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ISOLATION AND CHARACTERIZATION OF RAT INTESTINAL POLYRIBOSOMES AND RNA DURING ABSORPTION OF FAT. INCREASED TRANSLATION IN VITRO OF APO-AIV

M. PESSAH, C. SALVAT, N. AMIT AND R. INFANTE

Unité de Recherches d'Hépatologie (U.9)
Institut National de la Santé et de la Recherche Médicale
75571 PARIS CEDEX 12 FRANCE

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Polyribosomes were isolated from the intestinal mucosa of fasted and fat-fed rats in the presence of ribonuclease inhibitors. Polyribosomes from fat-fed rats were larger and more efficient in incorporating radioactive aminoacids into proteins than those from fasted rats.
Total RNA prepared by guanidine-HCl extraction, from the intestine of fasting and fat-fed rats was translated in vitro in a mRNA-dependent rabbit reticulocyte lysate system in the presence of ^{35}S-methionine. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and autoradiography of the synthesized peptides showed a relative increase in the radioactivity of some peptides of RNA from fat-fed animals and particularly a two fold increase in preapo-AIV indicating that the intestinal apo-AIV synthesis is under transcriptional regulation by the metabolic processes involved in fat transport, that is, triglyceride-rich lipoprotein production.

The intestine has been shown to be a major synthetic site of several apoproteins and an important source of plasma lipoproteins. During intestinal lipid absorption, triglyceride-rich lipoproteins (chylomicrons and VLDL) are formed and secreted into the lymphatic circulation. Active synthesis of intestinal apoproteins is necessary for normal lipoprotein formation. Protein synthesis inhibitors have been shown to cause, in animals, an accumulation of triglyceride droplets within the intestinal cell and a decreased lymphatic secretion of chylomicrons (1). In humans, lack of apoprotein-B synthesis in the genetic disease abetalipoproteinemia prevents lipoprotein formation and fat transport in the intestinal cells

ABBREVIATIONS

VLDL : very low density lipoproteins ; HDL : high density lipoproteins ; TCA : trichloroacetic acid ; TKM buffer : 50 mM Tris-HCl pH 7.5, 25 mM KCl, 5 mM $MgCl_2$; RNase : ribonuclease ; DOC : Deoxycholic acid ; SDS : sodium dodecyl sulfate ; NP_{40} : nonidet P_{40}.

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(2). Different studies in lymphatic duct cannulated rats have shown an increase in apo-B, apo-AI, apo-AIV secretion into the lymphatic circulation during triglyceride absorption (3, 4, 5). Estimates in the rat indicate that the intestine may contribute up to 60% of the daily synthesis of apo-AIV (6). Although apo-AIV is one of the major apolipoprotein of mesenteric lymph chylomicrons and plasma HDL, its function is unknown. It has a molecular weight of 46 000 daltons, an aminoacid composition and immunologic properties which clearly distinguish it from apo-B and apo-AI, the other major apolipoproteins synthesized by the enterocyte during fat absorption.

Many questions remain unanswered concerning the molecular regulation of apoprotein synthesis, lipoprotein formation and processing in intestinal epithelial cells.

The present report describes the isolation and properties of polyribosomes from rat intestinal mucosa, the isolation and translation in a cell free system of the intestinal mRNA from fasted and fat-fed rats, and the comparative synthesis in these two cases of the pre-apoprotein-AIV.

MATERIALS AND METHODS

Materials and animals

Guanidine hydrochloride (Gdn-HCl) and cesium chloride were purchased from Bethesda Research Laboratories, dithiothreitol and ethylene diamine tetraacetic acid (EDTA) were purchased from Sigma Chemicals Co (St-Louis, Md) ^{35}S-methionine (1330 Ci/mmole) and ^{14}C-leucine (318 mCi/mmole) were purchased from the C.E.A. (Saclay, France).

Wistar rats weighing 180-200 g were maintained on a standard diet until 18 hrs before the experiment. Fasted rats had free access to water; fat-fed rats received by gastric tube 2 ml of thick fresh cream containing more than 30 per cent triglyceride (about 1 mmole fatty acids/ml cream) three hours before sacrifice.

