Nucleic Acids Research

Non-polyadenylated 22 S ribonucleoprotein particle is insensitive to translational inhibitor RNA of cryptobiotic gastrulae of Artemia salina

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ABSTRACT

    A free cytoplasmic 22 S ribonucleoprotein particle exhibiting a major template activity in rabbit reticulocyte system has been identified in the cryptobiotic gastrulae of Artemia salina. This particle contains non-polyadenylated 9 S messenger RNA which codes primarily for a non-histone basic protein with an apparent molecular weight of 26 000 daltons.
    We have previously demonstrated the presence of a translational inhibitor RNA which is apparently responsible for transforming polyadenylated messenger ribonucleoproteins into a repressed state in the cryptobiotic gastrulae (Slegers et al., FEBS Letters 80, 390-394, 1977). This inhibitor RNA was found to be completely ineffective on the template activity of non-polyadenylated 22 S messenger ribonucleoprotein, confirming the specificity of this regulatory RNA for polyadenylate sequences.

INTRODUCTION

    The encysted cryptobiotic gastrulae of Artemia salina contain a significant quantity of stored polyadenylated messenger ribonucleoprotein particles (poly(A)$^+$-mRNP) sedimenting between 20 and 150 S (1). These mRNPs as well as mRNAs isolated thereof are inactive as templates in a cell-free translational system, owing to their bound translational inhibitor RNA, which can be dissociated in vitro from poly(A)$^+$ mRNP or mRNA by poly(U)-Sepharose affinity chromatography in the presence of 10 mM EDTA (2). Similar translational inhibitor RNAs associated with mRNPs have also been detected in the embryonic chicken muscle (3,4), duck erythroblasts (5), rat connective tissue (6) as well as in Artemia salina embryos (7).

    However, using a rabbit reticulocyte lysate translational system, we have observed a consistent template activity, in the absence of EDTA (buffer C), in the total postribosomal supernatant RNA or in its non-polyadenylated RNA fraction, prepared by oligo(dT)-cellulose affinity chromatography. Now we have identified and partially purified a major template active 22 S mRNP which contributes largely to the detectable messenger activity in these fractions.

In this communication, we demonstrate that the 22 S mRNP contains 9 S mRNA lacking polyadenylate sequences and that it is completely insensitive to added inhibitor RNA in a cell-free translational system.

A preliminary report has been presented at the Belgian Biochemical Society Meeting (8).

MATERIALS AND METHODS

Buffers. Buffer C, 10 mM sodium phosphate (pH 6.8)/5 mM $MgCl_2$/50 mM NaCl; buffer G, 10 mM Tris-HCl (pH 7.6)/100 mM NaCl; sodium dodecyl sulfate (SDS) buffer, 80 mM Tris-HCl (pH 7.8)/100 mM dithiothreitol/4% sodium dodecyl sulfate/10% glycerol; buffer L, 10 mM Hepes (N-2 hydroxyethylpiperazine-N'-2-ethane sulfonic acid, pH 7.2)/250 mM NaCl/1 mM EDTA; buffer M, 10 mM Na(OAC) (pH 6.0)/100 mM NaCl/5 mM $MgCl_2$.

Materials. Micrococcal nuclease was obtained from Boehringer Mannheim GmbH (Mannheim, FRG). Proteinase K and polyethylene glycol (PEG) were purchased from Merck (Darmstadt, FRG). Calf thymus histones were obtained from Sigma Chemical Co. (St. Louis, USA). [35S] Methionine was from Radiochemical Centre (Amersham, UK). Rabbit globin mRNA was obtained from Searle. All other materials were indicated in previous publications (1,2,9).

