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Cloning of a Complementary Deoxyribonucleic Acid Encoding a Portion of Rat Intestinal Preapolipoprotein AIV Messenger Ribonucleic Acid†

Jeffrey I. Gordon,* Dennis P. Smith, David H. Alpers, and Arnold W. Strauss

ABSTRACT: Apolipoprotein AIV is one of the principal apolipoproteins synthesized by the rat small intestine. We have cloned a cDNA encoding a portion of preapolipoprotein AIV mRNA. A kinetically fractionated cDNA probe highly enriched for the abundant intestinal mucosal mRNA sequences was used to screen a library of recombinants containing cDNA generated from total intestinal epithelial mRNA. The abundant class of mRNA sequences was defined by hybridization analyses. This frequency class had an aggregate complexity of 5300 nucleotides and represented 25% of accumulated mRNA sequences. The mRNAs comprising this class were identified by in vitro translation and included preapolipoprotein AIV, preproapolipoprotein AI, intestinal fatty acid binding protein, and liver fatty acid binding protein. A cDNA-containing clone derived from preapolipoprotein AIV mRNA was identified among probe-positive recombinants. This cDNA was used to establish that apolipoprotein AIV mRNA has a mass of 550 000 daltons (equivalent to 1780 nucleotides) and represents 0.013% of total cellular RNA in the fasting state. Acute feeding with triglyceride-rich meals resulted in a 2-fold increase in preapolipoprotein AIV mRNA after 4 h.

Apolipoprotein (apo)[1] AIV is one of the major apolipoproteins synthesized by the intestine (Wu & Windmueller, 1978; Imaizumi et al., 1978). Wu and Windmueller used in vivo perfusion with labeled amino acids to estimate that 59% of rat plasma apo AIV was derived from the intestine, with the rest being synthesized by the liver (Wu & Windmueller, 1979). The physiologic function of apo AIV is not known (Swaney et al., 1977). It is one of the principal plasma apolipoproteins in rats. Although this apoprotein is found in VLDL, HDL, and chylomicrons, at least 50% appears to be unassociated with any of the plasma lipoprotein density classes (Fidge, 1980). Nonetheless, using charge shift electrophoresis, Utermann and co-workers have shown that apo AIV binds detergents through hydrophobic interactions in the same fashion that other amphipathic apolipoproteins do (Beisiegel & Utermann, 1979; Helenius & Simons, 1977).

There have been divergent observations on the effects of triacylglycerol feeding on rat intestinal apo AIV biosynthesis. Windmueller and colleagues were unable to show any effect on intestinal apo AIV synthesis when rats were deprived of exogenous or endogenous (biliary) lipid for 16 h to 12 days (Windmueller & Wu, 1981). On the other hand, Krause et al. studied mesenteric lymph apolipoprotein concentrations and transport rates after infusion of lipid into the duodenum and found that transport of all classes of apolipoproteins was increased but only apo AIV displayed an increase in mesenteric concentration (Krause et al., 1981).

Apo AIV is initially synthesized in the intestine as a preprotein. We have recently shown that the early proteolytic processing of apo AIV is different from the other major intestinal apolipoprotein, AI (Gordon et al., 1982a,b). The primary translation product of rat intestinal apo AI mRNA is a preproprotein that contains a highly usual prosegment. This segment does not terminate with a pair of basic amino acids as most propeptides do but rather has a C-terminal Gln-Gln sequence. On the other hand, the initial product of apo AIV mRNA translation contains a 20 amino acid N-terminal extension which behaves in a cotranslational assay system as a prepeptide. No prosegment could be defined. There is a remarkable degree of amino acid sequence homology between the prepeptides of apo AI and AIV, although the N termini of the mature proteins are quite dissimilar.

In order to define apo AIV protein structure and to study regulation of apo AIV gene expression, we needed a pure complementary DNA. We have successfully cloned such a

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[1] Abbreviations: apo, apolipoprotein; HDL, high-density lipoprotein; $NaDodSO_4$, sodium dodecyl sulfate; SSC, NaCl (0.15 M)–sodium citrate (15 mM, pH 7.0); cDNA, complementary deoxyribonucleic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; AMV, avian myeloblastosis virus; Tc^r, tetracycline resistant; Ap^s, ampicillin sensitive.

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cDNA and measured apo AIV mRNA accumulation in the rat small intestinal epithelium before and after fat feeding.

Materials and Methods

Animals. Male Sprague-Dawley rats (250 g) were maintained on a standard rat chow diet.

Preparation of RNA. Total cellular RNA was isolated from scraped small intestinal epithelium by extraction with guanidine hydrochloride (Gordon et al., 1982a). Poly(A+) RNA was prepared by oligo[d(T)]–cellulose chromatography (Aviv & Leder, 1972).

Cell-Free Translation. Total cellular RNA was translated in a wheat germ cell-free system by using conditions previously outlined (Gordon et al., 1982a,b). Specific polypeptide synthesis was quantitated by immunoprecipitation with monospecific antibodies generated in rabbits. The methods for obtaining antibodies against apo AI and AIV have been described and the identity of the cell-free products immunoprecipitated by these antibodies confirmed by N-terminal sequence analyses (Gordon et al., 1982a,b). Dr. R. K. Ockner provided antisera against liver and intestinal fatty acid binding proteins. The specificity of all antibody preparations was established by Ouchterlony double immunodiffusion analysis. Methods for immunoprecipitation and quantitation of specific polypeptide synthesis in the cell-free translation system have been detailed elsewhere (Gordon et al., 1982a).

