Comparison of the Patterns of Expression of Rat Intestinal Fatty Acid Binding Protein/Human Growth Hormone Fusion Genes in Cultured Intestinal Epithelial Cell Lines and in the Gut Epithelium of Transgenic Mice*

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Jeffrey N. Rottman and Jeffrey I. Gordon†
From the Departments of Molecular Biology and Pharmacology and Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

The intestinal fatty acid binding protein gene (Fabpi) provides a good model system for studying how gene transcription is regulated in enterocytes as a function of their differentiation program and location along the duodenal-to-colonic axis. We have compared and contrasted the transcriptional activity of four fusion genes composed of elements from the 5'-nontranscribed domain of rat Fabpi linked to the human growth hormone gene (I-FABP/hGH) in transgenic mice and in five primate epithelial cell lines derived from intestine, liver, kidney, and cervix. Nucleotides -103 to +28 of rat Fabpi are able to direct appropriate lineage-specific and geographic patterns of hGH expression in transgenic mice. I-FABP-100 to -98/hGH is preferentially expressed in Caco-2 cells, which emulate some of the features of differentiated small intestinal enterocytes as they exit colonic crypts and enter villi. Correlation of their patterns of expression in vivo and ex vivo suggest that nonproliferating Caco-2 cells mimic some of the features of the transcriptional regulatory environment of enterocytes located in the upper crypt.

Nucleotides -103 to +28 of rat Fabpi contain one copy of a repeated 14-base pair element that is conserved in the orthologous mouse and human genes and represent in several other homologous and nonhomologous genes, which are expressed in villus-associated enterocytes. This element binds to two members of the steroid hormone receptor superfamily of transcription factors produced in enterocytes and Caco-2 cells: hepatic nuclear factor-4 (HNF-4) and apolipoprotein regulatory protein-1 (ARP-1). Co-transfection studies performed in Caco-2 cells and in a monkey kidney cell line (CV-1) that lacks endogenous pools of ARP-1 and HNF-4 suggest that ARP-1 and HNF-4 can function to activate I-FABP-100 to -98/hGH through their interactions with the 14-base pair element. This activation appears to be affected by elements located between nucleotides -277 and -104 and other transcription factors.

There are several reasons why the intestinal fatty acid binding protein gene (Fabpi) is a useful model for studying the mechanisms that regulate establishment and maintenance of cell lineage-specific patterns of transcription along the crypt-to-villus and duodenal-to-colonic axes of the gut. First, expression of mouse, rat, and human Fabpi is confined to the enterocyte (Sweetser et al., 1987; Green et al., 1992; Cohn et al., 1992), one of four principal epithelial cell types derived from the multipotent stem cell located near the base of each intestinal crypt (reviewed in Potten and Loeffler (1990) and Gordon et al. (1992)). Fabpi is activated in differentiating members of the enterocyte lineage after they complete their last passage through the cell cycle and are translocated from the upper crypt to the bases of adjacent small intestinal villi (Shields et al., 1986; Green et al., 1992; Cohn et al., 1992). Expression is sustained as enterocytes complete their differentiation program during a rapid, well organized, upward migration to the apical extrusion zone of each villus. Fabpi is also expressed in enterocytes as they exit colonic crypts and migrate to the colonic homolog of small intestinal villi, the hexagonal shaped surface epithelial cuff that surrounds the orifice of each colonic "gland" (Schmidt et al., 1985). Second, the steady state level of Fabpi's mRNA and protein products varies as a function of the location enterocytes occupy along the cephalocaudal axis of the gut. Highest cellular concentrations occur in the jejunal with levels falling progressively to the proximal duodenum and midcolon (Sweetser et al., 1988; Cohn et al., 1992). These axial differences in Fabpi expression are established at the time of the gene's induction during cytodifferentiation of the gut endoderm to an epithelial monolayer in fetal life (Rubin et al., 1989, 1991; Cohn et al., 1992). These regional differences in Fabpi expression are sustained throughout life despite continuous and rapid renewal of the intestinal epithelium (Al-Nafussi and Wright, 1982; Wright and Irwin, 1982). Third, studies with fetal jejunal, ileal, and colonic isografts indicate that the lineage-specific, differentiation-dependent, and regional patterns of rat and mouse Fabpi expression are not influenced by luminal contents (Rubin et al., 1991, 1992). Fourth, Fabpi is a member of a family of homologous genes that exhibit distinct cephalocaudal pat-

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† To whom correspondence should be addressed: Dept. of Molecular Biology and Pharmacology, Washington University School of Medicine, Box 8103, 660 S. Euclid Ave., St. Louis, MO 63110. Tel: 314-362-7243; Fax: 314-362-7058.

