CONSERVATION OF GLOBIN MESSENGER RNA IN RABBIT RETICULOCYTE MONORIBOSOMES AFTER SODIUM FLUORIDE TREATMENT.

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SUMMARY:

Following NaF treatment of rabbit reticulocytes, polyribosomes dissociate to monoribosomes with concomitant cessation of protein synthesis. mRNA was isolated from different fractions of the cell before and after NaF treatment and quantitated by assay for globin synthesis in a cell-free system prepared from Krebs ascites tumor cells. mRNA activity present in polyribosomes of control cells was recovered quantitatively from monoribosomes following NaF treatment. On removal of NaF, polyribosomes reform with mRNA activity similar to that of control cells. During polyribosome dissociation, no significant amount of mRNA is recovered from ribosomal subunits, the supernatant fraction or is lost by degradation.

Reticulocytes are a good model system for studying mechanisms of protein synthesis at the level of translation in mammalian cells; these cells do not synthesize RNA and more than 90% of the protein formed are hemoglobins. Previous reports from our laboratory demonstrated that treatment of reticulocytes with sodium fluoride (NaF), causes polyribosomes to dissociate into 80S ribosomes with cessation of protein synthesis (1,2). These effects of NaF are reversible. Within 2 to 3 minutes after removal of NaF, the rate of protein synthesis returns to normal and polyribosomes begin to reform(1). Thus, mRNA for globin is conserved during inhibition of protein synthesis by NaF. Further, these studies showed that the rate of protein synthesis is not directly dependent on the size or number of polyribosomes (2). NaF causes polyribosome dissociation in Hela cells (3) and ascites tumor cells (4), indicating that the effect of NaF is not specific for reticulocytes.

This paper demonstrates that after treatment with NaF, the mRNA initially associated with polyribosomes is recovered quantitatively in the monoribosomes region. On release from NaF inhibition, the mRNA again becomes associated

with polyribosomes. Following NaF induced dissociation of polyribosomes, no significant amount of mRNA is found in the supernatant fraction of the cell or associated with ribosomal subunits or is lost by degradation.

MATERIALS and METHODS

Krebs II Ascites tumor cells were kindly supplied by Dr. A.T.H. Burness, Kettering Laboratories, N.Y. The preparation of Krebs cell-free system and tRNA are described in a previous paper (5). Reticulocytes were obtained from New Zealand rabbits treated with phenylhydrazine and washed with 0.13 M NaCl, 7.4 mM $MgCl_2$ and 5 mM KCl (6). One volume of packed cells with 2 volumes of modified Krebs-Ringer-Bicarbonate buffer (2), containing 10 mM NaF, when required, were incubated at 37°C. After incubation, the cells were washed twice with washing medium, containing 3 mM NaF when cells were incubated with NaF, lysed and then an S-30 prepared as previously described (5).

The S-30 was centrifuged at 55,000 rpm in a Ti 60 Spinco rotor for 5 hr at 4°C to obtain total ribosomes. The ribosome pellets were dissolved in solution A[0.01 M Tris-HCl buffer, pH 7.4, 0.01 M KCl, 0.0015 M $MgCl_2$]; ribosomal patterns were obtained by centrifugation in SW 27 Spinco rotor at 27,000 rpm, 4°C, using sucrose density gradient centrifugation for the times indicated for each experiment. Different ribosomal fractions from sucrose gradients were pelleted separately by centrifugation at 60,000 rpm in a 65 Spinco rotor for 12 hr at 4°C and then processed to obtain mRNA. Two methods of preparing mRNA were used: (1) SDS-method: mRNA from the pelleted ribosome fractions was prepared by the SDS-method previously reported (6); (2) Phenol-SDS-method: mRNA from the total cells and the ribosome-free supernatant fractions were prepared as follows: 10 volumes of the Buffer K [0.6% SDS, 1 mM EDTA, 0.1 M NaCl, 0.01 M potassium acetate buffer, pH 5.0] were added to one volume of packed cells or to 5 volumes of the ribosomal-free supernatant fractions. An equal volume of water-saturated distilled phenol was then added and the mixture shaken at 4°C for 30 min The aqueous phase was removed and the phenol phase re-extracted with 5 volumes of Buffer K for 15 min at 4°C. The resulting aqueous phase was combined with the first aqueous phase and re-extracted again with an equal volume of phenol. The RNA was precipitated from the final aqueous phase by adjusting to 0.3 M NaCl, and the addition of 2 volumes of ethanol. The RNA was recovered by centrifugation at 27,000 g for 10 min. A 5 to 20% sucrose density gradient 5 mM Tris-HCl buffer, pH 7.4, was used to separate RNAs by spinning at 40,000 rpm for 14 hrs in a SW 40 Spinco rotor at 4°C (5,6). The 7S to 16S region of the gradient was pooled and precipitated RNA was collected by centrifugation and

