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Synthesis of Lens Protein *in vitro* Translation of Calf-Lens Messengers in Heterologous Systems

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Messenger ribonucleic acid was isolated from calf lens polyribosomes and fractionated. Previous identifications of the newly synthesized products have been extended in that the fidelity of the translation of the 14-S mRNA was assayed by fingerprinting analysis. The results suggest that lens messenger is translated with a high degree of fidelity in the cross systems even to the extent that the newly synthesized αA_2 chains contain an acetylated methionine residue in the N-terminal position like the native chains. There seems to be no requirement for cell-specific protein factors in order to obtain accurate translation. Evidence is accumulating that the acetylation is not involved in the initiation mechanism.

Lens messengers appear to possess interesting aspects for a better understanding of the process of protein biosynthesis in eukaryotes.

The eye lens is composed of an epithelial layer in which DNA, RNA and a restricted number of structural proteins are synthesized, and a cortical zone composed of fiber cells in which DNA and RNA synthesis has ceased but protein synthesis continues. Reeder and Bell [1] studying the effect of actinomycin D on cultured lenses have shown that in the epithelial layer RNA synthesis is a prerequisite for protein synthesis, whereas in the cortical zone it is not. This indicates that in the cortical zone the template is stabilized. Further, it has been shown [2] that in the epithelial layer as well as in the cortical zone predominantly one protein is synthesized, α -crystallin, which is an aggregate of molecular weight 800000, composed of four different polypeptide chains designated αA_1 , αA_2 , αB_1 and αB_2 with a molecular weight of approximately 20000 each [3,4].

The biosynthesis of α -crystallin polypeptides appeared to be also interesting for the elucidation of the initiation mechanism in eukaryotes, as all four chains contain an acetylated methionine residue in the N-terminal position [5]. This knowledge formed the background of our attempts to isolate mRNA from lens tissue and to assay its biological activity in cross systems.

Enzymes. Trypsin (EC 3.4.4.4); chymotrypsin (EC 3.4.4.5); subtilisin or subtilopeptidase A (EC 3.4.4.16); pronase (EC 3.4.4.-).

MATERIALS AND METHODS

All chemicals were analytical grade. ^{14}C -labeled amino acid mixture (54 Ci/atom) and [^{35}S]methionine (10 Ci/mmol) were purchased from the Radiochemical Centre (Amersham). Trypsin and chymotrypsin were obtained from Worthington, subtilisin from Nutritional Biochemical Corp. and pronase P from Serva.

Isolation of mRNA from Calf-Lens Polysomes

Calf lens polysomes were isolated as described earlier [6] except that only the cortex was used as starting tissue. From the polysomes messenger was obtained as described for the isolation of hemoglobin messenger by Lockard and Lingrel [7]. Polysomes were disaggregated by 1% sodium dodecylsulfate in 0.05 M Tris-HCl pH 7.5 for 5 min at 37 °C, diluted twice and applied on a sucrose gradient at a concentration of about 2 mg/ml.

The gradient was constructed by pumping 40% (w/w) sucrose, containing 0.05 M Tris-HCl pH 7.4 (freed from RNAase by boiling for 30 min with 0.02% diethylpyrocarbonate) into a mixing chamber of 800 ml containing buffer only. The chamber was connected with the edge of a B-XXIX zonal rotor of IEC. 180 ml of the gradient was used in the overlayer. Overlayer + sample volume comprised 500 ml. After centrifugation the gradient was displaced from the edge of the rotor by pumping in a heavy sucrose solution. The absorbance was measured at 260 nm with the aid of a Gilford spectrophotometer adapted

with a 2-mm flow cell of IEC, fractionated manually and precipitated with 1/10 vol. 2 M potassium acetate pH 5.0 and 2.5 vol. ethanol.

