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Distribution of Apolipoprotein A-I, C-II, C-III, and E mRNA in Fetal Human Tissues. Time-Dependent Induction of Apolipoprotein E mRNA by Cultures of Human Monocyte-Macrophages†

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ABSTRACT: Recently developed molecular probes for human apolipoprotein (apo) genes have been used to study the specificity of human tissue expression of the apo A-I, apo C-II, apo C-III, and apo E genes. We have found that apo E mRNA was present in all tissues examined. On the basis of total RNA concentration the relative abundance of apo E mRNA expressed as a percentage of the liver value is as follows: adrenal gland and macrophages, 74-100%; gonads and kidney, 12-15%; spleen, brain, thymus, ovaries, intestine, and pancreas, 3-9%; heart, 1.5%; stomach, striated muscle, and lung, <1%. The relative concentration of apo E mRNA in cultures of human peripheral blood monocyte-macrophages increases dramatically as a function of time in culture, and after 5 days, it compares to that of liver. The human tissues shown to synthesize apo E mRNA were also examined for their ability to synthesize apo A-I, apo C-II, and apo C-III mRNA. The relative abundance of apo A-I, apo C-III, and apo C-II mRNA expressed as a percentage of the liver value is as follows: apo A-I, intestine, 50%; apo A-I, pancreas and gonads, 12%; apo A-I, kidney, 4%; apo A-I, adrenal, 2.5%; apo A-I, ovaries and heart, 1%; apo A-I, stomach and thymus, <1%; apo C-III, intestine, 62%; apo C-III, pancreas, 7%; apo C-II, intestine, 3%; apo C-II, pancreas, <1%. The knowledge of tissue specificities in the synthesis of apolipoproteins is important for our understanding of the regulation of apolipoproteins and lipoprotein metabolism.

The definition of the sites of apolipoprotein synthesis has been the subject of extensive investigation during the last five years. On the basis of protein quantitation techniques, two organs in mammalian species, the liver and the intestine, were thought to produce most of the plasma apolipoproteins (Windmueller et al., 1973; Marsh, 1976; Hamilton et al., 1976; Glickman & Green, 1977; Green et al., 1978; Schonfeld et al., 1978; Wu & Windmueller, 1979; Windmueller & Wu, 1981). Recently, other tissues have been shown to synthesize some of the apolipoproteins (Blue et al., 1980, 1982, 1983; Basu et al., 1982). The development of probes for the human apolipoprotein genes (Breslow et al., 1982a,b, 1983; Karathanasis et al., 1985; Zannis et al., 1984; Jackson et al., 1984) permits a qualitative and quantitative comparison of the amounts of specific apolipoprotein mRNA present in different tissues. These studies show that multiple human tissues synthesize apo A-I, apo C-II, apo C-III, and apo E mRNA and that the synthesis of some of the mRNAs is tissue specific.

Experimental Procedures

Materials. Radiolabeled nucleotides [α-^{32}P]dATP, [α-^{32}P]dCTP, [α-^{32}P]dGTP, and [α-^{32}P]TTP were purchased from New England Nuclear. The Klenow fragment of DNA polymerase I and the M13 sequencing primer were purchased from New England Biolabs. DNase I was purchased from Sigma, and agarose and Bio-Gel P-60 were obtained from Bio-Rad. Nitrocellulose filters were obtained from Schleicher & Schuell, Inc. Medium 199 was purchased from M. A. Bioproducts.

Cell Cultures. Primary monolayer cultures of adherent human peripheral blood monocytes were established and maintained by using a modification of the method of Einstein et al. (1976). Briefly, peripheral blood leukocytes were isolated from ethylenediaminetetraacetic acid (EDTA) anticoagulated blood of adult human subjects by dextran sedimentation, washed, and incubated overnight at 4 °C to decrease adherence of polymorphonuclear leukocytes and lymphocytes. Mono-

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layers were established by adhering cell suspensions (approximately 9×10^6/mL) in medium 199 with 10% heat-inactivated (2 h, 56 °C) autologous serum to siliconized glass P-100 dishes in three applications. After adherence, monolayers were washed and maintained in medium 199 with 10% heat-inactivated fetal bovine serum. Monolayers of breast milk and bronchoalveolar lavage macrophages were established and maintained in a similar manner (Cole et al., 1982, 1983). The human hepatoma cell line HepG2 (Knowles et al., 1980), the human macrophage-like cell line U937 (Sunstrom & Nilsson, 1976; Ralf et al., 1976), and transformed human fibroblasts were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum.