RNA Extraction and Poly(U) Hybridization. The postmitochondrial or postribosomal supernatant fraction was prepared as described previously (9). The postribosomal supernatant was further divided by oligo(dT)-cellulose affinity chromatography into polyadenylated and non-polyadenylated fractions according to Slegers et al. (10). PEG precipitated material from the postmitochondrial , postribosomal supernatant or sucrose gradient fractions was dissolved in 1-2 ml buffer C containing 2% SDS to which an equal volume of phenol/chloroform/ isoamyl alcohol (50/50/1) was added. Deproteinization was carried out until no visible interphase was recognized. The RNA was precipitated from the last aqueous phase by adding one-tenth volume of 20% potassium acetate (pH 5.0) and 2 volumes of ethanol at -20°C. The RNA pellet was collected by centrifugation and dissolved in 500 μ l of 10 mM sodium phosphate buffer (pH 6.8), followed by a second ethanol precipitation. The final RNA pellet was dried in vacuo and redissolved in a minimal volume of redistilled water.

Alternatively, ribonucleoprotein particles were dissolved in buffer G containing 0.5% SDS and digested with proteinase K (50 μg /ml) overnight at room temperature. The digest was processed as above using phenol/chloroform/ isoamyl alcohol to isolate the RNA. Hybridization of RNA with [3H] poly(U) was performed as described (1).

Translational inhibitor RNA was prepared according to the published method (2) with some modifications which will be reported later.

In Vitro Protein Synthesis in Rabbit Reticulocyte Lysate. The nuclease treated rabbit reticulocyte lysate was prepared exactly as described by Pelham and Jackson (11). The reaction mixture (25 μ 1), consisting of 5 μ 1 template sample and 20 μ 1 treated lysate, contained 19.4 mM HEPES (pH 7.4), 1 mM ATP, 0.2 mM GTP, 2.5 mM MgCl$_2$, 90 mM KCl, 1 mM CaCl$_2$, 3.4 mM EGTA, 0.01 mM hemin, 0.1 mM 19 amino acids (except methionine), [^{35}S] methionine (ca. 500 Ci/mmol), 57 μ g/ml creatine phosphokinase and 14.6 mM creatine phosphate and was in-cubated at 30°C for 45 min. Aliquots (10 μ 1) were taken to determine the in-corporation of [^{35}S] methionine into protein (11).

Analysis of In Vitro Protein Products. An enlarged reaction mixture (90 μl) was diluted with redistilled water to 700 μl after incubation at 30°C for 45 min, and digested with pancreatic RNAase A (5 μg/ml) by incuba-tion at 37°C for 15 min. An equal volume of 20% trichloroacetic acid was added to the RNAase A-digest and the proteins were precipitated overnight at 4°C. The pellet collected by centrifugation was washed successively with 5% trichloroacetic acid (twice), ethanol, ethanol/diethyl ether (1/1), and finally with diethyl ether. The washed pellet was dissolved in SDS gel buffer. Electrophoresis was carried out in 10 or 15% gels (0.6 cm x 10 cm) essentially according to the procedure of Studier (12) which was slightly modified for the Cold Spring Harbor Laboratory Course. After electrophoresis for 4 h at 2 mA/gel, gels were frozen at -20°C, sliced and the [^{35}S] labelled proteins extracted with 1 ml of 0.1% sodium dodecylsulfate by continuous shaking overnight at 37°C. The radioactivity was determined in a Packard Tricarb 2450 scintillation spectrometer after the addition of 9 ml of scintillation liquid (1 1 toluene/0.5 1 Triton X-100/6 g PPO (2,5-diphenyl-oxazole)/150 mg POPOP (1,4-bis-2(5-phenyloxazol)-benzene)).

When protein products were analyzed by acid urea gels, the washed pellet (see above) was dissolved in 0.9 N acetic acid/15% sucrose and elec-trophoresed in 15% polyacrylamide gels (0.6 cm x 10 cm) at 2 mA per gel as described (13). The determination of the radioactivity in gel fractions was carried out as above.