Preparation of rat intestinal polyribosomes

Rats were anesthetized with diethylether and the jejunal and ileal segments of the small intestine were quickly removed and flushed with 0.13 M NaCl solution containing 100 µg heparin/ml at 4° C; mucosal cells were isolated using the technique of WEISER (7). Washed cells were suspended in rat liver cell sap (5 ml/g of cells) which is a potent inhibitor of ribonucleases, homogenized at 4° C and polysomes were isolated as described by EMTAGE (8). The polyribosome preparation had an E_{260}/E_{280} ratio of 1.70-1.80.

Density gradient analysis of polyribosomes

Rat intestinal polyribosomes were layered over a 12.5 ml linear density gradient of 15-45 % sucrose in TKM buffer and centrifuged for 100 min. at 34 000 rpm in a L3-40 Spinco ultracentrifuge (Beckman, Palo Alto. Cal.) with a SW41 rotor at 4° C.

Polyribosome patterns were obtained by measuring E_{254} in an automatic absorbance fluorometer monitor Isco Model UA-5 ; one mg of polyribosomal RNA gave an absorbance of 20 E_{260} units.

Polypeptide synthesis in vitro

Reaction mixtures containing 1 µmole of ATP, 0.4 µmole of GTP, 7 µmoles of creatine phosphate, 40 µg of creatine phosphokinase, 0.5 µCi of ^{14}C-leucine, 125 µl (2 mg protein) of rat liver cell sap (105.000 x g supernatant) and about 0.3 mg of polyribosomal RNA in a total volume of 1 ml, were incubated at 37° C for up to 60 min. The final ionic concentrations were 100 mM KCl, 4 mM $MgCl_2$, 25 mM Tris HCl pH 7.5 and 1 mM dithiothreitol. Trichloroacetic acid-precipitable radioactivity was measured as described by MANS and NOVELLI (9).

Preparation of rat intestinal RNA

Total cellular RNA was isolated from the small intestinal epithelium using the guanidine-HCl method (10).

In order to avoid ribonucleases activity, all glassware and aqueous solutions used in the preparation were treated with 0.1 % (v/v) diethyl pyrocarbonate and autoclaved at 120° C for 20 min. (10).

Isolation of HDL and of apo-AIV

HDL were isolated from rat plasma by sequential ultracentrifugation (11) and apo-AIV were prepared by column chromatography by modification of tetramethylurea method of KANE (12, 13).

Preparation of antibody

Antiserum against rat apo-AIV was obtained by injecting rabbits in both hind foot pads with 100 µg of apo-AIV emulsified with 1 ml of Freund's adjuvant. Three weeks later the same quantity of protein-adjuvant emulsion was injected subcutaneously at 10 different points along the back. Finally, the rabbits received 1 mg of apoprotein intravenously one week later. Blood was obtained one week after the last injection.

Antiserum specificity was tested by immunodiffusion and immunoelectrophoresis.

Cell free translation system and immunoprecipitation

RNA was translated in a protein-synthesizing system from rabbit reticulocyte lysate (14). Proteins were labelled by including 10 µCi of ^{35}S-methionine in the translation medium.

For immunoprecipitation, the translation medium was made 50 mM NaCl, 20 mM Tris pH 7.5, 0.5 % DOC (w/v), 0.5 % NP40 (w/v) and then 50 µl of Staphylococcus aureus suspension at 100 mg/ml was added in order to precipitate proteins that non-specifically adsorb to S. aureus (IgG Sorb, The Enzyme Center, Boston, MA). The S. aureus has been extensively washed and prepared according to KESSLER (15).

After 30 min., at 4° C, S. aureus was sedimented by low speed centrifugation and discarded. The newly synthesized ^{35}S-labelled apo-AIV was precipitated from the supernatant incubation with rat apo-AIV

antiserum at 4° C overnight. 50 µl of S. aureus (100 mg/ml) was then added and gently shaken at 4° C for 30 min.