Complexity Analyses. (*A*) *Synthesis of cDNA.* DNA complementary to total intestinal mRNA was synthesized by using the conditions of Monahan (Monahan et al., 1976; King et al., 1979). Fifty-microliter reactions contained total cellular RNA (75 μg), $d(T)_{12-18}$ (0.125 μg), AMV reverse transcriptase (45 units; Life Sciences, Inc., St. Petersburg, FL), [5-^{3}H]dCTP (25 Ci/mmol), and [5,8-$^3H_2$]dGTP (24.9 Ci/mmol). The specific activity of [^{3}H]cDNA synthesized was 83 dpm/pg. Its modal length was 700 nucleotides as determined by methylmercury–agarose gel electrophoresis (Bailey & Davidson, 1976).

(*B*) *RNA Excess Hybridizations.* Hybridizations were performed at 68 °C in 0.6 M NaCl (Deeley et al., 1977). Two-microliter reactions contained 5000 dpm of cDNA (60 pg) and 0.1–20 μg of total cellular RNA. This represented a minimum 1600-fold weight excess of total RNA and a calculated 32-fold weight excess of poly(A+) RNA. The fraction of cDNA in hybrid form was assayed by S1 nuclease digestion (Deeley et al., 1977). The results were evaluated by a computer program for least-squares analysis of hybridization data developed by Pearson et al. (1977).

(*C*) *Isolation of Abundant Class cDNA.* Preparative reactions (200 μL) containing 500 μg of total RNA and 0.5 μg of labeled cDNA were hybridized to R_0t = 5.0 mol s L^{-1}. Hybridized sequences were separated from unhybridized sequences by S1 nuclease digestion. Residual S1 nuclease was removed by incubation with proteinase K followed by phenol–chloroform extraction (Gordon et al., 1978). After removal of RNA by alkaline hydrolysis (Deeley et al., 1977), the cDNA was passed through a Sephadex G-75 column and the excluded fraction used for subsequent hybridization analyses (King et al., 1979).

Cloning Procedures. (*A*) *Synthesis and Cloning of Double-Stranded cDNA.* Double-stranded cDNA was prepared from total cellular small intestinal epithelial RNA by using procedures described earlier (Gordon et al., 1978). S1-digested double-stranded cDNA was inserted into the *Pst*1 site of plasmid pBR 322 by using the G-C tailing procedure (Bolivar et al., 1977). At the time these experiments were done, chimeric plasmids were used to transform *Escherichia coli* strain χ1776 or HB101 under P2 level of containment. This was in accordance with the prevailing NIH Guidelines for Recombinant DNA research. A library of 2000 Tc^r Ap^s recombinants was generated from unfractionated intestinal epithelial RNA. Transformants containing DNA complementary to abundant mRNAs were identified by colony hybridization with a kinetically fractionated cDNA probe. cDNA, highly enriched for abundant class sequences, was prepared as described above, except that [^{32}P]αdCTP was used as the labeled deoxynucleotide. Twenty-milliliter hybridization mixtures contained 4×10^6 dpm of abundant class [^{32}P]cDNA (specific activity 120 dpm/pg). Further details concerning colony hybridization conditions and washing stringencies can be found in Tiemeier et al. (1978).

(*B*) *Filter Selection.* Plasmid DNA was isolated from abundant cDNA probe-positive clones by using a rapid alkaline extraction procedure (Birnboim & Doly, 1979). Plasmid DNA (20–30 μg) was digested with *Eco*RI, denatured and bound to nitrocellulose filters by using a method outlined by Boothby and co-workers (Boothby et al., 1980). Hybridization reaction mixtures (200 μL) contained total cellular RNA (500 μg), deionized formamide (65% v/v), NaCl (0.4 M), Pipes (20 mM, pH 6.4), $NaDodSO_4$ (0.2%), and half of the plasmid–nitrocellulose filter. RNA was denatured just prior to hybridization by preincubating in 130 μL of 99% formamide at 50 °C for 15 min. The complete hybridization mixture was then incubated at 50 °C for 4 h. Filters were subsequently washed with 10 × 1-mL exchanges (1 min/exchange) of 1 × SSC/0.2% $NaDodSO_4$ preheated to 55 °C. This was followed by two higher stringency washes in 0.2 × SSC at 55 °C. Bound mRNA was eluted by boiling the filters for 2 min in 300 μL of 1 mM Na_2EDTA, pH 7.5. This RNA was precipitated with ethanol in the presence of 10 μg of wheat germ tRNA. Typically, half of the recovered mRNA was added to a 50-μL wheat germ cell-free translation reaction mixture containing [^{35}S]methionine. After a 2-h incubation, 10-μL aliquots were withdrawn for $NaDodSO_4$–polyacrylamide gel electrophoresis.

(*C*) *Hybridization of Cloned Preapo AIV cDNA to Northern Blots.* Total poly(A+) small intestinal epithelial RNA (5 μg) was separated by methylmercury–agarose gel electrophoresis and transferred to diazobenzyloxymethyl-cellulose paper (Alwine et al., 1977). DNA encoding a portion of apo AIV mRNA was purified from recombinant plasmids after *Pst*1 digestion, polyacrylamide gel electrophoresis, and electroelution. The isolated fragment (approximately 100 ng) was labeled by nick translation (Maniatis et al., 1975) to a specific activity of 50 cpm/pg by using [^{32}P]αdATP and hybridized to the RNA blot in the presence of dextran sulfate (Alwine et al., 1979).

