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Isolation and Characterization of Oat Globulin Messenger RNA[1]

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ABSTRACT

When polyadenylated RNA, isolated from membrane-bound polysomes extracted from developing oat (*Avena sativa* L.) seeds, was translated *in vitro* in the rabbit reticulocyte system, two polypeptides of about 58 and 60 kilodaltons were immunoprecipitated by anti-oat globulin antibody. No electrophoretic bands corresponding to the 40 and 20 kilodalton polypeptides of oat globulin were present. However, when *in vivo* labeled extracts were immunoprecipitated with anti-oat globulin antibody, three groups of polypeptides (60, 40, and 20 kilodaltons) were present. It therefore seems probable that the two large polypeptides (58 and 60 kilodaltons) were precursors of the 40 and 20 kilodalton polypeptides. When the polyadenylated RNA coding for these polypeptides was size fractionated on a sucrose density gradient, it sedimented near the 18S region of the gradient. Translation of the RNA from the gradient fractions and immunoprecipitation of translation products indicated that the template for the 58 to 60 kilodalton 'putative' precursors of oat globulin was probably the RNA which was approximately 18S in size.

The oat (*Avena sativa* L.) seed is unique among cereals because most of its storage protein is the salt-soluble globulin fraction and not prolamin or glutelin (13). In this respect, oat seeds are very similar to legume seeds in which the globulin fraction is a major portion of the total seed protein. At oat seed maturity, the globulin fraction comprises greater than 50% of the total seed protein (22). The native protein has a sedimentation coefficient of 11 to 12S and a mol wt of 320 to 322 kD (21). There are six of each α- and β-polypeptides in the holoprotein. When oat globulin is analyzed by SDS-PAGE[2], there are two major polypeptide bands at 21.7 (β) and 31.7 (α) kD; in addition, there are one to three minor bands near 56 kD (21). Luthe and Peterson (18) speculated that these minor bands might be unprocessed precursors of the α- and β-polypeptides. Recently, Brinegar and Peterson (3) determined that unreduced globulin yielded electrophoretic bands of 57 to 58 kD; whereas, reduced globulin showed multiple polypeptide bands with mol wt of 22 to 23.5 kD (β) and 33 to 38 kD (α). Walburg and Larkins (27) report the same phenomenon and refer to the α- and β-polypeptides as the 20 (β) and 40 (α) kD polypeptide groups. They determined that the holoprotein consisted of six disulfide-linked α- and β-polypeptides (27). Both groups (3, 27) have determined that the α- and β-polypeptides are acidic and basic, respectively.

These properties of oat globulin are very similar to the globulin glycinin found in soybeans (*Glycine max* L.) (24), and the globulin legumin found in pea (*Pisum sativum* L.) (9). Several groups have reported that glycinin (1) and legumin (4, 23) are synthesized *in vivo* as a precursor of 60 to 63 kD. The presence of this polypeptide in *in vivo* labeled extracts analyzed by SDS-PAGE under reducing conditions suggests that the precursor consists of covalently linked α- and β-polypeptides, which apparently, requires some type of posttranslational processing before it can be dissociated by reducing agents (4). The mRNA isolated from soybean (1, 26) and pea (5) codes for a similar 60 kD polypeptide, which cannot be dissociated by reducing agents but can be immunoprecipitated by the appropriate antibodies (1, 4, 26). Walburg and Larkins (27) have found that the antibody raised against either the 40 or 20 kD polypeptide group of oat globulin will immunoprecipitate the 60 kD polypeptides when oat polysomes are translated *in vitro*. Matashewski *et al.* (19) have also immunoprecipitated a 60 kD translation product from oat polysomes by using antibody prepared against total oat globulin. Consequently, it seems likely that these 60 kD polypeptides are precursors of the 40 and 20 kD polypeptides of oat globulin.

In this paper, we report the isolation of a poly(A)-RNA fraction that is enriched for mRNA coding for a 58 to 60 kD precursor of oat globulin.

MATERIALS AND METHODS

Plant Material. Spring oats (*Avena sativa* L., var Coker 6622) were grown on the Mississippi Agriculture and Forestry Experiment Station farm during the summers of 1981 and 1982. Oats were harvested between 12 and 15 d postanthesis. The seeds were hand dehulled, and the groats (dehulled seeds) were frozen in liquid N_2 and stored at −80°C.

