Translation of Silk Fibroin Messenger RNA in an Ehrlich Ascites Cell-free Extract

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RNA identified by its base composition and T, RNase oligonucleotide pattern as the message for silk fibroin was purified from mature posterior silk glands of Bombyx mori larvae and used to direct polypeptide synthesis in an Ehrlich ascites cell-free extract. Fibroin mRNA stimulated \[^{3}H\]alanine incorporation about 3- to 4-fold in the presence of 80 mM K\(^+\) and 4 mM Mg\(^{2+}\). The stimulation was reduced in the presence of 5 \times 10^{-5} to \times 10^{-4} \text{M aurintricarboxylic acid}, an inhibitor of the initiation of protein synthesis. The cell-free products were heterogeneous in size, including peptides as large as 100,000 daltons. They co-precipitated with carrier fibroin sequences after digestion with trypsin. A large fraction of the polypeptides synthesized in response to fibroin mRNA was precipitated by antisera directed against amino acid sequences in noncrystalline region polypeptides of fibroin. Furthermore, after digestion with chymotrypsin, a major fraction of the cell-free products specifically co-precipitated with crystalline region sequences of native fibroin. The size and amino acid composition of the fibroin crystalline region polypeptides isolated from the cell-free products were similar to those from native fibroin.

Several messenger RNA species have been identified from their cell-free translation products (e.g. 1–5). In contrast, the mRNA for silk fibroin of Bombyx mori has been identified solely by its characteristic base composition, and by certain unusual properties of its nucleotide sequence (6). Purified fibroin mRNA has been shown to stimulate amino acid incorporation in mouse Ehrlich ascites cell extracts (7), but the unusual properties of fibroin protein made impractical the application to the cell-free products of several of the common techniques for protein identification. Silk fibroin has a molecular weight of 3.5 \times 10^6 (8). Since cell-free synthesis of a complete molecule that is so large seemed unlikely, determination of the molecular weight of the cell-free products by polyacrylamide gel electrophoresis could not be used as a criterion of fibroin synthesis. Moreover, tryptic peptide mapping is not a practical method for the identification of fibroin, since digestion with trypsin results in precipitation of oligopeptides representing 90% of the molecule (9).

More than 90% of the amino acids in fibroin are glycine, alanine, serine, and tyrosine, in a ratio of 44.5:29.4:12.1:5.2. The molecule is organized into many repeats of two kinds of alanine, serine, and tyrosine, in a ratio of 44.5:29.4:12.1:5.2. Chymotrypsin digestion of fibroin releases crystalline region polypeptides, which crystallize and precipitate. Crystalline region polypeptides contain 60% of the amino acids in the protein, and have a sequence proposed by Lucas et al. (10) to be

Gly-Ala-Gly-Ala-Gly[Ser-Gly-(Ala-Gly)_n]-Ser-Gly-Ala-Ala-Gly-Tyr

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where \( n \) is usually 2 and has an average value of 2.

The major noncrystalline region sequences remain soluble after chymotrypsin digestion of fibroin, yet except for the more frequent occurrence of tyrosine, resemble those sequences which precipitate. Antibodies elicited by fibroin in rabbits seem to be directed against certain of these soluble peptides (11–13). For a review of the structural aspects of fibroin protein, see Lucas and Rudall (14).

We have taken advantage of the unusual features of this protein to detect the cell-free synthesis of fibroin polypeptides by specific precipitation after digestion with trypsin and by a chymotrypsin digestion, co-crystallization technique which specifically detects fibroin crystalline region polypeptides. An immune precipitation procedure which detects peptides in the noncrystalline region of fibroin has also been used.

**MATERIALS AND METHODS**

**Isolation and Identification of Silk Fibroin mRNA**—Diapause eggs of Bombyx mori were purchased from Turtox General Biological Supply House, Chicago, Illinois, and stored at 4\(^\circ\) until used. Eggs hatched after about 2 weeks at 25\(^\circ\). Animals were raised at 25\(^\circ\), and fed local mulberry leaves. On the 8th or 9th day of the fifth instar (just prior to cocoon spinning) larvae were immobilized on ice and dissected under ice-cold SSC.\(^1\) The posterior silk glands were dissected out, rinsed in cold SSC, and frozen in liquid nitrogen. Storage of frozen

