TRANSLATION OF EUKARYOTIC MESSENGER RNA IN VARIOUS HETEROLOGOUS SYSTEMS

H. BLOEMENDAL, A. BERNS, G. STROUS,
Department of Biochemistry, University of Nijmegen, The Netherlands,

M. MATHEWS,
M.R.C. Laboratory of Molecular Biology, Cambridge, England,

C.D. LANE,
Department of Zoology, University of Oxford, England.

INTRODUCTION

Our present view of protein biosynthesis in eukaryotic systems assigns a major role in this process to the messenger RNA involved. This paper deals mainly with some of the recent work on messenger RNA isolated from calf eye lenses. One may ask the question: why in particular lens mRNA? The answer is simple: the lens has several unique features which makes it particularly suitable for studies on protein biosynthesis and differentiation.
The lens is an isolated tissue without blood vessels and is composed entirely of an epithelial monolayer and a close package of fiber cells (Fig. 1). The fibers arise from the epithelial cells and represent the final stage of cellular differentiation.
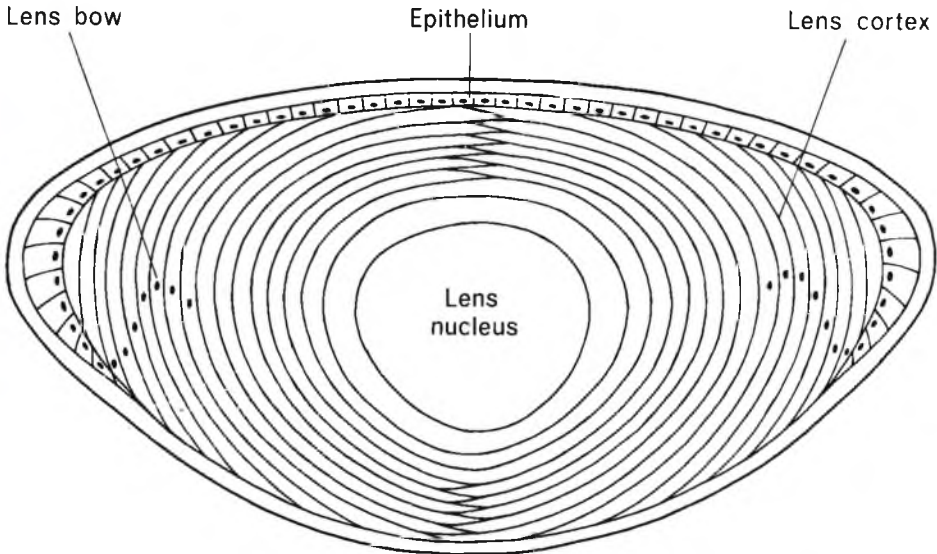

Fig. 1. Schematic representation of a cross section through a vertebrate eye lens.

Reeder and Bell (1965) presented evidence that in the intact lens DNA synthesis takes place exclusively in the epithelium, RNA synthesis in epithelium and the lens bow, whilst protein synthesis occurs in the epithelium and cortex. After treatment of cultured lenses with actinomycin D protein synthesis ceases in the epithelium while it continues in the lens body implying the activity of a messenger with long half-life.

The chemical composition of the lens is also exceptional. It contains approximately 35% of protein of which almost 90% is represented by the so-called crystallins. The crystallins have as far as it is known, no enzymic function. There are three classes of these water-soluble structural lens proteins: α, β, and γ-crystallin. At least in calf lens the insoluble protein is an altered form of α-crystallin. From a biosynthetic point of view α-crystallin is most interesting as both epithelial and cortical fiber cells are specialized for the synthesis of that protein. In the epithelium 60% of the newly synthesized protein is α-crystallin whereas in the cortex this amounts even to 75% (Delcour and Papaconstantinou, 1972).

From the foregoing it is clear that lens cells provide a good source for the isolation of stable and specific mRNA.

Some basic knowledge of lens proteins is required in order to enable the analysis of the products synthesized under direction of the lens messengers. The α-crystallin fraction consists of large aggregates with an average molecular weight of 800,000 dalton. All aggregates are composed of the same four kinds of polypeptide chains: αA_1, αA_2, αB_1, and αB_2 which migrate with different electrophoretic mobility in polyacrylamide gels containing 7 M urea at alkaline pH. αA_2 is the major component. In embryonic lenses the αA_1 chains are lacking. They arise gradually during aging, presumably by deamidation (Bloemendal et al. 1972a). The molecular weight of the individual polypeptide chain estimated in SDS or 7 M urea is approximately 20,000 dalton (Bloemendal, 1972). The NH_2-terminal sequence N-acetyl-Mer-Asp-Ile-Ala as well as the occurrence of 2 methionine residues are common features of all four polypeptide chains. These characteristics have been used as analytical tools for the identification of newly synthesized products under direction of lens messenger.

