

Correlation between riboflavin carrier protein induction and its mRNA activity in estrogen stimulated chicken liver and oviduct

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Abstract. Poly A enriched RNA from either liver or oviduct of estradiol-17 β treated immature chicks supported [3 H]-leucine incorporation into immunoprecipitable riboflavin carrier protein in a dose-dependent manner when translated in the rabbit reticulocyte lysate system. Primary translation product of riboflavin carrier protein had a molecular weight of 38,000 which on incubation with a stripped hepatic microsomal preparation was processed to a product with a size comparable to native riboflavin carrier protein. Poly A enriched RNA from both the liver and the oviduct of estrogen-treated birds stimulated [3 H]-leucine incorporation into riboflavin carrier protein and this was 2-3 fold higher during secondary stimulation *vis-a-vis* primary stimulation with the steroid. Poly A enriched RNA from the liver of progesterone-treated birds during secondary stimulation did not support riboflavin carrier protein synthesis. In contrast, poly A enriched RNA from the oviduct of the birds treated with progesterone during secondary (but not primary) stimulation did exhibit riboflavin carrier protein-mRNA activity which was comparable to that stimulated by estradiol-17 β .

Keywords. Primary translation product; cell-free translation; rabbit reticulocyte lysate; stripped microsomal membrane; progesterone; estradiol-17 β ; precursor; poly A⁺-RNA.

Introduction

According to current concepts of mechanisms of steroid hormone-induced specific gene expression in eukaryotic cells, one of the major events consequent on functional interaction of the hormone-receptor complex with the genomic regulatory sites on the chromatin is the initiation and accelerated transcription of protein-specific mRNA (Chan *et al.*, 1978). In line with the above is the finding that there exists excellent correlation between elevated levels of specific mRNAs and enhanced production of proteins encoded by them. This is amply illustrated by the estrogenized avian (Deeley *et al.*, 1975) and amphibian (Tata, 1976) liver expressing *de novo* the vitellogenin gene and the steroid-induced production of several egg white proteins by the differentiated chicken oviduct (Palmiter, 1975). Our earlier investigations on estrogen-induced *de novo* expression of the gene encoding riboflavin carrier protein (RCP) in the chicken

Abbreviations used: RCP, Riboflavin carrier protein; RIA, radioimmunoassay; poly A⁺-RNA, poly A enriched RNA; EGTA, ethylene glycol-bis (2-aminoethyl ether) N, N' -tetraacetic acid; DTT, dithiothreitol; HEPES, N-2 hydroxy ethyl piperazine-N' -2-ethane sulphonic acid; SDS, sodium dodecyl sulphate; PPO, 2,5-diphenyl oxazole; *M*, molecular weight; PAGE, polyacrylamide gel electrophoresis.

liver (Murthy and Adiga, 1978) and oviduct (Durga Kumari and Adiga, 1986) have clearly shown that, despite gross similarities between the inductive responses in the two tissues, there are subtle differences with regard to the kinetics and modulation of induction of this vitamin-carrier in the two hormone-responsive organs. However, these conclusions were arrived at largely by measurement of plasma and tissue levels of RCP by radioimmunoassay (RIA) which reflect, the total contents of this protein (which in turn may be influenced by the turnover rate) and hence indirectly the biosynthetic potency of the respective tissue. It was therefore considered desirable to assay the levels of RCP-mRNA in the two tissues to correlate the same with their RCP contents. Towards this end, the translational efficiency of isolated poly A enriched RNA (poly A⁺-RNA) preparations from the avian liver and oviduct in a heterologous cell-free system was exploited as a direct index of mRNA contents (Wetkam *et al.*, 1975). Incidentally, this also provided an opportunity to examine the nature of the primary translational product of RCP gene in the two avian tissues. The data presented in this paper reveal that the primary translational product of the RCP gene in the two estrogen-responsive avian tissues are grossly similar. Further, they indicate that there exists a significant correlation between the tissue mRNA levels and their capacity to elaborate RCP in response to hormonal stimuli.