RESULTS AND DISCUSSION

Isolation and characterisation of the 22 S mRNP. The oligo(dT)-cellulose unbound fraction (10) of the postribosomal supernatant was analysed by zonal sucrose gradient centrifugation in buffer C. As shown in Figure 1, a maximal
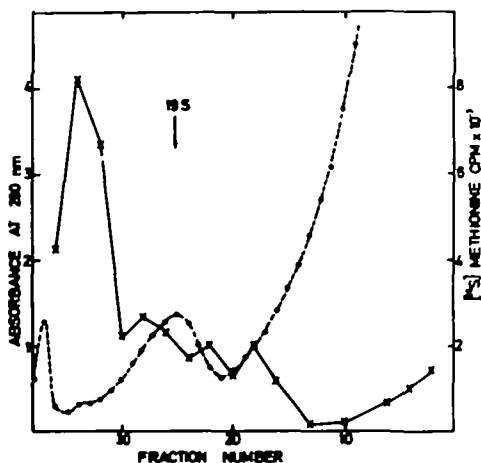
Figure 1 : Localization of template activity in the oligo(dT)-cellulose un-
bound fraction of the postribosomal supernatant. The postribosomal supernatant
from cryptobiotic gastrulae (100 g, dry weight) was concentrated to about 20 ml
by ultrafiltration (Amicon XM 50 filter) and loaded on a discontinuous sucrose
gradient in buffer C (90 ml of 15% (w/w), 145 ml 25%, 200 ml 30%, 200 ml 35%
and 100 ml 40%) in a Beckman Ti 14 zonal rotor. Centrifugation was carried out
at 157 200 X g for 45 h at 4°C. Fractions of 20 ml were collected and the ab-
sorbance at 280 nm was measured (●——●). 10 ml of the indicated fractions was
precipitated overnight with one-tenth volume of potassium acetate and two
volumes of ethanol. The precipitate was collected by centrifugation and dis-
solved in buffer C containing 2% SDS. RNA was then extracted as described in
Materials and Methods. The RNA precipitate was dissolved in 20 μl distilled
water and 3 μl was used for testing its template activity (X —— X) in a cell-
free system of rabbit reticulocyte as described in Materials and Methods.

template activity determined in a rabbit reticulocyte lysate was localized

at around the 22 S region of the gradient and a few very minor acti-

vity peaks sedimenting slower than 22 S were also seen. The ultraviolet-

absorbing peak marked 19 S (Figure 1) has already been characterised to con-

tain a pure protein complex of 0.6×10^6 daltons, consisting of a unique

27 000-Mr protein which is immunologically related to the elongation factor

(eEF) Ts (9).

An analogous 22 S zonal gradient fraction (see Figure 1) was reisolated

and further purified by repeated sucrose gradient centrifugations after concen-

tration of the pools by PEG, to obtain partially purified 22 S mRNP. Figure 2

illustrates some of the characteristics of the template activity elicited by

the 22 S mRNP and its mRNA component. The partially purified 22 S mRNP was
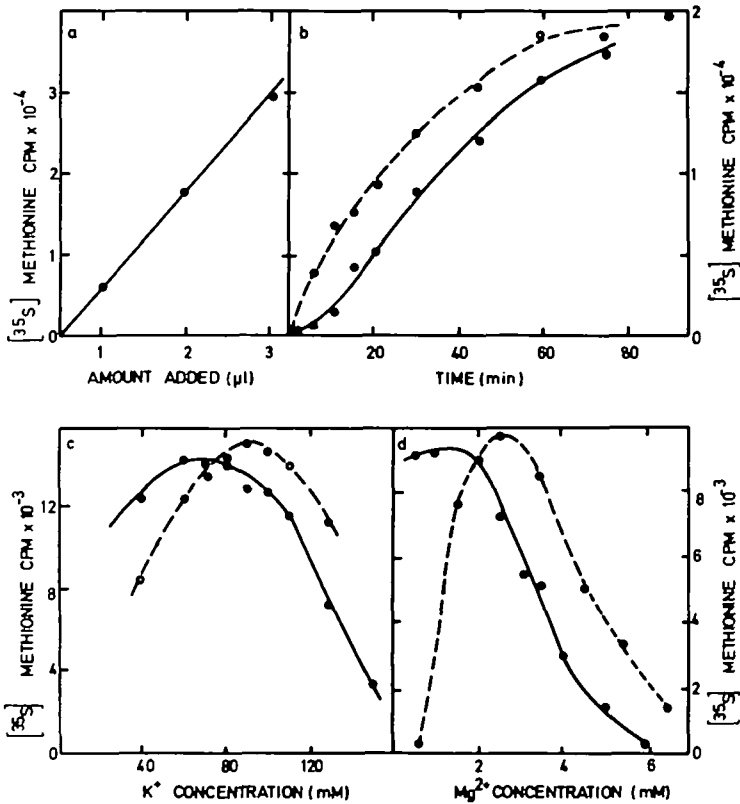
Figure 2 : Template characteristics of the 22 S mRNP and its RNA component.
The 22 S mRNP was purified as in the text and concentrated with PEG precipita-
tion and the precipitate was dissolved in 10 mM sodium phosphate buffer (pH
6.8). The RNA was purified from the 22 S mRNP as described in Materials and
Methods. The template activity was determined in a rabbit reticulocyte lysate
as in Figure 1. (a) Concentration dependence. (b) Time dependence. (c) Potassium
optimum and (d) Magnesium optimum. (●——●), 22S mRNP and (O---O) its mRNA
component.

