Evidence for the Translational Control of Storage Protein Gene Expression in Oat Seeds*

(Received for publication, January 23, 1992)

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We employed a rapid fractionation method coupled with a sensitive enzyme-linked immunosorbent assay to quantify the globulins and avenins in developing and mature oat seeds. On a molar basis, there is approximately 10–11 times as much globulin as avenin. Pulse labeling of endosperm proteins indicated that the rate of globulin synthesis is approximately nine times that of avenin. In addition, neither protein class showed any signs of degradation during this experiment. Analysis of the storage protein mRNAs indicates that both globulin and avenin transcripts are associated with membrane-bound polysomes and are found in similar concentrations within the membrane-bound polysome fraction. We found that avenin and globulin mRNAs are fully loaded with ribosomes, suggesting that initiation is not rate-limiting for translation of either protein. Rates of globulin and avenin synthesis were similar when synthetic storage protein mRNAs were translated in vitro. Translation of equimolar amounts of globulin and avenin mRNAs in the same reaction showed equivalent amounts of protein synthesized when compared with globulin and avenin mRNAs translated in separate reaction mixes. We propose that translation elongation or termination reactions are likely regulatory steps for controlling storage protein synthesis in oat endosperm.

Of all the cereals, oats are the best source of dietary protein. This is because the protein content of oat seeds is higher than other cereals (about 15 versus 12% in wheat and 10% in maize), and because the storage protein composition of oats is different from most other cereals. Approximately 70–85% of the protein in an oat seed is a globulin (1, 2) that is structurally similar to the storage globulins of legumes (3). Less than 15% of the seed protein is prolamine (called avenin), compared with 50–60% prolamine in most other cereals. The lower nutritional value of most cereals is a consequence of their high prolamine content, since this protein fraction is deficient in the essential amino acids lysine and tryptophan. Thus, the higher protein quality of oat seeds results from their high-globulin low-prolamine composition, which is in turn determined by the regulation of avenin and globulin gene expression during endosperm development.

The oat globulin shares structural features with the 11 S globulins of legumes and other dicots, although it is much less soluble, requiring almost 1.0 M sodium chloride (4). Sequence analysis of globulin cDNA and genomic clones has revealed details of the structure of these proteins (5). The globulin genes encode a precursor molecule of 518 amino acids, which includes a 24-amino acid signal peptide, a 289-amino acid acidic polypeptide (33 kDa, pI 6.5), and a 201-amino acid basic polypeptide (23 kDa, pI 9.0). By analogy with other storage globulins and the work of Walburg and Larkins (6), we hypothesize that the globulin protein is synthesized as a 55-kDa precursor whose signal sequence targets the protein to the endoplasmic reticulum. The protein is then proteolytically processed into the 35- and 23-kDa subunits, which are linked via disulfide bonds, before deposition in protein bodies.

Avenins, the prolamine proteins of oats, are a group of alcohol-soluble proteins of 10–23 kDa. Kim et al. (8) described three subfamilies within the avenin fraction called α, β, and γ, with γ being the most abundant. The avenin cDNA clones we isolated appear to be representative of the complexity of the γ-avenin polypeptides (5, 7). Despite the conservation of polypeptide structure, the avenin cDNA clones encode polypeptides ranging in molecular mass from 18.4 to 23.5 kDa. This size heterogeneity was also observed after separation of avenin polypeptides by polyacrylamide gel electrophoresis.

We determined the number of genes encoding the avenin and globulin proteins in various oat species and characterized the expression of these genes during endosperm development (7). Although there are about twice as many globulin genes as avenin genes (50 versus 25 per haploid genome), the steady-state concentration of avenin mRNAs is approximately equal to that of globulin mRNAs. This observation is surprising in light of the reports of 6- to 7-fold higher amounts of globulin than avenin protein in mature seeds (1, 2, 17). These results suggest that the differential accumulation of storage proteins in oats is determined primarily at the level of translation. Therefore, we established the rates of synthesis of the two classes of seed storage protein in vivo, determined whether protein turnover plays a role in the accumulation of storage proteins, and analyzed how translation affects the globulin to avenin ratio found in oat endosperm.

MATERIALS AND METHODS

Growth and Harvesting of Plant Materials—Avena sativa L. cv. Noble were grown in a greenhouse with supplemental lighting for 16 h a day. Developing (8–10 days after anthesis (DAA)) and mature (desiccated seed) florets were harvested and immediately frozen in liquid nitrogen, except for those used in the radioactive labeling of seed proteins. Plants used for the pulse labeling of seed proteins were treated the same way except that the day before the experiment the plants were transferred to a 25 °C growth chamber where both incandescent and fluorescent lighting was provided for 16 h a day.