Materials and methods

ATP, GTP, creatine phosphate, creatine phosphokinase, spermidine HCl, ethylene glycol-bis (2-amino ethyl ether) N,N'-tetraacetic acid (EGTA), dithiothriitol (DTT), N-2 hydroxy ethyl piperazine-N'-2 ethane sulphonic acid (HEPES) and *Micrococcal* nuclease were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Oligo (dT)-cellulose was purchased from Collaborative Research Inc., Waltham, Massachusetts, USA. [³H]-Leucine (specific activity, 136 Ci/m mol) was purchased from the Radio-chemical Centre, Amersham, Bucks, UK. All other chemicals and reagents were of analytical grade. The source of the White Leghorn chicks and their maintenance have been described earlier (Murthy and Adiga, 1978; Muniyappa and Adiga, 1980). The chicken RCP was purified to homogeneity from the egg white and specific anti bodies were raised in rabbits as described earlier (Murthy and Adiga, 1977).

Hormone regimen

A group of female chicks (2-week-old, 150g body wt) were injected (intramuscular) daily with estradiol-17 β (10 mg/kg body wt/day) in propylene glycol for 10 days during primary stimulation. After 21 days of hormone withdrawal, they were subdivided into two groups and were administered either estradiol- 17 β or progesterone, (both at 10 mg/kg body wt/day) respectively for 4 days (secondary stimulation).

Isolation of chicken liver and oviduct poly A⁺-RNA

Extraction of polysomal RNA and preparation of poly A⁺ -RNA: Polysomes were prepared by the procedure of Palmiter (1974). Birds were sacrificed 24h after the last hormone injection following both primary and secondary stimulations. Livers and

oviducts quickly excised from decapitated birds were homogenized in 9 volumes of 0.025 M Tris-HCl (pH 7.5) buffer containing 0.025 M NaCl, 5 mM MgCl₂, 2 % Triton X-100 and 1.0 mg/ml of heparin. After centrifugation at 27,000 g for 5 min at 4°C, the supernatant was mixed with an equal volume of the homogenising buffer containing 0.2 M MgCl₂. After standing for 2 h in the cold (4°C), the supernatant was layered over one half volume of 0.025 M Tris-HCl (pH 7.5) containing 0.025 M NaCl, 5 mM MgCl₂ and 0.5 M sucrose and centrifuged at 27,000 g for 20 min. The supernatant was aspirated and the polysomal pellet was used for the extraction of RNA.

RNA was prepared from the polysomes by extraction with phenol-chloroform (Palmiter, 1974).

Poly A⁺-RNA was isolated from the polysomal RNA by chromatography on oligo dT-cellulose (Krystosek *et al.*, 1975). Briefly, a column (2.5 ml bed volume) of regenerated oligo (dT)-cellulose was used and equilibrated with 20 ml of high salt-sodium dodecyl sulphate (SDS) buffer [0.01 M, Tris-HCl (pH 7.5), 0.5 % SDS, 0.5 M NaCl]. RNA dissolved in high salt-SDS buffer (Ca 15-20 A₂₆₀ units/ml) was applied to the column which was washed successively with the high salt- SDS buffer and then with 0.01 M Tris-HCl (pH 7.5) 0.5 M NaCl. The poly A⁺- RNA was eluted with autoclaved distilled water. The RNA in the effluent was precipitated with 2.5 volume of 95 % ethanol as before. It was dissolved in a minimal volume of autoclaved distilled water and stored at -20°C until used.

Protein synthesis in rabbit reticulocyte cell-free system

The reticulocytes were isolated (Palmiter, 1973) from rabbits rendered anaemic by treatment with phenylhydrazine hydrochloride. The washed cells were finally lysed with one volume of autoclaved distilled water and centrifuged at 10,000 g for 20 min at 4°C. Haemin (25 μM) and creatine phospho kinase (50 μg/ml) were added to the lysate and stored at -70°C.

Endogenous globin mRNA in the reticulocyte lysate was removed by digesting briefly (9 min at 20°C) with *Micrococcal* nuclease (Pelham and Jackson, 1976) in the presence of CaCl₂ (1 mM). The nuclease was inactivated with EGTA (2 mM) and the lysate directly used for the translation of chicken liver and oviduct poly A⁺-RNAs.