able to direct efficiently the in vitro protein synthesis which responded
linearly to the amounts added to each reaction mixture (Figure 2a). The
kinetics of protein synthesis by the 22 S mRNP indicated a linear incorporation
of amino acids over 1 h with a small initial lag phase, whereas those by the
isolated RNA component did not exhibit such a lag phase (Figure 2b). This lag
phase might have been caused by a possible interferance on the initiation step
by the contaminating 19 S complex still present in the 22 S mRNP preparation.

The potassium optimum, although broad, by the 22 S mRNP was below 80 mM and slightly lower than regularly observed (Figure 2c). On the other hand, mRNA-dependent potassium optimum was 90-100 mM as in other translational systems (Figure 2c). Furthermore, a broad magnesium optimum was observed between 0.5 and 2.5 mM by the 22 S mRNP-directed protein synthesis (Figure 2d), which was substantially lower than that (12 mM) reported for sea urchin maternal mRNP in a homologous cell-free system (14). However, a sharp magnesium optimum at 2.5-3 mM was attained with mRNA template in both Artemia and sea urchin (14) systems. Finally, the efficiency of translation of either mRNP or mRNA is very comparable for a given condition.

Since polyadenylated mRNP in the postribosomal supernatant was largely preeliminated by affinity chromatography on oligo(dT)-cellulose (10) before sucrose density gradients and since all purification procedures of the 22 S mRNP was carried out in buffer C, under which condition polyadenylated mRNP was in a repressed state by bound translational inhibitor RNA (2), the template active 22 S mRNA was anticipated to contain no polyadenylate residues. Experiments shown in Figure 3 and Figure 4a support that this is the case.

The RNA isolated from the postmitochondrial (Figure 3a) or postribosomal (Figure 3c) supernatant was fractionated by linear sucrose gradient centrifugation. Hybridisation of [^{3}H] poly(U) to fractions of the former gradient (Figure 3a) indicated the existence of a polyadenylate-deficient valley at around 9 S, which coincided with the maximal template activity in rabbit reticulocyte lysate (Figure 3b). A similar template activity profile was obtained by the oligo(dT)-cellulose unbound postribosomal supernatant RNA fractions (Figure 3d). The conclusive evidence for the absence of polyadenylate residues in the 22 S mRNP is demonstrated in Figure 4a. The 22 S mRNP was highly purified by pooling only the 22 S peak fractions of the last sucrose gradient and its RNA component was isolated. The RNA so prepared was found to lack completely the ability to hybridise with [^{3}H] poly(U) under conditions where polyadenylated mRNA could form a hybrid with poly(U) in a dose-dependent manner (Figure 4a).