\(^1\)The abbreviations used are: SSC, 0.15 M NaCl-0.015 M sodium citrate; TCA, tricarboxylic acid; ATA, aurintricarboxylic acid; EMC virus, encephalomyocarditis virus, TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Hepes, N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone.
incorporation by this extract was stimulated more than 5-fold by the addition of purified ascites tRNA. The stimulation was not improved by preincubation of a preincubated S-30, followed by sucrose gradient centrifugation in a Spinco SW 27 rotor at 85,000 x g for 21 hours. RNA in the 4 S marker RNAs was pooled, and precipitated with ethanol. The RNA was stored in ethanol at -20°. Before use for translation, the precipitate was washed with 70% ethanol-30% 0.1 x SSC, resuspended in water, and his RNase was freed from RNA by autoclaving or by treatment with diethylpyrocarbonate. The column was eluted with Buffer A, and the pooled aqueous phases were re-extracted twice with cold ethanol and three times with an equal volume of ether. One-half volume of each of the above, of a preincubated S-30, followed by sucrose gradient centrifugation in the Spinco SW 27 rotor at 53,000 x g for 15 hours, and then quick cooled, and layered on two 37.5 ml linear gradients of 15 to 80% sucrose (w/w) sucrose (Sigma) in Buffer A. Gradients were centrifuged in a Spinco SW 27 rotor at 53,000 x g for 15 hours, and then pumped through a flow cell in a Gilford spectrophotometer which monitored A260 RNA sedimenting at 41 (relative to B. mori 18 S and 4 S marker RNAs) was pooled, and precipitated with ethanol. The RNA was stored in ethanol at -20°. Before use for translation, the precipitate was washed and used once with 70% ethanol-0.1 x SSC.

For base composition analysis, RNA was hydrolyzed in sealed capillaries in 50 μl of 0.05 M KOH at 100° for 40 min (16), and then spotted directly onto Whatman No. 3MM paper. The 32P-labeled nucleotides and unlabeled 2':3'-mononucleotide standards were separated by electrophoresis on 10% acrylamide sodium dodecyl sulfate gels as described by Suzuki and Brown (6).

Cell-free Protein Synthesis—EMC viral RNA and a cell-free protein-synthesizing extract from 7-day Ehrlich ascites cells were prepared by the methods described in Eggen and Shattak (18). In brief, the cell-free extract is an S-30 preincubated for 1 hour to reduce cell-free synthesis, and passed over a Sephadex G-25 column. The standard reaction mixture contained 5 mg/ml of extract protein, 20 mM Hepes (pH 7.6), 1 mM ATP, 0.1 mM GTP, 0.6 mM CTP, 6 mM mercaptoethanol, and 5 mM magnesium acetate. The enzyme was used in experiments with pH 7.9 as the precursor. EMC viral RNA was used at 60 μg/ml, and the reaction mixture contained 120 mM KCl and 5 mM magnesium acetate. Fibroin mRNA was used at 100 μg/ml in a reaction mixture containing 80 mM KCl and 4 mM magnesium acetate. Labeled amino acids were added to a final concentration of 80 μM/ml. The extract was found to contribute 0.4 to 8.0 nmol of each amino acid/ml of final reaction mixture (data not shown). For base composition analysis, no unlabeled amino acids were added to the reaction mixture in most experiments. After addition of mRNA, reaction mixtures were incubated at 37°. Amino acid incorporation was assayed by spotting samples, usually 0.01 ml, onto Whatman No. 3MM filter paper circles which had been saturated with minimum essential medium amino acids (Grand Island Biological Co.) and dried. Filter papers were processed with hot TCA by the method of Mans and Novelli (19). For some experiments, RNA was removed from the S-30 by adsorption to DEAE-Sephadex in 0.3 M KCl before passage over G-25. In the presence of absence of added mRNA, [3H]alanine incorporation by this extract was stimulated more than 5-fold by the addition of purified ascites tRNA. The stimulation was not improved when B. mori tRNA was substituted for the ascites tRNA.

As previously reported by other workers (21), the S-30 fraction was prepared by sucrose gradient centrifugation in the Spinco SW 27 rotor at 85,000 x g for 21 hours. RNA in the 4 S region was precipitated with 2 volumes of ethanol, resuspended in 20 mM Tris (pH 8.5), and dialyzed against 2 M LiCl. After dialysis, the RNA was resuspended in 20 mM Hepes (pH 7.6), 120 mM KCl, 5 mM magnesium-acetate, and 0.2 M 2-mercaptoethanol, and stored at -20°.

Polyacrylamide Gel Electrophoresis—Radioactively labeled polypeptides in the reaction mixture, after 20 min incubation, were precipitated with TCA and then washed, alkylated, and subjected to electrophoresis on 10% acrylamide sodium dodecyl sulfate gels as described by Eggen and Shattak (18).

Preparation of Fibroin Solutions and Fractions—Fibroin was prepared from cocoons by autoclaving in distilled water four times for 2 hours each. The insoluble fibroin was then washed with water, ethanol, 0.02 N acetic acid, and water, and then air-dried. About 70% of the dry weight of the cocoon was recovered as fibroin. Fibroin solutions were initially fractionated by centrifugation and then stored in 10 ml LiBr which had been filtered through 0.2-μm pore size nitrocellulose membrane filters, followed by dialysis against four changes of 400 volumes of distilled water at 4°. Insoluble material was removed by centrifugation at 49,000 rpm for 90 min in the Spinco Ti-50 rotor.

Alternatively, fibroin solutions were prepared from the posterior segment of the middle silk gland by the method of Srividhara et al. (20). This consists of four successive 24-hour extractions at 4° with 50 mM 500 ml Tris, 8 mM MgCl2, 10 mM KCl, 10 mM NH4Cl, 1 mM 2-mercaptoethanol, followed by centrifugation, as described above. Amino acid analysis showed that the fourth supernatant contained only fibroin.