THE ISOLATION OF EUKARYOTIC MESSENGER RNA

For a rather long time the availability of mRNA was hampered by a lack of convenient techniques in order to separate the very small amount of functional messenger from the bulk of RNA. A breakthrough came with the introduction of zonal rotors which enabled the isolation of viable amounts of eukaryotic messenger fractions.

In most procedures polyribosomes are the starting material for messenger preparations. It stands to reason that polyribosomes from cells which manufacture only one or a very limited number of proteins are advantageous. In this connection reticulocytes and lens tissue have to be mentioned. However the isolation of one mRNA species from a population of different messengers is also possible. By means of immuno-precipitation a defined class of polyribosomes is isolated by virtue of the antigenic specificity of the nascent chains which are being synthesized. This procedure has e.g. been applied in studies upon the biosynthesis of albumin (Duerre, 1967), H and L chains of immunoglobulins (Williamson and Askonas, 1967; Schubert and Cohn, 1968; Boyd et al. 1971), myosin (Allen and Terrence, 1968), chick lens protein (Clayton et al. 1970) and catalase (Uenoyama and Ono, 1972). One has to keep in mind that unspecific

precipitation cannot always completely be avoided, which is a serious drawback of this elegant method.

The isolation of lens polyribosomes has been described earlier (Bloemendal et al. 1966). As many as 1000-1200 lenses are required for a yield of about 5 mg polyribosomal RNA. The polyribosomes are suspended in Mg^{++}-free medium and treated with sodium dodecyl sulfate. With the aid of equivolumetric gradients under strictly standardized conditions a separation as depicted in Fig. 2 is possible.
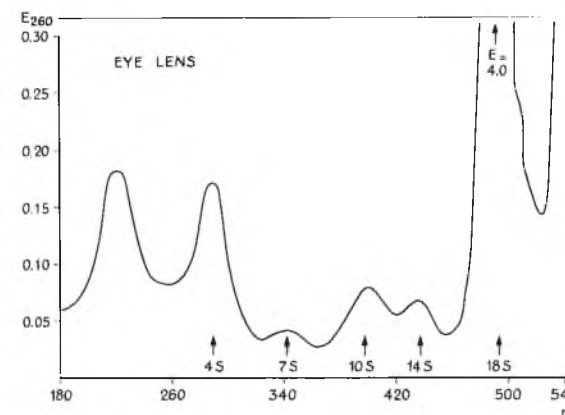

Fig. 2. Zonal centrifugation pattern of polyribosomal RNA from eye lens (centrifugation time 15 h.).

In this way approximately 50 µg mRNA can be obtained from 5 mg polyribosomal RNA. Recentrifugation in a swinging bucket rotor reveals that the separation in the zonal rotor yields fractions which seem to be rather homogeneous (Berns et al. 1971). Here only the 10S fraction is shown (Fig. 3).
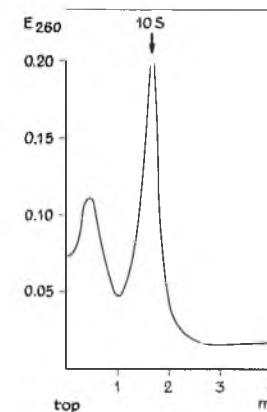
Fig. 3. Recentrifugation pattern of the lens messenger 10S fraction in a
swinging bucket rotor.