Cell-Free Protein Synthesis

Protein synthesis reaction in a rabbit reticulocyte lysate was performed as described by Lockard and Lingrel [7] using either [³⁵S]methionine (10 μM) plus the remaining 19 (50 μM) unlabeled amino acids or a ¹⁴C-labeled amino acid mixture (20 μM each) supplemented with the unlabeled six missing amino acids (asparagine, glutamine, methionine, tryptophan, cysteine, histidine) at 50 μM each. Lens messenger was added at 20 μg/ml. After 2-h incubation at 30 °C the reaction was terminated by incubation for 15 min at 37 °C with pancreatic RNAase (20 μg/ml) and 10 mM EDTA. In order to prepare biosynthetic products for characterization an acidic-acetone precipitation was performed.

Acetone Precipitation

Globin was prepared from reticulocyte lysates by adding the suspension dropwise to 15 vol. 2 1/2% oxalic acid in acetone at 0 °C under vigorous mixing. When globin was prepared from incubation mixtures for assaying lens messengers total lens crystallins (1 mg/ml incubation) were added as carrier before the acidic-acetone precipitation was carried out. The acetone precipitate was centrifuged at 3000 × g for 5 min, washed three times with cold acetone and once with ether. The precipitate was dried at room temperature.

Gel Electrophoresis

Electrophoresis on dodecylsulfate gels was carried out according to Laemmli [8] in cylindrical siliconized glass tubes (0.5 × 8 cm) on 12.5% polyacrylamide gels. Per gel 200 μg protein together with 20 μg carrier crystallins were applied. The gels were stained, sliced longitudinally and dried before autoradiography.

Electrophoresis on alkaline urea gels was performed as described earlier [9] except that the running buffer was replaced by 0.025 M Tris-glycine pH 8.5. Per gel 400 μg of the cell-free extract was applied together with 100 μg carrier α-crystallin. Gels were sliced and autoradiographed as described for dodecyl-sulfate gels. Gel electrophoresis on preparative alkaline-urea gels for the isolation of αA₂ crystallin chains was carried out in gels (9 × 1.3 cm) for 16 h at 100 V. The gels were prepared as described for the analytical urea gels. Per gel about 15 mg cell-free extract was applied, together with 1 mg carrier α-crystallin. After electrophoresis a thin longitudinal slice from each gel was stained and autoradiographed for 12 h. The desired component was cut out, minced and stirred for 16 h with 10 vol. buffer containing

6 M urea. The gel was removed by filtration and the remaining solution was dialyzed against distilled water containing 5 mM 2-mercaptoethanol, whereafter the extracted protein was lyophilized. By this procedure about 0.5 μCi of αA₂ labeled by a mixture of ¹⁴C labeled amino acids was obtained from a 1-ml incubation mixture to which 14-S messenger was added.

Enzymic Digestions

Pronase and subtilisin digestion was carried out as described earlier [10]. For tryptic digestion per 0.5 μCi αA₂, 30 mg carrier αA chains was added and tryptic digestion was carried out in 0.1 M NH₄HCO₃, adjusted to pH 8.9 by the addition of ammonia, for 5 h at 37 °C. Trypsin was added (1:50) at zero time and after 2 h of incubation. Chymotrypsin digestion was carried out for 16 h at 37 °C (1:50) in 0.1 M NH₄HCO₃.

Column Fractionation

After lyophilization of the tryptic digest 2 ml starting buffer (0.2 M pyridine-acetate, pH 3.1) was added. Part (400 μl) of the soluble peptides (200000 counts/min) were applied on a Bio-Rad Aminex A-5 column (0.4–17 cm) and eluted after washing with 15 ml starting buffer at 12 atm and 50 °C with a linear gradient (2 × 125 ml) ranging from pH 3.1 to 5.0 (pH 5.0 buffer: 2.0 M pyridine-acetate). 1.5-ml fractions were collected. 50 μl was mixed with 5 ml Instagel (Packard) and counted in a liquid scintillation counter. 50 μl was hydrolyzed with 3 M KOH, neutralized, stained with ninhydrin and the absorbance measured at 570 nm.

