In Vitro Synthesis of Wheat (Triticum aestivum L.) Storage Proteins

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ABSTRACT

Free and membrane-associated polysomes were isolated in approxi-
mately equal amounts from endosperm of wheat kernels harvested 20 days
after anthesis. The presence of heparin in the homogenizing buffer mini-
mized polysome degradation. Ribonuclease acid from the isolated polysomes,
when translated in vitro in a wheat germ system, yielded products ranging
in size from about 12,000 to about 30,000 daltons, including at least two
polypeptides that co-migrated with seed extract proteins in sodium dodecyl
sulfate polyacrylamide gel electrophoresis. The nature of the translation
products of free and membrane-associated RNA are distinctly different,
with membrane-associated RNA yielding a higher proportion of polypep-
tides in the size range of 30,000 to 37,000 daltons. Analysis of membrane-
associated 3'-terminal polyadenyl-containing RNA in vitro translation
products, by solubility in 70% ethanol and by immunoprecipitation, indi-
cates that the 33,000- to 37,000-dalton polypeptides contain gliadins, and
the analysis provides evidence that these proteins are synthesized in
association with membranous cell organelles. Gliadin polypeptides synthe-
sized in vitro are larger than authentic gliadins and probably are precursors
which, in vivo, undergo modification to yield the smaller final products.

In addition to its importance as a major source of nutrients for man (41), the endosperm of wheat is an attractive tissue in which
to study the regulation of protein biosynthesis: its genetics have been
well described (23), its polyploid nature facilitates the study
of gene dosage effects (1, 32), and its proteins have been ex-
tensively characterized (22). The endosperm protein of commercial
wheat varieties typically consists of 12 to 16% salt-soluble proteins
(albumins and globulins) and 30 to 35% proteins soluble in 70%
ethanol (prolamins or gliadins) (35). The gliadins contain large
amounts of glutamine and proline and small amounts of lysine,
and although most have mol w t near 36,000 daltons (22), they are
highly heterogeneous. They are coded by a multigene family of
more than 40 components (32, 47), can be grouped in subfamilies
having extensive sequence homology (2, 6), and are probably
under common regulatory control (31). The A-gliadins have been
especially well characterized (21). The gliadins and the glutelins
(multimeric protein complexes with gliadin-like amino acid com-
position), constitute the main storage proteins of wheat (22).
Gliadins, and possibly gliuteins, accumulate in the developing
wheat endosperm in concentrated deposits, termed "protein bod-
ies" (17), which have been proposed as the sites of storage protein
synthesis (33). Protein bodies have been isolated from maize, and

1 A preliminary account of this work was presented at the sixty-first
annual meeting of the American Society of Plant Physiologists, August,
1979, Columbus, OH.

2 Reference to a company and/or product named by the department is only
for purposes of information and does not imply approval or recom-
endation of the product to the exclusion of others which may also be
suitable.

3 Abbreviations: pmG, 7-methylguanosine 5'-phosphate; SDS-PAGE,
sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MB-RNA,
membrane-bound polysomal RNA; F-RNA, free polysomal RNA; PEP,
phosphoenolpyruvate; poly A(+), 3'-terminal polyadenyl-containing; B-
ME, β-mercaptoethanol; NaDOC, sodium deoxycholate; IgG, immuno-
globulin G; kd, kilodalton.

shown to incorporate labeled amino acids into zein (9). Likewise,
polysomes and RNA isolated from membranous seed tissues of
maize (26), barley (30), and oats (28) program the synthesis in vitro
of zein, hordein, and globulins, respectively.

There is considerable interest in improving the value of wheat
as a human food, and knowledge of the molecular biology of
protein synthesis may be useful in achieving this end. Although
such molecular studies have not been extensive in wheat, biochem-
ical investigations have shown that there is not a clear correlation
between non-protein nitrogen content and protein synthesis rates
in wheat grain; nor between grain RNA content and varietal
protein content (14). These phenomena may be regulated at finer
levels, such as transcription and translation. This paper reports the
isolation, from endosperm tissue of developing wheat, of RNA
capable of programming protein synthesis in vitro, and the dem-
stration that membrane-associated endosperm RNA contains a
relatively high proportion of gliadin mRNA.

MATERIALS AND METHODS

Cheyenne, CI 8885) and Hard Red Spring (Triticum aestivum L.,
cv. INIA 66R, CI 15328) wheats were grown in a greenhouse
with maximum temperature held below 25 C. Vernalization was in-
duced in the Cheyenne by incubation of shoots at 2 C. Kernels
were harvested 20 days after first anthesis: whole ears were severed,
frozen in liquid N2, and stored at −35 C until needed.