Preparation of stripped microsomal membranes

These were prepared by the method of Blobel and Dobberstein (1975) with slight modifications. Liver dissected from control (unstimulated) chicks were rinsed with and homogenized in the buffer containing triethanolamine (50 mM, pH 7.5), 0.25 M sucrose, 50 mM KCl, 5 mM MgCl₂ and 2 mM DTT. The homogenate was clarified by centrifugation at 10,000 g for 10 min and the supernatant was spun at 105,000 g at 4°C for 1 h. The microsomal pellet thus obtained was suspended in ice-cold 50 mM Tris-ethanolamine-HCl (pH 7.4)/50mM KCl to a concentration of 100 A₂₈₀ units/ml. EDTA was added to a final concentration of 1.5 μ mol/10.0 A₂₈₀ units and the suspension layered on 25 % sucrose in 50 mM triethanolamine-HCl (pH 7.4)/50 mM KCl buffer. After centrifugation for 2 h at 105,000 g, the pellet comprising of EDTA-stripped microsomes was resuspended in 50 mM triethanolamine-HCl (pH 7.4)/50 mM KCl and centrifuged again at 105,000 g for 1 h. The pellet was

suspended in 20 mM HEPES buffer, (pH 7.6) and was treated with *Micrococcal* nuclease as described earlier to remove the traces of RNA sticking to the membranes.

Translation of poly A⁺-RNA

The incubation mixture (100 μ l) contained 40 μ l of rabbit reticulocyte lysate, 0.02 M HEPES buffer (pH 7.6), 1 mM DTT, 1 mM ATP, 100 μ M GTP, 10 mM creatine phosphate, 375 μ M spermidine-HCl, 16 μ Ci of [³H]-leucine, 50 mM potassium acetate, 1 mM magnesium acetate, 50 μ M phenyl methyl sulphonyl fluoride and chicken liver or oviduct poly A⁺-RNA. The mixture was incubated at 37°C for 60 min. In some cases, the stripped microsomal membranes were included at a concentration of 5.0 A₂₈₀ units/ml. After 60 min, the tubes were centrifuged at 15,000 g for 5 min to remove any insoluble material. Aliquots were taken for the measurement of labelled amino acid incorporated into trichloroacetic acid-insoluble protein (Mans and Novelli, 1961) by liquid scintillation spectrometry.

Immunoprecipitation of radioactive RCP

After incubation, labelled lysate samples were adjusted to a final concentration of 0.2 % SDS, 10⁻⁴ M EDTA, 1% sodium deoxy cholate (w/v), 1 % Triton X-100 (v/v) and 0.1 M Tris-HCl (pH 7.5) in a total volume of 0.4 ml and the purified chicken RCP (10 μ g) was added as the carrier. To this, 0.1 ml RCP-antiserum was added, to provide a 2-fold excess of the antibody on the basis of the equivalence point. After incubation for 1 h at room temperature and for 12 h at 4°C, the immunoprecipitate was collected by centrifugation at 15,000 g for 5 min. It was washed 4 times with the buffer containing 1% sodium deoxycholate, 1% Triton X-100, 10⁻⁴ M EDTA and 0.1 M Tris-HCl (pH 7.5). The immunoprecipitate was suspended in buffer containing 10 mM Tris-HCl (pH 7.5), 2 \times 10⁻⁴ M EDTA and 150 mM NaCl. This was layered over 1 M sucrose cushion and spun at 15,000 g for 5 min. The pellet was washed twice with the same buffer. Immunoprecipitates were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 7 % gels (Laemmli, 1970).

Radioactivity measurements

After PAGE and staining with Coomassie brilliant blue R-250 the gels were sliced (2 mm) and digested with 0.5 ml of 30 (w/v) H₂O₂ at 55°C for 15 h. The immunoprecipitates to be counted directly were dissolved in 0.1 ml of 90% formic acid, transferred to Whatman No. 3 filter paper discs, dried and counted using toluene based scintillator containing 0.5 % (w/v) 2,5 diphenyl oxazole (PPO). For aqueous samples (gel digests) a mixture of 2-methoxy ethanol and toluene (1:1 v/v) containing 0.5 % PPO was used as the scintillation fluid. Samples were counted in a Rack beta liquid scintillation counter (LKB Produktor, Sweden).