Paper Chromatography

Fractions from the Aminex A-5 column were evaporated under reduced pressure at room temperature, solubilized in 50 μl distilled water and applied on Whatman 3-MM paper. The fractions were applied to the paper at distances of 0.6 cm. Descending chromatography was performed with *n*-butanol–acetic acid–pyridine–water (60:12:48:40, v/v/v/v) for 12 h. The chromatogram was stained with 0.5% ninhydrin and autoradiographed for 2 weeks.

RESULTS

Lens messengers have been isolated [11], translated and identified in several heterologous systems such as the ascites cell-free system [12], the reticulocyte lysate derived from rabbits [13] and ducks [14] and *in vivo* in the system of oocytes from *Xenopus laevis* [15]. Here we shall describe the more definite identification of one of the products synthesized in a rabbit reticulocyte lysate under the direction of one of the lens messengers.

Gel-Electrophoretic Analysis

The different RNA fractions in front of the 18-S ribosomal RNA were added to the cell-free system and their products identified by electrophoresis on dodecylsulfate gels. The fractionation of the RNA is visualized in Fig. 1, whereas the gel electrophoretic analysis of the products synthesized under the direction of the different fractions is shown in Fig. 2. From this figure it can be seen that subsequent RNA

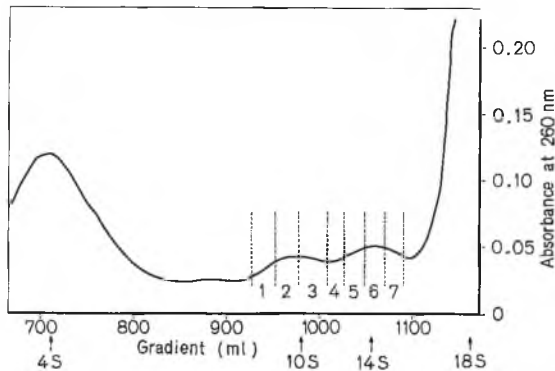


Fig. 1. Zonal centrifugation profile of 40 mg polysomal RNA from calf lens. Centrifugation was performed in a BXXIX zonal rotor of IEC for 25 h at 2 °C. Only part of the gradient is visualized. The numbers 1 to 7 refer to the fractionation of the RNA in the 8 to 15-S area.

fractions have differing coding properties, which illustrates the high-resolving power of zonal centrifugation as separation technique.

As the 14-S messenger fraction seemed to be freed from significant amounts of other messengers, the product of this messenger was further identified. Whereas gel electrophoretic analysis on dodecylsulfate gels revealed that the product was an A chain of α -crystallin, identification on alkaline gels in the presence of urea demonstrated that αA_2 was synthesized (Fig. 3) almost exclusively.

Characterization of the N-Terminus of Newly Synthesized αA_2

As all α -crystallin polypeptides possess the N-terminal sequence: Acetyl-Met-Asp-Ile-Ala, the most interesting aspect of the identification was to see whether the reticulocyte lysate, programmed with 14-S messenger was able to synthesize an αA_2 polypeptide with this N-terminal sequence.

Hence the N-terminus of the newly synthesized αA_2 chain was analyzed. After labeling with [35 S]-methionine, the αA_2 chains were separated from the endogenous proteins by electrophoresis on basic-urea gels. The labeled αA_2 polypeptide was extracted from the gel and the N-terminal peptide was identified after digestion of the extracted material with subtilisin and pronase: subtilisin releases the N-