Isolation of mRNA from Human Fetal Tissues. Human fetal tissues were obtained from 20–22-week human abortuses under a protocol approved by the Research Advisory Committee of the Brigham and Women's Hospital, Boston, MA. As described previously (Zannis et al., 1982), the tissues were collected within 1 hour following the abortion, were transported to the laboratory under liquid nitrogen, and were stored at −70 °C until further use. For isolation of the RNA, the tissues were homogenized to a fine powder under liquid nitrogen. The tissue powder was mixed with a solution of 4 M guanidinium thiocyanate, 0.025 M sodium citrate (pH 7.0), 0.5% sodium *N*-laurylsarcosinate, and 0.1 M β-mercaptoethanol. This mixture was forced successively through 20-, 23-, and 25-gauge needles, and 3 mL of the resultant mixture was layered on top of the 13-mL cushion of 5.7 M CsCl, 0.1 M EDTA, and 0.025 M sodium citrate (pH 7.0) in 16-mL tubes and centrifuged in a SW55 rotor at 35 000 rpm for 24 h at 20 °C. The pellet was then washed twice with 70% ethanol and dissolved in 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) and 1 mM EDTA (pH 7.55). The RNA was then ethanol precipitated, dissolved in double distilled water, and stored at −70 °C.

^{32}P Labeling of Apolipoprotein DNA Probes Using Single-Stranded M13 Clones as Templates and the M13 Primer. An aliquot of 1 μL of a solution containing 0.2–1 μg of the appropriate single stranded M13 apolipoprotein DNA template was mixed in an Eppendorf tube with 2.5 μL (2.5 pmol) of sequencing primer and 0.5 μL of a buffer composed of 30 mM Tris-HCl, pH 7.5, 50 mM NaCl, 15 mM $MgCl_2$ and 1 mM dithiothreitol. The mixture was heated at 65 °C for 30 min and then left at room temperature for 30 min. The above hybridization mixture was transferred to a new Eppendorf tube containing 50 pmol each of α-^{32}P-labeled dATP, dCTP, and TTP and 50 pmol of unlabeled dGTP that had been completely dried. The reaction cocktail was mixed with 0.5 μL of 10 mg/mL nuclease-free bovine serum albumin (BSA), and the mixture was vortexed well to resuspend the label. An aliquot of 1 μL (0.5 unit) of Klenow fragment of DNA polymerase I was added, and the mixture was then incubated for 30 min on ice and an additional 60 min at room temperature. Following incubation an aliquot of 25 μL of 10 mM Tris-HCl and 1 mM EDTA, pH 7.5, was added, and the radiolabeled probe was purified by phenol extraction and Bio-Gel P-60 chromatography. The single-stranded DNA templates used were as follows: (a) a clone containing the *Hpa*II to *Hpa*II fragment of the human apo A-I gene (Karathanasis et al., 1983b) inserted into the *Pst*I site of M13 mp11 vector (Messing et al., 1981); (b) a clone containing a full-length apo C-III (C-II-I-607) (Karathanasis et al., 1985) cDNA fragment inserted into the *Pst*I site of the M13 mp11 vector; (c) a clone containing the first 5′ upstream 0.45-kilobase (kb) *Pst*I–*Pst*I fragment of the apo E cDNA clone pE-368 (Zannis et al., 1984) inserted in the *Pst*I site of the M13 mp11 vector; and (d) a clone containing the *Pst*I to *Pst*I insert of an apo C-II cDNA clone (pC-II-711) inserted into the *Pst*I site of the M13 vector (Jackson et al., 1984).