washed twice with 70% ethanol containing 2% potassium acetate pH 5.5, followed by two more washing with 95% ethanol. The RNA was dissolved in 0.5 ml of H_2O and lyophilized. The RNA obtained was redissolved in a minimum volume of water for assay for biological activity. When indicated, 10S RNA was further purified by centrifugation in a 15 to 30% sucrose density gradient in a SW 40 Spinco rotor for 26 hrs at 40,000 rpm at 4°C (Figure 1). 10S RNA extracted by SDS or SDS-phenol methods had the identical stimulatory activity per $O.D._{260}$. The preparation of rabbit reticulocyte ribosomal wash fraction and the assay for protein synthesis in a Krebs ascites cell free system were performed as previously described (5). The incubation was performed at 37°C for 20 minutes,

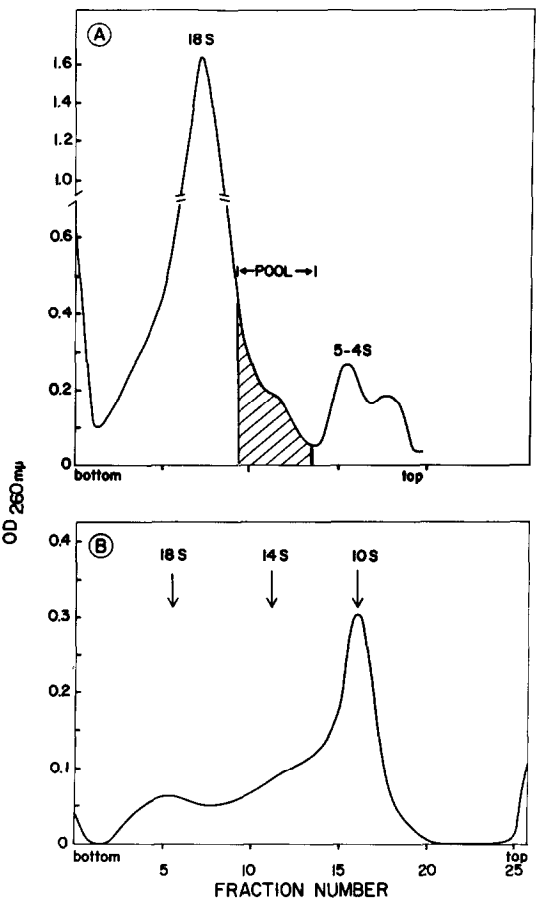

Fig. 1: Sucrose density gradient profile of RNA extracted from monoribosomes after NaF treatment. RNA was extracted from monoribosomes after NaF treatment and analyzed on sucrose gradients as described in "Methods" (A) Pattern obtained after 5-20% sucrose density gradient centrifugation. The shaded area including the 10S peak was assayed for biological activity. (B) Pattern obtained after 15-30% sucrose density centrifugation of the RNA present in the shaded area of (A).

and [^{3}H]leucine incorporation into acid-insoluble cpms were measured (5).

The small total amount of 10S RNA present in different ribosomal fractions makes it difficult to obtain an accurate determination of RNA content by direct optical density measurement. mRNA content was determined employing a standard curve relating biological activity (incorporation into protein per 20 min) to $O.D._{260}$ of purified 10S RNA added to the cell-free system. This standard curve (Figure 2) was determined for every set of experiments over a range of 0 to 10 pmole 10S RNA added to the 50 µl cell-free system assuming an average molecular weight of $2x10^5$ daltons (7). For each preparation of 10S RNA, three concentrations of the RNA fractions were assayed; at least two points were on the linear portion of the standard curve (Figure 2). The amount of 10S RNA present in a RNA preparation was taken as that corresponding to the level of incorporation for a known amount of purified 10S RNA on the curve.