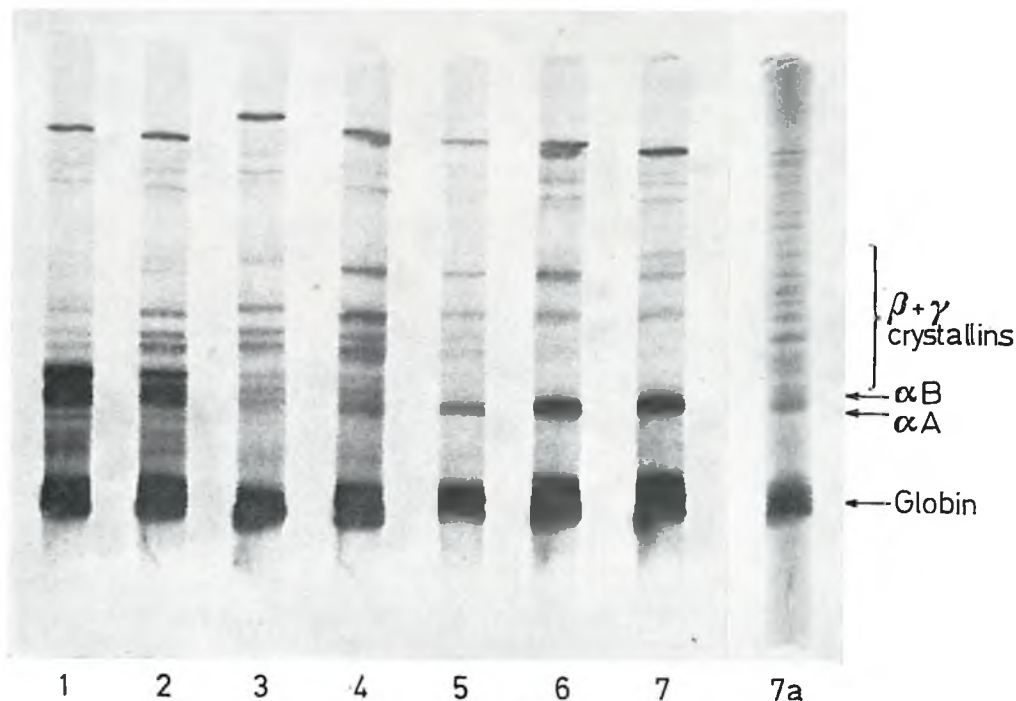


Fig. 2. Dodecylsulfate-gel analysis of the lens messenger-directed products in the reticulocyte lysate. Incubations and gel electrophoresis were performed as described in the method section. [35 S]Methionine was used as label. Autoradiography was carried out for 2 weeks. The numbers 1 to 7 refer to the RNA fractions 1 to 7 of Fig. 1. For comparison a stained gel is shown in 7a

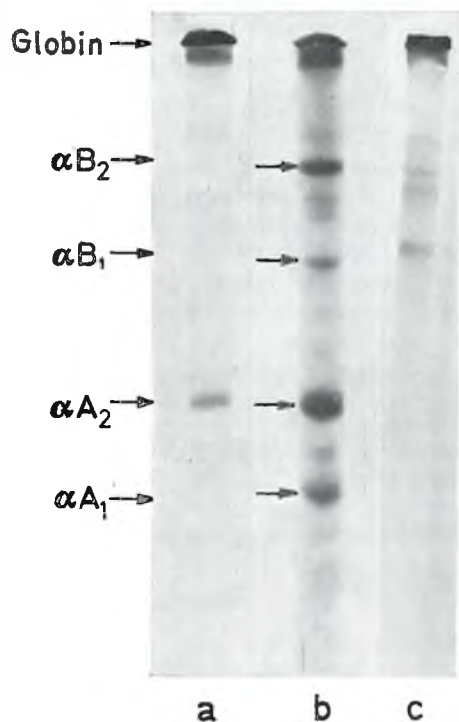


Fig. 3. Analysis of the 14-S directed product on alkaline-urea gels. Gels were prepared and run as described in the Methods section. [^{35}S]Methionine was used as label. Autoradiography was carried out for 2 weeks. (a) RNA fraction 6 added to the incubation mixture; (b) stained gel; (c) RNA fraction 1 added to the incubation mixture (control)

terminal tetrapeptide whereas pronase releases the N-terminal dipeptide. Digestion with subtilisin and pronase released peptides with an electrophoretic mobility identical to Ac-Met-Asp-Ile-Ala and Ac-Met-Asp, respectively. Evidence for N-terminal acetylation was further obtained by the behavior of the peptides on a Dowex-50 column: the dipeptide as well as the tetrapeptide were not retained indicating that no free NH_2 -group was accessible. That these peptides were indeed derived from the N-terminus has previously been shown by labeling experiments with different [^{35}S]Met-tRNAs. Only addition of [^{35}S]Met-tRNA^{Met} to the incubation mixture resulted in their labeling [16].