By either method, amino acid analysis of the fibroin obtained indicated that more than 90% of the residues were glycine, alanine, serine, and tyrosine with an average ratio of 44.9:30.7:11.3:5.4, very similar to the values (44.5:29.4:12.1:5.2) reported by Lucas and Rudall (14). Due to a low content of tryptophan and a high content of tyrosine, fibroin solutions absorb more strongly at 275 than at 280 nm (A280/A260 0.47; for a 1-mg/ml solution).

Fibroin crystalline region polypeptides were prepared by incubating a 1 to 10-mg/ml fibroin solution with 200 μg/ml of a-chymotrypsin ( Worthington) in 100 mM Tris (pH 7.9) at 37° for 15 hours. The reaction mixture was washed three times with distilled water, trypsin or trypsin, they were added to the reaction mixtures, and then digested for 15 min at 100°. The reaction mixtures by boiling water for 15 min, then washed with 10% TCA, ethanol, and ether.

When cell-free products were tested for precipitation with chymotrypsin or trypsin, they were added to the reaction mixtures, and then incubated and washed as described above. The final precipitates were counted in Aquasol (New England Nuclear).

Amino Acid Analysis of Fibroin—Fibroin fibrils were extracted from cell-free reaction mixtures by heating at 100° for 15 min in 0.5 N perchloric acid. The pellet was removed by centrifugation and washed twice with 0.5 N perchloric acid. If further concentration steps were necessary, cccaco acid was removed from the pooled supernatants by precipitation with KOH. Protein samples for amino acid analysis were hydrolyzed with 6 N HCl in sealed evacuated ampoules at 110° for 22 hours. Results were corrected for loss of serine, tyrosine, tryptophan, and cysteine during hydrolysis (21).

Unlabeled amino acids were measured using a JOELCO 6AH amino acid analyzer. Labeled amino acids were determined using a Beckman model 120C amino acid analyzer. Fractions of 1.5 ml were collected directly from the column into scintillation bottles, and counted in 15 ml of Aquasol. Under standard conditions, [3H]tyrosine eluted from resin UR 30 at 182 min. After in vitro incorporation into hot TCA insoluble material and hydrolysis, however, labeled tyrosine was not eluted until the column was washed with 0.35 M NaOH (data not shown). This change in elution behavior was noted only with tyrosine.
and was not prevented by the addition of 1 mM phenylthiourea to the reaction mixture (22). It was concluded that labeled tyrosine is modified by some component of the protein synthesis mixture in such a way as to alter its elution behavior on UR-30. The nature of the modification was not further investigated. After incorporation and hydrolysis, labeled tyrosine was determined at the new position.

**Immunoanalyses**—New Zealand albino rabbits were injected subcutaneously with 1 mg of fibroin in complete Freund’s adjuvant. Rabbits were injected twice more at 1-week intervals and occasionally thereafter. A single line of identity resulted when serum obtained from these rabbits and a sample of antifibroin serum prepared by Cebra (11) were compared by an Ouchterlony double diffusion test against purified fibroin (data not shown).

For immunoprecipitation, samples were suspended in 1 ml of 10 mM sodium borate buffer (pH 8.4), 140 mM NaCl. After addition of 0.1 ml of antifibroin or normal rabbit serum, mixtures were incubated at 37°C for 1 hour. An equivalence amount (0.7 ml) of goat antiserum to rabbit γ-globulin was added, and tubes were incubated at 37°C for 1 hour, then held at 4°C overnight. Precipitates were collected by centrifugation, washed twice with 1% deoxycholate, 1% Triton X-100, and once with 10 mM sodium borate buffer (pH 8.4), 140 mM NaCl. The precipitates were suspended in 1 ml 10% TCA, heated in boiling water for 15 min, then washed successively with 10% TCA, ethanol, and ether. The pellets were suspended in 1 ml of 0.95% acetic acid, and counted in Aquasol.

**RESULTS**

Isolation and Identification of Fibroin mRNA—Fibroin mRNA was extracted from posterior silk glands removed from *B. mori* larvae just prior to spinning, and purified by separation procedures based on its large size (6). Fig. 1a shows the fractionation obtained when whole posterior silk gland RNA is passed over a column of Sepharose 2B, which excludes polynucleotides larger than \(2 \times 10^6\) molecular weight. Thus, fibroin mRNA (5.5 to \(6.0 \times 10^6\) molecular weight) (23) is largely freed of other RNA in this single step. The RNA shown by the brackets in Fig. 1a was collected and heated in a solution of low ionic strength to disrupt aggregates before sedimentation in a sucrose gradient (Fig. 1b). Because they contain an internal nick, contaminating 28 S rRNA molecules sediment as two discrete fragments together with 18 S rRNA after denaturation (24, 25). Only small amounts of rRNA were removed by a second heating and sucrose gradient sedimentation (Fig. 1c).

The 47 S RNA from the first or second sucrose gradient was used to direct cell-free protein synthesis. This 47 S RNA has the sedimentation rate, base composition, and T1 RNase oligonucleotide pattern characteristic of fibroin mRNA molecules (6, 23). Molecular hybridization experiments indicate that fibroin mRNA prepared in this way is more than 95% pure.  