Analysis of Storage Protein Content in Developing Oat Seeds—

The abbreviations used are: DAA, days after anthesis; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; PCI, phenol:chloroform: isoamyl alcohol; PCR, polymerase chain reaction.

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Vol. 267, No. 24, Issue of August 25, pp. 17449-17457, 1992
Printed in U.S.A.
Total protein extracts were prepared by the procedure of Wallace et al. (9) with the following modifications. Flour was obtained by grinding dehulled oat groats of 8-10 DAA in liquid N\textsubscript{2}, defatting by 3 \times extraction with 10 volumes of ice-cold acetone, and lyophilizing until dry. Samples (5 mg) were digested only once, the digestes mixed with extraction buffer (0.0125 M sodium borate, pH 8.0, 1% sodium dodecyl sulfate (SDS), 2% mercaptoethanol). It was determined that additional digestions did not significantly improve the protein yield (data not shown).

The concentration of protein in these samples was determined after precipitating with trichloroacetic acid and resuspending the pellets in 0.1 N sodium hydroxide, 1% SDS. The protein concentration was estimated with a BCA protein determination kit from Pierce Chemical Co.

Storage proteins were separated into avenin and non-avenin fractions by starch gel electrophoresis. The protein concentration was adjusted to 70% ethanol and 2% mercaptoethanol, on ice for 1 h, and centrifuged at 10,000 \times g for 10 min in a microcentrifuge. The supernatant, which contained the alcohol-soluble avenins, was transferred to a new tube. Following lyophilization of both the pellet (non-avenin) and supernatant, the proteins were resuspended in an equal volume of extraction buffer and analyzed by SDS-PAGE and immunoblotting as described by Laemmli (10) and Knecht and Diamond (11), respectively.

The concentration of storage globulin in total protein extracts and non-avenin fractions was determined by ELISA analysis using a standard curve prepared with purified globulin protein. Similar to the concentration of avenin was determined by ELISA analysis of avenin fractions using a standard curve prepared with purified avenin protein. Isolation of purified avenin and globulin proteins and preparation of globulin- and avenin-specific antibodies were as described by Larkins et al. (12).

Pulse Labeling of Oat Seed Storage Proteins—Radiolabeling of the storage proteins in developing oat seeds was based on the procedure of Luthe (12). Individual 8-10 DAA florets (20 florets per time point) were cut from plants, immediately transferred to 1.5-ml microcentrifuge tubes containing 8 ml of \( ^{35} \text{S} \)SO\textsubscript{4} (2 mCi/ml, 929.8 Ci/mol, Amersham Chemical Co.) and incubated in the greenhouse for 2 h at 25 \textdegree C with continuous light. After this 2-h pulse, the florets were dipped in 250 ml of sterile water and blotted dry (to remove any radioactivity on the outside of the floret) and either frozen in liquid N\textsubscript{2} or transferred to new tubes containing 100 ml of Murashige and Skoog salts (GIBCO) 2% sucrose and incubated 5 days (4-, 8-, or 24-h chase samples, respectively). From the chase time points, the florets were cut from the time points were rinsed and frozen in liquid N\textsubscript{2} as described for the pulse time point.

After samples from all of the time points were gathered, the glumes were removed, the primary seeds were removed and defatted. The seeds for each time point were then ground to a fine flour in liquid N\textsubscript{2}, defatted as described previously, and either total protein or globulins and avenins extracted. Total protein, non-avenin, and avenin fractions were prepared as described in the previous section. Globulin and avenin were purified as described by Chesnut et al. (7). The purified avenin and globulin were radiolabeled in the greenhouse for 2 h at 25 \textdegree C with continuous light. After this 2-h pulse, the florets were transferred to new tubes containing 100 ml of Murashige and Skoog salts (GIBCO) 2% sucrose and incubated for an additional 4, 8, or 24 h (4-, 8-, or 24-h chase samples, respectively). From the chase time points, the florets were cut from the time points, and the proteins were resuspended in diethyl pyrocarbonate-treated water. RNA samples were resuspended in diethyl pyrocarbonate-treated water and the concentration determined by absorbance at 260 nm. Quantiﬁcation of storage protein mRNA was performed by Northern blot analysis as described by Chesnut et al. (7), except that the radioactivity bound to dot-blots ﬁlters was measured using the Betascan" (Betagen, Waltham, MA).

Distribution of Ribosomes on Storage Protein mRNAs—Membrane-bound polysomes were separated by size on continuous sucrose gradient as described by Larkins (14). Fractions of 0.4 ml were collected, extracted with PCI, precipitated with ammonium acetate and ethanol, and resuspended in diethyl pyrocarbonate-treated water. RNA samples were applied to duplicate dot-blot membranes as described by Chesnut et al. (7). After autoradiography, the dots were excised from the filters and radioactivity measured by liquid scintillation counting to determine the relative amount of globulin or avenin mRNA in each fraction.