Isolation of Polysomes. Membrane-bound polysomes were isolated from developing oat seeds by a procedure modified from that of Larkins *et al.* (14). Frozen oat groats were ground in a mortar and pestle as liquid N_2 was added to keep groats frozen. Extraction buffer (200 mM Tris·HCl [pH 8.5], 250 mM sucrose, 60 mM KCl, 50 mM Mg acetate, 20 mM EGTA, and 5 mM β-mercaptoethanol) was added to the oats at a buffer:tissue ratio of 3:1. After thawing, the mixture was homogenized in a mortar and pestle, strained through four layers of cheesecloth, and centrifuged at 1,300*g* for 10 min. The supernatant was centrifuged at 35,000*g* for 15 min. The 35,000*g* supernatant contained free polysomes, whereas the pellet contained membrane-bound polysomes. The membrane-bound fraction was resuspended by gentle vortexing in extraction buffer containing 1% (w/v) Triton X-100. All centrifugation steps were conducted at 4°C. After centrifugation at 35,000*g* for 10 min to sediment any insoluble material, both polysome fractions were layered over 8 ml of 1.5 M sucrose in 40 mM Tris·HCl (pH 8.5), 40 mM KCl, and 10 mM Mg acetate and pelleted by centrifugation at 88,000*g* in a Beckman Type 35 rotor for 3 h. The pellets were gently rinsed with double distilled H_2O, resuspended in buffer containing 10 mM Hepes (pH 7.6), 20 mM KCl, 1 mM Mg acetate, and 20% (v/v) glycerol, and stored at −80°C. For sucrose gradient analysis, 2 to 6 A_{260} units of polysomes were layered over a 10 to 40% (w/v) linear sucrose gradient

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[2] Abbreviations: PAGE, polyacrylamide gel electrophoresis; poly(A)-RNA, polyadenylated RNA; IgG, immunoglobulin G.

prepared in the same buffer as the 1.5 M sucrose (see above) and centrifuged in a Beckman SW 50.1 rotor at 120,000*g* for 90 min. The gradients were fractionated with an Isco model 185 Gradient Fractionator and scanned at 254 nm with a UA-5 Isco Absorbance Monitor.

Isolation of Poly(A)-RNA. Poly(A)-RNA was isolated from membrane-bound polysomes by affinity chromatography on oligo(dT)-cellulose (Collaborative Research Inc.) according to Tumer *et al.* (25) except that protease K was omitted. After ethanol precipitation, the RNA was dried under N_2 and dissolved in a small volume of sterile distilled H_2O. Prior to sucrose gradient analysis, the poly(A)-RNA (100 μg) was heated to 60°C for 4 min, rapidly chilled to 4°C, and layered on a linear sucrose gradient (10–40% [w/v] in 0.1 M NaCl, 10 mM Tris·HCl, [pH 7.5], 1 mM EDTA) gradient and centrifuged at 57,000*g* for 15 h in a SW 50.1 Beckman rotor. The gradient was fractionated as described above. Carrier tRNA (30 μg) was added to each fraction prior to ethanol precipitation. Dried RNA pellets were dissolved in 50 μl sterile distilled H_2O, and 10-μl aliquots from each fraction were electrophoresed on 1.5-mm-thick agarose (1.5% w/v) gels containing 5 mM methylmercuric hydroxide (Alfa Div., Ventron Corp.) (15). Electrophoresis was carried out at 7 v/cm until the bromphenol blue tracking dye reached the bottom of the gel. Gels were stained for 30 min in 1 μg/ml ethidium bromide in 0.5 M ammonium acetate.