As already indicated by results of Figures 3b and 3d, the major template activity was localised in the region of 9 S. This observation was further confirmed by the experiment in Figure 4b. The same RNA preparation as used in poly(U) hybridisation (Figure 4a) was analysed by a linear sucrose gradient and each gradient fraction was tested for its template activity in a rabbit reticulocyte lysate. As seen in Figure 4b, a reasonably sharp peak of the template activity was observed at about 9 S in the gradient, which coincided
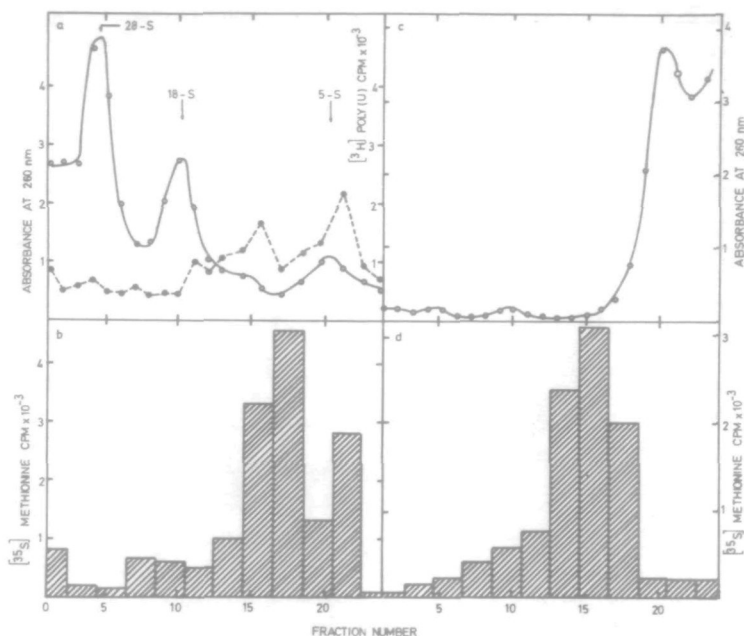
Figure 3 : <u>Sucrose gradient analysis of template active RNA.</u> The postmito-
chondrial and oligo(dT)-cellulose unbound postribosomal supernatants were
digested with proteinase K (50 µg/ml) at room temperature for 60 min in the
presence of 0.5% SDS. The RNA was extracted with phenol/chloroform/isoamyl-
alcohol (50/50/1) as described in Materials and Methods and dissolved finally
in buffer C. After centrifugation at 4°C on a linear 10-30% (w/v) sucrose
gradient in buffer C in a Beckman SW 27 rotor at 131 000 X g for 25 h, the
polyadenylate sequences were determined by hybridization with [^{3}H] poly(U)
as described previously (1). (a) 70 A_{260} units of the postmitochondrial RNA
was used; A_{260}, (O —— O); [^{3}H] poly(U) radioactivity (●---●). (b) Histogram
of template activity of gradient fractions of panel (a) measured in a rabbit
reticulocyte lysate as described and expressed as [^{35}S] methionine radio-
activity/µ gRNA. (c) 130 A_{260} units of the postribosomal RNA was used; A_{260}
(O——O). (d) Histogram of template activity of gradient fractions of
panel (c).

roughly to an optical density peak. These results indicate that the 22 S
mRNP contains primarily 9 S mRNA lacking any detectable polyadenylate
sequence.

   <u>Polyadenylate-specificity of translational inhibitor RNA.</u> As discussed
above and in our earlier publication (2), the translational inhibitor RNA
of the cryptobiotic gastrulae seems to be very specific for polyadenylated
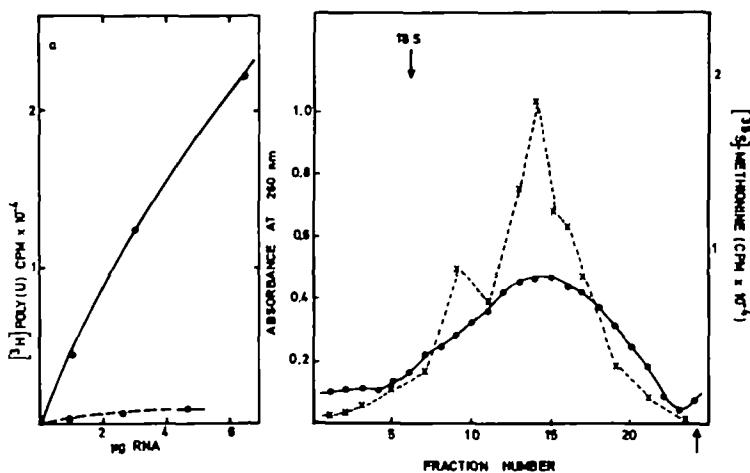mRNP or mRNA. Polyadenylated <u>Artemia</u> gastrula mRNA as well as rabbit globin