(*D*) *RNA Excess Hybridization of Cloned AIV cDNA.* A *Pst*1 fragment (approximately 200 ng) containing preapo AIV cDNA was purified and labeled by nick translation exactly as described above. Sense strand DNA (by this we mean the strand of DNA complementary to the corresponding mRNA) was then isolated by using a procedure developed by Wiskocil and co-workers (Wiskocil et al., 1980). Briefly, the labeled DNA fragment was hybridized to total poly(A+) RNA. We chose an amount of RNA that contained a 5-fold sequence excess of apo AIV mRNA. This calculation was based on cell-free translation data. High concentrations of formamide (80%) were employed to minimize DNA–DNA reassociation (Casey & Davidson, 1977). Unhybridized, antisense strand DNA was digested with S1 nuclease and the RNA hydrolyzed by alkali. Residual antisense strand was removed by per-

mitting the DNA to reanneal (in 0.6 M NaCl, 10 mM Hepes, pH 7.0, and 1 mM Na_2EDTA). The reaction mixture was then chromatographed on a hydroxylapatite column equilibrated with sodium phosphate (0.12 M, pH 7.0) and heated to 60 °C. The unbound fraction was collected. RNA excess hybridizations were performed in 2-μL reaction mixtures (Deeley et al., 1977) containing 20 pg of purified sense strand DNA (50 cpm/pg) and 10–20 μg of total cellular intestinal epithelial RNA.

(*E*) *Fat Feeding of Rats.* Animals previously maintained on a standard rat chow diet were fasted overnight. They were then fed 1 mL of corn oil by gastric intubation while fully conscious. At selected time points after feeding, animals were sacrificed (three animals per time point), epithelium was scraped from the small intestine, the scrapings were pooled, and RNA was extracted by using guanidine hydrochloride. The concentration of preapo AIV mRNA in these RNA preparations was assayed by using the cell-free translation system. Exactly the same amount of RNA from each time point (30 μg) was added to separate translation reaction mixtures (final volume 100 μL) containing [^{3}H]leucine (specific activity 140 Ci/mmol). The product of preapo AIV mRNA translation was isolated by immunoprecipitation, purified by $NaDodSO_4$–polyacrylamide gel electrophoresis, and recovered by extracting gel slices (Gordon et al., 1982a). The amount of radioactivity incorporated into preapo AIV was expressed as a fraction of total polypeptide synthesis. Translations were repeated on three separate occasions for all time points and the data analyzed by pairwise comparisons of least-squares means (Helwig & Council, 1979).

Results

Scheme for Cloning Rat Intestinal Apo AIV cDNA. In developing a strategy for cloning preapo cDNA from rat intestine, several factors had to be considered. We had previously used the wheat germ cell-free system to determine that preapo AIV mRNA was one of the most abundant mRNA species in the small intestinal epithelium, even though it comprised only 0.6% of the mRNA (Gordon et al., 1982a). Assuming that the frequency of a specific cloned cDNA species in a library of recombinants reflects the frequency of its mRNA in an RNA population, we would only have a 50% chance of finding *one* clone containing apo AIV cDNA if we screened 115 recombinants generated from unfractionated intestinal epithelial mRNA by filter selection (Ricciardi et al., 1979), hybrid arrest (Paterson et al., 1977), or DNA sequencing. On the other hand, partial purification of apo AIV mRNA prior to cloning did not seem feasible in view of its unremarkable size and the high level of extracellular ribonuclease present in intestine which limits recovery of intact polysomes (Alpers & Isselbacher, 1967) for immunoprecipitation (MacGillivray et al., 1980). We therefore felt that the easiest method of obtaining a preapo AIV cDNA clone would be to take advantage of the apparent *relative* abundance of this mRNA in intestine. A library of unfractionated cDNA containing clones could be screened with a cDNA probe enriched for these *abundant* class sequences. Recombinant clones reacting with this probe could be further characterized by using the methods outlined above. An analogous approach had been used earlier to successfully clone albumin mRNA from a partially purified avian liver RNA preparation (Gordon et al., 1978).

Analysis of Abundant mRNA Sequences in Small Intestinal Epithelium by Cell-Free Translation. Figure 1 shows the [^{35}S]methionine-labeled polypeptides synthesized when total cellular epithelial RNA was added to the wheat germ cell-free

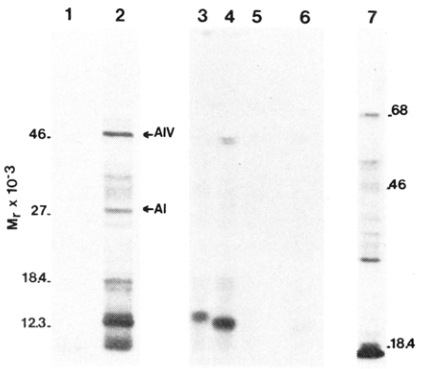

FIGURE 1: Cell-free translation of intestinal epithelial RNA. Total cellular intestinal epithelial RNA was translated in a wheat germ cell-free system. [^{3}H]Leucine-labeled polypeptides were separated by electrophoresis in a 12.5% polyacrylamide gel containing $NaDodSO_4$ (0.1%) (Laemmli, 1970). Gels were fluorographed and exposed at −70 °C. (Lane 1) Endogenous translation; (lane 2) translation of total intestinal RNA (15 μg/50 μL reaction); (lane 3) polypeptides immunoprecipitated from intestinal RNA translation by anti-intestinal fatty acid binding protein antibody; (lane 4) polypeptides immunoprecipitated from intestinal RNA translation by anti-liver fatty acid binding protein antibody; (lane 5) polypeptides immunoprecipitated from a translation of liver RNA by anti-intestinal fatty acid binding protein antibody; (lane 6) polypeptides immunoprecipitated from the liver translation reaction mixture by anti-liver fatty acid binding protein antibody; (lane 7) translation of total rat liver RNA (15 μg/50 μL reaction). The arrows indicate the positions of preproapo AI and preapo AIV (Gordon et al., 1982a,b). Protein standards used included plasma HDL associated apo AIV (46 kdaltons) and apo AI (27 kdaltons), lactoglobulin (18.4 kdaltons), and cytochrome *c* (12.3 kdaltons).

system. Four dominant bands were seen. Two of these bands have been identified by immunological methods and by N-terminal sequence analyses as apolipoproteins AI and AIV (Gordon et al., 1982a,b). The two lower molecular weight polypeptides made in abundance by the cell-free system have now been identified by immunoprecipitation with monospecific antisera. The M_r 14 500 peptide is intestinal fatty acid binding protein (Figure 1, lane 3), and the M_r 12 500 polypeptide is liver fatty acid binding protein (Figure 1, lane 4). As seen in lane 5 of Figure 1, antibody raised against intestinal fatty acid binding protein did not precipitate any of the polypeptides synthesized by a wheat germ cell-free system programmed with rat liver RNA. However, liver fatty acid binding protein was detected among the liver RNA translation products (lane 6, Figure 1), although it was present in lower concentration among liver mRNA sequences than among intestinal mRNAs (compare lane 4 with lane 6).

