Increased expression of apolipoprotein genes accompanies differentiation in the intestinal cell line Caco-2 (lipoproteins/transcription factor)

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ABSTRACT We have analyzed determinants of the synthesis and secretion of apolipoproteins including mRNA for apolipoproteins, in the human colon carcinoma cell line Caco-2 during differentiation in continuous culture. Significant increases in both cellular and secreted apolipoprotein A-I were observed early in the differentiation process. Increases in apolipoprotein B were limited to secreted protein and started later in the differentiation process. Levels of mRNA for apolipoproteins A-I, A-IV, B, C-III, and E increased significantly between the time cells reached confluence and 1 week postconfluence. The kinetics of mRNA accumulation were influenced by culture conditions. Nuclear extracts from postconfluent Caco-2 cells contained increased amounts of protein that bound to oligonucleotides containing the control regions of the apolipoprotein A-I and B genes. A competition experiment suggested that this protein recognized the control regions of both genes. We propose to name this protein DRIFT-1 (differentiation-related intestinal factor for transcription 1).

Synthesis of most human apolipoproteins occurs primarily in the liver and small intestine (1). Many of the apolipoproteins are expressed in the human colon carcinoma cell line Caco-2, which develops characteristics of small-intestinal epithelium such as brush-border microvilli, tight junctions, dome formation, and vectorial transport of both cations and anions when cultured continuously at confluence for about 2 weeks (2). The differentiated cells also express brush-border enzymes such as sucrase, alkaline phosphatase, and aminopeptidase (3) concomitant with these cellular differentiation markers. Under these conditions, particles corresponding to human plasma very low density lipoprotein, low density lipoprotein, and high density lipoprotein are found in the medium (4) and are observed to be transported vectorially through the basolateral membrane when the cells are cultured on semipermeable supports (5). In addition, the cells have been shown to synthesize and secrete apolipoprotein A-I (apoA-I), apoA-IV, apoB48 and apoB100, apoC-III, and apoE (4). mRNAs for apoA-I, apoC-III, and apoE (4) as well as apoB (6) were also detected.

In this paper we demonstrate that levels of protein and mRNA for several of the apolipoproteins increase substantially after confluence in Caco-2 cells. In addition, we show that the increase in apoA-I and apoB mRNA levels correlates with an increase in a Caco-2 nuclear protein binding to the control region of the genes.

METHODS

Cell Culture. Caco-2 cells in Program One were maintained in complete medium with 20% fetal bovine serum, as described (4). Medium was replenished daily. This program was designed to accelerate the differentiation process. Cells in Program Two were grown in Eagle's minimum essential medium with Earle's salts and glutamine, supplemented with antibiotics and 10% fetal bovine serum. Medium was changed twice weekly. This program mimicked conditions generally utilized when Caco-2 cells are transfected with DNA.

For metabolic labeling experiments, cell monolayers on six-well 35-mm plates were rinsed with methionine-free, serum-free medium, and then incubated for 8 hr in 1 ml with 75 μCi of [35S]methionine (>800 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq). After incubation, the labeled conditioned medium was centrifuged at 1000 × g for 30 min to remove debris prior to SDS/PAGE. Immunosprecipitations of apoA-I, apoB, and apoE were performed as described (4), except that 0.5 M NaCl was added to the wash buffer.

Assays. Total protein (7) and sucrase activity (8) were determined in total cellular extracts at various times after confluence, 24 hr after the medium was replenished. Non-competitive enzyme-linked immunosorbent assays (ELISAs) of apoA-I and apoB were performed as described (9).

RNA Preparation and RNA Blots. Cytoplasmic RNA was isolated by the Nonident P-40/phenol extraction procedure (10). Total cellular RNA was prepared as described (11). For slot blot analysis, RNA samples were suspended in 40 mM Tris acetate/1 mM disodium EDTA/1 M formaldehyde, mixed for 5 min, heated at 56°C for 15 min, and applied to Hybond-N nylon membranes (Amersham) in a slot blot apparatus. Cytoplasmic RNA samples from Caco-2 cells at 0, 3, 6, 9, 12, and 15 days postconfluence and from HepG2 human hepatoma cells (as a positive control) were applied at 0.01, 0.03, 0.10, 0.30, and 1.0 μg of RNA per slot. HeLa cell RNA was used as a negative control and showed no hybridization with probes for apoA-I or apoB. The slots were then rinsed twice with 0.5 ml of 40 mM Tris acetate/1 mM disodium EDTA and the filters were exposed to short-wave ultraviolet light for 2 min and stored dry until use. After hybridization (4), autoradiographs of the filters were quantitated with an Ultrascan laser densitometer (LKB). Values for Caco-2 apoA-I and apoB mRNAs were interpolated from standard curves constructed using HepG2 mRNA absorbance values and were expressed as "HepG2 equivalents," which reflect the ratio of the abundance of the particular Caco-2 mRNA to its cognate HepG2 mRNA.