1 The abbreviations used are: Fabpi, intestinal fatty acid binding protein gene; hGH, human growth hormone; MT, metallothionein; HNF-4, hepatic nuclear factor-4; ARP-1, apolipoprotein AI regulatory protein-1; kb, kilobase(s); bp, base pair(s).
terns of expression in the enterocytic lineage (Sacchettini et al., 1990; Cohn et al., 1992). These differences provide an opportunity to study how genes that have evolved from a common ancestor are able to modulate their expression in a particular cell lineage as a function of location along the duodenal-to-colonic axis.

Transgenic mice containing rat Fabpi/human growth hormone (hGH) fusion genes have been used to map cis-acting elements in Fabpi that control its cellular, geographic, and temporal patterns of expression (Sweetser et al., 1988; Cohn et al., 1992). The results of these studies are summarized in Fig. 1. Remarkably, nucleotides -103 to +28, which are highly conserved in the mouse, rat, and human Fabpi genes (Green et al., 1992), contain elements that are sufficient to appropriately initiate transgene expression in late fetal life, confine reporter production to members of the enterocytic lineage, and to generate a duodenal-to-colonic gradient of hGH mRNA and protein accumulation that mimics that of mouse Fabpi (Cohn et al., 1992). The shape of this cephalocaudal gradient is influenced by both positive and negative cis-acting sequences, e.g., elements positioned between nucleotides -1178 and -278 enhance expression in the ileum and proximal colon while those located between -277 and -185 function as suppressors in these segments of the gut (Cohn et al., 1992) (Fig. 1). Progressive deletions of the 5'-nontranscribed domain of Fabpi also disclosed elements that regulate differentiation-dependent transcription of this gene, e.g., I-FABP-277 to +28/hGH is appropriately activated in enterocytes as they exit intestinal crypts while removal of nucleotides -277 and -185 (yielding I-FABP-184 to +28/hGH) results in precocious expression of the hGH reporter in proliferating and nonproliferating epithelial cells located in the upper half of duodenal, jejunal, ileal, and colonic crypts (Cohn et al., 1992).

Although recent reports have described methods for preparing primary cultures of gut epithelial cells (Evans et al., 1993), none of these systems have been shown to be capable of recapitulating the complex geographic differences in the enterocytic differentiation program or the terminally differentiated state. We have now surveyed expression of a variety of Fabpi/hGH fusion genes in established epithelial cell lines derived from intestinal and extraintestinal tissues. This has provided an opportunity to compare and contrast the activities of a panel of recombinant DNAs in transgenic animals and in cultured cells and determine whether the latter contain a transcriptional regulatory environment that could faithfully support, e.g., in vivo, some features of Fabpi expression observed in vivo. In addition, we have used these cell lines to identify trans-acting factors that affect the transcriptional activity of nucleotides -103 and +28 of rat Fabpi.

**EXPERIMENTAL PROCEDURES**

**Construction of Recombinant DNAs—pIFhGH1 (Sweetser et al., 1988) encodes nucleotides -1178 to +28 of rat Fabpi (Sweetser et al., 1987) linked to the human growth hormone gene starting at its nucleotide +3 (Seeburg, 1982). This recombinant DNA was designated I-FABP-1178 to +28/hGH. I-FABP-277 to +28/hGH was obtained by digesting pIFhGH1 with EcoRI yielding a 2.4-kb fragment, which was cloned into the EcoRI site of pBlueScript SKII (Promega, Madison, WI). I-FABP-184 to +28/hGH was obtained from I-FABP-277 to +28/hGH by digesting I-FABP-277 to +28/hGH with BstEII, blunting the ends of the resulting reaction products with the Klenow fragment of DNA polymerase I, incubating the material with EcoRI and ligating the 2.3-kb restriction fragment to the Smal and EcoRI sites present in the polynucelotide of pBlueScript SKII. I-FABP-100 to +28/hGH was produced by cleaving I-FABP-277 to +28/hGH with SplI and subcloning the 1.9-kb fragment into the SplI site of pUC19 (Sambrook et al., 1989). I-FABP-327 to +28/hGH was produced by placing two copies of the sequence 5'-AGCTTTGAACTTCGAACTTA-3' into the HindIII site of the polynucelotide of I-FABP-277 to +28/hGH (this site is located 8 bases upstream from nucleotide -277 of rat Fabpi). The deoxyoligonucleotide sequencing method (Sanger et al., 1977) was used to confirm that the desired elements were present in the recombinant plasmid. MT/hGH and pRSV-β-Gal, containing the bovine growth hormone (hGH) and β-galactosidase (β-Gal) genes, respectively, were used as plasmids in transfection experiments. The DNA sequence encoding nucleotides -1178 to +28 of rat Fabpi (Sweetser et al., 1988) contains 1.8 kb of the mouse metallothionein promoter linked to the coding sequence of the hGH gene. C3P-TK-hGH was constructed by taking the HNF-4/ARP1/Ear3/COUP-TF binding site spanning nucleotides -86 to -74 of the human apolipoprotein (apo) CIII gene (Mietus-Snyder et al., 1992), adding HindIII linkers to both ends (yielding the sequence 5'-GCAGGTGACCTTTGCCCA-GCGC-3'), and placing the double-stranded cDNA oligonucleotide into the HindIII site beginning at base -109 of the Herpes simplex virus thymidine kinase gene contained in pTKGH (Nichols Institute). cDNAs encoding hepatic nuclear factor-4 (HNF-4, Sladek et al., 1990) and apolipoprotein regulatory protein (ARP-1, Ladas and Karathanasis, 1991) were subcloned as EcoRI fragments into the EcoRI site of the eukaryotic expression vector, pMT2 (Sambrook et al., 1989) yielding pMT2-HNF-4 and pMT2-ARP1, respectively (Ladas et al., 1992).

