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Heterologous *in vitro* Synthesis of Lens α-Crystallin Polypeptide

MESSENGER RNAs coding for α and β globin chains[1], for myosin[2] and for the mouse immunoglobin light chains[3] have been shown to be translated in a reticulocyte cell-free system. Messengers are also translated faithfully in the ascites tumour cell-free system[4] and in oocytes from *Xenopus laevis*[5].

We stressed earlier[6] that the lens is a unique tissue for the study of protein biosynthesis *in vitro* as it produces a class of highly specific proteins: the crystallins. α-Crystallin, which

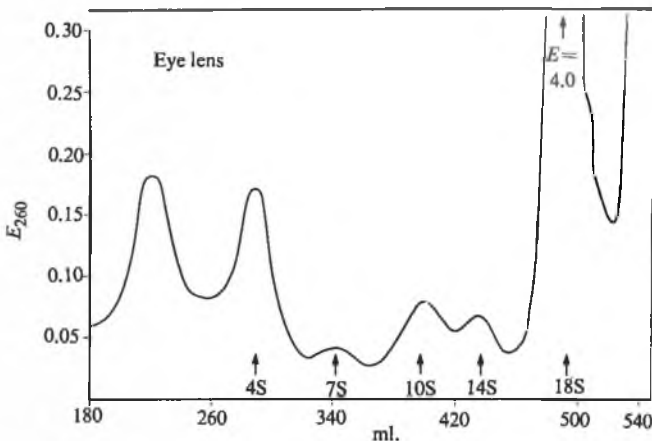

Fig. 1 Sedimentation profile of lens mRNA. Lens polyribosomes were suspended in a medium containing 6% sucrose 0.05 M Tris HCl, *p*H 7.4, and 1.0% SDS, incubated for 5 min at 37° C and diluted twice with the same medium in which SDS was omitted. The RNA was separated by zonal centrifugation using an exponential gradient from 8 to 28% (w/w) sucrose. All sucrose solutions were pretreated by boiling with 0.02% diethylpyrocarbonate for 30 min. The samples were applied in a volume varying from 10 to 25 ml., containing about 3 mg/ml. polyribosomes. Routinely an overlayer of 200 ml. was used. Centrifugation was performed at 2° C in an IEC B XXX zonal rotor for 15 h at 50,000 r.p.m. The profile was monitored at 260 nm in a Gilford spectrophotometer adapted with a 2 mm flow cell. The RNA fractions corresponding to 7S, 10S and 14S were precipitated with one-tenth volume of 2 M potassium acetate *p*H 5.0 and 2.5 volumes of ethanol for 16 h at −20° C. The precipitates were collected by centrifugation and dissolved in distilled water.