As the possibility existed that the blockade was due to a formyl group the dipeptide was exposed to 0.5 N HCl at 90 °C for 30 min. This treatment would result in removal of an N-terminal formyl group. Since the electrophoretic mobility of the dipeptide was not affected after this treatment the conclusion could be drawn that the blocking group was not removed.

Tryptic and Chymotryptic Identification

In order to obtain further information concerning the fidelity of the translation the 14-S mRNA-directed product was labeled with a ^{14}C -labeled amino acid mixture, separated from the endogenous reticulocyte proteins by gel electrophoresis on basic-urea gels and digested with trypsin. The soluble peptides

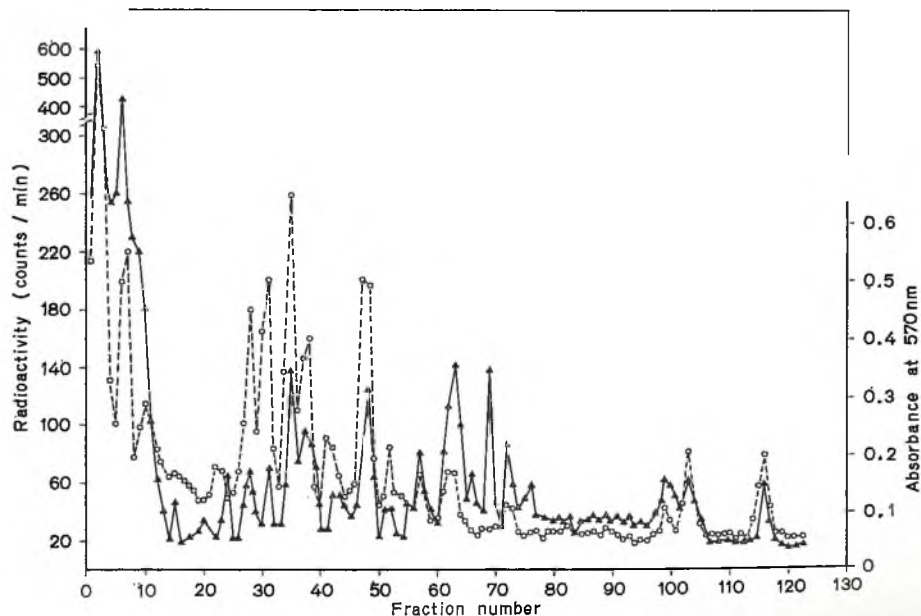


Fig. 4. Separation of the tryptic peptides of the αA_2 chain by ion-exchange chromatography. Tryptic digestion and column chromatography were carried out as described in the Methods section. (—) Absorbance at 570 nm; (---) radioactivity

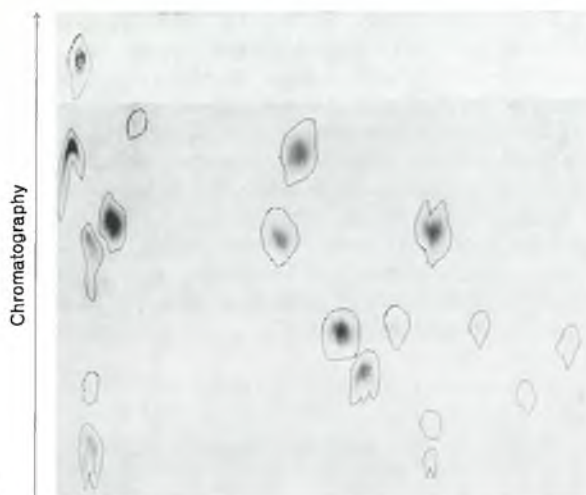


Fig. 5. Chromatographic identification of the tryptic peptidase in fractions 0 to 72 after ion-exchange chromatography. Chromatography was carried out as described in the Methods section. The autoradiogram is depicted and ninhydrin-positive areas are encircled



Fig. 6. Chromatographic identification of the tryptic peptides in fractions 72 to 120 after ion-exchange chromatography. Details as in Fig. 5

were applied on an Aminex A-5 column and eluted with a gradient ranging from pH 3.1 to 5.0.