Taking into account the average size of lens polyribosomes (Benedetti et
al. 1968) the 10S and 14S fractions (compare Fig. 2) seemed to be can-
didates for the role of messenger. These two RNA species can also be
isolated from RNP particles which arise upon treatment of lens poly-
ribosomes with EDTA. In Figs. 4A and B, the patterns of lens RNP par-
ticles and corresponding RNA fractions are visualized. For comparison
a parallel experiment with reticulocyte RNP is also shown (Fig. 4A and
4B(a)). A particle which sediments at 16S yields the 10S messenger
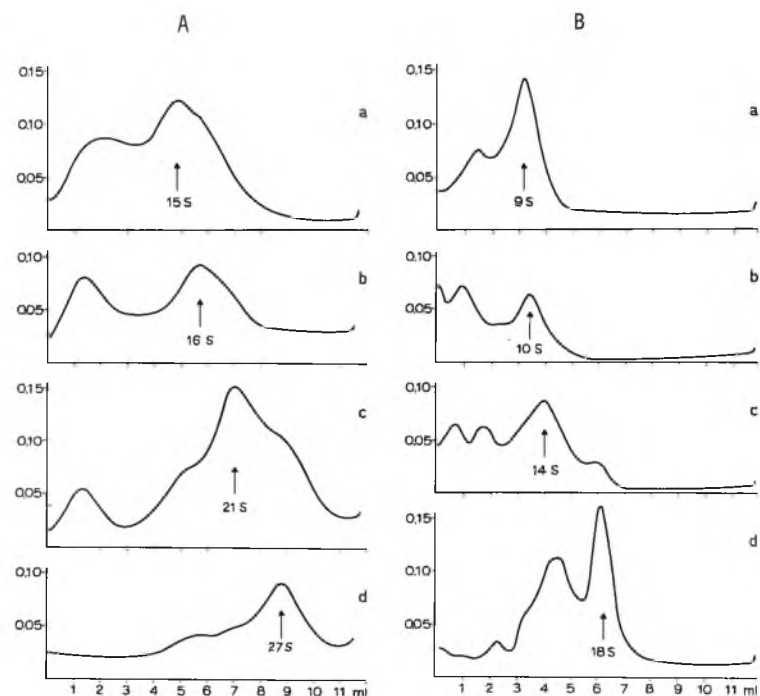(Fig. 4A and B(b)); a 21S particle the 14S messenger (Fig. 4A and 4B(c)).

Fig. 4. Zonal centrifugation patterns of RNP particles (A) and corres-
ponding RNA fractions (B).
a) from rabbit reticulocytes; b,c,d) from calf lens.

It is well established now that various mRNAs contain poly A-rich regions.
This property is the base of successful separation of biologically
active messenger from inactive RNA.
The poly A-rich fraction anneals to poly U cellulose (Kates, 1970;
Sheldon et al. 1972) or to oligothymidylic acid-cellulose columns
(Aviv and Leder, 1972). Preliminary experiments in collaboration with
Drs. Piperno and Bertazzoni (Institut de Biologie Moléculaire, Paris),
revealed that 20% of the messenger preparation isolated by zonal cen-
trifugation is retained by such dT-columns. After the purification step

the A content of the messenger fraction appeared to be approximately 31%.

THE CHARACTERIZATION OF MESSENGER RNA

In principle several approaches can be utilized for the characterization
of messenger.

1. A specific label

In pulse experiments there is label to be found in association with
polyribosomes. This label is predominantly due to the presence of mRNA.

2. A specific density

mRNP particles reveal a boyant density differing from the density of
ribosomal subunits or naked RNA.

3. The base composition

The significance of the base composition as specific property of mRNA
is questionable as long as this RNA species is not highly purified
and the composition of the corresponding polypeptide unknown.

4. Hybridization

In hybridization experiments one has to keep in mind that unspecific
annealing may occur.

5. Determination of nucleotide sequence

A prerequisite for sequentional studies on eukaryotic mRNA is the
availability of a homogeneous preparation with high specific activity.
It seems to be rather difficult to produce highly radioactive eukaryotic
mRNA by in vivo incorporation experiments. However, recently a promising
method was introduced in which the reversed transcriptase is utilized.
With the aid of the enzyme isolated mRNA is first transcribed into DNA.
This DNA can be retranscribed into mRNA by using polymerase and tri-
nucleotides of high specific activity.

6. Translation in cross systems

The ultimate proof of the messenger function is always: accurate trans-
lation upon addition of the presumptive messenger to a cross system.

ASSAY SYSTEMS FOR mRNA

Not only the appropriate isolation procedures for mRNA were lacking for
a rather long period of time but also efficient cell-free or in vivo
systems were unknown.
Recently investigations by Lockard and Lingrel (1969) and Mathews and
Korner (1970) demonstrated the usefulness of crude cell-free systems
derived from reticulocytes or ascites as assay system for exogenous
messenger activity. An additional very effective test system was obtained
from oocytes of Xenopus Laevis by Gurdon and coworkers (1971). It has to
be mentioned here that Schapira and coworkers have been the first to

show that an RNA fraction from rabbit reticulocytes sedimenting between 4S and 16S transmitted information to a reticulocyte extract derived from another species (Kruh et al. 1964; Schapira et al. 1968).
Up till now only a relatively small number of eukaryotic mRNAs has been identified in a functional assay and the complete translation achieved has practically been restricted to the three cross systems mentioned (Table 1).