**Cell-free Protein Synthesis**—Purified 47 S fibroin mRNA stimulated 3- to 4-fold the incorporation of \(^{14}C\)alanine into hot TCA-precipitable material by a mouse Ehrlich ascites cell-free extract. Optimum stimulation was obtained at a fibroin mRNA concentration of 100 μg/ml in the presence of 80 mM K⁺ and 4 mM Mg²⁺. Amino acid incorporation was linear for 40 min and continued for 120 min (Fig. 2).

The size of the reduced and alkylated product was determined by polyacrylamide gel electrophoresis to be heterogeneous, and to include polypeptides estimated relative to reovirus structural protein markers to be of up to \(10^9\) molecular weight (Fig. 3A). No polypeptides as large as native fibroin \((3.5 \times 10^9)\) (8) were detected in 10% gels. Glycine and alanine are enriched relative to leucine and valine in polypeptides greater than 20,000 molecular weight, a result consistent with the amino acid composition of fibroin, and the fact that the smaller peptides are synthesized in the same amount in the absence of added mRNA (Fig. 3B).

The protein synthesis inhibitor, aurintricarboxylic acid (ATA), inhibits the stimulation of amino acid incorporation significantly at concentrations between \(6 \times 10^{-8}\) M and \(10^{-4}\) M, but has little effect on endogenous incorporation (Table I), presenting further evidence that the stimulation represents initiation of polypeptides directed by exogenous fibroin mRNA rather than an increase in endogenous synthesis. At these concentrations, this inhibitor specifically prevents initiation of protein synthesis (26). ATA inhibition of fibroin mRNA translation parallels that of EMC virus RNA, on which initiation occurs correctly at a single locus in the Ehrlich ascites system (27, 28).

**Immunoprecipitation of Fibroin Polypeptides**—Although large quantities of purified antigen were available, the tendency of fibroin solutions to gel or form insoluble strands or films of protein made it very difficult to devise a reproducible direct immunoassay. Therefore, an indirect assay requiring no added
carrier fibroin was developed. Samples from a cell-free reaction mixture were incubated with excess rabbit antifibroin serum, and the resulting antigen-antibody complexes were precipitated by adding an equivalence amount of goat serum directed against rabbit γ-globulin.

Antifibroin serum precipitated about one-half of the labeled alanine incorporated in response to fibroin mRNA (Table II). Cell-free products formed in the absence of added RNA, or in the presence of EMC viral RNA, were precipitated poorly by either antifibroin serum or normal serum. Similar evidence of cell-free fibroin polypeptide synthesis was obtained (data not shown) in a fibroin mRNA-stimulated cell-free system prepared from wheat germ, as described by Roberts and Paterson (29).

Precipitation by Trypsin Digestion—When a solution of fibroin is digested with trypsin, oligopeptides containing 90% of the amino acids are precipitated (9). The cell-free products formed in response to fibroin mRNA are mixed with carrier fibroin and trypsin digested, the labeled peptides co-precipitate with the authentic fibroin sequences, but products formed in the absence of added RNA, or in the presence of EMC viral RNA are poorly precipitated (Table III). Thus, nonfibroin polypeptides are soluble after a treatment which precipitates both authentic fibroin polypeptides and the cell-free polypeptides directed by fibroin mRNA. Despite exhaustive washing and hot TCA extraction, in four such experiments, more than 100% of the hot TCA-precipitable radioactivity initially present was found in the tryptic precipitate of fibroin mRNA-directed cell-free products. Some of this precipitated radioactivity presumably reflects trapping of unincorporated labeled amino acids in the gelatinous precipitate formed from fibroin under these conditions (9).

Detection of Crystalline Region Polypeptides—The specific precipitation of fibroin crystalline region polypeptides after chymotrypsin digestion provided another method for the identification of fibroin sequences in the cell-free products. Fibroin mRNA was used to stimulate protein synthesis in the presence of [3H]alanine (34% of the crystalline region polypeptide amino acids are alanine) and [14C]leucine (absent from crystalline region polypeptides). Carrier fibroin was then added, and a precipitate formed by digestion with chymotrypsin. One-third of the incorporated labeled alanine precipitates together with the authentic fibroin crystalline region polypeptides, compared to only 3% of the leucine (Table IV). When the same experiment was performed with polypeptides formed in response to added EMC viral RNA, less than 2% of the incorporated alanine or leucine was precipitated. This demonstrates that a significant fraction of the cell-free synthesis consists of alanine-containing polypeptides which are similar enough to fibroin crystalline region polypeptides to co-crystallize and precipitate with them.