Northern Blotting Analysis and S1 Nuclease Mapping of RNA. S1 nuclease mapping of the RNA was performed as previously described (Berk & Sharp, 1977). For Northern analysis (Goldberg, 1980), the mRNA was electrophoresed in a 1% agarose gel prepared in a buffer containing 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.00, 5 mM sodium acetate, 1 mM EDTA, 0.23 M formaldehyde, and 10 μg/mL ethidium bromide. The electrophoresis solution contained the same buffer without formaldehyde and ethidium bromide. After electrophoresis, the RNA was blotted onto nitrocellulose filters, baked, and hybridized to the appropriate ^{32}P-labeled apolipoprotein gene probe. The filter was washed for 30 min at 65 °C in 0.1% sodium dodecyl sulfate (SDS), 0.15 M sodium chloride, and 0.02 M sodium citrate, pH 7.5, and autoradiographed. After hybridization with one of the probes (i.e., apo E), the radioactivity signal was erased by boiling the filter for 5 min in a solution of 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA, and the filter was then rehybridized with another probe (i.e., apo C-III). The relative concentration of apolipoprotein mRNAs was estimated in two different ways: (a) One method was by scanning densitometry of the autoradiograms containing the different apolipoprotein mRNA signals. For this purpose the concentration of the RNA analyzed and the exposure time of the film were chosen so that the radioactivity signal obtained was in the linear response range of the film. (b) The second method was by scintillation counting of the nitrocellulose filter bands corresponding to a specific mRNA signal. Prior to counting, the bands were incubated at 110 °C for 1 h in a buffer containing 10 mM Tris-HCl and 1 mM EDTA, pH 7.50. The latter quantitation is possible only for the abundant mRNA species.

Results

Several human tissues and cell cultures of human origin were analyzed for apolipoprotein mRNA synthesis. Figure 1 shows autoradiograms obtained from Northern blotting analysis of total RNA isolated from the various human tissues. Panels A and B represent autoradiograms following hybridization of the same nitrocellulose filter with apo E and apo C-III probes, respectively. Panels C and D show autoradiograms following hybridization of the same nitrocellulose filter with apo A-I and apo C-II probes, respectively. This analysis shows that apo E mRNA is present in all tissues studied. On the basis of total RNA concentrations the relative abundance of apolipoprotein mRNA expressed as a percentage of the liver value is as follows: apo E, HepG2 cells, 88%; apo E, adrenal, 74%; apo E, kidney, 15%; apo E, gonads, 12%; apo E, spleen, 9%; apo E, brain, 7%; apo E, ovaries, thymus, and intestine, 5%; apo E, heart, 1.5%; apo E, stomach, lung, striated muscle, and SV40 transformed fibroblasts, <1%; apo A-I, intestine, 50%; apo A-I, HepG2, 39%; apo A-I, pancreas and gonads, 12%; apo A-I, kidney, 4%; apo A-I, adrenal, 2.5%; apo A-I, ovaries and heart, 1%; apo A-I, stomach and thymus, <1%; apo C-III, intestine, 62%; apo C-III, pancreas, 7%; apo C-III, HepG2, 6%; apo C-II, HepG2, 35%; apo C-II, intestine, 3%; apo C-II, pancreas, <1%. The synthesis of apo E mRNA by cultures of human monocyte-macrophages increases as a function of time in culture. Figure 2A–C shows that the apo E mRNA concentration is very low on day 1, increases dramatically on day 3, and continues to increase further on days 5 and 15 (Figure 2C). High levels of apo E mRNA were found in 1-day cultures of breast milk and bronchoalveolar macrophages. The relative concentration of apo E mRNA of 1-day macrophage cultures of the 5-day monocyte-macrophage