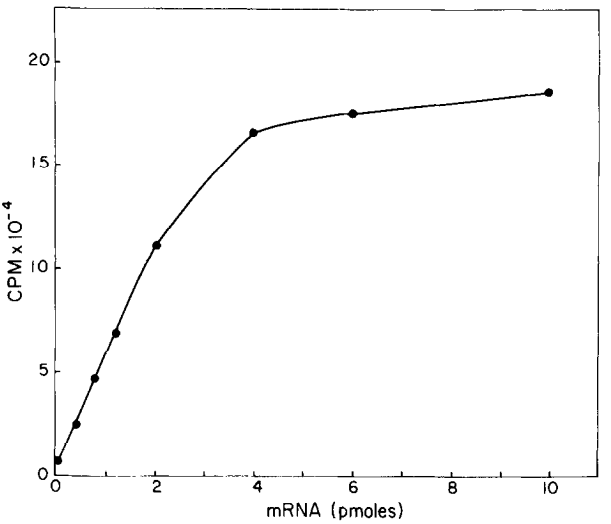

Fig. 2: Messenger RNA concentration curve. Purified rabbit 10S RNA prepared as described in "Methods" was added to the Krebs lysate cell-free system at different concentrations and assayed as described in "Methods' [^{3}H]-leucine (39C/mM) was used as the labelled precursor.

RESULTS:

Conditions of incubation of reticulocytes with NaF were employed which cause complete dissociation of polyribosomes to 80S ribosomes (Figure 3,4B). The 40S subunits are significantly decreased after NaF treatment (Figure 3). The cells were then washed to remove the inhibitor, and incubated at 37°C. In these cells, polyribosome reformation reached 20% of control levels within 10 min (4C) and was almost complete after 60 min (Figure 4D).

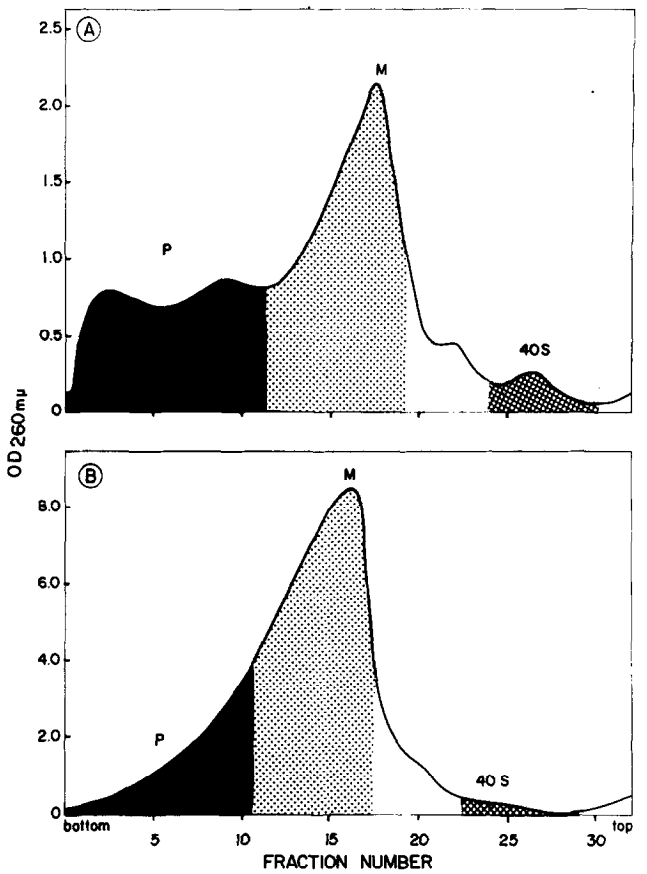

Fig. 3: Effects of NaF on ribosome pattern: (A) Ribosome pattern in cells prior to incubation with NaF. The fractions indicated by shaded areas P,M and 40S were assayed for mRNA activity and the results summarized in Table I, experiment II. (B) Ribosome pattern of cells recovered after incubation with 10 mM NaF for 60' at 37°C. The fractions indicated by shaded areas P, M and 40S were assayed for mRNA activity and the results summarized in Table I, experiment I. P represents polyribosome region, M represents monoribosome region, and 40S represents 40S ribosome subunit region.