Table 1

Eukaryotic mRNA translated in a cross system

source of mRNA	protein	system	reference
muscle (chick)	myosin	retic (chick)	Heywood (1969)
retic (rabbit)	hemoglobin	E. coli	Laycock & Hunt (1969)
retic (mouse)	hemoglobin	retic (rabbit)	Lockard & Lingrel (1971)
retic (mouse)	hemoglobin	ascites (mouse)	Mathews et al (1971)
retic (rabbit)	hemoglobin	oocytes (frog)	Gurdon et al (1971)
myeloma (mouse)	immunoglobulin (L chain)	retic (rabbit)	Stavnezer & Huang (1971)
oviduct (hen)	ovalbumin	retic (rabbit)	Rhoads et al (1971)
lens (calf)	α-crystallin (A chain)	retic (rabbit)	Berns et al (1972a)
lens (calf)	α-&β-crystallin	ascites (mouse)	Mathews et al (1972a)
lens (calf)	α-crystallin (A chain)	oocytes (frog)	Berns et al (1972b)

retic = reticulocyte

THE TRANSLATION OF LENS mRNA IN VARIOUS CROSS SYSTEMS

The isolated lens 10S and 14S fractions have been tested in the three cross systems mentioned above. In all cases it appeared that the 14S messenger directs the synthesis of the αA_2 chains whilst the 10S lens mRNA directs the synthesis of the αB chains and presumably β-crystallin chains. This is illustrated in Figs. 5 and 6 (compare also Fig. 8). No αA_1 seems to be made. This is in accord with previous findings that the αA_1 chain arises from αA_2 by deamidation (Bloemendal et al. 1972a). Deamidation of asparagine and glutamine, a posttranslational conversion process and presumably a more general aspect of aging, has previously been observed with rat cytochrome C (Flatmark and Sletten, 1968) and rabbit muscle aldolase (Lai and Horecker, 1971).
An intriguing observation is that the 14S messenger with a molecular weight of about 360,000 dalton apparently codes for the αA_2 chain with a molecular weight of about 20,000 dalton whereas αB and β-crystallin chains which have somewhat higher molecular weights than αA_2 are synthesized under direction of a 10S messenger with a calculated molecular weight of 260,000 dalton. This is clearly demonstrated by translation of lens mRNA in the reticulocyte system. First both messengers are collected in several fractions after a prolonged zonal run (Fig. 7, compare also Fig. 2). Thereafter the individual fractions are assayed in the cell-free system. The result is shown in Fig. 8. This picture allows the following conclusions:
1. The 14S RNA preparation codes exclusively for αA chains.
2. The 10S fraction codes for several crystallin polypeptides but not for αA chains.
3. For the 10S fraction it is true that a slightly higher sedimentation value of the RNA fraction corresponds with a higher molecular weight of the product synthesized under direction of this RNA species.
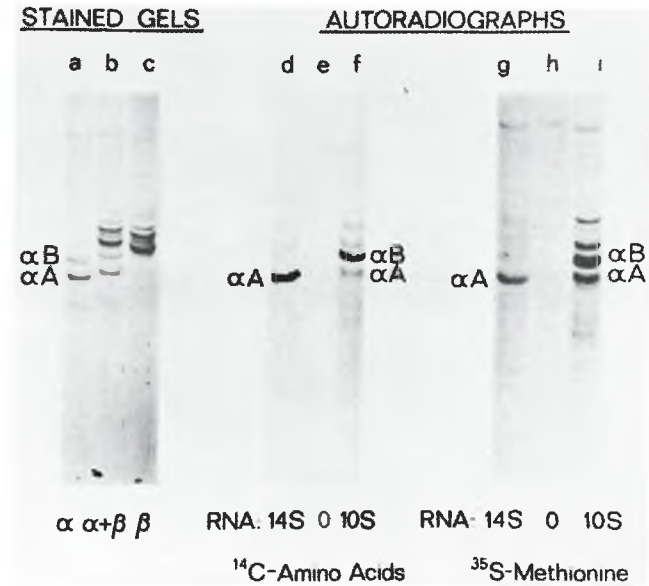