We calculated the amounts of these two fatty acid binding protein mRNAs by measuring the recovery of labeled amino acids in the primary translation products (Table I). Intestinal fatty acid binding protein mRNA represented 2% and liver fatty acid binding protein 3.3% of small intestinal epithelial mRNA. On the other hand, liver fatty acid binding protein comprised only 0.2% of liver mRNA. Intestinal fatty acid binding protein mRNA was not detectable in liver RNA.

Analysis of Abundant mRNA Sequences by Complexity Analyses. For definition and purification of a cDNA fraction that encoded these abundant mRNA sequences, complexity analyses were performed (Bishop et al., 1974). cDNA transcribed from the total mRNA population was hybridized back to its template under conditions of RNA excess and the kinetics of reassociation monitored. mRNA sequences present in highest frequency will hybridize most rapidly, while lower frequency class species will only form hybrids at higher R_0t values. Figure 2 shows the results of hybridizing cDNA

Table I: Measurement of Intestinal and Liver Fatty Acid Binding Protein mRNA Concentration in Small Intestinal Epithelial and Liver RNA Using the Cell-Free Translation System

RNA[a]	^{3}H-labeled amino acid[b]	binding protein mRNA (% total)[c] liver fatty acid	intestinal fatty acid
intestinal	leucine	3.0 ± 0.24	1.89 ± 0.2
	lysine	3.5 ± 0.42	2.0 ± 0.19
liver	leucine	0.16 ± 0.025	ND[d]
	lysine	0.2 ± 0.034	ND

[a] Total cellular RNA was isolated from both liver and small intestinal epithelium by guanidine hydrochloride extraction and translated in wheat germ lysates at the same concentration (300 μg/mL reaction mixture). [b] Added to cell-free system. [c] mRNA concentration was determined from the recovery of label in the primary translation products of these mRNAs. These polypeptides were isolated by immunoprecipitation and further purified by electrophoresis through $NaDodSO_4$–polyacrylamide gels, and the amount of radioactivity eluted was expressed as a percentage of total protein synthesis (Gordon et al., 1982a). [d] ND, none detected.

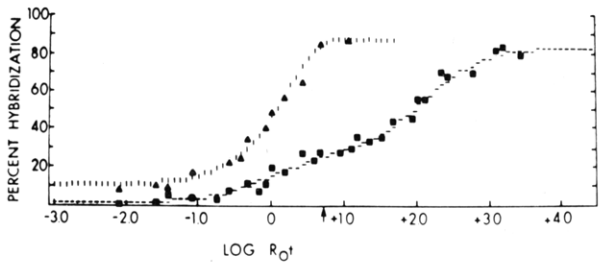

FIGURE 2: cDNA complexity analyses of intestinal RNA and isolation of abundant class cDNA. [^{3}H]cDNA (60 pg, 80 dpm/pg) transcribed from total cellular intestinal epithelial RNA was hybridized to 100 ng–20 μg of the RNA by using salt and temperature conditions specificed in the text. R_0t values have not been corrected for ribosomal RNA content or normalized to standard conditions (Britten et al., 1974). The extent of hybridization was assayed with S1 nuclease. Abundant class cDNA was isolated by preparative hybridization to R_0t 5.0 mol s L^{-1} (indicated by the arrow) and purified by using techniques described under Materials and Methods. This cDNA (60 pg) was then rehybridized to total cellular intestinal RNA (0.1–20 μg). The data shown are (■) total intestinal cDNA hybridized to total RNA and (▲) isolated abundant class cDNA to total intestinal RNA. A computer-generated least-squares best fit of the data is indicated by the dashed lines.

transcribed from total cellular small intestinal epithelial RNA back to its template. We transcribed cDNA from total rather than poly(A)-selected RNA, since we did not want to risk perturbing the frequency of a given mRNA species because of variable binding to oligo[d(T)]–cellulose. In addition, by conducting the hybridization analyses with total cellular RNA, we were able to more accurately estimate ribosomal RNA content. Hybridization occurred over a $10^{5.5}$-fold range of R_0t and proceeded to 85% completion. By use of a computer program for least-squares analysis of hybridization data (Pearson et al., 1977), a best-fit curve for the data was constructed. Three abundance classes could be described. The most abundant species were defined by a frequency class which represented 25% of the total intestinal sequences. They hybridized with an observed $R_0t_{1/2}$ of 0.855 mol s L^{-1}. When this observed $R_0t_{1/2}$ was corrected for the presence of ribosomal RNA (98% of the total) and the fractional representation of the abundance class (25%), a corrected $R_0t_{1/2}$ of 4.27×10^{-3} mol s L^{-1} was obtained. Under the salt and temperature conditions used, a pure mRNA species 7000 nucleotides long hybridizes to its cDNA with a $R_0t_{1/2}$ of 5.6×10^{-3} mol s L^{-1} (Deeley et al., 1977). Therefore, the aggregate complexity