In cells not incubated with NaF, over 75% of the mRNA activity recovered from all cell fractions, was isolated from the polyribosomes (Table 1). In cells treated with NaF, over 75% of the mRNA activity was recovered in the monoribosome region (Table 1, Figure 4B). In control and NaF treated cells, less than 3% of the total mRNA recovered from the cell fractions was extractable from 40S subunits or from the supernatant fraction. It was demonstrated that 10S RNA activity was not inhibited in preparations containing up to 95% 18S RNA.

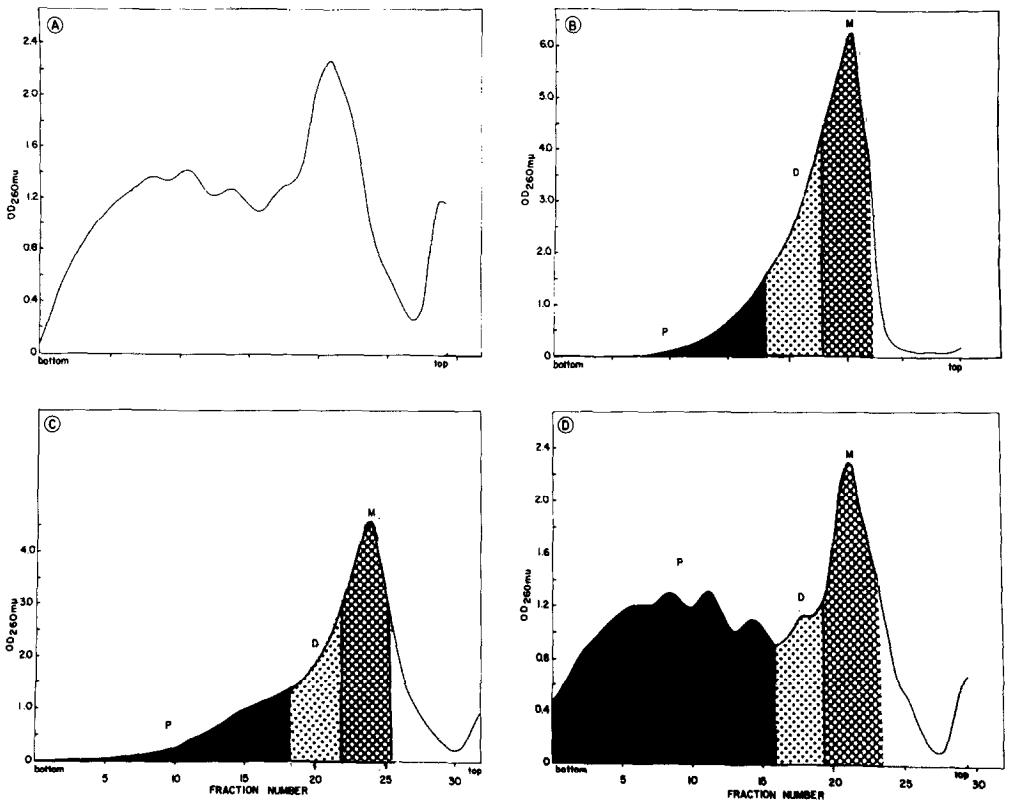

Fig.4: Reformation of polysomes after removal of NaF. 5 ml of packed cells were incubated with 10 mM NaF for 60 min as described in Table 1. After incubation the cells were washed with washing medium containing 3 mM NaF, lysed, ribosomes pelleted and analyzed by sucrose density gradient centrifugation for 3 hrs. (A) Ribosomal pattern obtained from 5 ml of packed cells not preincubated with NaF, following the above described procedure. (B) Ribosome pattern following NaF treatment for 60 min (C) and (D) 5 ml of packed cells were incubated as in (A), the NaF removed by 2x washing with washing medium, reincubated respectively for 10 (C) and 60 (D) min in the incubation medium (see "Methods"), then lysed and ribosome pattern obtained. P represents polyribosome region, D represents dimer region and M represents monoribosome region.