Reagents. Radiochemicals were purchased from New England
Nuclear. Agarose poly(U), type 6, and pmG3 were purchased from
P-L Biochemicals, Milwaukee, WI. Other biochemical re-
agents were purchased from Sigma Chemical Co. or Calbiochem-
Boehringer, San Diego, CA. Formamidine (Eastman) was deionized
with Dowex AG 501-X8(D) mixed bed resin (Bio-Rad) according to
Finder et al. (39). Glassware was autoclaved prior to use, and
solutions were either autoclaved or treated with diethyl pyrocar-
bonate (44). Phenol (Baker) was redistilled, saturated with 20%
(v/v) water, and stored at 2 C in the dark until needed.

Isolation of RNA. The extraction procedure was modified from
Davies et al. (13), with heparin addition based on Palmiter (37).
In a typical extraction, frozen kernels were cooled in liquid N2.

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and the embryos cut off with a razor blade. Kernels were allowed to thaw slightly, and endosperms extruded into liquid N₂ by gentle pinching of uncut ends of seeds. Frozen endosperms were ground with a small amount of solid CO₂ in a small blender-type coffee mill and held at -18°C for 60 min to allow residual CO₂ to sublime. Extraction operations were then carried out at 0 to 4°C. Homogenizing buffer A contained 200 mM Tris-HCl (pH 8.5), 200 mM sucrose, 25 mM MgCl₂, 60 mM KCl, 5 mM DTT, 2 mM AMP (mixed 2' and 3' isomers), and 1 mg/ml sodium heparin. After appropriate low speed centrifugations, and addition of Triton X-100 to 2% (v/v), free and initially membrane-bound polysomes were pelleted by centrifugation at 30,000g for 100 min. The ultracentrifuge pellets were gently rinsed and resuspended in buffer B (40 mM Tris [pH 8.5], 10 mM MgCl₂, 20 mM KCl), and aliquots taken for electron microscope analysis of polysomes (25).

Polysomal RNA was obtained from the pellets by treatment with SDS and NaOAc extraction, with phenol-chloroform, and ethanol precipitation, essentially as described by Palmiter (37). The resulting polysomal RNA fractions were designated MB-RNA and F-RNA, according to Larkins et al. (26). RNA yield was estimated from UV absorption spectra, assuming ε₂₆₀ = 20 (42). Agarose poly(U) chromatography of polysomal RNA was modified from the procedure of Jacobson et al. (20): RNA in high salt buffer (0.7 M NaCl, 1 mM NaEDTA, 10 mM Hepes [pH 7.5], 25% [v/v] formamide) was added to a jacketed column at 25°C and, after washing, the column was eluted at 50°C with low salt buffer (0.1 M NaCl, 1 mM NaEDTA, 10 mM Hepes [pH 7.5], 25% [v/v] formamide). RNA fractions were pooled and precipitated with 3 volumes ethanol. In vitro labeling of endosperm proteins was done with the culture method of Donovan and Lee (15). Heads of 20-day INIA 66R were labeled with 67 µCi [³²P]leucine for 30 min, incubated 210 min in unlabelled medium, frozen in liquid N₂, and stored at -80°C until needed.