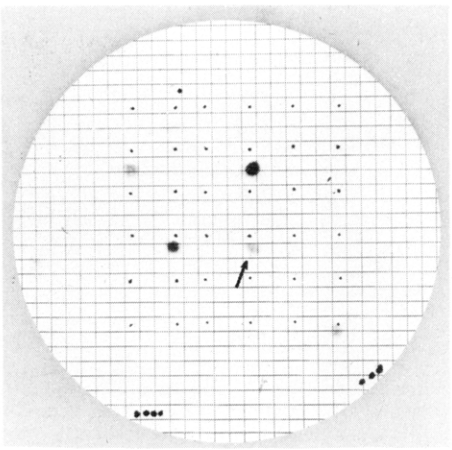

FIGURE 3: Colony hybridization using a kinetically fractionated cDNA probe enriched for abundanat class sequences. [^{32}P]cDNA (transcribed from total intestinal mRNA) was enriched for abundant class sequences by hybridization to a defined R_0t value as described in the text. This cDNA was subsequently used to probe a library of Ap^s Tc^r recombinants generated from unfractionated rat small intestinal epithelial RNA. Shown is an autoradiograph of a filter containing 36 clones probed with this cDNA. Clone 4, a transformant subsequently shown to contain a cDNA encoding a portion of preapo AIV mRNA, is indicated by the arrow.

of this abundance class was calculated by using the relationship (Chan et al., 1980)

$$[(R_0t_{1/2} \text{ of standard})/(R_0t_{1/2} \text{ of abundance class})] = [(\text{complexity of standard})/(\text{complexity of abundance class})]$$

A value of approximately 5300 nucleotides was obtained. This is more than enough sequence complexity to encode the four polypeptides identified in the cell-free translation system.

This most abundant class of intestinal sequences was clearly distinguishable by complexity analyses from an intermediate abundance class. This second class of mRNAs represented 45% of the accumulated small intestinal epithelial sequences and hybridized with a $R_0t_{1/2}$ of 118.4 mol s L^{-1}. The corrected $R_0t_{1/2}$ of 1.12 mol s L^{-1} corresponds to a complexity of 1.4×10^6 nucleotides or enough sequence to represent 700 mRNAs with an average length of 2000 nucleotides. Although a third class representing mRNAs of lowest frequency could be discerned, we were unable to accurately estimate its complexity because of the limited amount of the data in this portion of the reassociation curve.

These data were used to define a way of isolating a cDNA highly enriched for abundant class mRNA sequences. Total intestinal cDNA was hybridized back to its template under conditions of RNA excess to R_0t = 5.0 mol s l^{-1} (indicated by the arrow in Figure 2). This R_0t value was selected so that only abundant class sequences would have hybridized. Unhybridized, less abundant sequences were removed by S1 nuclease digestion. When the isolated abundant class cDNA was rehybridized to total intestinal RNA, the reassociation kinetics depicted by the closed triangles in Figure 2 were observed. The reaction went to 87% completion, obeyed pseudo-first-order reaction kinetics, and had a $R_0t_{1/2}$ virtually identical with that noted for the abundant class sequences during the cDNA complexity analyses.

This kinetically fractionated, ^{32}P-labeled abundant class cDNA probe was used to screen a library of unfractionated cDNA bearing recombinants (Figure 3) which were generated by using techniques described under Materials and Methods. Nineteen percent of 450 Tc^r Ap^s clones reacted with this probe. This number is close to the value predicted from the fractional

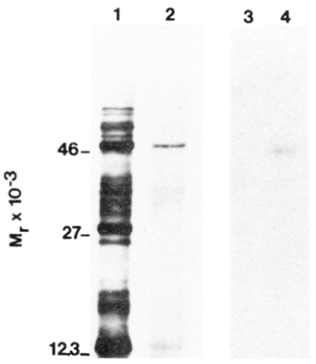

FIGURE 4: Plasmid DNA–nitrocellulose filter selection of apo AIV mRNA. Plasmid DNA (20 μg) purified from clone 4 was used in a filter selection assay as described under Materials and Methods. (Lane 1) Polypeptides synthesized when the wheat germ cell-free system was programmed with 15 μg of total small intestinal epithelial RNA; (lane 2) translation of mRNA bound and eluted from plasmid 4; (lane 3) immunoprecipitation of lane 2 with rabbit anti-rat albumin antibody; (lane 4) immunoprecipitation of lane 2 with anti-apo AIV antibody.

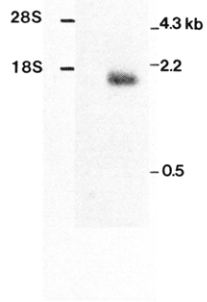

FIGURE 5: Sizing of apo AIV mRNA by hybridization of clone 4 plasmid DNA to total poly(A+) RNA. Total poly(A+) small intestinal epithelial RNA (5 μg) was denatured with methylmercury, electrophoresed through 1.5% agarose gels and transferred to diazobenzyloxymethyl paper as described in the text. The 120-nucleotide fragment generated by *Pst*1 digestion of clone 4 plasmid DNA was purified, labeled by nick translation, and hybridized to the RNA blot. An autoradiograph of the blot is shown. Markers run on parallel lanes of the agarose gel included 18S and 28S ribosomal RNA as well as restriction fragments generated from *Hin*dIII digestion of phage λ DNA.

representation of abundant class species in total intestinal epithelial nRNA (i.e., 25%). Using plasmid DNA purified from 16 abundant class probe-positive clones and the filter selection assay described above, we identified one recombinant that contained preapo AIV cDNA (Figure 4). A single dominant polypeptide was synthesized from RNA bound to clone 4 plasmid DNA (Figure 4, lane 2). The protein was immunoprecipitated by anti-preapo AIV antibody (lane 4), but not by anti-albumin antibody (lane 3). We have already used N-terminal amino acid sequence analyses to show that the polypeptide immunoprecipitated from the cell-free system by this antibody is preapo AIV (Gordon et al., 1982b).