**Cell Culture**—HepG2, CV-1, SK-Hep, HeLa, and COS-7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained at 37 °C under an atmosphere of 5% CO2/95% air. For hGH production in parallel cultures of cells co-transfected with MT/hGH and pRSV-β-Gal, 10% fetal calf serum was used. Cells were transfected with 6 µg of each plasmid DNA using the calcium phosphate co-precipitation method (Graham and van der Eb, 1973). To control for variations in transfection efficiency, 1 µg of pRSV-β-Gal DNA (Edlund et al., 1985) was co-transfected together with each promoter/hGH construct. Sixteen hours later, cell monolayers were washed twice in phosphate-buffered saline, and 2 ml of fresh medium were added. Aliquots of medium were withdrawn 48 h later and cells removed by centrifugation at 17,000 x g for 5 min. Levels of hGH production in parallel cultures of cells co-transfected with MT/hGH and pRSV-β-Gal were determined by RIA by direct comparison of results obtained with different transfected cell lines.

**Experiments Involving Stably Transfected Caco-2 Cells**—I-FABP/hGH DNA and pRSV-Neo DNA (Kim and Wold, 1986; molar ratio of 1:15) were used to transfected Caco-2 cells using the protocol described above. Cells were subsequently incubated with Dulbecco's modified Eagle's medium supplemented with fetal calf serum (20%) and G418 (final concentration, 800 µg/ml, Gibco/BRL). Once stably, G418-resistant populations were obtained, 104 pooled cells were plated in a 25 cm2 flask. Five-ml aliquots of culture medium were then subjected to centrifugation at 17,000 x g for 5 min. Cells were then lysed in situ using three cycles of freezing/thawing. Cell lysates were assayed for β-galactosidase activity according to a protocol described in Sambrook et al. (1989). hGH levels were measured in the medium using a radioimmunoassay kit (Nichols Institute). The shape of this cephalocaudal gradient could faithfully support, e.g., in vivo, some features of Fabpi expression observed in vivo. In addition, we have used these cell lines to identify trans-acting factors that affect the transcriptional activity of nucleotides -103 and +28 of rat Fabpi.
4 and ARP-1, pMT2-HNF-4 and pMT2-ARP-1 were introduced into COS-7 cells by calcium phosphate co-precipitation. Cell lysates were prepared 48 h later by subjecting cells to three cycles of freezing/thawing in a solution of phosphate-buffered saline containing 0.2 mM phenylmethylsulfonyl fluoride. Lysates were subjected to centrifugation at 17,000 × g to remove insoluble cellular debris. Protein concentrations were determined in the resulting supernatants using the method of Bradford (1976).

DNA fragments used for electrophoretic mobility gel shift assays were purified from agarose gels with GeneClean (Midwest Scientific, Valley Park, MO) and their 3′ ends labeled using [α-32P]dATP and the Klenow fragment of DNA polymerase I (Sambrook et al., 1989). The purified probe (25 fmol) was added to a reaction mixture (final volume, 15 μl) containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5% glycerol, 1 mM EDTA, 1 mM dithiothreitol, and 1 μg of poly(dI-dC), nuclear extract (1 μg of total protein), and, where indicated, an unlabeled oligodeoxynucleotide competitor (1.25 pmol). Following a 15-min incubation on ice, the mixture was fractionated by electrophoresis through non-denaturing polyacrylamide gels using the buffer conditions described in the legend to Fig. 3. Gels were dried and exposed to Kodak XAR film at −80 °C.