TABLE I

| Tube | Antifibroin serum | Normal serum | A/N |
|------|------------------|--------------|-----|
| EMC viral RNA | 6.9 | 13.6 | 0.5 |
| Fibroin mRNA | 6.9 | 13.6 | 0.5 |
| + Fibroin mRNA | 11.4 | 27.2 | 0.5 |
| - EMC viral RNA | 5.2 | 10.4 | 0.5 |
| + EMC viral RNA | 5.2 | 10.4 | 0.5 |
| - Fibroin mRNA | 5.2 | 10.4 | 0.5 |
| A/N | 44.4 | 9.6 | 4.7 |

FIG. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of cell-free polypeptides. Cell-free protein synthesis was conducted as described under “Experimental Procedure,” labeling with [3H]glycine and [3H]alanine (■), and with [14C]leucine and [14C]valine (O—O). Polypeptides present after 30 min of incubation were precipitated with TCA and then reduced, alkylated, and subjected to electrophoresis in 10% sodium dodecyl sulfate polyacrylamide gels. Reovirus structural proteins (X, p, and U) were used as molecular weight markers.
Chymotrypsin precipitation of cell-free polypeptides

Cell-free protein synthesis was conducted as described under "Experimental Procedure," labeling with both [3H]glycine and [14C]leucine, and adding either EMC RNA or fibroin mRNA. Incorporated radioactivity is the hot TCA-precipitable radioactivity before protease digestion, determined on an aliquot of the reaction mixture.

| Tube          | Incorporated radioactivity (cpm) | Precipitated radioactivity (pmol) | % precipitated |
|---------------|----------------------------------|----------------------------------|---------------|
| 1. EMC viral RNA | [3H]Glycine: 175,712            | 3,205                             | 1.9           |
|               | [14C]Leucine: 6,478              | 32                                | 0.5           |
| 2. Fibroin mRNA | [3H]Alanine: 35,105             | 11,384                            | 32.4          |
|               | [14C]Leucine: 1,575              | 47                                | 3.0           |

From the amino acid composition of intact fibroin and fibroin crystalline region, the fraction of the total amount of each amino acid in fibroin which precipitates with chymotrypsin can be calculated. This criterion was applied to the cell-free products by using fibroin mRNA to stimulate amino acid incorporation in a series of 12 tubes, each containing a different tritiated amino acid, but otherwise identical. In each case, total incorporation and the fraction of it that was precipitated after digestion were determined and compared to the prediction for native fibroin. The results indicate that cell-free polypeptides similar in amino acid composition to the authentic fibroin crystalline region polypeptides are precipitated by this technique (Table V). The eight amino acids not occurring in native fibroin crystalline region polypeptides precipitate poorly, but the fraction of incorporated glycine, alanine, serine, and tyrosine precipitated was close to the predicted value. This result suggests that polypeptides with an amino acid composition resembling that of authentic fibroin crystalline region polypeptides compose about the same fraction of the cell-free synthesis stimulated by fibroin mRNA as would be found if native fibroin were the primary product.

The actual amino acid composition of the cell-free crystalline region polypeptides could not be reliably calculated from the data of Table V because different amounts of products could have been synthesized in each tube. Consequently, the relative quantities of glycine, alanine, serine, and tyrosine present in the crystalline region polypeptides synthesized in a single large reaction were determined. The specific activity of each precursor amino acid was determined by amino acid analysis of a hot acid extract of an aliquot taken before synthesis began. After incubation for 120 min, the cell-free products were precipitated with chymotrypsin. The cell-free crystalline region polypeptides were hydrolyzed, and the radioactivity in each amino acid was quantitated. The amino acid compositions of fibroin and fibroin crystalline region polypeptides (15).

| Amino acid | % total | Predicted |
|------------|---------|-----------|
| Gly        | 14.950  | 14.343    | 0.96      | 0.64 |
| Ala        | 79.061  | 54.927    | 0.70      | 0.67 |
| Ser        | 1.503   | 1.318     | 0.88      | 0.75 |
| Tyr        | 33.087  | 4.514     | 0.14      | 0.20 |
| Asp H+     | 23.185  | 0.490     | 0.02      | 0.00 |
| Glu H+     | 3.990   | 0.135     | 0.03      | 0.00 |
| Pro        | 0       | 0.376     | 0.00      | 0.00 |
| Val        | 4.428   | 0         | 0.00      | 0.00 |
| Met        | 0       | 0         | 0.00      | 0.00 |
| Ile        | 4.923   | 0.310     | 0.01      | 0.00 |
| Leu        | 56.174  | 3.680     | 0.03      | 0.00 |
| Phe        | 8.284   | 1.25      | 0.02      | 0.00 |

Amino acids contained in chymotryptic precipitate of cell-free polypeptides

Cell-free protein synthesis was conducted as described under "Experimental Procedure," using the indicated amino acids as precursors. The results obtained from a tube to which no mRNA was added were subtracted from those obtained with mRNA, both after hot TCA precipitation and after chymotrypsin precipitation. The column labeled "predicted" contains the fraction of the total amount of the amino acid in fibroin which is in crystalline region polypeptides, calculated from the amino acid compositions of fibroin and fibroin crystalline region polypeptides (15).

| Amino acid | % total | Predicted |
|------------|---------|-----------|
| Gly        | 4.534   | 38.362    | 7.94      | 41.5  | 47.1  |
| Ala        | 4.805   | 28.539    | 5.94      | 31.1  | 34.6  |
| Ser        | 1.026   | 6.217     | 6.09      | 26.6  | 14.5  |
| Tyr        | 12.570  | 1.909     | 0.16      | 0.8   | 1.7   |