Sizing of Apo AIV mRNA. *Pst*1 digestion of this plasmid revealed a 120-nucleotide insert (data not shown). This was only 10% of the minimum size required to encode preapo AIV. We were able to determine the size of rat intestinal preapo AIV mRNA by hybridizing this 120 base pair insert to Northern blots of total poly(A+) intestinal RNA fractionated on methylmercury-agarose gels. As seen in Figure 5, the probe hybridized to a single mRNA species 1780 nucleotides long which corresponds to a molecular weight of 550 000. Thus,

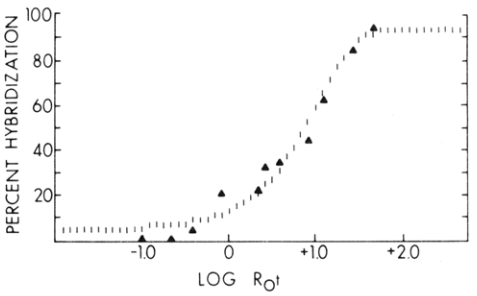

FIGURE 6: RNA excess hybridization of cloned apo AIV cDNA and total cellular intestinal epithelial RNA. Purified ^{32}P-labeled apo AIV sense strand DNA (50 cpm/pg, 20 pg/reaction) was incubated with 10–20 μg of total cellular RNA isolated from the entire length of the small intestine of rats fasted overnight. The extent of hybridization was assayed by S1 nuclease, and the results were analyzed according to Pearson et al. (1977). The sense strand DNA exhibited less than 10% self-association.

preapo AIV mRNA is 460 nucleotides longer than the minimum size necessary to code for the primary translation product.

Quantitation of Preapo AIV mRNA Levels in Small Intestinal Epithelium. As noted above, we had used an in vitro translation assay to determine that preapo AIV mRNA represented 0.6% of total small intestinal epithelial mRNA (Gordon et al., 1982a). For more precise quantitation of preapo AIV mRNA accumulation, we used the cloned cDNA as a probe in RNA excess hybridization analyses. Hybridization reaction mixtures contained unfractionated total cellular intestinal RNA isolated from the entire length of bowel after an overnight fast. Purified sense strand preapo AIV DNA hybridized to small intestinal RNA in a fashion consistent with pseudo-first-order reaction kinetics, the reaction going to completion over two decades of R_0t (Figure 6). The observed $R_0t_{1/2}$ was 10.7 mol s L^{-1}. Under the reaction conditions used, a pure mRNA species 1780 nucleotides long should hybridize to its cDNA with a $R_0t_{1/2}$ of 1.42×10^{-3} mol s L^{-1}. When the theoretical $R_0t_{1/2}$ for the reaction of purified preapo AIV mRNA with its cDNA is compared to the $R_0t_{1/2}$ observed for the reaction of cloned preapo AIV cDNA with fasted total cellular intestinal RNA, the rate of the latter reaction was found to be 7500 times slower. Therefore, preapo AIV mRNA represents $^1/_{7500}$ or 0.013% of total cellular RNA. Assuming that 2% of total cellular RNA is poly(A+), preapo AIV mRNA comprises 0.7% of the mRNA population. The calculation of intestinal preapo AIV mRNA abundance based on mRNA–cDNA hybridization analysis agreed to a remarkable degree with estimates derived from the cell-free translation system.

Effect of Triacylglycerol Absorption on Preapo AIV mRNA Concentration. As a first step toward identifying those factors that regulate intestinal preapo AIV gene expression, we examined the effects of triacylglycerol absorption on intestinal AIV mRNA concentration. For these experiments, only changes resulting from acute feeding were studied. Relative changes in preapo AIV mRNA concentration were first assayed in the cell-free system where equal masses of RNA derived from each time point were translated and the levels of preapo AIV mRNA calculated after immunoprecipitation of the cell-free products. Total cellular RNA isolated from animals sacrificed over an 8-h time period was translated on three separate occasions, and the results were analyzed by pairwise comparison of least-squares means (Table II). There was an increase in preapo AIV mRNA levels from 0.62% in fasted RNA to 1.33% in RNA isolated 4 h after triglyceride feeding. This difference was statistically significant ($p < 0.01$).

Table II: Effect of Triacylglycerol on Translatable Intestinal Epithelial Preapo AIV mRNA Concentration[a]

time after fat feeding (h)	preapo AIV mRNA (% total), least-squares mean
0	0.62
1	0.67
2	0.90
4	1.33[b]
6	1.0[b]
8	0.98[b]

[a] Total cellular intestinal epithelial RNA was isolated at various times after corn oil feeding groups of rats. Preapo AIV mRNA concentration was determined by purifying [^{3}H]leucine-labeled preapo AIV from the in vitro translation mixture (see Materials and Methods) and expressing the amount of label recovered as a percentage of total protein synthesis. All experiments were done in triplicate and the data analyzed by pairwise comparisons of least-squares means. [b] $p < 0.01$ compared to 0 h.

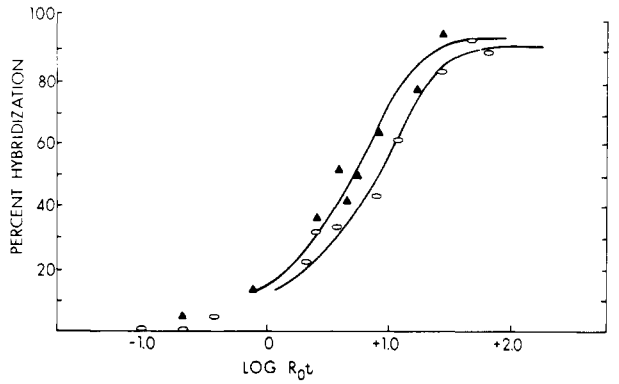

FIGURE 7: Small intestinal preapo AIV mRNA accumulation before and after fat feeding. Hybridization of cloned preapo AIV cDNA to total cellular intestinal epithelial obtained from fasted animals (○) and from animals 4 h after corn oil feeding (▲). Best-fit curves for the data were drawn by using a program for least-squares analyses. The root mean square of the fit for the 0-h data was 0.049 and the 4-h data 0.073.