***In Vitro* Translation.** Poly(A)-RNA was translated in rabbit reticulocyte lysate (Amersham) as suggested by the manufacturer. A typical reaction mixture (25 μl) contained: 70% (v/v) reticulocyte lysate (amino acid-depleted, nuclease-treated), 50 μM amino acids (minus methionine), 116 mM KCl, 1.6 mM Mg acetate, and 1.1 μCi [^{35}S]methionine (1157 Ci/mmol). The reaction was initiated by the addition of poly(A)-RNA (0.4–1 μg) and was incubated at 30°C for 60 min. Aliquots (2.5 μl) were removed at the appropriate time intervals and TCA-washed (18). When translation products were immunoprecipitated, the reaction was stopped by the addition of 1 ml of NET-2 buffer (50 mM Tris·HCl [pH 7.5], 0.15 M NaCl, 0.05% [v/v] Nonidet P40). The reaction mixture was 'precleared' by adding 100 μl IgG Sorb (The Enzyme Center, Boston, MA), and incubating at room temperature for 30 min. After centrifugation at 13,000*g* for 1 min in an Eppendorf Microfuge (Brinkman), an IgG fraction (300 μg) containing anti-globulin IgG (see below) was added to the precleared supernatant and incubated for 1 h at 37°C. The IgG-antigen complex was precipitated with 180 μl IgG Sorb (binding capacity, 1.74 mg IgG/ml). After incubation for 1 h at 4°C, the complex was pelleted by centrifugation at 13,000*g* for 1 min. The pellets were washed five times with NET-2 buffer and once with water. The pellet was dissolved in sample buffer (12), boiled for 10 min, and centrifuged. The supernatant was analyzed on 10% (w/v) polyacrylamide gels (12) with a 5% (w/v) stacking gel. Radioactive bands were visualized by fluorography in 1 M Na salicylate (6).

Isolation of Authentic Oat Globulin. Defatted oat flour (Coker 6622) was homogenized in 10 volumes of water with a Tekmar Tissumizer at full speed for 15 s. The homogenate was stirred at room temperature for 2 h to remove the albumin fraction. After centrifugation at 27,000*g* for 30 min at room temperature, the supernatant (albumin) was discarded. The pellet was extracted in globulin extraction buffer (1 M NaCl, 50 mM Tris·HCl [pH 8.5], and 1% (v/v) β-mercaptoethanol) (21). After stirring for 2 h at room temperature, the suspension was centrifuged at 27,000*g* for 30 min at room temperature. The supernatant (globulin) was dialyzed overnight (4°C) against several changes of distilled H_2O and lyophilized. The amount of protein in the globulin extract was determined by the Lowry method (17). The pattern of protein analyzed by SDS-PAGE is shown in Figure 1A.

Preparation of Anti-Oat Globulin IgG. Globulin was dissolved at protein concentration of 1 mg/ml in 50 mM Tris·HCl (pH 7.5) containing 200 mM NaCl. After samples of blood for preimmune serum were removed, two rabbits (a California-New Zealand cross) were injected intramuscularly with 1 mg globulin in Freund's complete adjuvant. Subsequently, the rabbits were injected at 1-month intervals with 1 mg globulin in Freund's incomplete adjuvant. When the titer reached 1/16, the rabbits were exsanguinated by cardiac puncture. Serum was prepared by standard immunological methods (11). The IgG fraction was purified using the DEAE-Affigel Blue (Bio-Rad) method (2). The IgG fraction cross-reacted with globulin extracted from Coker and Goodland oats; but it did not cross-react with albumin, prolamin, or glutelin fractions extracted from Coker oats when analyzed by the Ouchterlony double-diffusion assay.

Immunoprecipitation of *In Vivo* Labeled Globulin. Two panicles harvested 12 d postanthesis were labeled with 0.4 mCi [^{35}S]sulfate (43 Ci/mg S) in 2 ml of H_2O. After the solution was taken up by the panicles (2 h), the label was chased with sterile distilled H_2O for 2 h. Seeds were removed from the panicle, frozen in liquid N_2, and lyophilized. Five seeds were homogenized in 2 ml of seed extraction buffer (20), and centrifuged at 13,000*g*. An aliquot of the supernatant (equivalent to 300,000 TCA-insoluble cpm) was added to buffer A (16) to make a final volume of 1.4 ml. The IgG fraction (250 μg) was then added. After incubation for 1 h at 37°C and 24 h at 4°C, IgG Sorb was added and the mixture was incubated at 4°C for 1 h. The precipitate was washed and prepared for electrophoresis and fluorography as described above.