Results

Significant incorporation of [³H]-leucine into immunoprecipitable RCP could be clearly observed in the rabbit reticulocyte cell-free translation system when poly A⁺-

RNA from either the liver or the oviduct of hormone-treated, (but not control) chicks was used as the exogenous source of mRNA. When poly A⁺-RNA from either of the avian tissues was added, protein synthesis was linear upto 60 min (data not given). Under optimal conditions of translation, *i.e.*, 59 mM K⁺, 1 mM Mg²⁺ and incubation time of 60min, poly A⁺-RNA (8 μg/100μl) from hormone treated chicken liver stimulated [³H]-leucine incorporation into total proteins 8-10 fold while that from the oviduct 10-14 fold.

Labelled RCP immunoprecipitated using the specific antibody was analysed on SDS-PAGE to ascertain the size distribution of the primary translation product. It could be shown that RCP synthesized in the heterologous cell-free system had a molecular weight (*M_r*) of 38,000; (pre-RCP); there was no detectable labelling in the region of mature RCP (*M_r*, 36,000) (figure 1). It is significant that the total mRNA from the control chicks showed no detectable radioactivity at positions corresponding to either of these protein bands. The total poly A⁺-RNA fraction from estrogen-treated

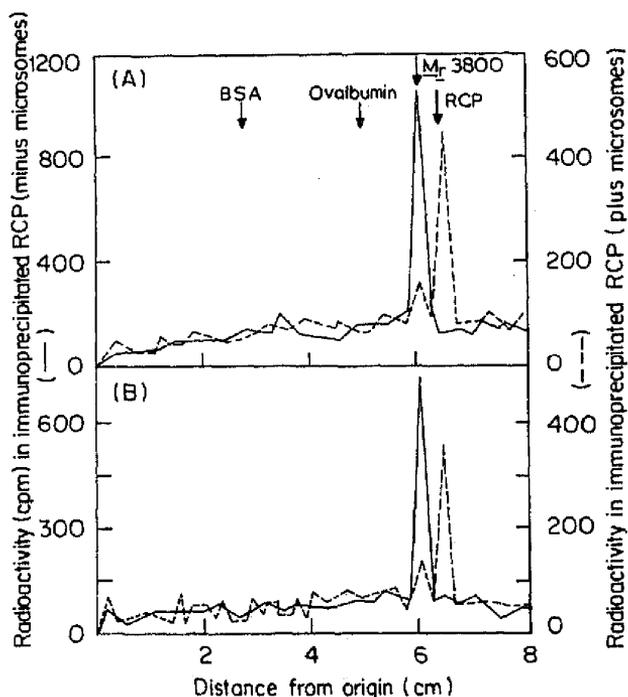


Figure 1. Characterization of [³H]-RCP synthesized in the reticulocyte lysate system programmed with poly A⁺-RNA from estradiol-17 β treated chicken oviduct and liver. Optimal concentrations of poly A⁺-RNAs isolated from chicken oviduct (A) and liver (B) were translated in the heterologous cell-free system and in the presence and absence of stripped microsomes. [³H]-RCP synthesized was immunoprecipitated using 2-fold excess of specific RCP antiserum. Washed immunoprecipitates were subjected to SDS-PAGE and gels stained and scanned for radioactivity. Bovine serum albumin, ovalbumin and RCP served as internal markers. For other details see 'materials and methods'. The positions of radioactivity peaks on the gels were fixed relative to RCP which obviated inter gel variations in the mobilities of protein species.

chicken oviduct during primary stimulation also showed a pattern similar to that elicited by the liver mRNA (figure 1). When the translation was carried out in the presence of 'stripped' microsomal membranes, from control chicken liver, there was a significant decrease in the radioactivity associated with M_r 38,000 pre-RCP species, with a concomitant increase in radioactivity in the region that moved marginally ahead of the native RCP. This slightly faster mobility of the microsomal membrane-processed translation product relative to native RCP may be attributed to the lack of covalently attached carbohydrate moiety. This is presumably due to the inability of the lysate system to glycosylate the primary translation product. A similar observation was made earlier in the case of ovalbumin mRNA (Rhodes *et al.*, 1971) translated in the reticulocyte lysate system.