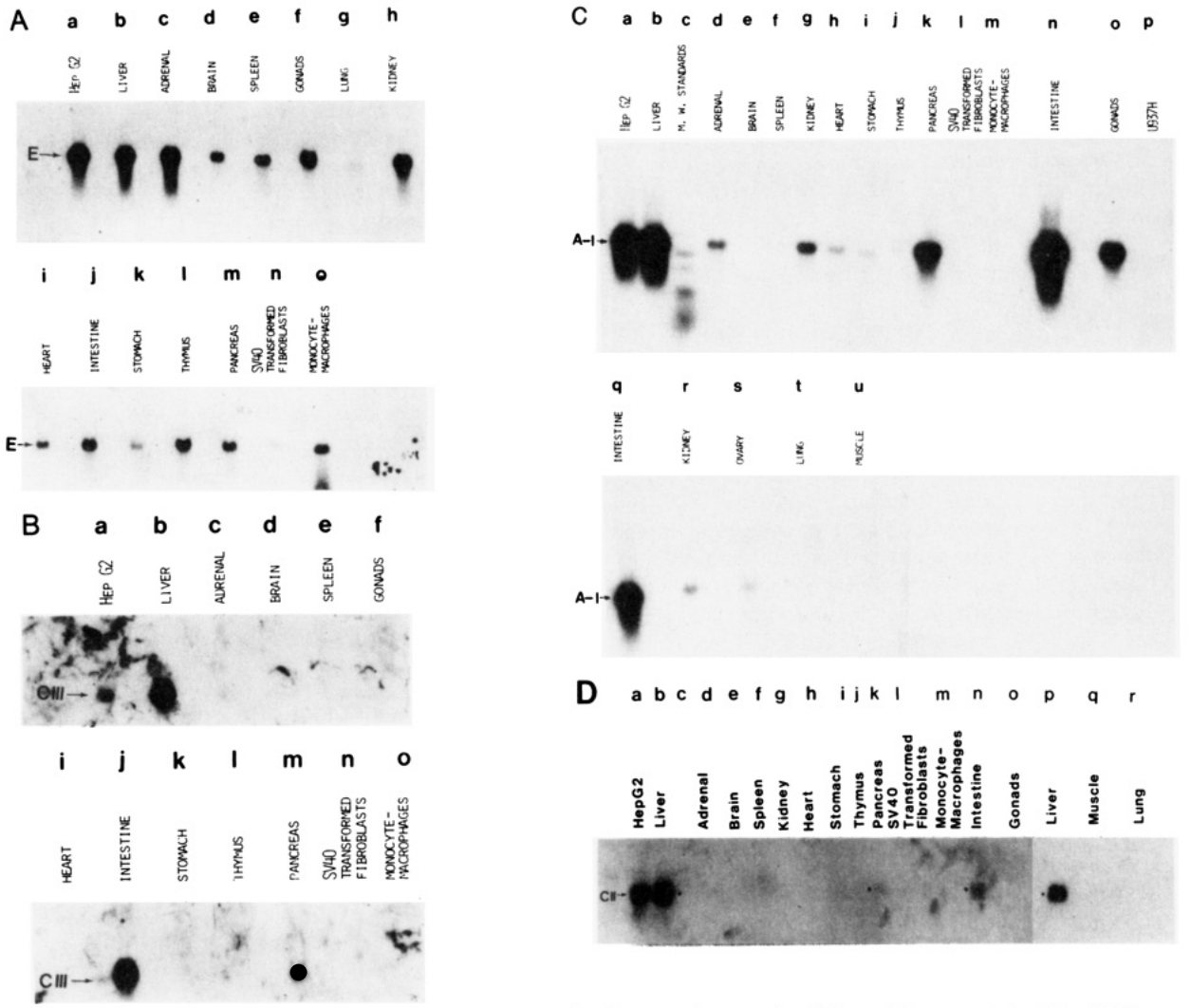

FIGURE 1: Northern hybridization of total RNA isolated from various fetal human tissues and cell lines of human origin. The RNA was electrophoresed on 1% agarose gels, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled M13 probe containing a *Pst*I–*Pst*I insert of the apo E cDNA clone pE-368 (Zannis et al., 1984). Panel A shows the autoradiogram obtained from this analysis. Lanes a–e contain 15 μg of total RNA isolated from (a) HepG2 cells, (b) liver, (c) adrenal gland, (d) brain, and (e) spleen. Lanes f–m contain 30 μg of total RNA isolated from (f) gonads, (g) lung, (h) kidney, (i) heart, (j) intestine, (k) stomach, (l) thymus, and (m) pancreas. Lane n contains 30 μg of RNA obtained from SV40 transformed human fibroblasts. Lane o contains 2.5 μg of RNA isolated from an 8-day culture of human monocyte-macrophages. Panel B shows an autoradiogram of the same nitrocellulose filter as in panel A except that the filter was hybridized with a ^{32}P-labeled M13 probe containing an insert of a full-length apo C-III cDNA clone (pC-III-607) (Karathanasis et al., 1985). Prior to hybridization, the apo E signal was erased by boiling the filter for 5 min in a solution of 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. Panel C shows the autoradiogram of a nitrocellulose filter that was hybridized with a ^{32}P-labeled probe containing a *Hpa*II–*Hpa*II insert of the human apo A-I gene (Karathanasis et al., 1983b). Lanes a, b, d–f, n, and q contain 15 μg of RNA obtained from (a) HepG2 cells, (b) human liver, (d) adrenal gland, (e) brain, (f) spleen, and (n and q) intestine. Lane c contains ^{32}P-labeled DNA of φX174 digested with *Hae*III. Lane m contains 5 μg of RNA obtained from an 8-day culture of human monocyte-macrophages. All other lanes contain 30 μg of human RNA obtained from (g) kidney, (h) heart, (i) stomach, (j) thymus, (k) pancreas, (l) SV40 transformed human fibroblasts, (o) gonads, (p) macrophage-like cell line U937, (r) kidney, (s) ovaries, (t) lung, and (u) muscle. Samples in lanes a–p and q–u were analyzed in different experiments. When the above filter was rehybridized with the apo C-III and apo E probes, we found that only liver, intestine, and pancreas contain apo C-III mRNA and that all tissues including ovaries and muscle synthesize apo E. Panel D, lanes a–o, shows an autoradiogram of the same nitrocellulose filter as in panel C except that the filter was hybridized with a ^{32}P-labeled M13 probe containing the *Pst*I to *Pst*I insert of the apo C-II cDNA clone pC-II-711 (Jackson et al., 1984). Lanes p, q, and r show the autoradiogram of a separate Northern analysis of 15 μg of RNA examined from the following fetal human tissues: (p) liver, (q) muscle, and (r) lung.