After fractionation an aliquot of each fraction was counted and the absorbance at 570 nm after reaction with ninhydrin determined. The elution pattern is shown in Fig. 4. This identification revealed a high degree of similarity between the newly synthesized chains and native αA_2 chains. In order to complete this analysis fractions were evaporated under reduced pressure, applied on paper and chromatographed. After staining with ninhydrin the chromatogram was autoradiographed for two weeks. The autoradiogram is depicted in Fig. 5 and 6. The ninhydrin-positive areas are encircled. The

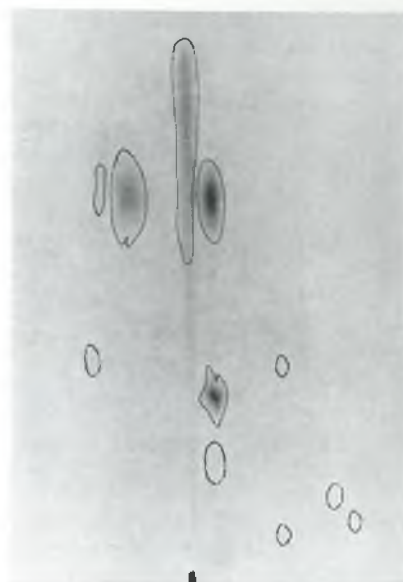


Fig. 7. Fingerprinting of the insoluble core peptides after chymotryptic digestion. Chymotryptic digestion, electrophoresis and chromatography were carried out as described in the Methods section. The autoradiogram is depicted and ninhydrin-positive areas are encircled

autoradiogram again reveals the high degree of fidelity of translation. Also the intensity of the spots is in accordance with our knowledge of the length of the different tryptic peptides.

Since part of the tryptic peptides were insoluble, these core peptides were digested with chymotrypsin. Fingerprinting of these peptides also demonstrated similarity although not all ninhydrin-positive spots had a counterpart in the autoradiogram (Fig. 7). This may be caused by the fact that several amino acids were absent in the ^{14}C -labeled mixture used or that not all ninhydrin-positive material was derived from αA_2 chains. However, it is worthwhile to emphasize that no radioactive material was found in positions in which no unlabeled peptides could be detected. Although minor substitutions cannot be excluded by this type of identification it lends support to the idea that the 14-S lens messenger, as other mammalian messengers [18-22], can be translated faithfully in heterologous systems. Recent identification of tryptic peptides derived from 14-S mRNA injected oocytes revealed the same high degree of similarity of oocyte-derived αA_2 and native chains.

Identification of Other Lens Messengers

The chains synthesized under the direction of the 10-S messengers are less extensively characterized. The partial identification of polypeptides synthesized in the reticulocyte lysate under the direction of these messengers confirmed the experiments with 10-S

messengers in the ascites cell-free system. One of the main products as determined by gel electrophoretic analysis seems to be the B₂ chain of α -crystallin. It is worth mentioning that the latter chain with an amino acid composition considerably differing from the A chains, but with the same N-terminal sequence Ac-Met-Asp-Ile-Ala, is acetylated too in the reticulocyte lysate.

Until now we have not been able to detect the synthesis of A₁ and B₁ chains. The absence of any newly synthesized A₁ supports the hypothesis of Palmer and Papaconstantinou [23] that the A₁ chain arises as the result of a post-translational modification of A₂. Although not very likely it cannot completely be excluded that a messenger coding for α A₁ exists. This hypothetical messenger could be lost during the isolation procedure or its translation might be blocked.

However the hypothesis of post-translational modification is fortified by the determination of the difference between the two A chains: a glutamine residue in A₂ has its counterpart in a glutamic acid residue in A₁ causing a difference between the two acidic polypeptide chains of α -crystallin [24, 25].