In Vitro Translation. In vitro protein synthesis was carried out by a procedure modified from Davies and Kaegbs (12); final concentrations in a standard 50 µl in vitro reaction mixture were: 25 to 30 A₂₆₀/µl wheat germ S-31000 extract (29), 26 mM Hepes, 2.5 mM ATP, 0.38 mM GTP, 10 mM PEP, 25 µM each of 19 unlabelled amino acids (but no leucine), 4 mM DTT, 86 µM spermine, 3 mM Mg(OAc)₂, 118 mM KOAc, and varying amounts of polysomal or poly A(+) RNA. Final pH was 7.5 to 7.6. Reactions were initiated with 6.25 µCi [³²P]leucine and continued for 90 min at 25°C. Reactions were terminated by the addition of 50 µl 50 mM Tris-Cl, followed by 1 ml 10% (w/v) TCA. Precipitates were collected and counted on Whatman GF/A filters. For extraction of gliadins after in vitro translation, 2.34 volumes of absolute ethanol were added, with stirring, to the reaction mixture. Following incubation at 25°C for 30 min, and centrifugation at 13,000g, the supernatant was mixed with 50 µg carrier BSA, and adjusted to a solvent composition of 10% (w/v) TCA and 10% (v/v) ethanol. After incubation in an ice bath for 30 min, the mixture was centrifuged, the precipitate was washed with absolute ethanol, and dissolved for electrophoresis in sample buffer (60 mM Tris-HCl [pH 6.8], 1.9% [w/v] SDS, 5% [w/v] β-ME, and 5% sucrose with 0.033% pyronin Y tracking dye). For electrophoresis of total translation products, reactions were stopped with 50 µl 2X sample buffer. SDS-PAGE was carried out using the buffer system of Laemmli (24) in a 17% (w/v) acrylamide/0.0765% (w/v) bisacrylamide gel (Payne et al. [38]). Fluorography (8) was carried out with Kodak XR-5 film. A-gliadin prepared according to Bernardin et al. (5) and [¹⁴C]carboxymethylated according to O'Donnell et al. (34), was used as secondary mol wt standards of 32,500 and 33,900 daltons.

Immunological Methods. New Zealand White rabbits were sensitized by subcutaneous injection of A-gliadin with Freund's adjuvant. Immunoprecipitation of gliadin proteins was done essentially according to the direct method of Rhoads et al. (42) or the double antibody method of Shapiro et al. (43) in the presence of 1% (w/v) NaDOC (11), with IgG prepared according to Palacios et al. (36). The immunoprecipitates were washed with 1% (w/v) NaDOC and dissolved in sample buffer for electrophoresis.

RESULTS

Isolation and Translation of Endosperm RNA. Whole kernels were used in initial attempts to isolate a translatable fraction of mRNA from developing wheat, and a number of protocols were tested with little success, including isolation of protein bodies (low yield) and hot phenol/SDS extraction (low activity, interference from phenolics and from starch gelatinization). In the protocol described in this report, (2' + 3')-AMP was added, and the Mg concentration in the homogenizing buffer chosen to enhance polysome stability at the expense of RNase activity (25). The most significant improvement in yield of polysomal RNA occurred with the inclusion of 1% (w/v) heparin in the homogenizing buffer. Endosperm RNA extracted in the absence of heparin was present mainly as monosomes or small polysomes. In the presence of heparin, the extracted population was shifted to larger polysomes.

This is shown in Figure 1 for preparations of Cheyenne RNA.

In experiments with INIA 66R, a typical yield of endosperm polysomal RNA was 25 µg RNA/20-day endosperm. This is approximately 35% of the total RNA extractable by homogenization in 1% (w/v) SDS. The absorption maximum of the isolated polysomal RNA was 258 nm, and it had an A₂₆₀/A₂₈₀ ratio of 2.0. Approximately 55% of the recovered polysomal RNA was isolated from the membrane fraction and 45% from the free cytoplasmic fraction. The proportion of membrane-bound RNA may be higher in vivo than these data indicate, however, as heparin may release polysomes from RER (16).

The products of translation, at 160 µg/ml of MB-RNA and F-RNA, after SDS-PAGE, are shown in Figure 2 (lanes 1, 2). The most noticeable electrophoretic differences between the translation products of the two RNA types occurred in the putative gliadin region, where polypeptides of 33,300, 34,000, and 36,600 daltons were much more prominent in products from the MB-RNA than from the F-RNA (see "Analysis of In Vitro Translation Products" for further discussion).

When poly A(+) RNA was isolated from MB- and F-RNA and translated, a selective dependence of translation on RNA concentra-


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were indicated under the 25 sucrose, were stained with 2.5% uranyl acetate (16). A: kernels homog-

FIG. 1. Effect of heparin on polysome yield. Twenty-day Cheyenne kernels were homogenized in buffer (200 mM Tris-HCl [pH 8.5], 200 mM sucrose, 25 mM MgCl₂, 60 mM KCl, 5 mM DTT, 2 mM (2' + 3')-AMP) in the presence or absence of 1% (w/v) heparin, and polysomes isolated as indicated under “Materials and Methods.” Electron micrograph samples were negatively stained with 2.5% uranyl acetate (16). A: kernels homog-