The translatable RCP-mRNA activities from estrogenized chicken liver and oviduct during primary stimulation and from the corresponding tissues from estrogen-primed but secondarily stimulated with either estrogen or progesterone for 4 days were assayed to assess the magnitude of stimulation of RCP gene expression. The results of figure 2 indicate that at equal RNA concentrations ($8 \mu\text{g}/100\mu\text{l}$), the poly A⁺-RNA from

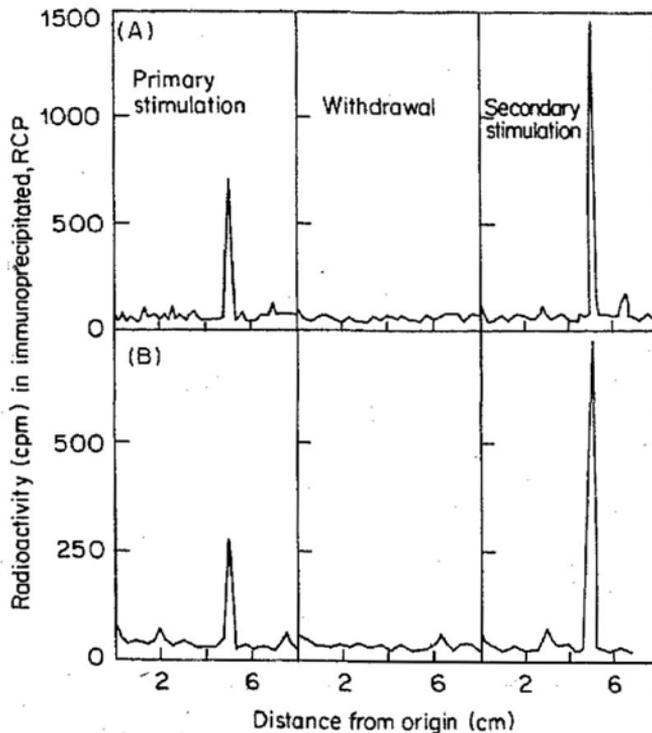


Figure 2. Distribution of radioactivity in electrophorograms following resolution of immunoprecipitated [³H]-RCP on SDS-PAGE. [³H]-RCP synthesized in the rabbit reticulocyte lysate system programmed with equivalent amounts of poly A⁺-RNA from estrogen-treated chicken oviduct (A) and liver (B) during primary stimulation, hormone withdrawal and secondary stimulation were immunoprecipitated and subjected to SDS-PAGE. Gels were scanned for radioactivity and protein stain. Radioactivity in the region corresponding to M_r 38,000 was quantitated. For other details see legend to figure 1.

oviducts of chicks treated with estrogen during secondary stimulation promoted pre-RCP synthesis almost 2-fold more than that from the oviducts of chicks during primary stimulation (table 1). Similarly liver poly A⁺-RNA isolated from birds, secondarily stimulated with estrogen enhanced RCP synthesis by 2.7 fold more than that obtained during primary stimulation (figure 2). Interestingly, there was no detectable synthesis of pre-RCP, when poly A⁺-RNA from livers or oviducts of either control birds or birds withdrawn from the hormone were used (figures 1 and 2).

The significance of the finding was that poly A⁺-RNA from liver treated with progesterone during secondary stimulation failed to support pre-RCP synthesis to any discernible extent whereas poly A⁺-RNA from the oviducts of estrogen-primed chicks stimulated secondarily with progesterone did support pre-RCP synthesis more or less to the same extent as that from oviducts of estrogen-primed chicks treated again with estrogen during secondary stimulation (table 1).