Figure 4 : Further analysis of the RNA component of the 22 S mRNP. 22 mRNP
and its mRNA component were prepared as in Figure 2. Polyadenylated mRNP and
mRNA were prepared using buffer L as described by Slegers et al. (9).
(a) Hybridization of RNA with [^{3}H]poly(U) was performed as described (1).
(O——O) poly(A)$^+$ RNA, (●——●) RNA from the 22 S mRNP.
(b) Sedimentation analysis of RNA isolated from the 22 S mRNP on a linear
10-30% (w/v) sucrose gradient in buffer M; A$_{260}$ (●---●). Each fraction was
precipitated with two volumes of ethanol and dissolved in 20 μl distilled
water; 3 μl was used for testing its template activity (X——X) in a rabbit
reticulocyte lysate.

mRNA were effectively inhibited to a similar extent by the inhibitor RNA in

rabbit reticulocyte lysate (Figure 5a). On the other hand, the 22 S mRNP

(or 9 S mRNA, not shown) was completely insensitive to this inhibitor RNA

in the same concentration range (Figure 5b). We therefore conclude that the

translational inhibitor RNA of Artemia salina is highly specific for poly-

adenylated mRNP or mRNA and is unable to exert its effect on non-polyadeny-

lated RNP or mRNA. In addition, it appears that the effect of this inhi-

bitor RNA is not restricted to polyadenylated mRNA of homologous origin,

but also to those of heterologous origins (Figure 5a). Thus, it might be

possible that non-polyadenylated mRNPs be preferentially utilised immedia-

tely after disruption of the cryptobiosis or in the early phase of postgas-

trula development. Furthermore, during the postgastrula development of Ar-

temia salina the polyadenylate-specific translational RNA might be able to

control the availability of specific polyadenylated mRNA stored in the

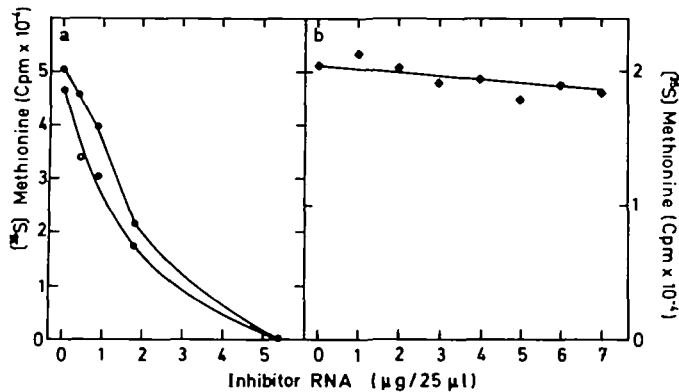cryptobiotic gastrula at a specific differentiation period when certain

Figure 5 : <u>Specific effect of translational inhibitor RNA</u>. The effect of
translational inhibitor RNA (amounts indicated in the figure) was tested
in rabbit reticulocyte lysate as described in Materials and Methods on (a)
polyadenylated or (b) non-polyadenylate template. (O—O) , <u>Artemia salina</u>
poly(A)$^+$ mRNA (2 µg); (●—●), rabbit globin mRNA (0.4 µg); (◆—◆), 22 S
mRNP (2 µl equivalent of the sucrose gradient fraction as used in Figure
2a).

mRNA species are required. A possible mechanism of the postulated reacti-

vation <u>in vivo</u> of stored, repressed polyadenylated mRNP is totally unknown.