Fig. 5. SDS gel analysis of the newly synthesized products obtained in the ascites cell-free system under direction of lens messengers.
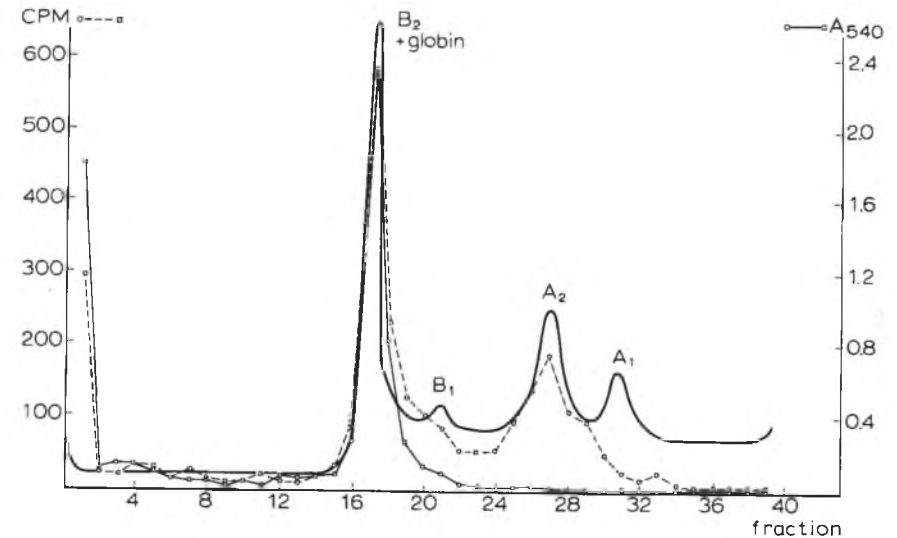

Fig. 6. Tracing of a polyacrylamide-urea gel electrophoretic pattern of the newly synthesized products in the reticulocyte cell-free system under direction of 14S lens messenger.
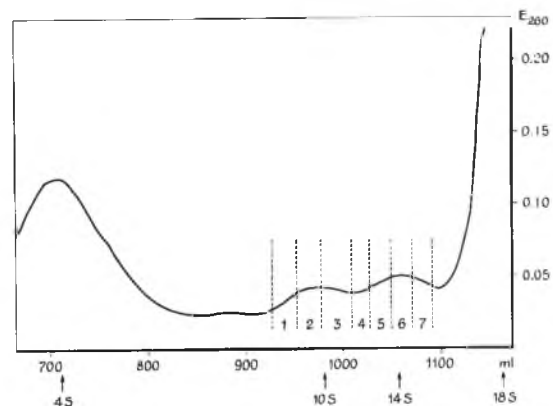
Fig. 7. Zonal centrifugation pattern of polyribosomal RNA from calf lens (centrifugation time 25 h.).
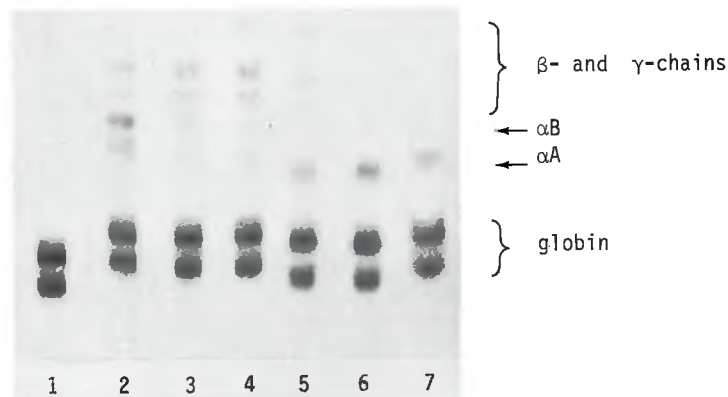

Fig. 8. Autoradiography of the SDS gel pattern of the newly synthesized products obtained in the reticulocyte system under direction of the RNA fractions 2-7 visualized in Fig. 7.

REQUIREMENT AND EFFECT OF PROTEIN FACTORS

There are conflicting reports concerning the requirement of specific recognition factors. Heywood (1970) stated that protein factors isolated from chick muscle ribosomes are necessary for the translation of mRNA derived from embryonic chick muscle. According to this author especially initiation factor IF_3 reveals this tissue specificity. The majority of reports including our experiments with lens messenger RNA in 3 different systems exclude a stringent necessity of cell- or messenger specific proteins for accurate translation. A possibility to explain the discrepancies may be that the requirement for specific factors becomes only evident under certain well-defined ionic conditions. At any rate for the time being the experimental evidence is in favor of those authors who found that several cross systems contain the complete machinery required for the translation of a foreign messenger.