For Northern blots, 10 μg of total cellular RNA was electrophoresed in formaldehyde agarose gels, transferred to nitrocellulose paper, baked, and hybridized as described (12).

Probes for RNA Detection. The probes for apoA-I were either a 2.2-kb genomic Pst I fragment (13) or a 50-base
oligonucleotide: 5'-CGTTCTCGACGGACGAATCCTGACGCTCTTCCTCAGGGGGTGCCTGGGA-3'. The last 34 nt (underlined) are complementary to the 5' end of human apoA-I mRNA. Several cDNA probes were used for apoB: I2, I10, and LB1.5 (14) from Andrew Protter (Scios, Mountain View, CA) and pB8 from Li-Shin Huang (Rockefeller University, New York) (15). All gave identical patterns of hybridization. The probe for apoC-III was the cDNA clone pCHI-655-2 (16). The probe for apoE was 5'-GCAACGCAGCCCACAGAACCTTCCTTCCCTGCCT-3', an oligonucleotide complementary to nt 58-92 of the mature mRNA sequence (17). The probe for α-tubulin (bovine) was a gift from Gill Diamond (Children's Hospital of Philadelphia, Philadelphia).

**Probes for DNA-Binding Proteins.** The oligonucleotide used for detecting the protein binding to the control region of the apoA-I gene is referred to as A-I ds-210, and contains the following sequence.

5'-'CTAGACTGAACCTTGACCCTCTGCTGCA-3'
3'-'TGACTGGAGACCGGTGGACGG-5'

The double-stranded portion corresponds to nt -210 to -189 relative to the transcriptional start of the apoA-I gene. An oligonucleotide corresponding to nt -174 to -144 of the apoA-I gene, A-I ds-174, was used as a non-specific competitor.

5'-'CTTGCTGTTTGCCCACTCTATTGCCCAGCC-3'
3'-'AACCGAACCGCCCTTGCACCTGC-5'

The double-stranded oligonucleotide used to detect protein binding to the control region of the apoB gene, B ds-84, was a gift of Hriday K. Das (University of Tennessee, Memphis).

5'-'GATCGGGAGCCCGCTTTGGACATTTC-3'
3'-'CCCTCCCGCGGAAAACCTGGAACGCTAG-5'

The double-stranded portion of the sequence corresponds to nt -84 to -60 of the apoB gene. The double-stranded oligonucleotide used to detect protein binding to the control region of the apoC-III gene, C-III ds-85, corresponds to nt -85 to -64 of the human apoC-III gene.

5'-'AGGGAGCCCTTTGGACGCGCCGG-3'
3'-'TCCACTGGGAAACGGGTGGCCCG-5'

**Electrophoretic Mobility-Shift Assays.** Nuclear extracts were prepared according to Dignam et al. (18). Oligonucleotides were end-labeled with T4 polynucleotide kinase. Between 0.1 and 1 ng of labeled DNA (≈10⁶ cpmp) was mixed with 5 μg of nuclear extract protein and 2-4 μg of poly(di-dC)poly(dI-dC) and incubated in binding buffer (19) containing 8 mM MgCl₂. Competition experiments used a 100-fold molar excess of the unlabeled oligonucleotide. The mixture was incubated for 30 min on ice and then analyzed by PAGE in 0.25 × TBE buffer (22.25 mM Tris/22.25 mM boric acid/0.25 mM EDTA, pH 8.3) at 10 V/cm. Gels were autoradiographed.

**RESULTS**

**Cell Growth and Differentiation.** Cells cultured under Program One began dividing within 24 hr, reached confluence within 2–3 days, and continued to divide until the monolayer was tight, ≈6 days later (Fig. 1A). The increase in cell number was paralleled by an increase in protein content (Fig. 1B), which reached a maximum value of ≈0.28 mg/cm² on day 12 postconfluence. The protein content was 0.49 ± 0.08 mg per 10⁶ cells (n = 36), a value which did not change significantly through the experimental period and which corresponds to values of 0.48 and 0.49 mg per 10⁶ cells reported for Caco-2 cells at 5 and 25 days of culture, respectively (20).

Sucrase activity displayed a 15.8-fold increase in total activity and a 7.7-fold increase in activity relative to cellular protein levels (Fig. 1C). This increase was typical of the induction of sucrase, alkaline phosphatase, and aminopeptidase reported for Caco-2 (21-24).