10 minutes after removal of NaF and incubation of cells in a NaF free medium, 40% of the mRNA is found in the dimer region (Table 2,Figure 4C). 60 minutes after NaF removal, 70% of the mRNA activity, a value comparable to control, is recovered from polyribosomes (Table 2, Figure 4D).

TABLE 1

Distribution of mRNA in NaF treated rabbit reticulocytes.

		Total Cells	T.R.P.	P.	M	40	Sup.
[I] NaF	VOLUME [ml]	40	-	-	-	-	240
	$O.D._{260}$	-	450	30	214	1.6	-
	mRNA [pmole]	510	350	40	311	14	48
	VOLUME [ml]	40	-	-	-	-	240
	$O.D._{260}$	0	450	203	53	4.2	-
	mRNA [pmole]	490	340	248	57	15	7.2

[I] = NaF treated cells; [II] = cells not preincubated with NaF. Messenger RNA was obtained with the "SDS-method" from fractions P, M and 40 as indicated in Fig.3, and from the total ribosomal pellet (T.R.P.) as described in the "Methods". The SDS-Phenol- method was used to prepare 10S RNA from the ribosome-free supernatant (Sup.). The same method was used to obtain 10S RNA fraction from total cells. The $O.D._{260}$ represents the amount present in the ribosome fractions prior to mRNA isolation. pmoles of mRNA are obtained as described in "Methods".

TABLE 2

Distribution of mRNA in NaF treated reticulocytes after NaF removal.

	Messenger RNA [pmoles]		
	P	D	M
[I] Fluoride	4.8	8.4	31.0
[II] 10 min after NaF removal	6.2	21.0	25.0
[III] 60 min after NaF removal	35.0	8.2	8.4

Messenger RNA was obtained from the polyribosome region (P), dimer region (D) and monoribosome region (M) of sucrose gradients B, C and D shown in Fig.4. Biological activity was assayed as described in "Methods".

DISCUSSION:

NaF induced inhibition of protein synthesis in reticulocytes is associated with polyribosome dissociation and accumulation of 80S ribosomes (1). A 10S RNA fraction has been shown to be present in the total ribosomal pellet of NaF-treated cells (8). The present studies provide the first direct evidence that the mRNA in NaF-treated cells is almost totally conserved in association with 80S monoribosomes under conditions of cessation of protein synthesis. There is no detectable loss of biologically active mRNA into the supernatant or by degradation.

The demonstration that biologically active mRNA can be conserved in

reticulocytes without being translated may be analogous to the presence of "masked mRNA" which has been suggested to exist in several other animal cells (9). In sea urchin eggs, maternal mRNA stored in the unfertilized egg is expressed only after fertilization (10,11). Studies on erythropoiesis also indicate that mRNA may be synthesized and stored for variable periods without being translated (12,13). It is possible that mRNA in these cells may be "masked" or rendered temporarily inactive by inhibitory factors (14) whose actions result in effects similar to those of NaF in preventing expression of potentially biologically active mRNA.

Although the rate of protein synthesis has returned to a level comparable to that in untreated cells within 10 min of removal of NaF (2), less than 40% of the mRNA has become associated with polyribosomes and less than 40% of the ribosomes are in polyribosomes (2). These data indicate that the rate of protein synthesis in these cells is not limited by the amount of mRNA associated with polyribosomes. It has been shown that there is a more rapid rate of translation of mRNA when there is only partial reformation of polyribosomes following recovery from NaF inhibition (2). NaF specifically inhibits the initiation of new polypeptide chains while not affecting chain elongation or release (3,15). In addition, the present study confirms previous reports (3) demonstrating a decrease in 40S subunits of NaF treatment. Taken together, the present data and those obtained using cell-free systems (16-18) are compatible with NaF acting to inhibit initiation of protein synthesis by a mechanism which cannot be further defined at present.

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