Analysis of in vitro synthesized protein coded by the 22 S mRNP. Pro-

tein products synthesised in a rabbit reticulocyte lysate by either 22 S

mRNP or 9 S mRNA, were analysed by electrophoresis in two gel systems.

Both templates synthesised one major protein product with an apparent

Mr 26 000, as estimated in SDS gels (Figures 6b and 6d). This protein

was found to be a quite basic one, migrating slightly faster than calf

thymus H1 histone in acid-urea gels (Figures 6a and 6c). However, this

basic protein does not appear to be a <u>Artemia</u> histone on the following grounds

(A. Van Broekhoven et al., unpublished results); (a) <u>Artemia</u> H1 histone mi-

grates with a Rf value of 0.44 while calf thymus H1 histone does with that

of 0.50, when both <u>Artemia</u> and calf thymus H4 histones have migrated to Rf

value of 0.75, (b) under the same conditions, the modified minor component

(possibly phosphorylated) of <u>Artemia</u> H1 migrates slightly slower than the

major component by the Rf difference of 0.01, (c) estimated molecular weight

of <u>Artemia</u> H1 histone is 23 000, on the basis of published value (Mr 21 000)

of calf thymus H1 histone (13), (d) other <u>Artemia</u> histones (H3, H2A, H2B

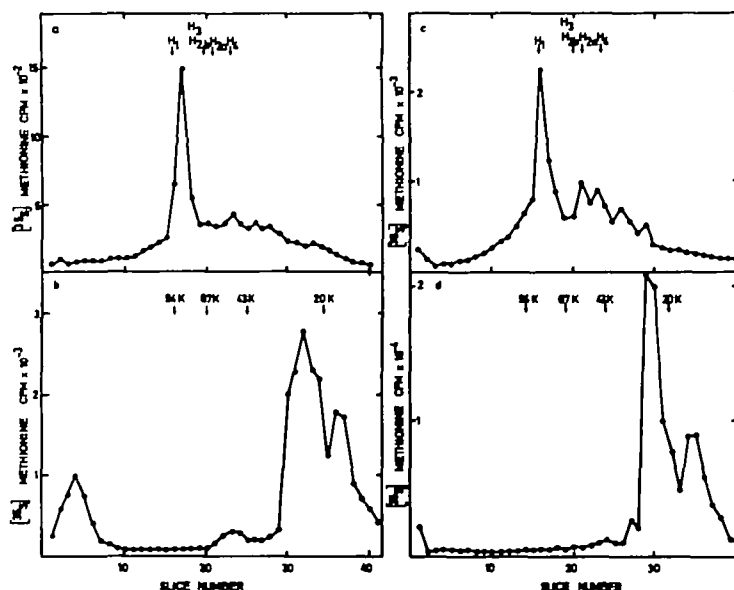and H4) migrate with similar Rf values as do the corresponding calf thymus

Figure 6 : Electrophoretical analysis of the in vitro synthesized proteins. [^{35}S] labelled proteins directed by the 22 S mRNP (a,b) and by the 9 S mRNA component, (fraction 14, in Figure 4c) (c,d) were analysed by SDS polyacryl-amide gels (b,d) and by acid-urea gels (a,c) ; the [^{35}S] radioactivity of 2 mm-thick slices was determined as described in Materials and Methods. Protein markers used were rabbit muscle phosphorylase b (Mr 97 000), bovine serum albumin (Mr 68 000), egg white ovalbumin (Mr 43 000) and soybean trypsin inhibitor (Mr 20 100) for SDS polyacrylamide gel electrophoresis and calf thymus histones for the acid-urea gel electrophoresis.

histones and have almost identical molecular weights (Mr 14 000, 13 000, 11 500, respectively) to those of their calf thymus counterparts. Therefore, the 26 000-Mr basic protein synthesised by non-polyadenylated 9 S mRNA does not seem to be identical to any one of Artemia histones. The possible func-tion of this basic protein remains to be investigated.

Aanmoediging van het Wetenschappelijk Onderzoek in Nijverheid en Landbouw),
respectively.

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