FIG. 2. Synthesis of endosperm proteins in vivo and in vitro. Analysis by SDS-PAGE and fluorography. 1: in vitro translation products of F poly A(+) RNA from 20-day endosperm; 2: in vitro translation products of MB poly A(+) RNA from 20-day endosperm; 3: SDS-β-ME extract of endosperm labeled in vivo at 20 days after anthesis; 4: SDS-β-ME extract of unlabeled 20-day endosperm; 5: 70% ethanol extract of 20-day INIA 66R endosperm. Proteins of lanes 4 and 5 are stained with Coomassie blue. The arrows refer to polypeptides discussed in the text.

products from membrane-derived and free cytoplasmic polysomal RNA, Figure 2 shows SDS-PAGE patterns of peptides labeled in vivo in 20-day INIA 66R endosperm, and extracts of unlabeled 20-day INIA 66R endosperm (lanes 3 to 5). The most intensive polypeptide labeling in vivo occurs in the putative gliadin region, in two broad bands centered at 31,700 and 34,200 daltons. In the total endosperm extract, this region is similar to that labeled in vivo, but is resolved into four bands. In the in vitro translation products, bands in the 31,000- to 37,000-dalton region are represented most prominently in the products from membrane-derived RNA at 33,000, 34,000, and 36,000 daltons. An additional polypeptide of 31,200 daltons is prominent in the free RNA translation products.

The prominent 12,600- and 13,300-dalton in vitro translation products have no obvious counterparts in the in vivo labeled proteins, but may be represented in the total seed extract. The SDS-PAGE pattern of polypeptides accumulated in the endosperm in the 20 days following anthesis differs from the above short term synthesis patterns in that, in addition to the polypeptides in the 30,000 to 50,000 region, it contains significant amounts of species in the 50,000- to 120,000-dalton range. Prominent polypeptide bands near 42,000 and 67,000 daltons are present in all samples, as are polypeptides of 39,800 and 40,800 daltons. These were prominent in aqueous buffer (200 mM Tris-HCl [pH 8.5], 200 mM sucrose, 60 mM KCl, 5 mM DTT) extracts of 20-day INIA 66R endosperm, but only minor components of a 70%
ethanol extract of endosperm tissue (Fig. 3). They are probably albumins and/or globulins, but not gliadins or storage proteins. They are also more prominently represented in the in vitro translation products of free polysomal RNA than of membrane-derived polysomal RNA (Fig. 2).

Detection of Storage Proteins. The identities of the 31,000- to 37,000-dalton polypeptides, and the possible relations between those synthesized in vivo and in vitro were explored by immunoprecipitation and by ethanol solubility experiments, followed by SDS-PAGE (Fig. 4). The 70% (v/v) ethanol extract of in vivo labeled endosperm contains polypeptides of 32,800, 34,200, and 36,600 daltons (lane 7, Fig. 4). Polypeptides immunoprecipitated from the ethanol-soluble fraction by anti-A-gliadin IgG include those contained in a major band that co-migrates with the 34,200-dalton species and in a minor one that co-migrates with the 36,600-dalton component (lane 6). Gliadin components of 32,800, 34,200, and 36,600 daltons can thus be identified among the proteins being synthesized in vivo in endosperm of INIA 66R at 20 days after anthesis.

In the products from in vitro translation of membrane-derived poly A(+) RNA of 20-day INIA 66R endosperm, ethanol-soluble polypeptides are prominent at 33,300, 34,400, and 36,600 daltons, and co-migrate with equivalent components from the total translation mixture (lanes 2 and 1, respectively). An additional polypeptide is apparent at 42,000 daltons. Direct immunoprecipitation of in vitro synthesized gliadin polypeptides, using unlabeled A-gliadin as a carrier, yielded major polypeptides with mol wt of 35,400, 36,700, and 39,000 daltons; minor polypeptides of 33,000, 34,700, and 39,000 daltons were also present (lane 4). Smaller amounts of these polypeptides were immunoprecipitated from translation products of the free cytoplasmic poly A(+) RNA (data not shown). Polypeptides immunoprecipitated by the double antibody method (lane 3) include species similar to those revealed by the direct method. By the criteria of ethanol solubility and antigenicity, these 33,000 to 39,000 polypeptides are gliadins. Gliadin-like translation products may also be present in a prominent polypeptide of 13,500 daltons that is precipitated by the double antibody method, and which may consist of uncompleted gliadin molecules with single antigenic determinants. Material of this size is also present in the ethanol-soluble fraction of the in vitro translation products.