Table 1. Effect of estradiol-17 β and progesterone of immature female chicks on translatable RCP mRNA activity in the oviduct and liver during primary and secondary stimulations.

Group	Source of poly A ⁺ -RNA Tissue	Protein synthesis (cpm)		RCP (% of total protein) synthesized
		Total ($\times 10^{-4}$)	RCP	
E _I	Liver	20.00	2400	1.2
	Oviduct	24.10	2000	0.83
E _I \rightarrow E _{II}	Liver	23.20	7130	3.1
	Oviduct	28.00	5880	2.1
E _I \rightarrow P _{II}	Liver	16.20	20	0.0
	Oviduct	24.10	4800	2.0

Three groups of 5 female chicks (15 day old) were administered with estradiol-17 β (10 mg/kg body wt/day) for 10 consecutive days (primary stimulation). One group was sacrificed and their livers and oviducts were processed for poly A⁺-RNA. The remaining two groups were withdrawn from estrogen for 21 days and each of these groups were administered (secondary stimulation) either estradiol-17 β or progesterone (each at 10 mg/kg body wt/day) for 4 consecutive days. Poly A⁺-RNA prepared from their oviducts and livers were translated in the rabbit reticulocyte lysate system and [³H]-RCP immunoprecipitated and quantified as described under 'materials and methods'. E_I E_{II} represent primary and secondary stimulations with estradiol-17 β while P_{II} represents secondary stimulation with progesterone.

These results are in good agreement with the corresponding data obtained by measurements of liver or oviductal cytosolic RCP levels quantitated either by RIA or labelled amino acid incorporation into tissue explants (Durga Kumari and Adiga, 1986). This provides confirmatory evidence that progesterone could stimulate RCP mRNA activity only in the differentiated oviduct but not in the liver.

Discussion

The first indication, that RCP mRNA does in fact accumulate intracellularly in the steroid hormone-stimulated avian tissues, stems from the earlier observation that both the relative and absolute rates of RCP syntheses increase with time after hormonal

dosing (Durga Kumari and Adiga, 1986). Motivated by a desire to obtain a direct correlation between the extent of RCP induction and intracellular levels of mRNA activity, the versatile heterologous cell-free translation system *viz.*, the rabbit reticulocyte lysate, was recruited for mRNA quantification. The availability of a potent and highly specific RCP-antibody was exploited to measure RCP synthesized *in vitro* by immunoprecipitation. It could be clearly established that the lysate system responds to poly A⁺-mRNA preparation from either chicken liver or oviduct in dose-dependent manner. Poly A⁺-RNA preparations from the unstimulated (control) birds fail to support RCP synthesis in the cell-free system which confirms our earlier finding that RCP gene is not constitutively expressed, but induced *de novo* in response to the hormonal stimuli (Murthy and Adiga, 1978).

It is significant that assayable RCP-mRNA activity becomes clearly manifest in both the tissues during primary stimulation with estrogen, but not with progesterone and this activity declines to undetectable levels upon hormone withdrawal (figure 2). It is also clear (table 1) that equivalent amounts of poly A⁺-RNA from the oviducts of estrogen-primed birds given either estrogen or progesterone during secondary stimulation, support RCP synthesis 2-fold higher than mRNAs extracted from the tissues during primary stimulation. This clearly confirms the results obtained with the measurement of the oviduct content of RCP by RIA (Durga Kumari and Adiga, 1986) which not only showed the equipotency of the two steroids in terms of stimulating RCP synthesis/unit wt. of the tissue, but also correlated the magnitude of the amplified response elicited during secondary stimulation with estrogen (Murthy and Adiga, 1978). It is noteworthy that progesterone administration either during primary or secondary stimulation did not elicit any detectable RCP mRNA activity in the liver which is in conformity with the total lack of inductive response as reflected by undetectable plasma levels of RCP. It is also pertinent to mention at this juncture that earlier investigations with the chicken oviduct from hormone-withdrawn birds did reveal the presence, though in vastly reduced amounts of mRNAs specific to both conalbumin and ovalbumin (McKnight and Palmiter, 1979) (as measured by cDNA hybridization technique) but were inactive, since they were not associated with the polysomes. But the present study employing a cell-free translation system failed to reveal detectable amount of RCP-mRNA activity in both the liver and the oviduct under conditions of hormone withdrawal (figures 1 and 2). Most probably, this may be related to the fact that RCP represents a much smaller proportion (1-2 %) of the total protein synthesized both in the oviduct and the liver (Durga Kumari and Adiga, 1986) than conalbumin (10%) or ovalbumin (50-60%). Alternatively, mRNA translation assay is not as sensitive as the cDNA-hybridization technique earlier employed to quantitate ovalbumin and conalbumin mRNAs (McKnight and Palmiter, 1979).