RESULTS AND DISCUSSION

Membrane-bound polysomes from oat groats were used for the isolation of poly(A)-RNA. Membrane-bound polysomes were chosen because they were less degraded than free polysomes and because it is likely that oat globulin is sequestered in protein bodies (10). Poly(A)-RNA isolated from membrane-bound polysomes was about five times more active in the wheat germ translation system than poly(A)-RNA from free polysomes. In addition, when the template was poly(A)-RNA from membrane-bound polysomes about 14-fold more counts were immunoprecipitated by anti-oat globulin IgG fraction than when an equivalent amount of poly(A)-RNA from free polysomes was translated (Luthe, unpublished data).

Because oat globulin comprises about 75 to 80% (Luthe, unpublished data) of the protein synthesized between 12 and 15 d postanthesis, we expected the poly(A)-RNA population to be enriched for oat globulin mRNA. When poly(A)-RNA collected from one pass over oligo(dT)-cellulose was analyzed on methylmercuric hydroxide gels, there were two bands: one migrating slightly faster than 18S RNA and another comigrating with 26S RNA. It appeared that there were no other distinct RNA species. After three passes over oligo(dT)-cellulose, the 26S rRNA band had greatly diminished in intensity, but there appeared to be less diminution of the band migrating slightly faster than 18S RNA. Because the mRNA for glycinin was about 18S in size (26), we felt that RNA migrating near 18S might be a good candidate for oat globulin mRNA.

To test this, the poly(A)-RNA fraction was translated in the reticulocyte lysate system. The immunoprecipitated translation product of poly(A)-RNA from membrane-bound polysomes is shown in Figure 1C. The major translation products were two polypeptides with mol wt of 58 and 60 kD. These *in vitro* products appeared to be close to the same size as the large polypeptides (58 and 60 kD) of authentic globulin (Fig. 1A). No peptides corresponding to the 40 and 20 kD polypeptides were labeled *in vitro*. To test the unlikely situation that the antibody prepared against unreduced oat globulin was only specific for 58 to 60 kD polypeptides and, consequently, would not precipitate the 40 and 20 kD polypeptides, the antibody was added to an *in vivo* labeled seed extract (Fig. 1B). Both the 40 and 20 kD polypeptides were indeed