Amino acid composition of cell-free and authentic crystalline region polypeptides

Cell-free protein synthesis was conducted as described under "Experimental Procedure," using [3H]glycine, [3H]leucine, [3H]serine, and [3H]tyrosine as precursors in a single 1.2-ml reaction. The initial specific activity of each amino acid was determined on a hot acid extract of an aliquot taken before synthesis began. After incubation for 120 min, the cell-free products were precipitated with chymotrypsin. The cell-free crystalline region polypeptides were hydrolyzed, and the radioactivity in each amino acid was quantitated. Results were calculated in terms of picomoles by using the specific activities, and then were converted to per cent values. The authentic per cent total was calculated from the total amino acid composition of crystalline region polypeptides prepared from cococon fibroin (data not shown).
However, about 40% of the 47 S mRNA molecules bind to poly(U) under conditions in which poly(A) is bound (7). Either cellulose nitrate filters or glass filters impregnated with LiBr. About 75% of the labeled polypeptides eluted in a major peak slightly ahead and 15% in a shoulder which chromatographed similarly. The bound and unbound polynucleotides were counted in 3 ml of Aquasol. Molecular weight markers, run separately, were: insulin, 5,800; RNase A, 13,700; chymotrypsinogen, 25,000; cocoon fibroin, 60,000 to 200,000, excluded.

The average molecular weight for the unlabeled crystalline region polypeptides was estimated to be about 6800. The mechanism of this protection is unknown. The messenger RNA for silk fibroin was the first mRNA to be identified by chemical means. Its characteristic base composition and unusual nucleotide sequence properties were both predicted from the structure of fibroin protein (6). Support for this identification of fibroin mRNA is provided by the demonstration of fibroin polypeptide synthesis directed by the mRNA in a mouse Ehrlich ascites cell extract.

**TABLE VII**

**Translation of fibroin mRNA fractionated by oligo(dT)-cellulose chromatography**

Fibroin 47 S mRNA was isolated as shown in Fig. 1, and passed over a 3-ml oligo(dT)-cellulose column in 0.5 M NaCl, 0.01 M Tris-Cl, pH 7.5. Unbound mRNA was that which was not retained by the column after three passages, bound mRNA was eluted with 0.01 M Tris, pH 7.5.

Cell-free protein synthesis, chymotrypsin digestion and precipitation, and antisera precipitation were as described under "Experimental Procedure."

| Per cent of product precipitated after chymotrypsin digestion | 22.6 | 18.8 |
|---------------------------------------------------------------|------|------|
| Per cent of product precipitated by antisera                   |      |      |
| Normal serum                                                  | 19.8 | 22.1 |
| Antifibroin serum                                             | 51.0 | 38.4 |
| Antifibroin/normal                                            | 2.58 | 1.74 |
| II. Stimulation of amino acid incorporation                   |      |      |
| Alanine                                                       | 3.1  | 2.9  |
| Leucine                                                       | 1.7  | 1.6  |
| Alanine/leucine                                               | 1.8  | 1.8  |
| III. Saturation of [3H] alanine incorporation                 |      |      |
| 50 &mu;g/ml of mRNA                                          | 1.6  | 1.7  |
| 100 &mu;g/ml of mRNA                                         | 2.0  | 2.3  |
| 150 &mu;g/ml of mRNA                                         | 2.7  | 3.0  |

The messenger RNA for silk fibroin was the first mRNA to be identified by chemical means. Its characteristic base composition and unusual nucleotide sequence properties were both predicted from the structure of fibroin protein (6). Support for this identification of fibroin mRNA is provided by the demonstration of fibroin polypeptide synthesis directed by the mRNA in a mouse Ehrlich ascites cell extract.

*L. P. Gage, unpublished observations.
Three separate techniques have been used to demonstrate the cell-free synthesis of fibroin peptides in response to added mRNA. An immunoprecipitation technique detected noncrystalline region fibroin peptides. Two other tests were based on the tendency of fibroin peptides to form specific precipitates when digested with proteases.

Cebra (13) analyzed the structure of the antifibroin-binding site by quantitative inhibition studies employing fibroin peptides. He concluded that the binding site for rabbit antifibroin antibodies was closely approximated by or was identical to a dodecapeptide composed of glycine and alanine and some tyrosine, much like the noncrystalline region sequences left in solution after chymotrypsin digestion of fibroin (12). Cebra's results led to an estimate of about 20 antibody binding sites/molecular weight of 400,000, and there are just a sufficient number of these noncrystalline region peptides per monomer (34) to account for the number of antigenic sites. The rabbit antifibroin serum used in the present work was shown to be identical to a sample of Cebra's antifibroin serum by an Ouchterlony double diffusion test against fibroin. Thus, formation of specific antiserum-precipitable material is evidence for the cell-free synthesis of noncrystalline region fibroin peptides.