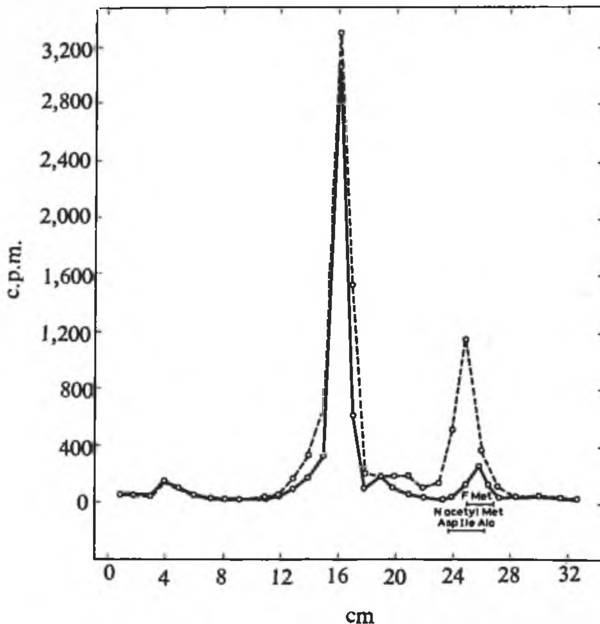

Fig. 2 Characterization of N-terminal peptides. The incubation mixture was as described by Lockard and Lingrel[1]. Messenger RNA was added in a concentration of 20 µg/ml. Incubations were performed at 37° C for 75 min in the presence of 0.5 µCi formyl ^{35}S Met-tRNA$^{Met}_{f}$. After incubation 50 µl. of 0.25 M EDTA, 20 µg pancreatic RNAase and 300 µg carrier α-crystallin were added per ml. and incubation was continued for 0.5 h at 37° C. A 0.5 ml. aliquot of this mixture was precipitated with 5% trichloroacetic acid (TCA) washed three times and the TCA removed from the sediment with acetone. The N-terminal peptides were obtained by incubating the precipitate with 500 µg subtilisin in 2 ml. 0.1 M ammonium bicarbonate at 37° C for 6 h. If no complete solubilization of the precipitate occurred a second subtilisin treatment was performed, 1.5 ml. of a 'Dowex 50' suspension was added and the mixture centrifuged. The supernatant was removed and the resin was washed with 2 ml. of distilled water. After centrifugation the two supernatant fractions were combined and lyophilized. The peptides excluded from the 'Dowex 50' were dissolved in 50 µl. distilled water and 10 µl. was used for high voltage electrophoresis. Electrophoresis was performed on 'Whatman 3 MM' paper at *p*H 6.5 for 2 h at 35 V/cm. Strips of 1 cm were cut out and radioactivity was counted in a liquid scintillation counter using a toluene based scintillator. Reference peptides were stained for methionine using platinic iodide. ○—○, Distribution of radioactivity without mRNA; ○ - - - ○, distribution of radioactivity with added mRNA.