Construction of Plasmids for in vitro Transcription of Storage Protein mRNAs—The pT7A18 plasmid was constructed such that transcripts synthesized in vitro would resemble their in vivo counterparts with the introduction of a minimum number of extraneous nucleotides. This plasmid contains a T7-prm&eacute;phore and a poly(A) tail in a pUC18-based plasmid (Fig. 6A). A double-stranded oligonucleotide containing the T7 promoter modified to incorporate a Stul restriction site was ligated into HindIII-digested pUC18 to create the plasmid pUC7A18. A polynucleotide- and avenin cDNA clone pAVT16. This cDNA clone was used as a template for polymerase chain reaction (PCR) amplification of a 65-residue polyadenylation sequence (pA65). The primers used in the PCR amplification incorporated Stul and EcoRI sites. The pA65 PCR product and the pT7e18 plasmid were digested with Stul and EcoRI and ligated to form the plasmid pT7A18 (see Fig. 6A). The Stul and SstI sites were used for the insertion of globulin and avenin DNA fragments. Cloning into the Stul restriction site adds only one guanosine to the 5' end of the transcript; therefore, in vitro transcripts have only one additional nucleotide at the 5' end when comparing them to their respective in vivo mRNAs.

Three different avenin mRNA-encoding plasmids were constructed by PCR amplification of avenin genomic sequences. Gene-specific primers were designed to produce an avenin-encoding DNA fragment beginning at the transcription start site and ending at the "poly(A)" addition site. The 5' primer produced a blunt-end for cloning into the Stul site, and the 3' primer encoded the Stul restriction site used in the directional cloning of amplified DNA fragments into the pT7A18 plasmid. The three avenin transcription plasmids designated pAVT6, pAVT21, and pAVT32 were derived from the avenin gene mRNAs (see Fig. 6C). The pGFT2 plasmid was used to generate in vitro transcripts representative of globulin mRNAs.

In vitro transcription reactions were performed as described in the Protocols and Applications Guide (Promega Corp., Madison, WI) with the following exceptions. Following the final PCR extraction, the transcripts were precipitated with 0.5 volumes of 1 M ammonium acetate and 2 volumes of ethanol. The resulting RNA was resuspended in diethyl pyrocarbonate-treated water. Synthetic mRNAs were not "capped," since we found that capped RNAs were not required for efficient translation in the wheat germ cell-free translation system (data not shown).

In Vitro Synthesis of Oat Seed Storage Proteins—The synthetic avenin and globulin mRNAs were translated in a wheat germ cell-free translation system (Promega Corp.) according to the manufacturer's instructions using [\(^{35} \text{S}\) ]methionine (800 Ci/mmol, Amersham Chemical Co.) and sodium monovalent ions. The in vitro rates of synthesis of avenin and globulin proteins were determined as follows. Identical 50-ml reactions were prepared...
with equimolar amounts of either avenin or globulin synthetic mRNAs. Portions (5 μl) were removed to separate tubes containing 2.5 μl of 3 x sample buffer (1 x = 62.5 mM Tris, pH 6.8, 2.4% SDS, 10% glycerol, 0.05% 2-mercaptoethanol, and 0.1% bromphenol blue), mixed, and placed on ice at 0, 5, 10, 15, 20, 25, 30, 45, and 60 min. Samples from each time point were either subjected to SDS-PAGE and fluorography or analyzed to determine the incorporation of [35S]methionine into storage protein. The incorporation of [35S]methionine into protein was determined by spotting duplicate filters with portions of the samples from each time point and boiling the filters in trichloroacetic acid as described previously (15). The percent incorporation was determined from the trichloroacetic acid-insoluble counts (incorporated counts) and the total counts (spotted filters that were not subjected to trichloroacetic acid treatment). Since avenin transcripts encode more methionine than globulin transcripts, the percent incorporation values were corrected for the percent methionine (as determined from the deduced amino acid sequence) and plotted against time.

To determine whether the simultaneous translation of avenin and globulin transcripts would affect the yield of each protein, equimolar amounts (1 picomol) of the two mRNAs were translated in vitro and the polypeptide products separated by SDS-PAGE. Two control reactions contained either 1 picomol of avenin mRNA or 1 picomol of globulin mRNA. The third reaction contained 1 picomol each of avenin and globulin mRNAs, in order to determine whether the two mRNAs would compete for “translation factors.” All three reactions contained 100 mM potassium acetate. The products from these reactions were analyzed by SDS-PAGE and fluorography.