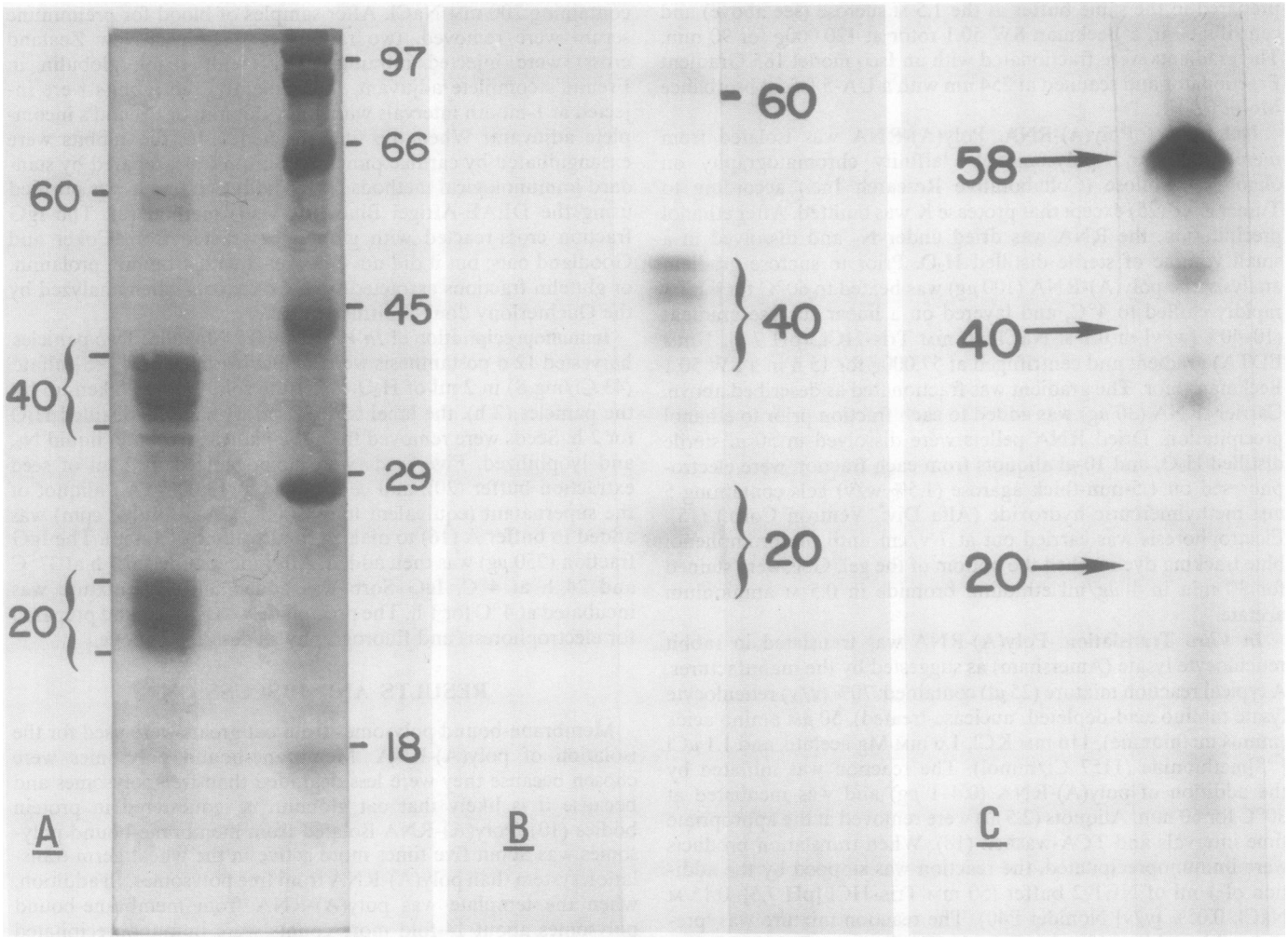

FIG. 1. SDS-PAGE of authentic oat globulin, and immunoprecipitated products from the *in vivo* labeled seed extract and the *in vitro* translation of poly(A)-RNA. A, Authentic oat globulin. Markers refer to the 60 kD pair of polypeptides with mol wt of 58 and 60 kD; the 40 kD polypeptides (α) with mol wt range of 36 to 40 kD; and the 20 kD polypeptides (β) with mol wt range of 20 to 23 kD. The right lane contains mol wt markers; there is no sample in the middle lane. B, Immunoprecipitated *in vivo* labeled polypeptides. C, Immunoprecipitated *in vitro* translation products. Markers refer to the migration of authentic globulin on the same gel. Radioactive polypeptides were visualized by fluorography in Na salicylate (B and C).

immunoprecipitated. A small amount of a 60 kD polypeptide was also immunoprecipitated. If the seeds had been pulse-labeled and the label not chased, it may have been possible to detect the unprocessed 60 kD precursor. In two other cases (19, 27), it has been shown that the *in vitro* translation products of oat polysomes were 60 kD polypeptides, and not the 40 and 20 kD polypeptides. The results are in conflict with those of Luthe and Peterson (18), who suggested that both α- and β-polypeptides were synthesized when oat polysomes were translated *in vitro*. However, in that case, the translation products were not identified by immunoprecipitation. They did see, however, a relatively large number of counts incorporated into protein that comigrated with the large (56–58 kD) polypeptides (18).

It is interesting to compare the results for oat globulin with those for glycinin (1, 25) and legumin (12). In both cases, the 40 and 20 kD polypeptides were labeled *in vivo* but not *in vitro*. This suggests that some posttranslational processing must occur before reducing conditions will dissociate the precursors. Recently, it was shown that glycinin had a 1 to 2 kD 'leader peptide' removed from the N-terminus when its mRNA was translated in the oocyte system (25). The remaining polypeptide consisted of the 40 and 20 kD polypeptides connected by a small peptide (25). The authors (25) speculate that another processing step must cleave the 'precursor' before the 20 and 40 kD polypeptides could be seen under reducing conditions. Since all samples (Fig. 1, A–C) were boiled in sample buffer (12) containing β-mercaptoethanol prior to electrophoresis, it seems possible that the 58 to 60 kD polypeptides seen in the authentic globulin (Fig. 1A), in the *in vivo* labeled globulin (Fig. 1B), and in the *in vitro* translation products (Fig. 1C) may also be unprocessed precursors of oat globulin.