Eight hours after fat feeding, there was a persistent elevation in apo AIV mRNA (0.98%). During the same time period there was no difference in apo AI mRNA (0.87, 0.81, and 0.89% at 0, 4, and 8 h, respectively). These changes in the amount of apo mRNA were confirmed when purified sense strand apo AIV DNA was hybridized to RNA isolated 4 h after fat feeding. The $R_0t_{1/2}$ of the reaction (6 mol s L^{-1}, Figure 7) indicated an almost 2-fold increase in apo AIV mRNA levels compared to the fasting state ($R_0t_{1/2}$ = 10.7 mol s L^{-1}). Although these differences are difficult to measure when RNA excess hybridization analyses are used, the agreement between the translation and hybridization data supports our conclusion that preapo AIV mRNA accumulation increases after triacylglycerol feeding.

Discussion

As a first step toward studying apo AIV gene expression, we have cloned a cDNA encoding a portion of apo AIV mRNA. The strategy we used for cloning this sequence had to take into account the estimated abundance of the mRNA (0.6–0.7%), its unremarkable size, the difficulty in obtaining polysomes from the ribonuclease-rich environment of the bowel epithelium, and the lack of an appropriate (known) amino acid sequence from which to make an oligodeoxynucleotide primer (Agarwal et al., 1981). Our cloning strategy relied upon screening a library of unfractionated cDNA-containing recombinants with a probe highly enriched for abundant class cDNAs. This cDNA probe was easily generated once the results of the complexity analyses were known. This approach can be used for sequences in any abundance class, especially if there is a change in concentration of a specific sequence as a result of some physiologic or pharmacologic stimulus (King et al., 1979).

Having identified a preapo AIV cDNA among probe-positive recombinants, we were able to accurately determine the steady-state levels of this mRNA in intestinal epithelium using RNA excess hybridization analyses. The measurement of preapo AIV concentration in the mucosa of fasted animals [0.013% of total cellular RNA or 0.7% of poly(A+) RNA] represents an average of its distribution along the entire length of small bowel. Large differences in concentration among segments of bowel might remain undetected. Moreover, differences in gene expression existing between populations of immature enterocytes in the crypt and more mature forms that have migrated up the villus would also be missed. These factors must be considered not only when interpreting the hybridization data but also when considering our estimates of the concentrations of the other abundant intestinal epithelial mRNA sequences.

Our use of the cell-free translation system to assay the effects of triacylglycerol absorption on preapo AIV mRNA accumulation represents a direct approach to studying those factors which regulate apo AIV biosynthesis in vivo. Measurements of apolipoprotein levels within the enterocyte have been particularly difficult to interpret because of contamination from the lipoproteins in the submucosa (Schonfeld et al., 1980). The finding of a 2-fold increase in preapo AIV mRNA levels 4 h after acute fat feeding without an associated change in preproapo AI mRNA contrasts with the results reported by Windmueller & Wu (1981). They used in vivo regional perfusion with labeled amino acids and assayed changes in apolipoprotein synthesis by changes in the incorporation of label into plasma lipoprotein associated apolipoproteins. Their data suggested that the intestine synthesized 50% of plasma apo AI and AIV whether or not fat was withheld for 16–20 h. Our data show that acute triacylglycerol feeding produces an increase in apo AIV synthesis, as measured by mRNA levels. It is possible that the rapidity of this response and its magnitude would not have been detected by the methods used by Wu and Windmueller. Our findings, however, do agree with the observations of Krause and co-workers (Krause et al., 1981). They found that after fat feeding rats, the rate of transport of several apolipoproteins from the intestine into mesenteric lymph increased, with the apo IV increase (400%) exceeding that of all other apoproteins including apo AI (86%). Our data are also consistent with observations reported by Green and colleagues (Green et al., 1980). They measured intracellular apo AIV levels in human intestinal biopsies obtained from subjects who were fasted or who had been given corn oil and found a marked increase in immunofluorescence after lipid feeding. Plasma apo AIV levels also increased (by 20%) after corn oil ingestion. In addition, apo AIV was found to be a major apolipoprotein species in intestinal triglyceride-rich lipoproteins, representing approximately 10% of chylomicron-associated and 30% of intestinal VLDL-associated apolipoproteins. Corn oil ingestion by a patient with a mesenteric lymphatic-urinary fistula resulted in coincident increase in urinary triglyceride and apo AIV excretion. All these data suggest that apo AIV may play an important role in the formation, transport, and/or metabolism of intestinal triglyceride-rich lipoproteins.

The importance of the intestinal epithelium in fat metabolism is further emphasized by our characterization of the four most translationally active mRNAs. Two have an apparent role in the transport of fat from the bowel lumen to the mesenteric lymph (apo AI and apo AIV), and two may be involved in fatty acid transport and cholesterol synthesis (the fatty acid binding proteins). Intestinal fatty acid binding protein (Ockner & Manning, 1974, 1976) binds long chain fatty acids, having higher affinity for unsaturated as opposed to saturated fatty acids. It may act as an intracellular transport protein or have a role in the intracellular utilization of fatty acids. Liver fatty acid binding protein (Ockner et al., 1982) has an amino acid composition that distinguishes it from intestinal fatty acid binding protein. The p*I* (7.0) and amino acid composition of this liver fatty acid binding protein are remarkably similar to proteins previously isolated by others and designated as Z-protein (Ketterer et al., 1976) and DEAE-sterol carrier protein (Dempsey et al., 1981). Another set of liver fatty acid binding proteins [SCP2 (Noland et al., 1980), CM2 (Bloj et al., 1978), band D (Billheimer & Gaylor, 1980), and DEAE peak I (Dempsey et al., 1981)] differ in p*I* (8.6–9.0) and amino acid composition. Although both groups of proteins are known to bind a variety of ligands and affect several enzymes involved in cholesterol biosynthesis, their physiologic functions are not proved.