cultures is 75–100% of that of liver, whether the comparison is based on total cell number or total micrograms of RNA analyzed (Figure 2A,B). There were no detectable amounts of apo A-I or apo C-III mRNA in either the human peripheral blood monocyte-macrophages or the human macrophage cultures. In contrast to human monocyte-macrophages and macrophage culture, the human macrophage-like cell line U937 (Sunstrom & Nilsson, 1976; Ralf, 1976) does not have detectable amounts of apo E mRNA. Various treatments were employed in order to induce the U937 cells to synthesize apo E mRNA. These included growth of the cells for 6 h in fibroblast-conditioned medium as well as growth of the cells for 5 days in MEM in the presence of 0.5 and 2% Me_2SO, 10^{-8}–10^{-4} M cAMP, 10^{-7}–10^{-5} M dexamethasone, 2.5×10^{-5} and 5×10^{-5} M 5′-deoxy-5-bromouridine, 10 μg/mL insulin, 1–10 μg/mL glucagon, 1 μg/mL thyroxine, and 0.4 mM sodium butyrate. All these treatments gave negative results (data not shown). The results of Northern analysis pertaining to apo A-I and apo E mRNA synthesis were confirmed by S1 nuclease mapping of the various RNA preparations. The probes used for this preliminary analysis were not designed to investigate the start site of the apo A-I and apo E mRNA, and thus the data are not shown (see paragraph at end of paper regarding supplementary material). The estimated sizes of

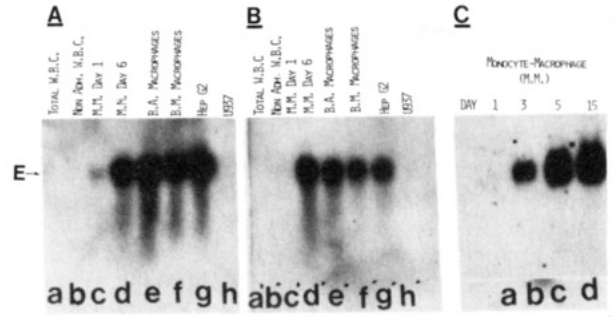

FIGURE 2: Northern hybridization of total RNA isolated from cultures of peripheral blood human monocyte-macrophages and human macrophages. RNA was analyzed as described in Figure 1. The nitrocellulose filter was hybridized with ^{32}P-labeled pE-301 apo E cDNA probe (Breslow et al., 1982a,b, 1983) and autoradiographed. Panel A, lanes a–h, contains RNA obtained from 10^6 cells as follows: (a) total white blood cells, (b) nonadhering white blood cells, (c) 1-day and (d) 6-day cultures of human peripheral blood monocyte-macrophages, (e) bronchoalveolar macrophages, (f) breast milk macrophages, (g) HepG2 cells, and (h) U937 cells. Panel B, lanes a′–h′, contains 20 μg of RNA obtained from the same sources as the corresponding lanes a–h of panel A. Panel C, lanes a–d, contains 6 μg of RNA isolated from 1-, 3-, 5-, and 15-day cultures, respectively, of human peripheral blood monocyte-macrophages.