It may however be emphasized that the accumulation of RCP-mRNA, encountered during the different hormonal stimulations, may reflect both the enhanced transcription of RCP gene and significant cytoplasmic stabilization as has been observed earlier during vitellogenin induction in the avian (Gruber *et al.*, 1976) and amphibian liver (Brock and Shapiro, 1983) as well as during egg white protein production by the chicken oviduct (McKnight and Palmiter, 1979). The relative contributions of these two phenomena to the overall accumulation of mRNA activity are unknown at present and await further study.

The approach of mRNA quantification using the heterologous cell-free translation system (with no apparent post-translational modification) also afforded an opportunity to compare the nature of the primary translation product of RCP mRNAs from the two avian tissues. The finding that there is no gross numerical or size differences in the immunoprecipitable-labelled protein recovered, supports the earlier premise (Farrell *et al.*, 1966) that a single structural gene encodes this specific protein in two different cell types. Similar conclusions have been arrived at regarding translational and transcriptional products of transferrin gene in the two avian tissues (McKnight *et al.*, 1980). It would appear therefore, that tissue specific differential hormonal regulation of RCP gene expression may not be attributable to the recruitment of different copies of the same gene family as has been demonstrated in the case of genes coding for MUP proteins (Shaw *et al.*, 1983) and α_2 μ globulin (Laperche *et al.*, 1983) in murine liver and submaxillary glands.

The information regarding the primary translational product of RCP has some relevance in the context of the proposed secondary structure of RCP. It was hypothesized earlier (Clagett, 1971) that like insulin, RCP consists of two unequal subunits held covalently by two disulphide bonds and hence its biosynthesis might involve a precursor (pro-RCP?) which in turn may be processed proteolytically to native RCP on lines demonstrated for proinsulin \rightarrow insulin conversion (Steiner and Oyer, 1967). The above translation data (figure 1) no doubt reveal that RCP synthesized in the rabbit reticulocyte lysate has a larger (M_r 38,000) size, which clearly distinguishes it from the native RCP. While this accords with the postulates of Clagett (1971) regarding involvement of a precursor in RCP synthesis, it is rather doubtful whether further processing of this slightly larger translation product proceeds on the lines suggested for insulin biosynthesis. Infact, the data recorded here seem to favour the view that the primary translation product of RCP represents pre-RCP rather than pre-pro-RCP (based on the nomenclature adopted for intermediates in insulin biosynthesis), since on incubation with stripped microsomal membrane preparation, it could be readily converted to an entity with a size comparable to native RCP (figure 1) without the carbohydrate rather than to a molecule with an expected size intermediate between the two, as has been observed during insulin processing. In other words, the processing of the primary translation product on incubation with the 'stripped' microsomal membranes follows Blobel hypothesis and is more akin to the cleaving of the 'signal peptide' from the new synthesized secretory proteins (Blobel and Dobberstein, 1975). It may also be emphasized that our repeated attempts to separate the two putative subunits of RCP by SDS-PAGE under reducing conditions have borne negative results (Murthy *et al.*, 1976). Thus bulk of our current evidence seem to suggest that this specific protein is biosynthesized as 'pre-RCP' but not as 'pre-pro-RCP' unlike as envisaged earlier (Clagett, 1971). In line with these observation is the recent finding that both chicken egg yolk and white RCP are single chain polypeptides with identical amino sequence and hence are products of a single structural gene (Narioka *et al.*, 1985).

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