RESULTS AND DISCUSSION

Epithelial Cell Type-specific Expression of I-FABP/hGH+3 Constructs—Five different primary epithelial cell lines were included in our study. Caco-2 cells are derived from a human colon adenocarcinoma. After achieving confluence they are able to complete a differentiation program that results in the acquisition of some features of human small intestinal enterocytes (Pinto et al., 1983; Roussel 1986). CV-1 cells are derived from the kidney of an African Green monkey (Jensen et al., 1964). HepG2 cells are derived from a human hepatoma and express a broad range of gene products that are also produced in normal human hepatocytes (Aden et al., 1979). Human SK-Hep-1 cells are of hepatic origin but have a less differentiated phenotype than HepG2 cells (Fogh and Trempe, 1975). The HeLa cell line was established from a human cervical adenocarcinoma (Gey et al., 1964). CV-1 cells were re-fed with fresh medium after each sampling. Under the conditions of this experiment, any changes in the level of hGH observed in medium harvested from a population of nonreplicating, stably transfected cells should reflect changes in reporter production associated with differentiation.

The concentration of hGH in the medium of cells containing I-FABP-1178 to +28/hGH+3 increased 3-4-fold within 3 days after reaching confluence (Fig. 2). These cells assume many characteristics of the differentiated phenotype between 3 and 12 days after confluence (as defined by the appearance of brush border-specific sucrase-isomaltase and tight junctions). During this interval, the concentration of hGH fell progressively, reaching levels that were 50% of those noted on day 0 (Fig. 2). In contrast, the relative levels of hGH production in Caco-2 cells stably transfected with I-FABP-1178 to +28/hGH+3 or MT/hGH+3 remained essentially constant during this 12-day period (Fig. 2).

The behavior of I-FABP-1178 to +28/hGH+3 in Caco-2 cells is consistent with the high levels of expression of I-FABP-1178 to +28/hGH+3 observed in crypt epithelial cells that...
**Fabpi Expression in Cultured Cell Lines and in Transgenic Mice**

![Diagram](image)

**Fig. 1.** Summary of the I-FABP/hGH* DNA sequences characterized in transgenic mice and used for transfection of cultured primate epithelial cell lines. Domains I–III represent three highly conserved elements present in the orthologous rat, mouse, and human Fabpi genes (Green et al., 1992). Gel shift mobility assays identified a 24-bp sequence spanning nucleotides −241 to −188 of rat Fabpi that binds proteins present in colonic but not proximal small intestinal nuclear extracts (Cohn et al., 1992). DNase I footprinting studies employing Escherichia coli-derived C/EBPα (Landschulz et al., 1988) and nucleotides −277 to +28 of rat Fabpi disclosed a single protected site between nucleotides −188 and −167 (Cohn et al., 1992). Computer-assisted sequence analyses of the three orthologous Fabpi genes revealed several copies of a 14-bp element (Sweetser et al., 1987). This element is present in the homologous Cry2 gene whose lineage-specific and regional patterns of expression mimic those of Fabpi. This figure has been adapted from Cohn et al. (1992).

**Table I**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Cell line</th>
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<tbody>
<tr>
<td>MT/hGH*</td>
<td>Caco-2 100%*</td>
</tr>
<tr>
<td>I-FABP−1178 to +28/hGH*3</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>I-FABP−277 to +28/hGH*3</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>I-FABP−184 to +28/hGH*3</td>
<td>110 ± 26</td>
</tr>
<tr>
<td>I-FABP−103 to +28/hGH*3</td>
<td>490 ± 125</td>
</tr>
</tbody>
</table>

* 100% = 5–51 ng of hGH/ml of supernatant.  
| 100% = 5–60 ng of hGH/ml of supernatant.  
| 100% = 4–21 ng of hGH/ml of supernatant.  
| 100% = 8–14 ng of hGH/ml of supernatant.  
| 100% = 7–40 ng of hGH/ml of supernatant.  

**Fig. 2.** Expression of I-FABP/hGH* DNA sequences in stably transfected Caco-2 cells as a function of time after achieving confluence. Caco-2 cells were co-transfected with the indicated DNA and pRSV-Neo. Following a selection for resistance to the aminoglycoside G418, 10⁶ cells were plated in T25 flasks. Day 0 represents the day at which confluence was achieved. Medium was subsequently replaced every 2 days. The level of I-FABP/hGH* expression was determined by measuring the concentration of hGH in medium at the time it is replaced. Hormone levels are referenced to the levels observed on day 0 in the same flask.

*Disregarding changes in protein turnover the predicted level of accumulation of hGH in these cells should be related to the integral of the transient expression (defined by the interval between medium changes).
this human cell line approximates but does not fully recapitulate the differentiation program of normal human enterocytes, it may produce "inappropriately" high levels of negative-acting transcription factors that serve to repress I-FABP-1178 to +29/hGH+4 and Fabpi expression.