apo E, apo A-I, apo C-III, and apo C-II mRNA are 1.2, 1.05, 0.70, and 0.70 kb, respectively.

Discussion

Lipoprotein metabolism is a complex pathway that begins with the synthesis of apolipoproteins and the secretion of nascent lipoprotein particles and ends with the catabolism of mature lipoprotein particles by extrahepatic and hepatic tissues. To date, our knowledge of the contribution of the various tissues to the plasma apolipoprotein and lipoprotein pool is incomplete. Hepatic synthesis of all the major apolipoproteins has been demonstrated in rats (Wu & Windmueller, 1979; Windmueller & Wu, 1981). Similar studies have shown that small intestine synthesizes large amounts of the apo A-I, apo A-IV, and apo B (Glickman & Green, 1977; Schonfeld et al., 1978; Wu & Windmueller, 1979; Windmueller & Wu, 1981), but only small amounts of the apo C peptides and little or no apo E (Schonfeld et al., 1978; Wu & Windmueller, 1979; Windmueller & Wu, 1981). A quantitative study in rats indicated that the liver contributes 81% and the intestine 19% to the total apolipoprotein pool. The percent contribution of the intestine to individual apolipoproteins was as follows: apo A-IV, 59%; apo A-I, 56%; apo B, 16%; apo C's, 5%; apo E, <1% (Wu & Windmueller, 1979). Apolipoprotein synthesis has also been demonstrated by liver cell cultures (Tarlow et al., 1977; Davis et al., 1979; Dashti et al., 1980; Kempen, 1980; Zannis et al., 1981) and by organ cultures of human intestine and liver (Zannis et al., 1982). In avian species, apo B synthesis has been reported in kidney (Blue et al., 1980) and apo A-I synthesis in a variety of peripheral tissues (Blue et al., 1982). Finally, in man, apo E synthesis has been demonstrated in kidney, adrenal gland, and reticuloendothelial cells (Basu et al., 1982; Blue et al., 1983). This finding indicates that the mRNA is translatable in these tissues. We confirmed the above findings with organ cultures of fetal human adrenal gland, kidney, and heart (data not shown). More systematic studies are required to determine whether the apolipoprotein mRNAs observed in Figure 1A–D are likewise translatable into proteins.

An important finding that emerges from this study is that apo E is a ubiquitous protein that is apparently synthesized by practically every tissue of the human body. It remains to be established whether this observation is a clue to the physiological significance of this protein and the proposed role of apo E in the reverse transport of cholesterol. Recent studies indicate that high-density lipoprotein (HDL) free of apo E binds to specific HDL receptors and promotes cholesterol efflux from cells (Biesbroeck et al., 1983). Other studies have shown that macrophages can secrete apo E and cholesterol by two independent pathways (Basu et al., 1983). Finally, in vitro experiments have shown that apo E (and cholesterol) secreted by these cells is incorporated into HDL, which produces HDL particles enriched in cholesterol, cholesteryl ester, and apo E (Gordon et al., 1983). These lipoprotein particles can deliver their cholesterol content both to hepatic tissues through the apo E and B/E receptors (Carella & Cooper, 1979; Hui et al., 1981) and to extrahepatic tissues through the B/E receptor (Bersot et al., 1976; Pitas et al., 1979). It is reasonable to propose that apo E, which is synthesized by the peripheral tissues in vivo, is likewise incorporated into HDL and serves to redistribute the cholesterol of the body as it is required. In future studies it will be interesting to assess how changes in plasma and tissue cholesterol content may affect the tissue concentrations of apo E mRNA.