To determine which size class of poly(A)-RNA directed the synthesis of the 58 to 60 kD polypeptide, the poly(A)-RNA from membrane-bound polysomes was centrifuged on a linear (10–40% w/v) sucrose gradient (Fig. 2A). The RNA sedimented as a broad band with a peak slightly less than 18S. When fractions (6–15) from the gradient were translated in the reticulocyte system (but not immunoprecipitated), the results in the histogram (Fig. 2A) were obtained. Fractions 11 and 12 were the most active in the translation system. The immunoprecipitated translation products of the fractions in Figure 2A are shown in Figure 2B. Two polypeptides with mol wt of 58 and 60 kD were present in the translation products of fractions 9 to 14. The polypeptides were most abundant in fractions 11 and 12. Again, no polypeptides of 40 and 20 kD were present. The small immunoprecipitated peptides in Figures 1B and 2B may be the result of the translation of small RNA fragments.

When aliquots of the gradient fractions were analyzed on methylmercuric hydroxide gel, the results in Figure 2C were

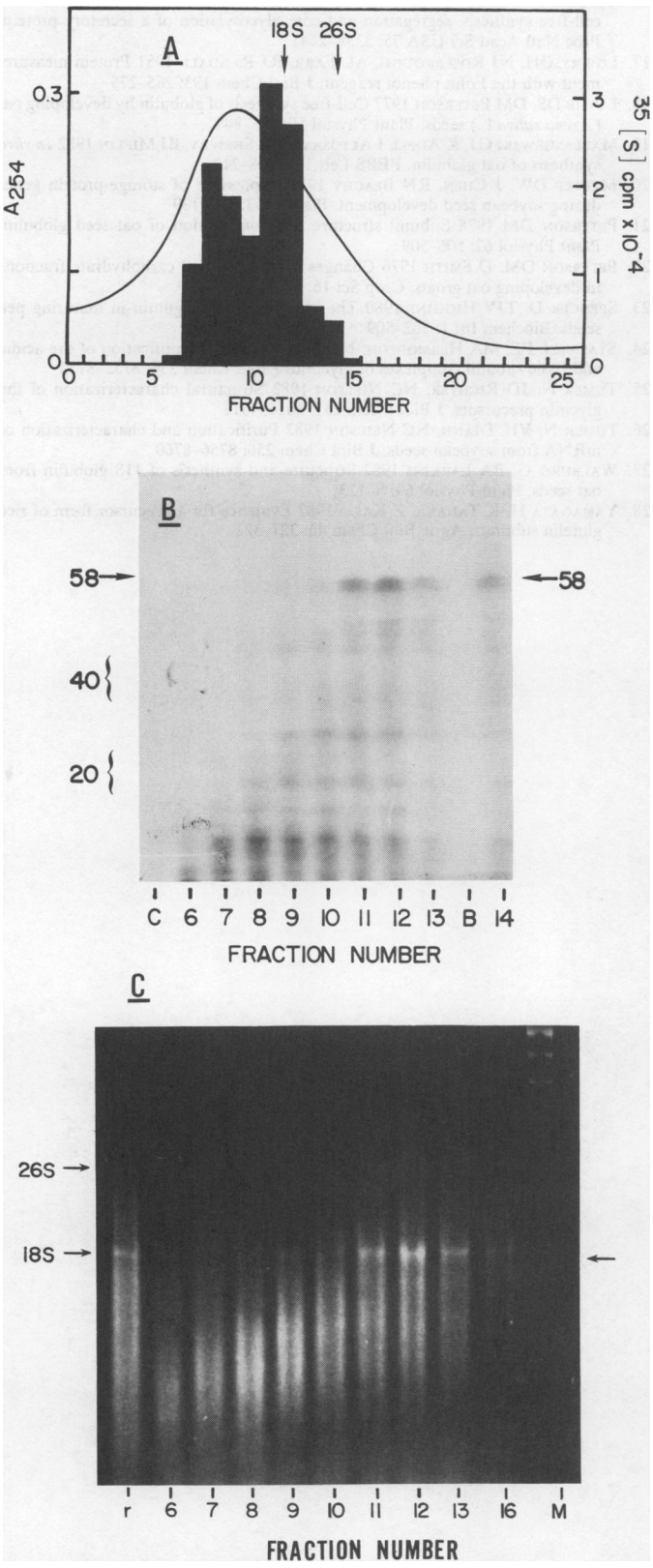

FIG. 2. Sucrose gradient analysis of oat poly(A)-RNA. A, Poly(A)-RNA (100 μg) isolated after three cycles of oligo(dT)-cellulose chromatography was centrifuged and fractionated as described in "Materials and Methods." Equivalent volumes of RNA from the peak fractions were translated (but not immunoprecipitated) in the reticulocyte system (histogram). Counts in histogram were corrected for zero time, and minus RNA controls. Markers refer to 18S and 26S rRNA centrifuged on a parallel gradient. B, Immunoprecipitated products from the *in vitro* translation of RNA from the sucrose gradient. Aliquots of gradient fractions were translated, immunoprecipitated, and analyzed by SDS-PAGE as described in "Materials and Methods." Products were visualized by fluorography. (Lane C), Control (no mRNA); (lane B), blank; remaining lanes are numbered as respective gradient fractions. C, Analysis of poly(A)-RNA fractions from the sucrose gradient (A) on methylmercuriç hydroxide agarose gel. Aliquots of RNA from each gradient fraction were electrophoresed as described in "Materials and Methods." (Lane r), rRNA standard; (lane M), λ-DNA digested with Hind III; remaining lanes are numbered as respective gradient fractions.