**Apolipoprotein Production During Differentiation.** The accumulation of apoA-I in the medium from cells in Program One during the differentiation period rose sharply from day 0 to plateau at ≈4.5 μg/mg of cell protein until day 15, when it decreased (Fig. 2A). In contrast, cellular apoA-I levels peaked on day 3 postconfluence and then decreased (Fig. 2B). The ratio of medium apoA-I to cellular apoA-I followed

**FIG. 1.** Caco-2 cell number (A), cellular protein (B), and sucrase activity (C) at various times after confluence. Results are the means ± SD of six 35-mm dishes.

**FIG. 2.** Production of apoA-I by Caco-2 cells. Caco-2 conditioned medium (A) and cells (B) were assayed for apoA-I by ELISA at various times after confluence. The ratio of medium apoA-I mass to cellular apoA-I mass is also given (C). Results are the means ± SD of six 35-mm dishes.
a pattern similar to sucrose activity, rising to a maximum at day 12 (Fig. 2C).

The production of apoB in Program One was very low until day 6 postconfluence; apoB levels then increased to a peak at day 12 at a level almost 8-fold that at day 0 (Fig. 3A). In spite of the increase in the net secretion of apoB, cellular levels detected by ELISA did not change in a concordant pattern (Fig. 3B). Reflecting the rise in secreted apoB, the ratio of secreted to cellular apoB rose sharply, in a manner similar to sucrase activity, to peak at day 12 (Fig. 3C).

In contrast to the changes observed for apoB and apoA-I, the production of most secreted proteins did not change appreciably during the differentiation (Fig. 4). Note especially the 400-kDa protein. These data point to the specificity of the response of individual proteins to the differentiation process.

Changes in Levels of Apolipoprotein mRNAs in Caco-2 Cells. Levels of both apoA-I and apoB mRNA peaked relatively early in cells in Program One and then began a slow decline (Fig. 5). Results obtained for apoC-III and apoA-IV were similar to those for apoA-I (data not shown). Under conditions of Program Two, the differentiation process appeared to be slower. The results of a typical experiment (Fig. 6) indicate that the levels of mRNA for apoA-I, apoB, apoC-III, and apoE were low before confluence (lane 1) and at confluence (lane 2), but by 1 week after confluence there was an extensive increase which persisted for at least 2 weeks (lanes 2, 3, 4, 5, and 6).
Fig. 7. Proteins from nuclear extracts of Caco-2 cells that bind to the human apoA-I control sequence. End-labeled oligonucleotide A-I ds-210 was incubated with 5 μg of nuclear extract protein. The incubated mixture was electrophoresed in a 4% polyacrylamide gel. Lane 1, preconfluent; lane 2, confluent; lane 3, 1 week postconfluent; lane 4, 2 weeks postconfluent; lane 5, 3 weeks postconfluent. Arrow indicates the shifted band of DNA.

3–5). These results suggest that the major increase in apolipoprotein mRNAs is an early step in Caco-2 cell differentiation.

Protein Factors in Caco-2 Cell Nuclei That Bind to the Control Region of the apoA-I Gene. The region of the apoA-I gene between 210 and 189 nt upstream from the transcriptional start binds nuclear proteins from HepG2 cells (26–28). Deletion of this region decreased expression in Caco-2 cells by a factor of about 9 (29).

Nuclear extracts prepared from Caco-2 cells in Program Two were analyzed by electrophoretic mobility-shift assays. The results (Fig. 7) indicate that in Caco-2 cells, a major increase in binding to this region occurs by 1 week after confluence, corresponding to the time when the major increase in apolipoprotein mRNA levels takes place. The increased binding persists until at least 3 weeks after confluence (Fig. 7). Competition against an excess of unlabeled oligonucleotide A-I ds-210 resulted in loss of the shifted band. However, competition against an excess of unlabeled oligonucleotide A-I ds-174, which binds a different protein (27, 28), did not significantly diminish the shifted band (data not shown).

Since levels of apoB mRNA also increased with time in continuous culture, oligonucleotide B ds-84 was also tested. The sequence of this oligonucleotide corresponds to a region containing a DNA element which has a strong, positive effect on transcription in both HepG2 and Caco-2 cells (30–32).

The results demonstrated that there was an increase in binding of protein from postconfluent cells to the apoB element (Fig. 8, compare lanes 3, 5, and 7 with lane 1). In addition, excess unlabeled A-I ds-210 competed for binding to this protein(s) (Fig. 8, lanes 4, 6, and 8). This suggests that the same protein factor may be involved in the increased expression of both apoA-I and apoB in differentiating Caco-2 cells.