Another important observation is that cultures of peripheral blood monocyte-macrophages contain traces of apo E mRNA after 24 h in culture, whereas breast milk and bronchoalveolar macrophages have relative apo E mRNA concentrations comparable to that of liver. It is interesting that the induction of scavenger [acyl low-density lipoprotein (acyl LDL)] receptors in cultures of peripheral blood monocyte-macrophages is a function of time in culture and reaches a plateau on day 6 (Fogelman et al., 1981). It is possible that the synthesis of apo E may be related to the maturation of monocyte into macrophage. If this assumption is correct, apo E could serve as a marker to study various agents that may modulate this differentiation process.

In contrast to our findings with apo E, there is tissue specificity in the synthesis of apo A-I, apo C-II, and apo C-III mRNA. Apo A-I mRNA is present in numerous tissues including intestine, liver, gonads, pancreas, kidney, adrenal gland, ovaries, heart, stomach, and thymus but absent from brain, lung, muscle, and macrophages. Contrary to the findings with human tissues, recent studies have shown that apo A-I mRNA is present in the striated muscle of avian species and is developmentally regulated (Shackelford & Lebhertz, 1983). Apo C-III mRNA is present in large concentrations in liver and intestine and in low concentrations in pancreas. Apo C-II mRNA was found mainly in the liver and in much lower concentrations in the intestine and pancreas. Previous studies have shown the presence of apo C-III mRNA and the synthesis of apo C-III by rat intestine (Schonfeld et al., 1980; Tanaka et al., 1982). However, quantitative studies have indicated small contribution by the small intestine in the overall plasma apo C-III pool (Wu & Windmueller, 1979). It remains to be established whether there is a translational control in the synthesis of apo C-III by the intestine.

Recent studies on apolipoprotein mRNA synthesis have been limited to human liver (Cheung & Chan, 1983; Sharpe et al., 1984; Knott et al., 1984). In addition, in the baboon, apo A-I mRNA was found in liver and intestine but not in skeletal muscle, kidney, and spleen (Cheung & Chan, 1983). Similar studies in our laboratory confirmed the presence of apo A-I mRNA in adult cynomolgus monkey liver and intestine and its absence from a variety of other tissues including kidney, skeletal muscle and spleen. The observed difference between human and monkey tissues may reflect species differences in the expression of the apo A-I gene. Alternatively, they may

indicate developmentally regulated expression of the apo A-I gene since the human and monkey specimens originated from fetal and adult tissues, respectively.

Several factors may affect the degree of expression of a specific apolipoprotein gene in a particular tissue. An inverse relationship has been found between the degree of cytosine methylation in the vicinity of a gene (usually at CpG sites) and the transcriptional activity of the gene (Razin & Riggs, 1980). Several humoral factors, cell receptors, and small molecules may also be involved in the regulation of expression of the apolipoprotein genes. These aspects will be the topic of future investigations.

We have recently shown that the apo A-I and apo C-III genes are closely linked and convergently transcribed (Karathanasis et al., 1983a). The observation that several tissues can synthesize apo A-I but not apo C-III mRNA suggests that the transcription of the apo A-I and apo C-III genes is regulated by different factors. In conclusion, the specificity of various tissues in the synthesis of apolipoproteins documented in this paper provides the basis for our further understanding of the regulation of apolipoprotein and lipoprotein metabolism.

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SUPPLEMENTARY MATERIAL AVAILABLE

Three figures showing restriction maps of the human apo E and apo A-I genes, S1 nuclease mapping of human apo E and apo A-I mRNA, restriction map of clone pE-368 of apo E and strategy used for its DNA sequence analysis, and Northern hybridization of total RNA isolated from tissues of cynomolgus monkey (5 pages). Ordering information is given on any current masthead page.

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Mechanism of $CaCl_2$-Induced Actin Polymerization

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ABSTRACT: The $CaCl_2$ concentration dependence of the rate of actin filament elongation and of the actin monomer concentration at steady state with actin polymer (the critical actin concentration) has been investigated. A relative rate of actin filament elongation from actin polymer intermolecularly cross-linked with *N,N'-p*-phenylenebis(maleimide) showed a sigmoidal dependence on the concentration of $CaCl_2$ used to induce actin polymerization. This result is shown to be consistent with a model in which only actin monomer containing five equivalently bound Ca^{2+} ions (K_a = 2 mM^{-1}) is capable of addition to actin polymer. A relative dissociation rate constant for actin monomer removal from polymer was calculated from the product of the critical actin concentration and the relative elongation rate constant and was found to be virtually independent of $CaCl_2$ concentration. The relationship between Ca^{2+} binding sites on actin and the $CaCl_2$ concentration dependence of the kinetics of actin filament elongation is discussed.