After washing 3 times with 0.5 ml of 50 mM NaCl, 20 mM Tris pH 7.5, 0.5 % DOC, 0.5 % NP40, 0.1 % SDS, the immunoprecipitated protein was released from the bacteria by incubation in the mixture : 30 mg DTT, 24 mg Tris base, 0.2 ml 10 % SDS, 0.8 ml 50 % glycerol, 1 ml distilled H_2O at 95° C for 5 min. After centrifugation, the supernatant was analyzed by SDS-PAGE.

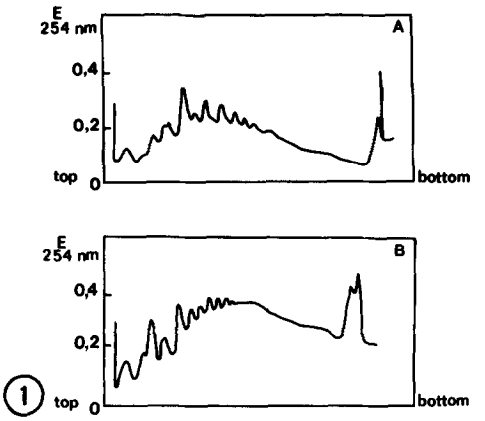
Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE)

Total translation products and immunoprecipitated proteins were electrophoresed on an 8 % polyacrylamide slab-gel using the LAEMMLI buffer systems (16).

Autoradiography of dried slab gels was performed using X-ray films (Kodak x-omat). Standard proteins of known molecular weight, as well as native apo-AIV from rat plasma HDL, were run on the same gel and relative positions of radioactive bands were compared to those of standard proteins.

RESULTS AND DISCUSSION

Hydrolysis of RNA by ribonucleases has always hindered the preparation of polyribosomes or RNA from intestinal cells homogenates. Thus, particular attention was paid to controling endogenous and exogenous ribonuclease activity, all through the experiments. Rats were therefore fasted for 18 h before use, to decrease the secretion of RNase-rich pancreatic juices. Furthermore, the duodenum with the pancreas attached was discarded and mucosal cells were obtained only from jejunal and ileal segments. In addition to using sterile materials and solutions, intestinal mucosa cells were homogenized in rat liver cell-sap, which is known to possess anti-ribonuclease activity. Finally, the high concentration of guanidine hydrochloride used for RNA extraction is also a good inhibitor of RNAse activity. All these factors contribute to obtaining polysomal profiles showing few monosomes or small polysomes and containing a high proportion of heavy polysomes. This distribution fits in well with the native organization of ribosomes in the intestinal epithelial cells as observed by electron microscopy. Intestinal polysome profiles are close to those observed in liver homogenates and are compatible with the high protein synthesis activity in both organs. In the case of fat-fed rats, we could noticed an increase in heavy polysomes compared with fasted rats