**RESULTS**

**Determination of the Ratio of Globulin to Avenin in Developing and Mature Oat Seeds**—The quantity of avenin and globulin in oat seeds is traditionally estimated by measuring the weight or nitrogen content of these protein fractions (1, 2). We attempted to simplify this by using an ELISA-based procedure following separation of avenin and globulin fractions. Total protein was prepared from flour of developing (8-10 DAA, mid-development) and mature (dry) grains by extraction with SDS-borate buffer containing 2-mercaptoethanol. The extract was then adjusted to 70% ethanol to precipitate non-avenin proteins. Analysis of the resulting fractions showed that only avenins are soluble in ethanol; the globulins are among the ethanol-insoluble proteins (Fig. 1). Immuno-blotting with anti-globulin (Fig. 1B) and anti-avenin (Fig. 1C) antibodies revealed that a small amount of avenin contaminated the alcohol-insoluble proteins, but no globulin contaminated the alcohol-soluble proteins. We also detected a trace amount of nonspecific binding of avenin antibodies to bands that appear to comigrate with globulin polypeptides in the total protein and non-avenin protein fractions (Fig. 1C, lanes T and N, respectively). This nonspecific binding is presumably due to the large amount of globulin present in total protein extracts and non-avenin protein fractions.

We estimated the amount of avenin and globulin in developing and mature seeds by comparing the intensity of the ELISA reaction from the appropriate protein fraction with a standard curve constructed with known amounts of purified protein. Roughly equal concentrations of globulin were found in developing and mature seeds, when comparisons were made of equal amounts of total protein. In addition, no significant differences in quantity of globulin were observed when comparing total protein extracts to non-avenin protein fractions (data not shown). Concentrations of avenin in developing and mature seeds were also comparable. As shown in Table I, the ratio of globulin to avenin is similar in developing and mature seeds when expressed on either a weight or molar basis.

**Synthesis and Stability of Storage Proteins in Oat Seeds**—Several studies have shown that both globulins and avenins accumulate simultaneously in developing seeds (7, 12); however, there are no data regarding their synthesis and stability. Because these factors determine the ratio of avenin and globulin in the mature seed, we measured the rate of avenin and globulin synthesis in vivo.

We investigated the relative rates of avenin and globulin synthesis in developing oat seeds by radiolabeling the storage proteins in detached florets with [35S]SO4. Individual florets were cut from the plants at 8-10 DAA and incubated in [35S]SO4 for 2 h, after which the florets were transferred to nutrient media for 4 to 24 h. Avenins and globulins were subsequently extracted from these seeds, and the percent incorporation of [35S]into protein was determined. After correcting the incorporation values for the relative content of sulfur-containing amino acids in each protein, the rate of synthesis of avenins and globulins was determined by plotting the normalized percent incorporation against time. The kinetics of radioactivity incorporated into storage proteins is shown in Fig. 2. The rates of incorporation of radioactivity into both avenin and globulin fractions was linear until the 4th h of chase, after which the rates declined. A comparison of the incorporation rates showed an 8-9-fold higher amount of globulin than avenin synthesis.

**Table I**

| Globulin:avenin ratio | Developing seeda | Mature seedb |
|-----------------------|------------------|--------------|
| Weight basis          | 29:1             | 26:1         |
| Molar basisc          | 11:1             | 10:1         |

|                          | Developing seed  | Mature seed  |
|-------------------------|------------------|--------------|
| Weight basis            | 29:1             | 26:1         |
| Molar basis             | 11:1             | 10:1         |

a Developing seeds (8-10 DAA).

b Mature seeds (dry seeds).

c Estimated using Mf values of 55,000 for globulin and 20,000 for avenin.
In addition to the globulin precursor, other high molecular weight proteins were labeled, and they also appeared to accumulate throughout the experiment. Since no degradation of either avenin or globulin was observed, we assumed that both avenin and globulin proteins are stable following synthesis. The apparent stability of both protein classes and the 8–9-fold higher amount of globulin than avenin synthesis suggests that translation of the two proteins dictates the high globulin to avenin ratio.

**In Vivo Distribution of Avenin and Globulin mRNAs—**Roughly equivalent amounts of avenin and globulin mRNAs were found in total RNA of developing oat seeds (7), but the subcellular distribution of these mRNAs was not determined. Both proteins are synthesized on rough endoplasmic reticulum; consequently, the efficiency of loading of avenin and globulin mRNAs into membrane-bound polysomes could influence the rate of protein synthesis. To investigate the subcellular distribution of avenin and globulin mRNAs, total RNA was isolated from whole tissue, free polysomes, and membrane-bound polysomes. The RNA samples were immobilized on nylon membranes using a dot-blot apparatus and hybridized to either an avenin or a globulin cDNA clone (Fig. 4, A and B, respectively). Quantitation of each type of mRNA (Table II) was based on reconstruction hybridization with mRNAs transcribed in vitro. The standard curves generated in these experiments were linear for both avenin and globulin standards (correlation coefficients of 0.998 for both). Similar concentrations of avenin and globulin mRNA were observed in total RNA samples prepared from whole seeds (less than a 2-fold difference was observed). Very little avenin mRNA or globulin mRNA was found in free polysomes. The majority of avenin and globulin mRNA was associated with membrane-bound polysomes, and within this fraction, both storage protein mRNAs were found in comparable concentrations. However, when a combination of two avenin cDNAs (pAV10 and