obtained. Fractions 11 and 12 were enriched for RNA migrating near the 18S RNA. Translation of RNA from these fractions also produced the most intense 58 to 60 kD polypeptide bands (Fig. 2B). Although we cannot rule out the possibility that the 18S RNA is rRNA, it seems likely that some RNA species, sedimenting in the 18S region of the gradient, and abundant in fractions 11 and 12, codes for the 58 to 60 kD precursor. If one assumes that 18S RNA contains about 1,950 nucleotides (26) and that a 60 kD polypeptide contains about 500 amino acids, a mRNA of 1,950 nucleotides should be large enough to code for a 60 kD polypeptide with about 450 nucleotides remaining for noncoding and poly(A) sequences. The length of glycinin mRNA has been reported to be between 1,950 (26) and 2,050 (1) nucleotides; legumin mRNA was reported to be 2,200 nucleotides (5). Larkins and Walburg (personal communication) have also found poly(A)-RNA which codes for oat globulin precursor to be slightly smaller than 18S.

The precursor-product relationship between the 60 kD and the 40 and 20 kD polypeptides of oat globulin still needs to be rigorously proven. However, data from several laboratories (3, 19, 27), immunoprecipitation data (Fig. 1), and the analogy with the legume globulins (1, 4, 5, 7, 8, 23, 25–27) provides some evidence that the 60 kD polypeptides are precursors of oat globulin. It is not known if the precursor is synthesized on ER and then transported to protein bodies as is the case for legumin in pea (7, 8). The fact that membrane-bound polysomes are enriched for the mRNA for the precursor suggests that either the ER or protein body fraction is the site of synthesis.

We find it interesting that oats, a member of the *Gramineae*, have a major storage protein fraction and, possibly, a protein processing mechanism very similar to that found in the *Leguminosae*. It has been reported that the 38 and 22 kD polypeptides of rice glutelin arise from a 57 kD precursor (28). It appears that this mechanism of storage protein processing may be highly conserved throughout evolution.

Note Added in Proof. After the revised manuscript was accepted for publication, we became aware of the following two references, which also pertain to oat globulin. (1) Brinegar AC, DM Peterson 1982 Synthesis of oat globulin precursors. An analogy to legume 11S storage protein synthesis. Plant Physiol 70: 1767–1769. (2) Brinegar AC, DM Peterson 1982 Separation and characterization of oat globulin polypeptides. Arch Biochem Biophys 219: 71–79.

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