A Nuclear Factor or Factors from Caco-2, but Not CV-1 Cells, Bind(s) to a 14-bp Repeated Element Present in Fabpi—The remarkable lineage specificity of I-FABP-103 to +28 expression observed in vivo led us to ask whether it binds gut-specific factors in nuclear extracts of small intestinal or colonic epithelial cells. When such extracts were prepared using a modification of the procedure of Gorski et al. (1986), we were able to identify a 24-bp element spanning nucleotides -212 to -188 that binds factors present in colonic but not small intestinal cells (Cohn et al., 1992). Our functional mapping studies in transgenic mice indicated that this sequence is contained in a region of rat Fabpi that suppresses expression in ileum and colon (see Fig. 1 and Cohn et al. (1992)). When nuclear extracts were prepared from spleen, liver, kidney, proximal small intestine, and colon and used for electrophoretic mobility gel shift assays with labeled nucleotides -103 to +28, we were not able to identify any reproducible gut-specific binding patterns, despite the fact that control studies with the 24-bp sequence confirmed our earlier observations (data not shown).

Because nucleotides -103 to +28 of rat Fabpi maintain a striking degree of lineage specificity in the epithelial cell culture experiments and because of the difficulties in preparing active nuclear extracts from the gut due to contaminating luminal (pancreatic) proteases, we reasoned that nuclear extracts derived from cell lines that support or prohibit I-FABP-103 to +28/hGH+3 expression could be used to identify functionally important transcription factors that interact with its promoter elements. Therefore, extracts were prepared from Caco-2 cells and CV-1 cells immediately after they achieved confluency. Caco-2 extracts produced several retarded complexes with a labeled DNA fragment representing nucleotides -103 to +28 of rat Fabpi while no complexes were observed with nuclear extracts prepared from CV-1 cells (Fig. 3A, compare lanes 2 and 6). Binding of Caco-2 nuclear factors to I-FABP-103 to +28 was competed by a 50-fold molar excess of an unlabeled double-stranded oligodeoxynucleotide spanning bases -103 to -50 (Fig. 3A, lane 3) but not by a 50-fold molar excess of an oligodeoxynucleotide representing bases -49 to +1 (lane 4).

Nucleotides -103 to -50 of rat Fabpi contain a 14-bp element between bases -82 and -69 that is present in a comparable position in mouse and human Fabpi (Sweetser et al., 1987; Green et al., 1992). Each of these orthologous genes contains 2–3 additional copies of this element (consensus, 5'-TGAACCT(T/C)GAACCT-3') located in similar positions of their 5' nontranscribed domains (Green et al., 1992; Cohn et al., 1992; cf. Fig. 1). This 14-bp sequence is also found in the promoter regions of the homologous rat cellular retinol binding protein II gene and in several nonhomologous genes that are expressed in differentiating members of the enterocytic lineage (Demmer et al., 1987; Sweetser et al., 1987). An unlabeled oligodeoxynucleotide representing bases -82 to -69 of rat Fabpi was able to block binding of Caco-2 nuclear proteins to labeled I-FABP-103 to +28 DNA (Fig. 3A, lane 5).

By changing the buffer conditions used for the electrophoretic fractionation of these I-FABP-103 to +28 DNA-Caco-2 nuclear protein complexes, we were able to identify three distinct labeled bands. The formation of all three labeled complexes was blocked by a 50-fold molar excess of an oligodeoxynucleotide representing bases -103 to -50 or -82 to -69 of rat Fabpi but not by an oligodeoxynucleotide spanning bases -49 to +1 (Fig. 3B, compare lanes 1–5).

The Conserved 14-bp Element Spanning Nucleotides -82 to -69 Binds Hepatic Nuclear Factor-4 and Apolipoprotein Regulatory Protein-1—Previous searches of a data base of known transcription factor binding sites (Ghosh, 1990) with the 14-bp sequence had not revealed any significant matches. However, recent reports (Tian and Schibler, 1991; Kuo et al., 1992) noted that the 5' nontranscribed domain of the mouse and rat hepatic nuclear factor-1 (HNF-1) gene contains a sequence that binds hepatic nuclear factor-4 (HNF-4), a member of the steroid hormone receptor superfamily (Sladek et al., 1990). This site in the HNF-1 gene (5'-GACTGAACTTTGGACTT-3') contains 4 of 12 bases that differ from a previously reported consensus sequence for HNF-4 binding (5'-GGCAAAGTTGTGGACTT-3'). Three observations led us to investigate whether HNF-4 or a related transcription factor could bind to the 14-bp sequence present between nucleotides -82 and -69 of rat Fabpi and what the functional consequences of such binding might be on expression of I-FABP-103 to +28/hGH+3 in Caco-2 and CV-1 cells. First, Mietus-Snyder et al. (1992) demonstrated that the human apolipoprotein (apo) CIII gene contains a sequence spanning nucleotides -86 to -74 that functions as a binding site for HNF-4 and two other closely related members of the steroid-thyroid hormone receptor superfamily of zinc finger transcription factors: apolipoprotein regulatory protein-1.