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**Fig. 2. In vivo rates of oat storage protein synthesis.** Pulse-labeled proteins were extracted from developing oat seeds and analyzed for the percent of the total 35S incorporated. These values were normalized using the percent of sulfur-containing amino acids deduced from avenin and globulin DNA sequences. Time points are hours of chase; the 0 h of chase samples were taken after the 2-h pulse labeling.

**Fig. 3. SDS-PAGE of pulse-labeled oat storage proteins synthesized in vivo.** Oat seed proteins were pulse-labeled with 35S, extracted with the appropriate buffer, separated by SDS-PAGE, and subjected to fluorography. Identical amounts of flour were extracted for each time point, and the gels were loaded with equal volumes of the appropriate extract. The fluorograms show the alcohol-soluble avenin proteins in A and the saline-soluble globulin proteins in B. Lanes P show proteins extracted after 2 h of pulse labeling; lanes 4, 8, and 24 show proteins extracted after 4, 8, or 24 h of chase, respectively. Lanes M contains 14C-labeled molecular mass standards.

Avenin and globulin protein stability was analyzed by SDS-PAGE of the pulse-labeled proteins (Fig. 3). The radioactivity incorporated into proteins in the avenin fraction increased throughout the experiment. Bands corresponding to α, β, and γ avenins were detected, with the γ avenins most intensely labeled. Although some labeled proteins were detected after the 2-h pulse, most of the label was incorporated into protein by 4 h. Some alcohol-soluble high-molecular weight bands present after 4 h of chase disappeared by 24 h; these bands do not correspond to avenin proteins, however. The bands corresponding to mature avenin proteins all increased in intensity throughout the chase period, suggesting that the disappearance of the high molecular weight bands may correspond to protein processing rather than degradation.

The incorporation of 35S into globulin proteins was similar to that of avenin proteins. After 2 h of labeling, only the high-molecular weight globulin precursor was evident. This protein was present throughout the experiment, but by 4 h of chase the processed acidic and basic polypeptides were also detected.
TABLE II
Measurement of the concentration of storage protein mRNAs in subcellular fractions of oat endosperm

| Source of total RNA | Avenin concentration (fmols/μg) | Globulin concentration (fmols/μg) |
|---------------------|---------------------------------|----------------------------------|
| Total seed RNA      | 0.138 ± 0.014§                  | 0.194 ± 0.014§                   |
| Free polysomal RNA  | <0.031*                         | <0.031*                          |
| Membrane-bound      | 0.288 ± 0.022§                  | 0.455 ± 0.044§                   |
| polysomal RNA       |                                 |                                  |

§ Femtomoles of storage protein mRNA/μg of total RNA in each fraction.

* Standard deviation.

Below the lowest value in the standard curve.

pAV31, Ref. 7) was used to probe dot-bLOTS like those shown in Fig. 4, the detection of avenin mRNAs was enhanced 3-fold (data not shown). Thus, the estimate of the molar amount of avenin mRNA shown in Table II may be conservative. These results suggest that there are no major differences in the initiation of translation of avenin and globulin mRNAs nor in the mRNA’s ability to associate with the rough endoplasmic reticulum.

Determination of the Number of Ribosomes on Avenin and Globulin mRNAs—We observed that there are equivalent concentrations of avenin and globulin mRNAs in membrane-bound polysomes but an 8-9-fold difference in the rate of concentrations of avenin and globulin synthesis. This suggested that there may be gross differences in the efficiency with which the two types of mRNA are translated. To determine whether there are gross differences in the translation of these mRNAs, we compared the number of ribosomes bound to avenin and globulin transcripts. Membrane-bound polysomes were separated by sucrose density gradient centrifugation and the location of storage protein mRNAs within the gradient fractions was determined by dot-blot hybridization to avenin- or globulin-specific probes. With this technique, we were able to resolve polysomes containing at least 8 to 10 ribosomes (Fig. 5, fraction 8, the highest peak). However, this method did not clearly separate polysomes containing higher numbers of ribosomes. RNA dot-blot hybridizations of the gradient fractions showed that avenin and globulin mRNAs were first detected in significant amounts in fraction 4 (Fig. 5, B and C), which corresponds to mRNAs with three ribosomes. The highest concentrations of both mRNAs were found in gradient fractions 7-10, which corresponds to seven or more ribosomes per mRNA.