The spectrum of in vitro synthesized gliadin polypeptides that is revealed by ethanol solubility is different from those revealed by the immunoprecipitation methods. This is probably due to differences in the specificities of the two types of identification procedures: whereas the 70% (v/v) aqueous ethanol solubility of gliadins is a general property and should equally reveal all components, the immunological methods are based on the properties of a subset of gliadins (A-gliadins) and may emphasize their presence relative to others.

As a group, the polypeptides synthesized in vitro are larger (33,300–39,000 daltons) than those synthesized in vivo (32,500–36,600 daltons). In vivo, the larger group is probably converted to the smaller by co- or post-translational modifications that result in cleavage of a peptide of about 2,000 daltons. Additional evidence in support of in vivo modification of nascent gliadin polypeptides is provided by analysis of the products of translation of
total poly A(+) RNA from 25-day Cheyenne wheat, which is prominent in the parentage of the variety (Scout 66) used to prepare the A-gliadin antigen (40). A sample of in vitro translation products provided by Dr. T. W. Okita of this laboratory was immunoprecipitated with anti-A-gliadin IgG using the direct method. Approximately 19% of the incorporated [3H]leucine was recovered in the immunoprecipitate. The major SDS-PAGE band from this precipitate had an apparent molecular size range of approximately 33,500 to 35,000 daltons, compared to 32,500 to 34,000 daltons for the S-[14C]carboxymethylated A-gliadin (Fig. 5). This difference is consistent with the in vitro synthesis of polypeptides that, in vivo, would be further processed to smaller products.

DISCUSSION

The results presented here indicate that poly A(+) RNA from developing wheat endosperm is rich in mRNAs for gliadins, a major class of wheat storage protein. The mRNAs are apparently capped, and the storage protein message is probably associated with membranous elements of endosperm cells in vivo, in a fashion similar to maize, barley, and oats. Isolation methods designed to purify protein bodies and associated RNA from maize were unsuccessful with wheat, however, indicating that the properties of the membranous organelles or ribonucleases of the two plant endosperms are different. In the present investigation, the use of heparin in the extraction buffer was of critical importance to the isolation of undegraded polysomes, and to their fractionation into membrane-derived and free cytoplasmic RNA. This protective action of heparin is consistent with its ability to inhibit ribonucleases (37, 42). The extremely poor yield of polysomes in the absence of heparin is an indication that wheat endosperm tissue of at least two wheat varieties has high endogenous ribonuclease activity. Additional research will be required to determine whether this is a general property of wheat endosperm, or whether there exists in wheat a wide range of ribonuclease activity, as reported in maize (46). In the absence of such information, it is prudent to use ribonuclease inhibitors or denaturing methods (10, 19) in the isolation of mRNA from wheat endosperm.

The gliadins synthesized in vitro from both INIA 66R and Cheyenne wheats are larger than the proteins synthesized in vivo, which indicates that some co- and post-translational processing probably occurs in vivo to produce the final product. Similar phenomena occur in barley and maize, and all are consistent with the involvement of events such as those proposed in the "signal hypothesis" of Blobel and Dobberstein (7). The possible occurrence of additional in vivo post-translational modifications, or of differences between in vivo and in vitro translational efficiencies of individual gliadin mRNAs is suggested by the differences in the respective SDS-PAGE patterns of Figure 2.

The proportion of complete gliadin polypeptides synthesized in vitro is adversely affected by high mRNA concentrations, and declines selectively as the incubation mixture approaches mRNA saturation, similar to observations of Matthews and Miflin (30) on the in vitro synthesis of barley storage proteins. In wheat, in addition, the proportion of gliadin synthesis relative to production of the low mol wt components is a sensitive function of the K concentration of the in vitro synthesis mixture (see 3, 45). Since some of these low mol wt products may be incomplete gliadin translation products, the gliadin message may not be translated with optimal efficiency in the in vitro system being used. One possible reason for such inefficiency is interference from protein-protein interactions, such as formation of fibrillar and related multimeric aggregates under conditions of neutral pH and moderate ionic strength as reported by Bernardin and Kasarda (4); or aggregation of zein-synthesizing polysomes through interactions among nascent polypeptide chains, as observed in the zein system by Larkins and Tsai (27). Other possible causes of inefficient translation of gliadin mRNAs in vitro may derive from specific structural features of the mRNAs, or from a requirement for endosperm-specific translation factors; finally, some degradation of mRNA may also be involved. Considering their physical properties, the confinement of wheat and other cereal storage proteins in membranous organelles may serve to prevent disruptions of protein synthetic and other metabolic activities of the endosperm cell.

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