Whereas there is no apparent absolute need for exogenous protein factors in the translation process as studied in mixed systems there is an effect upon addition of these factors. The addition of crude initiation factors to an incubation mixture containing ribosomes and enzymes from ascites tumor cells and globin messenger increases the incorporation of amino acids into protein about 3-fold (Mathews et al. 1972b). Fig. 9 shows an SDS-gel pattern in which the effect of heterologous initiation factors on messenger directed cell-free protein synthesis can be seen.
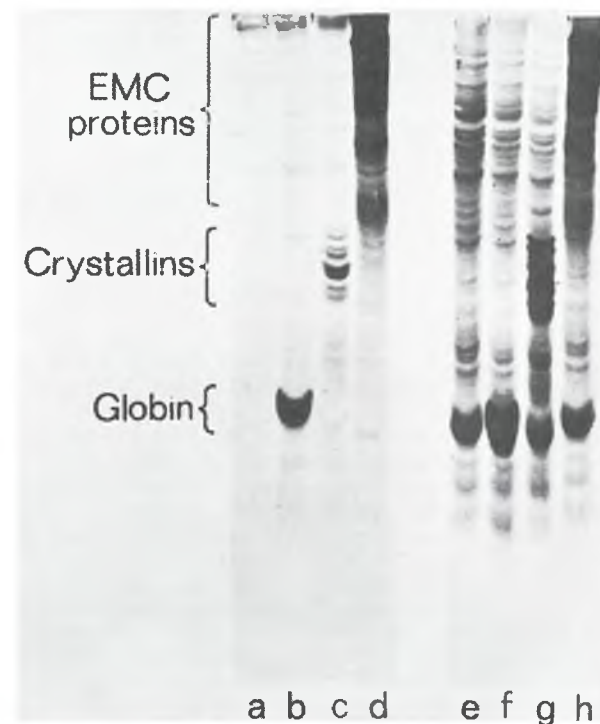

Fig. 9. SDS gel analysis of newly synthesized products under direction of endogenous and exogenous messengers in the Krebs ascites cell-free system.
a-d: Biosynthesis in the absence of added 0.5 M KCl wash from reticulocytes;
e-h: in the presence of the KCl wash.

a, e: no messenger added
b, f: with globin mRNA
c, g: with 10S lens mRNA
d, h: with EMC-RNA.

Comparison of Fig. 9c with 9g reveals the considerable stimulation brought
about by the crude factors from reticulocytes in a system containing
ascites S30 lysate and lens 10S messenger. From Fig. 9e it can be concluded
that in the 0.5 M KCl wash procedure also globin messenger from the
reticulocyte polyribosomes is released. This messenger activity can be
eliminated by digestion of the KCl wash with RNase under mild conditions.
The stimulating ability of the factors appears to be maintained.
A stimulating effect of reticulocyte factors on the translation of en-
dogenous or exogenous messenger in the ascites system has also been
reported recently by Metafora et al. (1972).

IDENTITY OF NEWLY SYNTHESIZED AND NATIVE αA_2 CHAINS

In order to determine the fidelity by which 14S lens messenger is trans-
lated an NH_2-terminal analysis and a complete peptide map of the tryptic
digest of newly synthesized polypeptide was carried out. In Table 2
the NH_2-terminus analysis is briefly summarized.

Table 2

Enzyme	Amino-acid sequence
Pronase	acetyl-Met-Asp
Subtilisin	acetyl-Met-Asp-Ile-Ala
Trypsin	acetyl-Met-Asp-Ile-Ala- -Ile-Gln-His-Pro- -Trp-Phe-Lys-(Arg)

In all cases co-electrophoresis or co-chromatography revealed identity
of peptides released from native and newly synthesized lens polypeptide
chains. A purified carrier αA_2 chain digest gives rise to about 20
ninhydrin positive spots. The radioactive spots obtained from the product
obtained under direction of the 14S lens messenger coincide with the
ninhydrin stained peptides. This result indicates the high degree of
fidelity with which lens mRNA is translated (Berns and Bloemendal, 1972).
The extent of identity of the newly synthesized chains as compared to
native αA_2 chains goes even further. In our first experiments formyl-
$[^{35}S]$Met-tRNA^{fMet} was used as radioactive precursor and as expected a
polypeptide with a blocked NH_2-terminus was obtained (Berns et al.1972a).
However, to our surprise when in the reticulocyte system programmed with
14S lens mRNA $[^{35}S]$ methionine was used as label the newly synthesized
polypeptide carried an acetyl group in N-terminal position exactly like
native αA_2 (Strous et al. 1972).

This was again proven by the analytical procedures summarized in Table 2.
1. Subtilisin treatment released a compound with identical electrophoretic
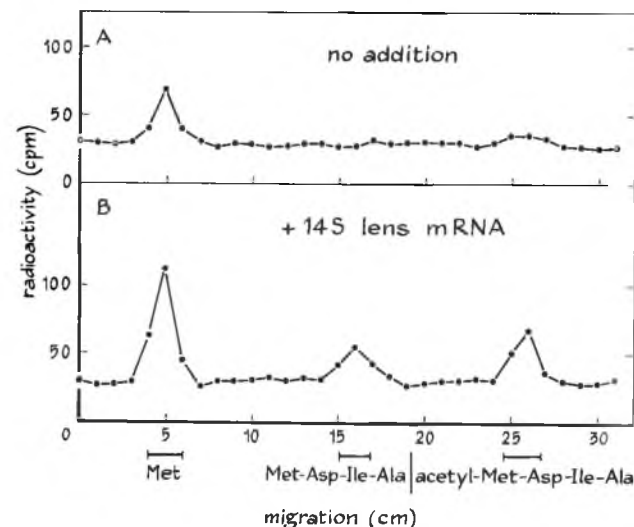behavior as the native NH_2-terminal tetrapeptide (Fig. 10).