When a solution of fibroin is digested with trypsin, a gelatinous precipitate forms which contains 90% of the amino acids (9). This procedure precipitates cell-free polypeptides directed by fibroin mRNA, but not those directed by EMC virus RNA, or those synthesized in the absence of added RNA. This unusual behavior upon trypsin digestion demonstrates the similarity of authentic fibroin polypeptides and the cell-free polypeptides directed by fibroin mRNA. Furthermore, the behavior of both is distinct from that of polypeptides with a more typical amino acid composition such as those synthesized in the presence of EMC virus RNA or on endogenous templates.

As shown by their different sizes, the cell-free crystalline region polypeptides and the average crystalline region polypeptide produced from authentic fibroin are not identical. The in vitro synthesized crystalline region polypeptide has an estimated average molecular weight of 8200, and thus, contains about 88 amino acids. The apparent average molecular weight of the authentic crystalline region polypeptides is 6800, corresponding to 79 amino acids. Thus, the major cell-free crystalline region polypeptide is nine amino acids longer than the corresponding to 79 amino acids. Thus, the major cell-free crystalline region polypeptide is nine amino acids longer than the average authentic crystalline region polypeptide.

Lucas et al. (10) have suggested that the length of the crystalline region polypeptides may not be rigidly fixed. There are at least 30 copies of this polypeptide per fibroin molecule, but only about one-quarter of the entire molecule was synthesized in the cell-free extract. If initiation occurs properly, the slightly larger size of the crystalline region polypeptides synthesized in vitro may indicate a bias toward larger units of this repeated sequence near the NH₂-terminal end of the fibroin molecule. The chymotrypsin-digested, precipitated material excluded from the G-50 column may represent the initial crystalline region polypeptide, substantially larger than the others because it contains a part of the amino-terminal sequence which lacks tyrosine.

Qualitatively, of 12 amino acids polymerized in fibroin mRNA-directed assays, only glycine, alanine, serine, and tyrosine were found to co-precipitate with authentic crystalline region polypeptides. The quantitative amino acid composition of the cell-free chymotryptic precipitate was found to be very similar to that formed from native fibroin. Both cell free and authentic crystalline region polypeptides are approximately 80 to 90 amino acids long, and contain more than 35 glycine residues, and more than 25 alanine residues.

Specific inhibition by ATA suggests that the stimulation of polypeptide synthesis by added fibroin mRNA is initiation-dependent. In the experiments described in Table II, 44.4% of the hot TCA-precipitable [³H]alanine was precipitated by antifibroin serum. The stimulation of amino acid incorporation above the endogenous level in this experiment was 3.8-fold. Since endogenous synthesis appears to continue in the presence of added mRNA (Fig. 2), the results indicate that at least 60% of the [³H]alanine specifically incorporated in response to fibroin mRNA is incorporated into fibroin sequences.

In the experiment described in Table IV, 32.4% of the hot TCA-precipitable [³H]alanine was precipitated after digestion with chymotrypsin. In this experiment, the stimulation of alanine incorporation above the endogenous level was 3.3-fold. Assuming that the endogenous synthesis also occurs in the presence of added mRNA, and that the radioactivity precipitable with chymotrypsin represents 67% of the total alanine incorporated into fibroin, 70% of the [³H]alanine incorporation in response to fibroin mRNA is incorporated into fibroin polypeptides. The average value for four similar experiments was that 79% of the [³H]alanine incorporated in response to fibroin mRNA is incorporated into fibroin polypeptides. Thus, cell-free synthesis was usually initiated in frame on fibroin mRNA, and elongated correctly for the duration of synthesis.

Since globin mRNA is often used for cell-free protein synthesis at a concentration of approximately 5 µg/ml (35, 36), the requirement for 100 µg/ml of fibroin mRNA for maximal stimulation of polypeptide synthesis may seem unusually large. However, the molecular weight of globin β chain mRNA is only 2.27 x 10⁴ (37), compared to 5.5 to 6.0 x 10⁴ for fibroin mRNA (23). Thus, if there is one initiation site per molecule, 100 µg of fibroin mRNA contain the same number of initiation sites as 4 µg of globin β chain mRNA.

However, whereas complete globin chains are synthesized by various cell-free systems, only partial fibroin chains are synthesized. In rabbit and duck reticulocyte cell-free systems, approximately two mouse globin chains are synthesized per mRNA molecule in 30 min (38). Assuming that the crystalline region polypeptides described in Table VI represent 60% of the total fibroin synthesized in 1.2 ml, a similar calculation indicates that one fibroin chain of 50,000 molecular weight is synthesized/350 mRNA molecules in 30 min. Thus, globin chains are initiated about 700-fold more frequently per mRNA molecule than are fibroin chains.

In the mature silk gland, a single cell synthesizes about 1 µg of fibroin in 30 min (39), about 400-fold more than 1 ml of mouse Ehrlich ascites cell-free system. However, since a single silk gland cell contains only 170 ng of fibroin mRNA (40), the cell is more than 10⁴-fold more efficient than a 1-ml cell-free system.

It should be noted that synthesis of this unusual protein by the ascites cell extract occurred without addition of any specific initiation factors or tRNAs from the silk gland. The specific translation of an insect mRNA in a mammalian cell-free system again demonstrates the universality of the genetic code and the mechanisms of its expression.

