at least two SAA gene products transcribed during the acute phase response.

Serum amyloid A (SAA) is one of two major inducible human acute phase proteins. SAA is named for the insoluble $\beta$-pleated sheet fibril protein amyloid A (AA) that is probably derived from SAA by proteolysis at both its amino and carboxyl ends [as reviewed by Kushner et al. (1982), Skinner & Cohen (1983), and Kisilevsky (1983)]. AA varies in length from 45 to 83 residues and is found only in tissues. Originally the low molecular weight ($M_r$ 12000) species of SAA obtained under denaturing conditions was called SAAL (Linke et al., 1975; Benditt et al., 1980) to distinguish it from the approximately 160000-dalton SAA complex detected under physiologic conditions. However, because of association of SAA with the $HDL_3$ subclass of serum lipoproteins (Benditt & Eriksen, 1977), the term apoSAA is now frequently employed (Benditt et al., 1982; Marhaug et al., 1982).

Two major isotypes of SAA have been recognized in human, rabbit, monkey, mink, and mouse serum; in human four additional minor variants have been described (Benditt & Er-

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