**DISCUSSION**

The differentiation of Caco-2 cells in culture provides a unique system for studying the regulation of apolipoprotein gene expression. We have shown that this differentiation is accompanied by an increase in secretion of apoA-I and apoB, although with different time courses. Additionally, we have found increases in mRNA for apolipoproteins A-I, A-IV, B, C-III, and E. The process of differentiation occurred without any changes in the conditions of culture and therefore may reflect an internal pattern of cellular responses to confluence.

We have chosen to culture our cells on plastic. A publication (33) which appeared after our experiments had been completed concluded that differentiation of Caco-2 cells growing on Millicell filters is more rapid than that on plastic, based upon the levels of apolipoprotein mRNA and protein and the ratio of apoB-48, the intestinal form, to apoB-100, the hepatic form. However, at day 1 postconfluence, markers such as secreted apoB, apoA-IV mRNA and protein, and apoA-I protein were higher in cells grown on plastic and substantial amounts of apoB-48 were detected. Our experiments showed much larger increases in mRNA for apoA-I and apoB than were reported in that study. We note that a previous publication (6) reported a 17-fold increase in apoB mRNA as Caco-2 cells differentiated on plastic, in substantial agreement with our results.

The increase in apolipoprotein mRNA may involve increased transcription, since the efficiency of expression of a transfected apoA-I promoter construct relative to a simian virus 40 control was >30-fold higher in Caco-2 cells cultured for 2 weeks postconfluence as compared with cells 1 day postconfluence (34). The coordinated changes in apolipoprotein mRNAs in Caco-2 cultures suggest common transcriptional regulation. The increase in apolipoprotein mRNA levels is accompanied by an increase in protein binding to upstream DNA sequences of the apoA-I and apoB genes which have been shown to facilitate expression in HepG2 and Caco-2 cells. Our results suggest that control of both apoA-I and apoB expression in differentiating Caco-2 cells may involve the same protein. A comparison of the oligonucleotides used for the apoA-I and apoB control regions reveals a number of conserved nucleotides which are also present in the control region of the apoC-III and apoE genes. A comparison of these four regions results in a consensus sequence for protein binding (Fig. 9). We propose to call this protein DRIFT-1 (differentiation-related intestinal factor for transcription 1). The apoC-III sequence contained in oligonucleotide C-III ds-85 fully matches the consensus sequence. This oligonucleotide is more efficient in binding DRIFT-1 than the oligonucleotide A-I ds-210 (data not shown), which differs from the consensus sequence at several positions.

Several different protein factors have been reported to bind to the apoA-I region corresponding to A-I ds-210. Transcription factors AID1 and AID2 from rat liver both bind to sequences which include this region (28); however, their recognition site appears to extend further upstream than the site required for DRIFT-1. Protein(s) from HepG2 cell nuclei also bind to this region of the apoA-I gene (27), as do several cloned proteins including HNF-4, a positive regulator of transcription (27), ARP-1, a negative regulator of transcription (37), and RXRa, a retinoid receptor (38). A rat liver transcription factor designated NF-BA1 binds to the apoA-I and apoB regions and apoC-III sequences denoted in Fig. 9 (39). Most of these proteins have been identified in intestinal tissue or hepatic cells; their presence in cells of intestinal
<table>
<thead>
<tr>
<th>ACGGAGCCTTGGGACCGTTTGAC</th>
<th>APO A-I (−210 TO −189)</th>
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<tr>
<td>ACGGAGCCTTGGGACCGTTTGAC</td>
<td>APO B (−81 TO −60)</td>
</tr>
<tr>
<td>AGGTGACCCCGCCAGCCGCC</td>
<td>APO C-III (−85 TO −64)</td>
</tr>
<tr>
<td>AGAGTGCTTTGACACCCCGC</td>
<td>APO E (−160 TO −181)</td>
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**CONSENSUS**

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**Fig. 9.** Consensus sequence of regions binding DRIFT-1. The sequences for apol A-I (35) and apol B (30) are the actual sequences of the binding oligonucleotides. The sequences for apoC-III (36) and apoE (17) were selected on the basis of sequence homology. The apoE sequence occurs on the noncoding strand. The consensus sequence contains nucleotides conserved in at least three of the four sequences. Positions conserved in all four sequences are indicated by stars.

origin needs further investigation. For example, it was reported that Caco-2 cell extracts do not contain NF-BA1 (39); however, it is likely that the cells used to prepare those extracts were not fully differentiated. It has been shown that Caco-2 cells contain HNF-4 and that HNF-4 binds to the region from −90 to −59 of the apoC-III gene (40). Further studies would be needed to determine whether any of the liver-specific factors identified as binding to the apolipoprotein control regions are involved in intestinal differentiation as well.

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