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REFERENCES

1. Shapiro, G., Padieu, P., Maleknia, N., Krub, J., Dreyfus, J. C., Alexandre, Y., Denrubi-Dumont, M., Reibel, L., Thireau, A. M., and Tichonickv, L. (1966) J. Mol. Biol. 20, 427-446
2. Berns, A. J. M., Strous, G. J. A. M., and Bloemendaal, H. (1972) Nature New Biol. 236, 7-9
3. Jacobs-Lorena, M., Baglioni, C., and Borun, T. W. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 2095-2099
4. Means, A. R., Comstock, J. P., Rosenfeld, G. C., and O'Malley, B. W. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 1146-1150
5. Sarker, S., and Mukherjee, S. P. (1973) Prep. Biochem. 3, 583-604
6. Suzuki, Y., and Brown, D. D. (1972) J. Mol. Biol. 63, 409-429
7. Greene, R. A., Morgan, M., Shatkin, A. J., and Gage, L. P. (1973) Proc. Fed. Am. Soc. Exp. Biol. 32, 455
8. Sprague, K. U. (1948) Biochemistry, 14, 925-931
9. Shaw, J. T. B. (1964) Biochem. J. 93, 45-54
10. Lucas, F., Shaw, J. T. B., and Smith, S. G. (1957) Biochem. J. 66, 468-470
11. Cebra, J. J. (1961) J. Immunol. 86, 190-196
12. Cebra, J. J. (1961) J. Immunol. 86, 197-204
13. Cebra, J. J. (1961) J. Immunol. 86, 205-214
14. Lucas, F., and Rudall, K. M. (1968) In Comprehensive Biochemistry (Florkin, M., and Stotz, E. H., eds), Vol. 26, Part B, pp. 475-558, American Elsevier, New York
15. Barlow, M. J., Mathias, A. P., Williamson, R., and Gammack, D. B. (1965) Biochim. Biophys. Res. Commun. 13, 61-66
16. Bock, R. M. (1967) Methods Enzymology 12, 218-221
17. Sebring, E. D., and Salzman, N. P. (1964) Anal. Biochem. 8, 126-135
18. Eggen, K. L., and Shatkin, A. J. (1972) J. Virol. 9, 636-645
19. Mans, R. J., and Novelli, G. D. (1961) Arch. Biochem. Biophys. 94, 45-53
20. Sridhara, S., Prudhomme, J. C., and Daillie, J. (1973) Arch. Biochem. Biophys. 156, 168-175
21. Moore, S., and Stein, W. H. (1962) Methods Enzymol. 8, 199-301
22. Ilan, J. (1968) J. Biol. Chem. 243, 5859-5866
23. Lizardi, P. M., and Brown, D. D. (1974) Cold Spring Harbor Symp. Quant. Biol. 38, 701-706
24. Appelbaum, S. W., Elstein, R. P., and Wyatt, G. R. (1966) J. Mol. Biol. 21, 29-41
25. Suzuki, Y., Gage, L. P., and Brown, D. D. (1972) J. Mol. Biol. 70, 637-649
26. Stewart, M. L., Grollman, A. P., and Huang, M. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 97-101
27. Öberg, B. F., and Shatkin, A. J. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 3589-3593
28. Smith, A. E. (1973) Eur. J. Biochem. 33, 301-313
29. Roberts, B. E., and Patterson, B. M. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 2330-2334
30. Williamson, R. (1973) Carnegie Year Book Wash DC 72, 19
31. Williamson, R., Crossley, J., and Humphries, S. (1974) Biochimica 13, 709-719
32. Soreq, H., Nudel, U., Salomon, R., Revel, M., and Littauer, U. Z. (1974) J. Mol. Biol. 88, 233-245
33. Huez, G., Marboix, G., Hubert, E., Lederq, M., Nudel, U., Soreq, H., Salomon, R., Lebleu, B., Revel, M., and Littauer, U. Z. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 3143-3146
34. Lucas, F., Shaw, J. T. B., and Smith, S. G. (1962) Biochem. J. 83, 164-171
35. Molinoff, P. B., Gorski, J., and Lingrel, J. B. (1972) Biochem. Biophys. Res. Commun. 49, 775-781
36. Sampson, J., Mathews, M. B., Osborn, M., and Borchetti, A. F. (1972) Biochemistry 11, 3636-3640
37. Gould, H. J., and Hamlyn, P. H. (1973) FEBS Lett. 30, 301-304
38. Lockard, R. E., and Lingrel, J. B. (1972) J. Biol. Chem. 247, 4174-4179
39. Takizato, Y., Morimoto, T., Matsuura, S., and Nagata, S. (1968) J. Cell Biol. 38, 574-588
40. Suzuki, Y., and Suzuki, E. (1974) J. Mol. Biol. 88, 393-407
Translation of silk fibroin messenger RNA in an Ehrlich ascites cell-free extract.
R A Greene, M Morgan, A J Shatkin and L P Gage

J. Biol. Chem. 1975, 250:5114-5121.

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