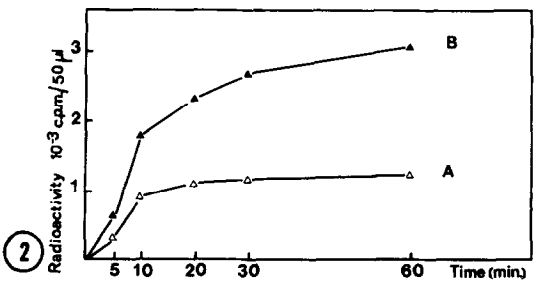


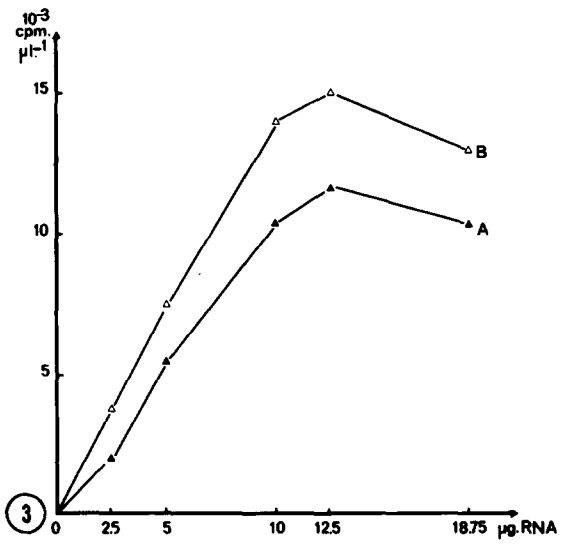
Figure 1 : Sucrose density-gradient analysis of polyribosomes (10 A_{260} units) isolated from the intestine of fasted (A) and fat-fed (B) rats.

Figure 2 : Kinetics of ^{14}C-leucine incorporation by intestinal polyribosomes from fasted (A) and fat-fed (B) rats.

(Fig. 1). These results were confirmed by the fact that intestinal polysomes from fat-fed rats were more efficient in incorporating ^{14}C-leucine into proteins than intestinal polysomes from fasted rats. The kinetics of incorporation of ^{14}C-leucine into TCA-precipitable protein by intestinal polyribosomes is shown in Fig. 2. Incorporation of amino acids, under optimum conditions, was linear for the first 10 min. and then reached a plateau after 30 min. We noticed an increase in ^{14}C-leucine incorporation into proteins in the case of intestinal polyribosomes from fat-fed rats. It seems of interest to precise to what this increase in synthesis corresponds.

RNA was isolated, as described, from the small intestinal epithelium of fat-fed and fasted rats and was further translated in the presence of ^{35}S-methionine in a mRNA-dependent protein synthesizing system derived from rabbit reticulocyte lysates. Initial experiments were carried out in order to optimize the system. Maximal protein synthesis was obtained by addition into the assay of 12.5 µg RNA.

Under optimal conditions, using equivalent amounts of intestinal RNA from fasted or fat-fed rats, the latter had a higher efficiency in ^{35}S-methionine incorporation into proteins (Fig. 3) suggesting a higher mRNA content in fat-absorbing intestinal cells.



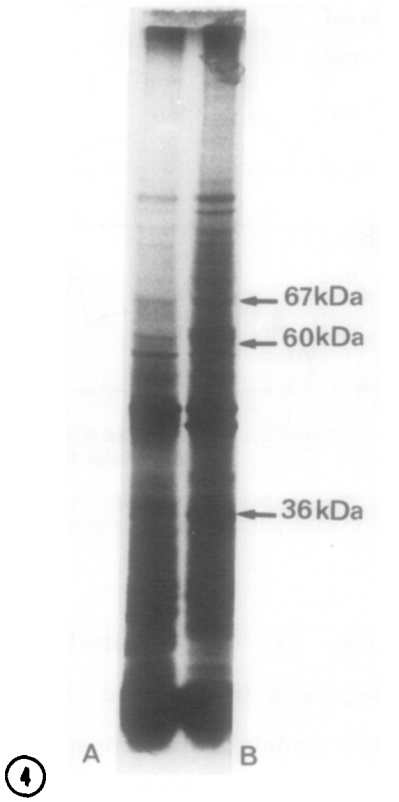


Figure 3 : Protein synthesis in the rabbit reticulocyte lysate system supplemented with various concentrations of RNA isolated from the intestine of fasted (A) and fat-fed (B) rats. Means of three determinations.

Figure 4 : Autoradiography of PAGE slabs of translation products of intestinal RNA from fasted (A) and fat-fed (B) rats.

Electrophoretic analysis of the total translation products on SDS polyacrylamide gels, showed that intestinal mRNA directed the synthesis of many products ranging in molecular weight from 14 000 to 94 000 daltons with differences in intensity of some bands between the two RNA preparations (Fig. 4). Although these peptides have not been identified it can be hypothesized that stimulation of their synthesis could be related to the turnover of lipid synthesizing enzymes and membrane proteins.