In Vitro Translation of Synthetic Avenin and Globulin mRNAs—DNA sequence analysis of avenin and globulin clones suggested that the codon usage of the respective mRNAs, as well as putative mRNA secondary structures, may influence the translation of storage protein transcripts (5). To investigate these possibilities, we compared the in vitro translation of avenin and globulin mRNAs in a wheat germ protein synthesizing system.

The most accurate reconstitution of storage protein synthesis in vitro would require the use of native mRNAs. However, the complexity of the globulin and avenin mRNAs makes it difficult to assess their translational efficiencies. Consequently, transcripts derived from cloned sequences (Fig. 6) were used for in vitro protein synthesis.

The globulin sequences are highly homologous, but there is considerable variation among avenin mRNAs. Therefore, it was necessary to compare the relative translational efficiencies of different avenin transcripts. When the translational efficiencies of three synthetic avenin mRNAs (pAVT16, pAVT21, and pAVT32) were compared, all three yielded an equivalent amount of protein (data not shown). Accordingly, the plasmid pAVT16 was used for all subsequent experiments.

Since monovalent cations influence the yield of full-length translation products, the translation of avenin and globulin mRNAs was evaluated at concentrations of 100 and 200 mM potassium acetate. Fluorograms of in vitro translation products separated by SDS-PAGE showed that more full-length polypeptides were produced with 200 mM potassium (Fig. 7, C and D) than with 100 mM potassium (Fig. 7, A and B). However, more [35S]methionine was incorporated into protein at 100 mM potassium acetate than at 200 mM potassium acetate.

Because the avenin transcript encodes more methionine than the globulin transcript, more radioactivity was incorporated into avenin than into globulin polypeptides. However, comparable rates of avenin and globulin synthesis occurred at both potassium acetate concentrations when the radioactive labeling was corrected for the percent methionine and plotted against time to obtain normalized translation rates for the avenin and globulin mRNAs (Fig. 8, A and B). Heating the transcripts to 65°C for 15 min to reduce RNA secondary structure immediately before translation had no effect on the rate of protein synthesis (data not shown).
Translational Control of Storage Protein Gene Expression

**Fig. 6.** Transcription plasmids for the synthesis of oat storage protein mRNAs. Plasmids to prepare synthetic avenin and globulin transcripts were constructed as described under "Materials and Methods." A shows the parent plasmid, pT7A18, whereas the avenin-encoding (pAVT16) and globulin-encoding (pGBT2) transcription plasmids are shown in B and C, respectively. Restriction endonuclease recognition sites are designated as follows: E = EcoRI, H = HindIII, H' = partial HindIII (compatible site), Ss = SsI, and St = StuI. The boxes labeled PL-E, Avenin, and Globulin are the pUC18 polylinker minus the EcoRI site, avenin gene sequences, and globulin gene sequences, respectively. Cross-hatched boxes show the position the pA65 polyadenylation sequence. The solid boxes show the position of the T7 RNA polymerase promoter with the transcription initiation sites and the direction of transcription indicated by arrows.

**Fig. 7.** SDS-PAGE of in vitro translation products from synthetic avenin and globulin mRNAs. One picomole of synthetic avenin or globulin mRNA was incubated for 0-60 min (lanes 0-60) in a wheat germ cell-free protein synthesizing system with potassium acetate concentrations of either 100 mM (A and B) or 200 mM (C and D). An aliquot from each time point was analyzed by SDS-PAGE and fluorography. The fluorograms show translation products synthesized from avenin transcripts (pAVT16) in A and C and from globulin transcripts (pGBT2) in B and D, with full-length avenin (A and C) or globulin (B and D) translation products indicated by arrowheads. Lane M contains the [14C]-labeled marker proteins of molecular mass 97.4, 69.0, and 30.0 kDa used as standards.

To determine whether the simultaneous translation of avenin and globulin mRNAs would affect the yield of products, equimolar amounts of the two mRNAs were translated in vitro and the polypeptide products separated by SDS-PAGE. Equimolar amounts of avenin and globulin transcripts were translated either separately (Fig. 9, lanes 1 and 3) or together (Fig. 9, lane 2). As shown in Fig. 9, avenin and globulin transcripts yielded approximately equal amounts of labeled protein when translated together or separately (cf. Fig. 9, lanes 1 and 2 and lanes 3 and 2). Thus, the different translation efficiencies found in vivo do not appear to be solely a consequence of mRNA secondary structures.