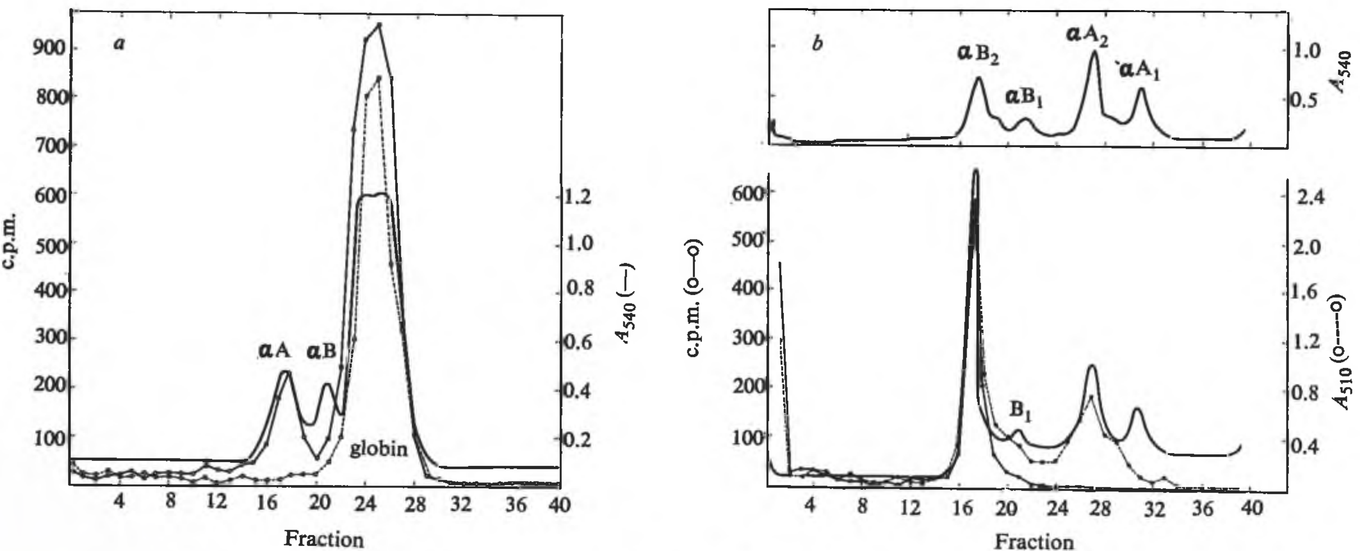

Fig. 3 Gel electrophoresis of the radioactive chains. Gel electrophoresis was performed on 8.0×0.5 cm polyacrylamide gels in 6 M urea as described[7]. To each sample 100 μg carrier α-crystallin was added. The gels were stained with Amido black and monitored at 540 nm in a Gilford spectrophotometer adapted with a gel scanner. Thereafter the gels were cut into 1.5 mm slices and, after solubilization with NCS, counted in a liquid scintillation counter. The positions of the different chains are indicated in the figure. *a*, Electrophoresis at *p*H 3.0; ——, absorbance at 540 nm; ○ - - - ○, distribution of radioactivity without added mRNA; ○—○, distribution of radioactivity with added 14S mRNA. *b*, Electrophoresis at *p*H 8.9; ——, absorbance at 540 nm; ○—○, distribution of radioactivity without added mRNA; ○ - - - ○, distribution of radioactivity with added 14S mRNA.

comprises about 35% of the total protein content, is composed of only four polypeptide chains designated αA_1, αA_2, αB_1 and αB_2 of which αA_2 is the major component[7]. We have isolated two mRNA fractions from bovine lens polyribosomes and one of these fractions codes for the synthesis of αA_2 in a cell-free reticulocyte system. The newly formed polypeptide was identified by electrophoresis in acidic and basic urea gels, in SDS gels and by analysis of the N-terminal peptides. Both the lens mRNA fractions can be translated in the Krebs ascites cell-free system[8].

Polyribosomes which were isolated from bovine lens, as described earlier[9] were the source of 10S and 14S mRNA preparations. The resolution achieved after zonal centrifugation is shown in Fig. 1. As all crystallin polypeptide chains have molecular weights of 19,000 to 25,000[10,11], the 10S and 14S RNAs might represent the messengers coding for these polypeptide chains. RNA with a sedimentation value of about 10S and 14S can also be extracted from 16S and 21S ribonuclear protein (RNP) particles prepared by dissociation of the lens polyribosomes by EDTA treatment. Moreover, 10S and 14S RNA have base compositions clearly different from lens ribosomal[6] and tRNA[12]. We shall describe here only the 14S used as a source of mRNA.

A lysate system of rabbit reticulocytes prepared according to Lockard and Lingrel[1] was used. Addition of lens messenger to this system resulted in a significant inhibition of the total amino-acid incorporation. As about 60–75% of the newly formed chains in organ culture are α-crystallin chains[13] and all chains of α-crystallin contain N-acetyl-Met-Asp-Ile-Ala at the N-terminus[14], we analysed the N-terminal peptides of the *in vitro* products. Since we could not expect N-terminal amino-acetylation in the rabbit reticulocyte system, ^{35}S Met-$tRNA_f^{Met}$ (derived from lens tissue and formylated with the aid of a crude *E. coli* enzyme preparation[15]), was used as radioactive precursor. Formyl-Met-$tRNA_f^{Met}$ donates formyl-Met for the N-terminal sequence formyl-Met-Asp of α-crystallin polypeptides in a lens lysate[15]. The electropherogram in Fig. 2, shows that addition of the 14S RNA to the cell-free system resulted in a radioactive spot located at a position identical to formyl-Met-Asp-Ile-Ala. Pronase digestion of this material released a peptide with an electrophoretic mobility identical to formyl-Met-Asp. Deformylation of the latter compound resulted in a peptide with the characteristics of Met-Asp.

We conclude that addition of the 14S RNA to the lysate results in the formation of polypeptides with an N-terminus identical to α-crystallin polypeptide chains. Since all four α-crystallin polypeptides have this N-terminal sequence, this

analysis does not allow any conclusion concerning the type of polypeptide chains synthesized. Moreover it does not provide evidence for the completion of the chains. For this reason gel electrophoresis on basic and acidic urea gels and on SDS gels was carried out with the biosynthetic product.