![Fig. 3. Formation of complexes between nuclear proteins present in Caco-2 cells and nucleotides -103 to +28 of rat Fabpi.](image-url)
According to Devereux repeated in the proximal promoter regions of rat, mouse, and human HNF-1 and apoCIII genes with the ARP-1/HNF-4 binding site in the apoCIII gene and revealed that 10 of 14 bases are identical (Fig. 4). TF (Miyajima consensus sequence of the 14-bp elements in rat conducted in HepG2 and Caco-2 cells showed that HNF-4 and ARP-1 bind to this cis-acting element in the human bryos revealed that HNF-4 and ARP-1 are co-expressed in 4 and ARP-1 have opposing effects on apoCIII expression; transcription (Mietus-Snyder et al., 1992). Second, studies conducted in HepG2 and Caco-2 cells showed that HNF-4 and ARP-1 bind to this cis-acting element in the human apoCIII gene (known as C3P) with similar affinities (Mietus-Snyder et al., 1992). Co-transfection studies indicated HNF-4 and ARP-1 have opposing effects on apoCIII expression; HNF-4 functions as a C3P-dependent transcriptional activator in Caco-2 and HepG2 cells while ARP-1 binding represses transcription (Mietus-Snyder et al., 1992). HepG2 and Caco-2 cells contain more HNF-4 than ARP-1 while CV-1 cells lack HNF-4 and ARP-1 (Mietus-Snyder et al., 1992). Finally, in situ hybridization studies using late gestation mouse embryos revealed that HNF-4 and ARP-1 are co-expressed in hepatocytes and in epithelial cells located in intestinal crypts and villi (Mietus-Snyder et al., 1992).

With these observations in mind, lysates were prepared from COS-7 cells transfected with a plasmid that directs expression of HNF-4 or ARP-1. Lysates prepared from cells transfected with the vector without insert DNA produce no retarded complex when incubated with nucleotides -103 to +28 of rat Fabpi (Fig. 5, lane 4). Lysates prepared from COS-7 cells transfected with the HNF-4 expression vector produce a single retarded complex that co-migrates with one of the three complexes produced by Caco-2 nuclear extracts (Fig. 5, compare lanes 5 and 2). Lysates prepared from COS-7 cells transfected with the plasmid encoding ARP-1 produce two distinct retarded complexes that co-migrate with the other two bands generated with Caco-2 nuclear extracts (Fig. 5, lane 9). Nucleotides -103 to -50 of rat Fabpi and an oligodeoxynucleotide derived from the TRH site in the HNF-1 gene block formation of the HNF-4-I-FABP-103 to +28 DNA complex (Fig. 5, lanes 7 and 8) and the ARP-1-I-FABP-103 to +28 DNA complexes (lanes 11 and 12). A 50-fold molar excess of an oligodeoxynucleotide derived from bases -49 to +1 of rat Fabpi has no effect on formation of complexes 1, 2, or 3 (lanes 6 and 10 of Fig. 5).

When cell lysates prepared from COS-7 cells transfected with the HNF-4 or ARP-1 plasmids are mixed together and incubated with nucleotides -103 to +28 of rat Fabpi, three complexes are formed that have electrophoretic properties which mimic those produced with Caco-2 cell extracts (Fig. 5, lanes 13 and 14). These co-migrating complexes suggest, but do not prove, that HNF-4 and ARP-1 contained in Caco-2 extracts bind to nucleotides -82 to -69 of rat Fabpi. By using a methylation interference assay, we were able to demonstrate that the factor(s) responsible for forming the Caco-2 DNA complexes produce a pattern of protection of I-FABP -82 to -69, which is identical to that produced by the HNF-4- and ARP-1-containing COS-7 cell lysates (Fig. 6).

Co-transfection of Caco-2 and CV-1 Cells with I-FABP/ hGH+DNA s and Plasmids Encoding either HNF-4 or ARP-
The functional consequences of HNF-4 or ARP-1 binding to the 14-bp sequence were investigated. Because transcriptional effects can depend upon promoter context and transcriptional environment, several I-FABP/hGH+3 DNAs were introduced into Caco-2 and CV-1 cells together with the plasmids specifying HNF-4 or ARP-1. I-FABP-277 to +28/hGH+3, I-FABP-184 to +28/hGH+3 and I-FABP-103 to +28/hGH+3 were used plus an additional construct containing two copies of the 14-bp element placed immediately upstream of nucleotide −104 to −277 of rat Fabpi (yielding I-FABP−277 to +28/hGH+3). This construct was designed to “amplify” any transcriptional effects specific to the 14-bp element and, in some sense, recreated the three repeats of this element found in I-FABP-277 to +28/hGH+3 (Fig. 1). A parallel set of experiments was performed using a recombinant DNA that contained the 14-bp element located between nucleotides -82 and -277 of rat Fabpi.