Since the mRNAs that accumulate to highest concentration within the intestinal mucosa are involved in the absorption and transport of fat, it will be interesting to note whether the expression of their genes is coordinated within the enterocyte. Furthermore, since apo AI, AIV, and liver fatty acid binding protein are synthesized in both liver and intestine, the question as to whether regulation of specific gene expression in these two tissues is coordinated remains open.

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Maturation of Nucleosomal and Nonnucleosomal Components of Nascent Chromatin: Differential Requirements for Concurrent Protein Synthesis†

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ABSTRACT: The DNA of newly replicated chromatin is comprised of two components, distinguishable by their solubility characteristics and requirements for maturation. One of these components possesses core histones, typical nucleosomal structure, a nuclease-resistant core containing 146 base pairs (bp) of new DNA, and all the nucleosomal species found in bulk chromatin (due to bound histone H1 and high mobility group proteins). In addition, this class of nascent chromatin exhibits a shortened repeat length of ~165 bp, as opposed to the 188-bp repeat of bulk chromatin. Within 10 min of DNA synthesis, the spacing of mature chromatin is established; the spacing maturation can occur in the absence of protein synthesis. The second class of nascent DNA is distinguished from the nucleosomal component by its insolubility, lack of discernible nucleosomal organization, and dependence on protein synthesis to attain typical subunit structure. This unassembled component is not free DNA, as demonstrated by its intermediate resistance to nucleolytic degradation. The structural properties and maturation requirements of this material suggest that it is the site of de novo nucleosome assembly.

During the replication of nuclear DNA, the structure of chromatin is transiently altered. Among the effects of such conformational modification is an increased sensitivity of nascent chromatin to nuclease digestion, such that newly replicated chromatin DNA is both cleaved to mononucleosomes and digested to acid solubility more rapidly than bulk chromatin (Seale, 1975, 1976, 1981; Hildebrand & Walters, 1976; Levy & Jakob, 1978; Schlaeger & Klempnauer, 1978; Worcel et al., 1978; Klempnauer et al., 1980; Annunziato et al., 1981). Another structural alteration is observed as a shortened repeat length for the nucleosomes on new DNA (Levy & Jakob, 1978; Murphy et al., 1978, 1980; Seale, 1978a, 1981; Galili et al., 1981; Jackson et al., 1981). Lastly, we have recently shown that in the vicinity of the replication fork approximately 40% of the new DNA lacks typical nucleosomal organization (Annunziato et al., 1981). Similar findings have been reported for replicating SV40 minichromosomes (Herman et al., 1979; Cusick et al., 1981).

One useful approach in dissecting the mechanisms of chromatin replication and maturation has been to block concurrent protein synthesis with the inhibitor cycloheximide. DNA replication continues at a reduced rate in the absence of protein synthesis (Weintraub & Holtzer, 1972; Seale & Simpson, 1975), but chromatin DNA so produced is approximately twice as sensitive to digestion to acid solubility with DNases than control chromatin, indicating that approximately half of the new DNA lacks nucleosomes (Weintraub, 1973, 1976; Seale & Simpson, 1975; Seale, 1976, 1978a; Schlaeger & Klempnauer, 1978; Roufa, 1978). In contrast, regularly spaced nucleosomal multimers are observed on the nuclease-resistant fraction of new DNA, as predicted for the segregation of parental histone octamers to the growing daughter DNA molecules (Seale, 1976, 1978a; Weintraub, 1976; Schlaeger & Klempnauer, 1978; Riley & Weintraub, 1979; Seidman et al., 1979). In this paper, we have examined the subunit structure and nucleosomal periodicity of chromatin replicated in either the presence or the absence of protein synthesis, in order to gain an understanding of the processes of chromatin maturation following DNA replication. We provide evidence that the nucleosomal and nonnucleosomal classes of nascent DNA can be distinguished, not only by their structures but also by their differential requirements for protein synthesis for maturation.

Experimental Procedures

Cell Culture and Labeling. HeLa cells were maintained in spinner culture at 37 °C in Eagle's minimal essential medium supplemented with 5% calf serum.

Long-term labeling of cells with [^{14}C]thymidine (50 mCi/mmol, Schwarz/Mann) was performed at 0.01 μCi/mL for one generation (24 h). For pulse-labeling experiments, cells were harvested by centrifugation (250*g*, 1.5 min) when at ~4 × 10^5 cells/mL. Pulse labeling with [*methyl*-^{3}H]thymidine (60 Ci/mmol, New England Nuclear) was performed by concentrating cells 20-fold (0.5-min pulse) or 5-fold (5- to 60-min pulse) in prewarmed whole medium followed by equilibration at 37 °C for 5 min. For 30-s labeling, cells were incubated with radioactive thymidine at 50–60 μCi/mL; for longer labeling periods, thymidine was added at 2.5–6.7 μCi/mL.

For measurement of protein synthesis, cells were prelabeled for 24 h with [^{14}C]thymidine and incubated with [^{3}H]lysine (60 Ci/mmol, New England Nuclear) for the times indicated at 25 μCi/mL, in culture medium depleted 80% in lysine.

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