Electrophoresis of the biosynthetic product on acidic urea gels (Fig. 3*a*) reveals that αA polypeptides and no αB chains are synthesized as no significant radioactivity is located in the αB region of the gel. Electrophoresis on basic urea gels (Fig. 3*b*) indicates that of the αA polypeptides only the αA_2 chain is synthesized. To obtain an estimate of the molecular weight of the product, slices of the alkaline urea gels were subjected to SDS gel electrophoresis. The segments containing the globin and B chains and the segments corresponding to the A_2 chains were applied to 15% acrylamide gels in 0.1% SDS. From the gel patterns it can be deduced that the molecular weight of the radioactive material originating from the αA_2 band is approximately 19,000, which is identical to the molecular weight of the native αA polypeptides[16]. Electrophoretic separation of the mixture containing αB and globin on SDS gels revealed that no detectable radioactivity is located in the αB region. These findings in combination with the N-terminal analysis clearly indicate that the 14S mRNA isolated from calf lens polysomes directs the synthesis of a lens polypeptide chain in a heterologous system. It is worth mentioning that a messenger from a highly differentiated organ as the lens can be translated faithfully in a reticulocyte lysate of another species. This in combination with the observations of other authors indicates that, even in a highly differentiated cell, mRNA recognition factors do not exhibit stringent tissue specificity as suggested by Heywood[17].

Quite remarkable is the fact that a mRNA of 14S with a molecular weight of about 360,000, as estimated by the method of Boedker[18] is required to direct the synthesis of a polypeptide of a molecular weight of only 19,000. Two possibilities may be considered.

(1) The messenger does contain untranslated regions of considerable length. The occurrence of large poly A pieces, as reported for Hela mRNA[19], mouse ascites mRNA[20] and globin mRNA[21,22] is not very likely, however, as the AMP content of lens mRNA is extremely low[6]. (2) The 14S lens mRNA might be bicistronic, either with repeating nucleotide sequences for A_2 or a sequence for A_2 and (an)other unknown polypeptide(s). This problem is under investigation.

Our experiments may also shed some light on the initiation of translation of lens crystallin mRNA. As an acetylated methionine occurs at the N-terminal position of all α-crystallin polypeptide chains, the following initiation mechanisms might

be considered. (1) Initiation takes place with initiator $tRNA_f^{Met}$ charged *in vivo* with N-acetylmethionine. (2) The N-terminal Met is derived from $Met\text{-}RNA_f^{Met}$. Cleavage is prevented by the nature of the adjacent amino-acid residue(s), which might determine the specificity of the splitting enzyme. Such a specificity has been reported for ribosome-bound leucine aminopeptidase in *E. coli*[23]. An alternative explanation might be that a specific acetylation mechanism prevents the removal of the N-terminal methionine derived from $Met\text{-}tRNA_f^{Met}$. (3) The mRNA does code for a starting sequence Met-Met-Asp in which the first Met, derived from $Met\text{-}tRNA_f^{Met}$ is removed in the usual manner and the second, derived from $Met\text{-}tRNA_M^{Met}$ is acetylated in a later phase.

The first possibility is unlikely as the presence of an acetylated Met-tRNA in lens tissue could not be demonstrated[15]. The third possibility may be excluded by our results, as no formyl-Met-Met-Asp was found at the N-terminus of the biosynthetic A_2 chains. Therefore the acetylated N-terminal methionine present in α-crystallin polypeptide chains is derived from $Met\text{-}tRNA_f^{Met}$. The same conclusion could be drawn from initiation studies in a lens lysate system[15].

Note added in proof: Meanwhile we were able to show that frog oocytes programmed with 14S messenger also synthesize αA_2 chains. Berns, Kraaikamp Bloemendal and Lane, Manuscript submitted for publication).

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