Introduction of a HNF-4 expression vector into proliferating Caco-2 cells produced no significant change in the levels of hGH production compared with the level observed in cells containing C3P-TK-hGH+3 alone. In contrast, co-transfection with pMT2-ARP1 and C3P-TK-hGH+3 resulted in a 6-7-fold reduction in expression (Fig. 7A). Finally, co-transfection of proliferating Caco-2 cells with pMT2-ARP1 (or pMT2-HNF-4) and MT-hGH+3 produced no changes in hGH production (data not shown).

Together, these results support the notion that pMT2-ARP1 changes the level of ARP-1 in proliferating Caco-2 cells and confirms the known response of the C3P element to this transcription factor (i.e. repression). The data also indicate that the 14-bp element located between nucleotides −82 and −69 of rat Fabpi can serve as a positive element when it binds ARP-1, although this latter effect appears to require and/or be influenced by sequence elements located between nucleotides −104 and −277 of rat Fabpi. While positive as well as negative transcriptional regulation by COUP-TF, a close relative of ARP-1, has been described (Wang et al., 1987), prior co-transfection studies have only demonstrated a negative effect with ARP-1 (Mietus-Snyder et al., 1992; Wi- dom et al., 1992; Cooney et al., 1992; Tran et al., 1992; Ladias et al., 1992; Ladias and Karathanasis, 1991).

Unlike proliferating Caco-2 cells, pMT2-HNF-4 produces modest but significant increases in expression of I-FABP-277 to +28/hGH+3 and I-FABP−277 to +28/hGH+3 in proliferating CV-1 cells, (levels of hGH production rise by 2-3-fold, p < 0.005; Fig. 7B). Removal of nucleotides −277 to −185 abolishes this stimulatory effect (Fig. 7B) while removal of nucleotides −277 to −104 converts the effect to a negative

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FIG. 7. Co-transfection of proliferating Caco-2 and CV-1 cells with I-FABP/hGH+3 DNAs and plasmids encoding either HNF-4 or ARP-1. hGH levels were determined in culture medium 64 h after transfection and expressed relative to the amount of β-galactosidase activity present in cell lysates prepared at the time medium was harvested. (RSV/β-Gal DNA was used as an internal standard to control for differences in transfection efficiency between experiments.) All experiments were repeated 4 times and the mean ± 1 S.D. plotted. Results are normalized to those obtained after co-transfecting cells with I-FABP/hGH+3 plus pMT2-null (the expression vector used to direct synthesis of ARP-1 or HNF-4).
one (hGH production falls 2-3-fold). The marked stimulatory effect of ARP-1 on 1-FABP-184 to +28/hGH+3 observed in proliferating Caco-2 cells was not observed in CV-1 cells where no change in hGH production was detectable (Fig. 7B). Additional control experiments demonstrated that co-transfection of proliferating CV-1 cells with pMT2-ARP1 and C3P-TK-hGH+4 produced a degree of relative suppression of hGH production similar to that observed in proliferating Caco-2 cells (Fig. 7, A and B). Finally, co-transfection with pMT-HNF-4 and C3P-TK-hGH+4 resulted in a significant (2-fold) stimulation of hGH production in proliferating CV-1 cells whereas no significant change was noted in proliferating Caco-2 cells (compare panels A and B in Fig. 7). These results suggest the following conclusions. First, there appears to be a sufficient pool of HNF-4 present in proliferating Caco-2 cells so that any increase in the level of this transcription factor produced by pMT-HNF-4 has little detectable stimulatory effect on either C3P-TK-hGH+4 or 1-FABP/hGH+277 to +28/hGH+3 expression. Such a pool of functionally available HNF-4 does not appear to be present in CV-1 cells. Alternatively, Caco-2 cells may produce other factors, not represented in CV-1 cells, that can compete with HNF-4 for binding to C3P and a site or sites in 1-FABP/277 to +28/hGH+3. Competition for binding to identical or highly conserved transcriptional regulatory sequences has been noted among various members of the steroid receptor superfamily of transcription factors that contain conserved, zinc finger DNA binding domains (Mietus-Snyder et al., 1992; Widom et al., 1992; Cooney et al., 1992; Ladisa et al., 1992). Second, the fact that ARP-1 stimulates 1-FABP/277 to +28/hGH+3, 1-FABP/277 to +28/hGH+3, and 1-FABP-184 to +28/hGH+3 in proliferating Caco-2 but not CV-1 cells may reflect the presence or absence of these other competing transcription factor(s) and/or the fact that an endogenous ligand or ligands for this orphan receptor are present in one cell type but not another. Third, ARP-1 and HNF-4 appear to function as positive factors when bound to the conserved 14-bp element spanning nucleotides -82 to -69 of rat Fabpi. However, expression of such effects appears to require the participation of additional cis-acting elements located between nucleotides -277 and -104 and/or other transcription factors. Synergistic or cooperative effects between nearby elements have been demonstrated in a number of promoters (e.g. Kuo et al. (1990), Widom et al. (1991), and Strahle et al. (1988)). It is likely that a similar mechanism may be involved with Fabpi, i.e. 1-FABP-184 to +28/hGH+3 but not 1-FABP-103 to +28/hGH+3 is up-regulated by ARP-1 in Caco-2 cells even though both constructs contain a single copy of the 14-bp sequence. Nonetheless, based on results obtained in transgenic mice, these postulated sequences located between nucleotides -104 and -277 are not sufficient by themselves to support Fabpi expression in enterocytes; analysis of 1-20-week-old mice belonging to 10 pedigrees containing nucleotides -277 to -104 linked to a neutral promoter/reporter (the hGH gene beginning at its nucleotide -84) failed to disclose any hGH production in any intestinal cell lineage distributed along the duodenal-to-colonic axis (or any one of nine extraintestinal tissues, Cohn et al. (1992)). Perspective—Functional mapping studies of the homologous rat Fabpi and liver fatty acid binding protein (Fabpl) genes in transgenic mice have indicated that distinct cis-acting elements regulate their patterns of expression along the crypto-villus and duodenal-to-colonic axes (Cohn et al., 1992, Simon et al., 1993). Moreover, these mapping studies have revealed that remarkably compact sequences located close to the start site of transcription (nucleotides -103 to -1 in Fabpi and nucleotides 132 to -1 in Fabpl) are apparently sufficient to establish and maintain appropriate cephalocaudal gradients of expression in enterocytes. However, comparative analysis of these sequences has failed to reveal any conserved domains other than their TATA boxes.