Actin is a major protein of eukaryotic cells and has essential roles in cell structure and motility and an involvement in a wide range of other cellular activities [see reviews by Clarke & Spudich (1977), Pollard (1981), and Korn (1982)]. This protein reversibly polymerizes from a monomer or G-actin to long double-helical actin filaments or F-actin containing many hundreds of actin protomers. Many actin-related activities of nonmuscle cells may require the disassembly and reassembly of actin filaments at specific sites and times within a cell. The degree and rate of actin polymerization in a cell are probably influenced by factors such as pH, bivalent cations, ionic strength, temperature, adenine nucleotides, and actin binding proteins, all of which affect the in vitro polymerization properties of actin [reviewed by Korn (1982)]. Thus, an understanding of cellular activities involving this protein may first require detailed knowledge of the influence of these factors on the state of actin in vitro.

G-Actin can be induced to polymerize into F-actin by millimolar concentrations of bivalent cations and/or physiological ionic strengths. The overall rate of actin polymerization is remarkably sensitive to the concentration of bivalent cations required to induce polymerization (Kasai et al., 1962; Kasai, 1971; Oosawa & Kasai, 1971; Pollard & Mooseker, 1981; Rouayrenc & Travers, 1981; Frieden, 1983; Tobacman & Korn, 1983; Lal et al., 1984). Although this sensitivity has been well documented, its origin remains obscure.

The present study reports the $CaCl_2$ concentration dependence of the kinetics of actin filament elongation from actin polymer intermolecularly cross-linked with *N,N'-p*-phenylenebis(maleimide) and of the concentration of actin monomer at steady state with actin polymer (the critical actin concentration). A simple model is proposed which describes the $CaCl_2$ concentration dependence of these results.

Experimental Procedures

Rabbit skeletal muscle actin was isolated and purified according to the procedure described by Pardee & Spudich (1982) with the gel filtration (Sephadex G-150) modification of MacLean-Fletcher & Pollard (1980). G-Actin was in a buffer consisting of 2 mM tris(hydroxymethyl)aminomethane (Tris),[1] 200 μM ATP, 200 μM $CaCl_2$, and 1.5 mM NaN_3, pH 8.0 (G buffer), and used within 4 days of G-150 Sephadex chromatography. Before all experiments, the G-actin was centrifuged (3 h, 4 °C, 96000*g*) to remove any actin oligomers. Actin concentrations were measured spectrophotometrically at 290 nm using an extinction coefficient of 0.63 $mL \cdot mg^{-1}$ (Houk & Ue, 1974) and a molecular weight of 42300 (Elzinga et al., 1973).

N-Pyrenylactin was prepared as previously described (Tellam & Frieden, 1982) and dialyzed exhaustively against G buffer at 4 °C. This chemically modified G-actin was centrifuged (178000*g*, 30 psi, Beckman airfuge) before use. The concentration of *N*-pyrenylactin was determined by using the Bradford (1976) protein assay with unmodified G-actin as a standard. The ratio of dye to protein was approximately 0.70–0.85.

All fluorescence measurements were made in a Perkin-Elmer Model LS-5 spectrofluorometer at 25 °C. Incorporation of *N*-pyrenylactin into actin polymer results in appoximately a 25–26-fold fluorescence enhancement of the labeled actin at an emission wavelength of 407 nm and an excitation wavelength of 365 nm (Kouyama & Mihashi, 1981). The time course of fluorescence increase of trace levels (<10% of the

[1] Abbreviations: G buffer, 2 mM Tris, 200 μM ATP, 200 μM $CaCl_2$, and 1.5 mM NaN_3, pH 8.0; *N*-pyrenylactin, (*N*-pyrenylcarboxamidomethyl)actin; Tris, tris(hydroxymethyl)aminomethane.

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