Immunoprecipitation of the labelled polypeptides with apo-AIV antiserum revealed a single protein band in the two cases (Fig. 5). It had an apparent molecular weight of 48 000 daltons, which is 2 000 daltons greater than mature apo-AIV from plasma HDL, (17). Our findings are in agreement with a previous report (18) showing that the primary translation

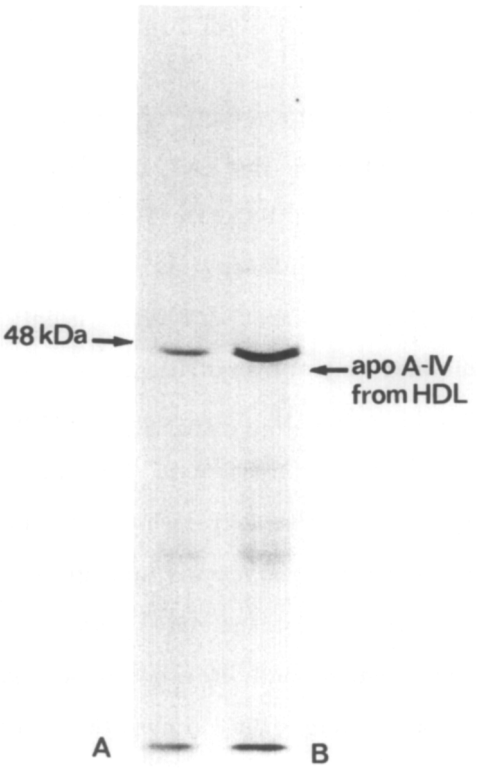


Figure 5 : Autoradiography of ^{35}S-methionine-labelled preapo-AIV synthesized in a cell-free system by intestinal mRNA from fasted (A) and fed-rats (B). The protein was immunoprecipitated by a rat anti-apo-AIV antiserum. The position of migration of plasma HDL-associated apo-AIV is indicated.

product of intestinal mRNA apo-AIV is a pre-protein containing an NH_2 -terminal extension of 20 amino acids.

Comparison of the immunoprecipitated proteins from fasted and fat-fed rats showed an important increase in synthesized apo-AIV in fat-absorbing intestinal cells (Fig. 5).

Calculations of mRNA abundance based on translational data are inaccurate because of incomplete recovery of labelled polypeptides and differences in amino acid composition.

However, for comparative purposes we have calculated the abundance of apo-AIV mRNA in epithelial RNA isolated from the small intestine of fasted and fat-fed rats, based on recovery of label from immunoprecipitable proteins purified from SDS polyacrylamide gels. Apo-AIV mRNA of fasted

and fat-fed rats comprised 0.7 % and 1.3 % of translatable RNA, respectively.

In contrast with the stimulatory effect of fat absorption on apo-AIV synthesis, it has been reported that fat feeding did not result in an increase in enterocyte apo-AI mRNA (5). However, a significant apo-AI secretion was shown in glucose infused rats suggesting that the synthesis of apo-AI and apo-AIV, two apolipoproteins mainly produced in the intestine are under distinct control by nutritional factors.

The present results indicates that de novo synthesis of apo-AIV is necessary for triglyceride-rich lipoprotein formation in the course of intestinal fat absorption in close agreement with in vivo experiments showing a relationship between triglyceride and apo-AIV secretion into the intestinal lymph (5). Indeed more translatable mRNA is present both in the total polyribosome preparation and in the RNA extract from fat-fed intestine, strongly supporting the assumption that apoprotein-AIV synthesis in the intestine is under transcriptional regulation by fatty acids. Further studies are necessary to elucidate whether the synthesis of other important constituents of chylomicrons and VLDL (apo-B, apo-AI, apo-C) is under control by nutritional factors, since increasing evidence indicates that excessive lipoprotein production by intestine could be an important etiological factor in some human hyperlipemias. Further work is required to determine the molecular mechanisms involved in the induction of specific mRNA synthesis by the flux of fats, carbohydrates, etc, through intestinal epithelial cells.

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