**DISCUSSION**

The relative amounts of avenin and globulin in oat seeds was previously estimated at 10-15% avenin and 70-85% globulin (1, 2). Our analysis of the concentration of these proteins by ELISA yielded a considerably higher ratio of globulin to avenin than those documented previously (1, 2, 8). We found that there is over 26 times as much globulin as avenin when comparisons are made on a weight basis and slightly greater than a 10 to 1 ratio when expressed on a molar basis (Table I). The difference between these values and those previously reported could be the result of several factors. First, a variety of oat cultivars were used in the previous studies. For this study we selected a high-protein cultivar of Avena sativa called Noble. Peterson (17) found that the increase in protein content in high-protein oats is due entirely to an increase in globulin, which could explain why our globulin to avenin ratio is considerably higher than those of previous reports. In addition, our extraction methods are different and, perhaps, more efficient than the sequential extraction techniques used in the earlier studies. Finally, the ELISA analysis is highly specific and may be less prone to the variation in measurement associated with previous methods.

A comparison of the in vivo rates of storage protein synthesis indicated an 8-9-fold higher level of globulin than avenin production (Fig. 2). These values are in accordance with the levels of steady-state protein determined by ELISA. Since the steady-state levels of avenin and globulin mRNA are approximately equal (7), translational or post-translational control mechanisms must be largely responsible for determining the quantity of avenin and globulin proteins in mature oat seeds.

It is possible that the stability of the avenin and globulin proteins could play an important role in determining the
accumulation of these proteins. If, for example, the avenin protein was less stable than the globulin protein, the globulin to avenin ratio would increase over time. However, we found no evidence for turnover of either avenin or globulin protein during the course of our experiment (Fig. 3). It is not surprising that both proteins are stable, since the function of storage proteins is to sequester amino acids for later use. It would be very inefficient for the endosperm cells to produce these proteins only to have them degraded before they are needed (during germination). This result demonstrates that the high globulin to avenin ratio is probably not a consequence of avenin “turnover” in developing oat seeds; therefore, the translation of storage protein mRNAs must control avenin and globulin accumulation.

Most models describing the regulation of protein synthesis (e.g. Ref. 18) are based on the assumption that initiation is the rate-limiting step in mRNA translation. Gene sequence analysis revealed that the translation initiation sequences in avenin and globulin mRNAs are different. The globulin sequence (5'-AAUC AUG GC-3') is a closer match with the consensus for plant mRNAs (5'-AAACA AUG GC-3'; Ref. 19) than are the avenin sequences (5'-CACC AUG AA-3' and 5'-UACC AUG AA-3') (5). Adenines found at positions +4 and +5 of the avenin mRNA are rare in plant mRNAs but common in animal mRNAs (20). Lütcke et al. (19) suggested that the strong preference for G at position +4 and C at position +5 in plant mRNAs indicates that nucleotides +4 and +5 may modulate initiation codon selection in plants as has been shown for nucleotide −3 in animals (21). Nevertheless, it does not appear that these differences are responsible for the dramatic difference in avenin and globulin content, since avenin and globulin mRNAs occur in comparable concentrations and are largely found in membrane-bound polysomes (Ref. 7, Fig. 4, and Table II). If translation initiation is the rate-limiting step for avenin synthesis, we would expect a larger proportion of avenin mRNA in free polysomes or in the post-polysomal supernatant (mRNA which is not found in polysomes). Therefore, these results suggest that translation initiation is not rate-limiting for avenin and globulin synthesis.

Secondary structures in mRNAs have also been shown to affect rates of translation (reviewed by Laz et al. (22)). The Bergmann and Lodish (18) model predicts that secondary structures near the 5' end of mRNAs could cause stalling of ribosomes, slowing initiation, and lowering the overall rate of protein synthesis. Kozak (23) suggested that hairpin loops of moderate strength immediately downstream from the AUG codon can cause ribosome stalling. Thus, it may be significant that all avenin genes characterized to date contain inverted sequence repeats with the potential to form hairpin loops (Fig. 3). Should these mRNA secondary structures exist in vivo, they could cause ribosomes to pause within these regions, resulting in lower rates of avenin synthesis.

The number of ribosomes associated with polysomes has been measured for several mRNAs. There are, for example, five ribosomes for leghemoglobin (140–150 amino acids) (24), eight or nine for maize zeins (230–245 amino acids) (25), and 15 for β-conglycinin (454 amino acids) (26). Therefore, ribosomes are spaced approximately every 90 nucleotides (30 amino acids) along the mRNA. Using this information, one would predict that the oat globulin mRNA (500–520 amino acids) would accommodate 16–18 ribosomes, whereas, the avenin mRNA (180–220 amino acids) would bind six to eight ribosomes. We expect that if ribosomes are pausing near the 5' end of avenin mRNAs, the avenin mRNAs would be found in small polysomes (i.e. mRNAs with few ribosomes bound).
However, our results show that at least avenin mRNAs, and probably globulin mRNAs, are fully loaded with ribosomes (Fig. 5). Therefore, the potential RNA secondary structures in avenin mRNAs apparently do not cause ribosome stalling and presumably do not affect translation.