Fig. 10. Identification of the N-terminal tetrapeptide of the αA_2 chain by
          high voltage paper electrophoresis.
          A. Without added 14S lens mRNA to the reticulocyte cell-free
             system.
          B. Incubation in the presence of 14S lens mRNA.

2. Trypsin treatment released two large methionine containing peptides
   with similar chromatographic properties as the peptides released from
   native αA_2 chains (Fig. 11A).
The fast moving tryptic peptide yielded acetyl-Met-Asp after pronase
digestion. From Fig. 11 it may also be concluded that the NH_2-terminal
methionine residue is exclusively donated by Met-tRNA$^{fMet}_1$ (Fig. 11B).
Either Met-tRNA$^{Met}_1$ or Met-tRNA$^{Met}_2$ are responsible for the insertion of the
internal methionine (Fig. 11C and D). Acetylation of the NH_2-terminus of
αA_2 is also observed after microinjection of 14S mRNA into living oocytes
(Berns et al. 1972b). Moreover the B chains of α-crystallin become acetyl-
ated in N-terminal position too (unpublished results).
Keeping these observations in mind the conclusion seems to be justified
that the acetylation reaction is a more general phenomenon in eukaryotic
tissues. The gradually expanding list of NH_2-terminally acetylated proteins
in eukaryotes is in favor of this idea (Table 3). In this connection it
has to be mentioned that also several plant viral proteins carry the
acetyl group in NH_2-terminal position. It is striking to observe that
again the blocking group is attached to the same limited kinds of NH_2-

terminal amino acid residues. In the case of α-crystallin one can think out a reason for the NH_2-terminal acetylation. The eye lens is a good if not the best source for the isolation of the enzyme leucine aminopepti-dase. The acetyl group protects the protein against degradation by this enzyme. However, it cannot be concluded yet that protection against enzymic attack is the general meaning of NH_2-terminal amino acetylation. The question: does acetylation take place either short or later after initiation, the nascent chain still being attached to ribosomes or following the release of the completed polypeptide is a problem which is under investigation now.
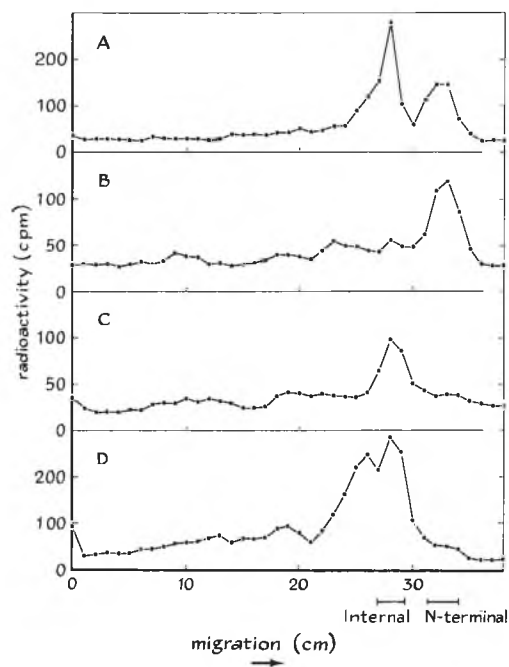

Fig. 11. Paper chromatography of the methionine containing tryptic peptides of the αA_2 chain.

A. Peptides obtained after $[^{35}S]$ methionine labeling

B. Peptides obtained after labeling with $[^{35}S]$ Met-tRNA^{fMet}

C. Peptides obtained after labeling with $[^{35}S]$ Met-tRNA$_1^{Met}$

D. Peptides obtained after labeling with $[^{35}S]$ Met-tRNA$_2^{Met}$

Table 3

Eukaryotic and viral proteins labeled in NH_2-terminal position.

