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

Ton J.M. Berns, Victor V.A.M. Schreurs, Marjolijn W.G. van Kraakamp, and Hans Bloemendal
Department of Biochemistry, University of Nijmegen
(Received October 21/December 18, 1972)

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 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 αA1, αA2, αB1 and αB2 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.

**MATERIALS AND METHODS**

All chemicals were analytical grade. 14C-labeled amino acid mixture (54 Ci/atom) and [35S]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 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 (free of RNAase by boiling for 30 min with 0.02% diethyldiisocyanurate) 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 [\(^{14}C\)]methionine (10 \(\mu M\)) plus the remaining 19 (50 \(\mu M\)) unlabeled amino acids or a \(^{14}C\)-labeled amino acid mixture (20 \(\mu M\)) supplemented with the unlabeled six missing amino acids (asparagine, glutamine, methionine, tryptophan, cysteine, histidine) at 50 \(\mu M\) each. Lens messenger was added at 20 \(\mu g/\text{ml}\). After 2-h incubation at 30 °C the reaction was terminated by incubation for 15 min at 37 °C with pancreatic RNAase (20 \(\mu g/\text{ml}\)) 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 16 vol. 21/\(\%\) 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 \(\times g\) 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 \(\times\) 8 cm) on 12.5/\(\%\) polyacrylamide gels. Per gel 200 \(\mu g\) protein together with 20 \(\mu g\) 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 \(\mu g\) of the cell-free extract was applied together with 100 \(\mu g\) carrier \(\alpha\)-crystallin. Gels were sliced and autoradiographed as described for dodecylsulfate gels. Gel electrophoresis on preparative alkaline-urea gels for the isolation of \(\alpha\)A2 crystallin chains was carried out in gels (9 \(\times\) 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 \(\alpha\)-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 \(\mu g\) of \(\alpha\)A2 labeled by a mixture of \(^{14}C\) 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 trypsin digestion per 0.5 \(\mu g\) \(\alpha\)A, 30 mg carrier \(\alpha\)A chains was added and tryptic digestion was carried out in 0.1 M NH\(_4\)HCO\(_3\), 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 NH\(_4\)HCO\(_3\).

Column Fractionation

After lyophilization of the trypptic digest 2 ml starting buffer (0.2 M pyridine-acetate, pH 3.1) was added. Part (400 \(\mu l\)) 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 \(\times\) 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 \(\mu l\) was mixed with 5 ml Instagel (Packard) and counted in a liquid scintillation counter. 50 \(\mu l\) 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 \(\mu l\) 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 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 αA2 was synthesized (Fig. 3) almost exclusively.

Characterization of the N-Terminus of Newly Synthesized αA2

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 αA2 polypeptide with this N-terminal sequence.

Hence the N-terminus of the newly synthesized αA2 chain was analyzed. After labeling with [35S]-methionine, the αA2 chains were separated from the endogenous proteins by electrophoresis on basic-urea gels. The labeled αA2 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-
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 NH₂-group was accessible. That these peptides were indeed derived from the N-terminus has previously been shown by labeling experiments with different [³⁵S]Met-tRNAs. Only addition of [³⁵S]Met-tRNA⁰Met 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 ¹⁴C-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. 3. Analysis of the 14-S directed product on alkaline-urea gels.** Gels were prepared and run as described in the Methods section. [³⁵S]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).

**Fig. 4. Separation of the tryptic peptides of the αA₂ chain by ion-exchange chromatography.** Tryptic digestion and column chromatography were carried out as described in the Methods section. (—) Absorbance at 570 nm; (——) radioactivity.
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.

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 \( \alpha \text{A}_4 \) 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 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 \( ^{14} \text{C} \)-labeled mixture used or that not all ninhydrin-positive material was derived from \( \alpha \text{A}_4 \) 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 \( \alpha \text{A}_4 \) 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 B chain of \( \alpha \)-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 [28] that the A1 chain
arises as the result of a post-translational modifica-
tion of A2. Although not very likely it cannot com-
pletely be excluded that a messenger coding for \( \alpha A1 \)
exists. This hypothetical messenger could be lost
during the isolation procedure or its translation might
be blocked.

However the hypothesis of post-translational modi-
fication 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 \( \alpha \)-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
acytlation of the chains.

The heterologous systems tested are able to recog-
nize and translate a messenger originating from a
highly specialized tissue such as the lens without re-
quiring 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 re-
sults 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 messen-
gers. The occurrence of those factors should not be
restricted to one tissue or species.

It is quite remarkable that the 14-S lens messen-
ger fraction, with an estimated molecular weight of
about 360000, is required to direct the synthesis of
a polypeptide of molecular weight 20000 only, while
the 10-S lens messenger fraction with an average
molecular weight of about 260000 directs the syn-
thesis 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
(Bens, 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 molecu-
lar weight of the messenger had to be somewhat
higher than the estimated values. Nevertheless this
does not exclude bicistronic mRNA as the determina-
tion 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
\( \alpha A2 \)-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 protein-
ase 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 \( \alpha \)-crystallin
chains enables the following initiation mechanism.

a) Lens messengers require initiation with Ac-
Met-tRNA\(^{\text{Met}}\).

b) Initiation takes place with Met-tRNA\(^{\text{Met}}\) 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 acetylcyte
mechanism prevents the N-terminal methionine
from being split off.

c) The lens messengers coding for \( \alpha \)-crystallin
polypeptide chains code for a starting sequence
Met-Met-Asp in which the first amino acid, derived
from Met-tRNA\(^{\text{Met}}\) is split off in the usual manner
and the second methionine, derived from Met-
tRNA\(^{\text{Met}}\) 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 experi-
ments with fMet-tRNA\(^{\text{Met}}\), Met-tRNA\(^{\text{Met}}\) and Meta-
tRNA\(^{\text{Met}}\) [18]. After incubation with fMet-tRNA\(^{\text{Met}}\)
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.

The present investigations have partly been carried out under auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid of the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

REFERENCES


T. J. M. Berns, V. V. A. M. Schreurs, M. W. G. van Kraaijkamp, and H. Bloemendal

Laboratorium voor Biochemie der Universiteit, Geert Grooteplein Noord 21, Nijmegen, The Netherlands

1 to whom requests for reprints should be addressed.