The results from the translation of synthetic avenin and globulin mRNAs in a heterologous in vitro protein synthesis system show that there is no inherent difference in the translatability of these two mRNAs (Figs. 8 and 9). Although the conditions for in vitro translation are not comparable with those found in vivo, these results support the argument that the difference in avenin and globulin translation efficiencies in vivo are not a consequence of mRNA secondary structures. They also infer that the wheat germ translation system does not contain the factor or factors required for preferential translation of globulin mRNAs.

It appears that the elongation or the termination phases of mRNA translation are the mostly likely regulatory steps for controlling avenin and globulin synthesis. The avenin and globulin genes of oats have considerably different codon usage patterns, with a different subset of preferred synonymous codons to encode most amino acids (Ref. 5; Table 1). For example, 25 of the 33 codons for alanine in the globulin gene are GCT or GCA, but 13 of the 15 alanine codons in the avenin gene are GCC or GCG. The prevalent hydrophobic amino acids leucine, isoleucine, and valine are encoded by 41% codons ending in C or G in the globulins, but by 84% codons ending in C or G in the avenins. These differences are observed throughout the two gene families with the globulin genes containing 46% C + G and the avenin genes 56%. In addition, there is a pronounced difference in the third position of the codons, where globulin genes contain 43% C + G and avenin genes contain 72% C + G.

The difference in codon usage patterns between avenin and globulin genes may influence the rate of translation of storage protein mRNAs because of the tRNA population in developing oat endosperm. Differences in codon distribution have been postulated to affect rates of translation in several organisms (27-31). One model explains this phenomenon by the distribution of iso-accepting tRNAs in several cell types parallel to the codon usage pattern of the most actively expressed genes (28, 32-34). This model implies that the transcription of genes for needed tRNA isoacceptors is coordinated with the transcription of highly expressed genes and that limiting tRNAs modulate the rate of translation. However, at least in E. coli the abundance of tRNA isoacceptors does not appear to be correlated with the rate of translation elongation (35). In E. coli, transcription and translation are coupled, and therefore, may not be directly comparable with eucaryotic systems where transcription and translation are uncoupled.

A second model states that energetic interaction between codons and anticodons are critical in determining translation rates with synonymous codons translated at different rates (36, 37). It has been postulated that codon-anticodon interactions of intermediate strength are maximized in highly expressed genes so that translation elongation may proceed at a steady rate (38). According to this model, if globulin genes use more favorable codons than do avenin genes, or if the globulin genes use fewer avoided codons than do the avenin genes, the rate of globulin synthesis would be higher than avenin synthesis. Although the results presented in this manuscript are consistent with this model, we do not have enough evidence to state whether codon usage within oat storage protein genes influences the translation rates of avenin and globulin mRNAs.

The efficiency of translation termination could also play a role in modulating the amounts of avenin and globulin synthesis. Plant genes, as shown by Angenon et al. (39), have a nonrandom distribution of the three stop codons used to terminate translation as well as a bias in the context of these codons (i.e. the nucleotides before and after the termination codons). Sequence analysis of avenin and globulin genes reveals that avenin genes terminate translation with a nonfavored UAA(G) sequence, whereas globulin genes terminate with a favored stop codon UGA(A). We hypothesize that since UAA(G) is not a favored stop codon, there is a reduced binding affinity for a release factor (similar to RF2 described by Caskey (40)) and the UAA(G)-ribosomal complex. The reduced binding affinity causes the ribosome to stall at the UAA(G), resulting in the queuing of the ensuing ribosomes on the avenin mRNA and slowing the overall rate of avenin synthesis. Our observations that avenin mRNAs are found in large polysomes and that the in vivo rates of synthesis of avenins are significantly less than globulins are consistent with a model where translation termination is rate-limiting for avenin synthesis. However, based on our results, we are unable to state that translation termination plays a role in regulating avenin gene expression.

In summary, we have shown that the storage protein content of oat seeds appears to be controlled by several modes of regulation. First, the developmental timing and amounts of steady-state transcripts for avenins and globulins are controlled transcriptionally (7). Second, protein synthesis, and not protein turnover, is the primary determinant of the high ratio of globulin to avenin protein found in developing seeds. Third, the difference in translational efficiencies appears to result from events after translation initiation. We are currently conducting experiments to elucidate which of the processes following translation initiation are involved in modulating avenin and globulin synthesis. These experiments will allow us to further refine our understanding of the regulation of storage protein synthesis in developing oat seeds.

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