PROTEIN	ACETYLATED NH_2 = TERMINAL AMINO ACID RESIDUE
Cytochrome C (plant)	Ala
Enolase	Ala
LDH	Ala
Keratin	Ala (?)
Actine	Asp NH_2
Trypomyosin	Asp NH_2, Met
Ovalbumin	Gly
Cytochrome C (vertebrate)	Gly
α-crystallin	Met
Histone	Ser
Myosin	Ser
Apoferritin	Ser
α-MSH	Ser
Mitochondrial proteins	Ser
Fibrinogen	Thr
Cucumber virus	Ala
TYMV	Met
TMV	Ser

ACKNOWLEDGEMENT

REFERENCES

Allen, E.R. and Terrence, C.F. (1968). Proc. Nat. Acad. Sci. 60, 1209.

Aviv, H. and Leder, Ph. (1972). Proc. Nat. Acad. Sci. 69, 1408.

Benedetti, E.L., Zweers, A., and Bloemendal, H. (1968). Biochem. J. 108, 765.

Berns, A.J.M., De Abreu, R.A., van Kraaikamp, M., Benedetti, E.L. and Bloemendal, H. (1971). FEBS Letters 18, 159.

Berns, A.J.M., Strous, G.J.A.M. and Bloemendal, H. (1972a). Nature New Biol. 236, 7.

Berns, A.J.M., van Kraaikamp, M., Bloemendal, H. and Lane, C.D. (1972b). Proc. Nat. Acad. Sci. 69, 1606.

Berns, A.J.M. and Bloemendal, H. (1972). Communication at the Cold Spring Harbor Symposium.

Bloemendal, H., Berns, A.J.M., van der Ouderaa, F. and de Jong, W.W.W. (1972a). Exp. Eye Res. 14, 80.

Bloemendal, H. (1972). Acta Morph. Neerl.Scand. (in the press).

Boyd, S.L., Delovitch, T.L., Holme, G. and Sehon, A.H. (1971). Biochem. J.
        125, P17.
Clayton, R.M., Truman, D.E.S. and Campbell, J.C. (1970). Biochem.J.117,63P.
Delcour, J. and Papaconstantinou, J. (1972). J. Biol. Chem. 247, 3289.
Duerre, J.A. (1967). Nature 213, 885.
Flatmark, T. and Sletten, K. (1968). J. Biol. Chem. 243, 1623.
Gurdon, J.B., Lane, C.D., Woodland, H.R. and Marbaix, G. (1971). Nature
        233, 177.
Heywood, S.M. (1969). Cold Spring Harbor Symp. Quant. Biol. 34, 799.
Heywood, S.M. (1970). Proc. Nat. Acad. Sci. 67, 1782.
Kates, J. (1970). Cold Spring Harbor Symp. Quant. Biol. 35, 743.
Kruh, J., Dreyfus, J.C. and Schapira, G. (1964). Biochim. Biophys. Acta
        87, 253.
Lai, C.Y. and Horecker, B.L. (1971). J. Cell Physiol. 76, 381.
Laycock, D. and Hunt, J.A. (1969). Nature 221, 1118.
Lockard, R.E. and Lingrel, J.B. (1969). Biochem. Biophys. Res. Commun.
        37, 204.
Lockard, R.E. and Lingrel, J.B. (1971). Nature New Biol. 233, 204.
Mathews, M.B. and Korner, A. (1970). Eur. J. Biochem. 17, 328.
Mathews, M.B., Osborn, M. and Lingrel, J.B. (1971). Nature 233, 206.
Mathews, M.B., Osborn, M., Berns, A.J.M. and Bloemendal, H. (1972a). Nature
        New Biol. 236, 5.
Mathews, M.B., Pragnell, B., Osborn, M. and Arnstein, H.R.V. (1972b).sub-
        mitted for publication.
Metafora, S., Terada, M., Widow, L., Marks, P.A. and Bank, A. (1972). Proc.
        Nat. Acad. Sci. 69, 1299.
Reeder, R. and Bell, E. (1965). Science 150, 71.
Rhoads, R.E., McKnight, G.S. and Schimke, R.T. (1971). J. Biol. Chem. 246,
        7407.
Schapira, G., Dreyfus, J.C. and Maleknia, N. (1968). Biochem. Biophys.
        Res. Commun. 32, 558.
Schubert, D. and Cohn, M. (1968). J. Mol. Biol. 38, 273.
Sheldon, R., Jurale, Ch. and Kates, J. (1972). Proc. Nat. Acad. Sci. 69,
        417.
Stavnezer, J. and Huang, R.C. (1971). Nature New Biol. 230, 172.
Strous, G.J.A.M., Berns, A.J.M., van Westreenen, H. and Bloemendal, H.
        (1972), in the press.
Uenoyama, K. and Ono, T. (1972). J. Mol. Biol. 65, 75.
Williamson, A.R. and Askonas, B.A. (1967). J. Mol. Biol. 23, 201.