That no B₁ synthesis could be detected may be explained by the fact that the B₁ chain occurs only in very small amounts [26]. Hence it would be reasonable to assume that the amount of messenger coding for B₁ is below the detection level. However, a similar explanation as given for the absence of A₁ synthesis cannot be excluded, although the kinetics of synthesis of B₁ in cultured lenses seem to be in favor of direct synthesis.

DISCUSSION

From the present results it may be concluded that lens messenger can be translated faithfully in heterologous systems even to the extent of N-terminal acetylation of the chains.

The heterologous systems tested are able to recognize and translate a messenger originating from a highly specialized tissue such as the lens without requiring additional lens-specific protein factors. This is in contrast with results obtained with myosin messenger [28] but in accordance with results obtained with other messenger RNAs [7, 12, 18–20, 29–33]. The results indicate that tissue-specific recognition factors, if they are involved, are not obligatory. The discrepancy in observations might be explained by assuming the existence of different types of initiation factors which exhibit varying affinities towards different messengers. The occurrence of those factors should not be restricted to one tissue or species.

It is quite remarkable that the 14-S lens messenger fraction, with an estimated molecular weight of about 360 000, is required to direct the synthesis of a polypeptide of molecular weight 20 000 only, while

the 10-S lens messenger fraction with an average molecular weight of about 260 000 directs the synthesis of polypeptides with significantly higher molecular weights.

Three possibilities may be considered:

a) The 14-S messenger contains untranslated regions of considerable length. We have evidence that the messenger contains poly(A) tracks as it is retained on oligo(dT)-cellulose and poly(dT)-cellulose (Berns, van Kraaikamp, Bloemendal and Piperno, unpublished results). Whether or not the poly(A) segment(s) can account for the excess of nucleotides awaits elucidation.

b) The messenger might be bicistronic. However, since a poly(A) track seems to be present the molecular weight of the messenger had to be somewhat higher than the estimated values. Nevertheless this does not exclude bicistronic mRNA as the determination of the molecular weight of the messenger may be influenced by the contaminating RNAs which pass the oligo(dT) column.

c) The initial product may be a longer precursor molecule which is proteolytically trimmed to yield α A₂ crystallin. Although no labeled high-molecular-weight components were detected in either of the cell-free systems after incubation in the presence of 14-S messenger, experiments in which several proteinase inhibitors are added to the incubation mixture will be necessary to verify this possibility.

This work with lens messengers may also shed some light on the initiation process of crystallin synthesis. The presence of an acetylated methionine residue in N-terminal position in all α -crystallin chains enables the following initiation mechanism.

a) Lens messengers require initiation with Ac-Met-tRNA^{fMet}.

b) Initiation takes place with Met-tRNA^{fMet} and cleavage of the methionine residue is prevented by the nature of the adjacent amino acid(s), which might determine the specificity of the splitting enzyme. Such a specificity has been reported for ribosome-bound leucine aminopeptidase [34]. An alternative explanation might be that a specific acetylation mechanism prevents the N-terminal methionine from being split off.

c) The lens messengers coding for α -crystallin polypeptide chains code for a starting sequence Met-Met-Asp in which the first amino acid, derived from Met-tRNA^{fMet} is split off in the usual manner and the second methionine, derived from Met-tRNA^{fMet} is acetylated in a later phase.

The first possibility is unlikely: no acetylated methionyl-tRNA could be detected in the lens [16] or the reticulocyte system [35].

The third possibility is excluded by the experiments with fMet-tRNA^{fMet}, Met-tRNA^{fMet} and Met-tRNA^{fMet} [16]. After incubation with fMet-tRNA^{fMet} only fMet-Asp can be detected, whereas after incuba-

tion with the unblocked Met-tRNAs only the initiator tRNA donates methionine in the N-terminal position. As it has been shown earlier that in the reticulocyte system no blocking group is present during initiation [35], the presence of the acetyl group is most likely due to an acetylation mechanism working after initiation or even after release of the polypeptide from the ribosome. The synthesis of acetylated α -crystallin chains in the heterologous systems described lends support to the idea that the machinery responsible for this acetylation is a common equipment of a number if not all eukaryotic cells.

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