Further functional studies of these proximal promoter domains conducted in Caco-2 cells will have to be cautiously interpreted. Our comparison of the relative activities of 1-FABP-1178 to +28/hGH+3, 1-FABP-184 to +28/hGH+3, and 1-FABP-103 to +28/hGH+3 in transgenic mice and Caco-2 cells emphasizes the differences in the transcriptional regulatory environments present in proliferating preconfluent and differentiated postconfluent Caco-2 cells and those present in members of the enterocytic lineage during their migration-associated differentiation program.

Formation of duodenal-to-colonic or crypt-to-villus gradients of Fabpi expression likely reflects a complex system that includes competition of multiple factors such as HNF-4 and ARP-1 for common cis-acting sequences.3 Other transcriptional factors might also share this target sequence specificity. The conserved 14-bp element is quite similar to a direct repeat of the sequence TGACCT that serves as half of a "canonical" COUP-TF element (Kadowaki et al., 1992). COUP-TF can compete with retinoic acid, thyroid hormone, and vitamin D3 receptors for DNA binding and inhibit transcriptional activation (Cooney et al., 1992; Tran et al., 1992; Kliever et al., 1992; Widom et al., 1992). Some of these other members of the transcription factor superfamily may also interact with the 14-bp site. Lineage-specific, differentiation-dependent, and regional differences in the relative abundance of these transcription factors could yield a rich repertoire of patterns of expression mediated by such proteins. Heterodimer formation, which is common among members of this transcription factor family (Forman and Samuels, 1990), would add exponentially to the flexibility of such a system. Furthermore the distribution of transcriptional co-factors (such as S300-II for COUP-TF, Tsai et al. (1987)) or endogenous ligands for these "orphan" receptors could affect their transcriptional properties. In addition, epigenetic modifications may contribute to Fabpi's pattern of expression in the gut. We have found a gradient in the degree of methylation of Fabpi that parallels the level of its expression along the cephalocaudal axis.4 Such epigenetic changes could represent an economical way to establish and maintain position information, e.g. by modifying the affinity of cis-acting elements for all of their cognate factors or for some specific subset of them. These considerations suggest that a combination of in vitro, cell culture, and in vivo experimental systems will be required to characterize exactly how the activity of this remarkably compact Fabpi promoter is regulated.

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3 A Drosophila homolog of HNF-4 has been described recently that is strikingly similar in structure and distribution to the mammalian protein (Zhong et al., 1993). Deletion of the chromosomal region containing this gene prevents midgut development (Zhong et al., 1993). These data underscore the central role that HNF-4 and related factors likely play in gut development and gene expression.

4 J. N. Rottman and J. L. Gordon, unpublished observations. These differences in methylation are based on results obtained after MspI and HpaII digestion of DNA prepared from intestinal mucosa harvested at different positions along the duodenal-to-colonic axis.
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