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The *cis*-element *CE-LPH1* of the rat intestinal lactase gene promoter interacts in vitro with several nuclear factors present in endodermal tissues

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Abstract We have shown by electrophoretic mobility shift assays that the nucleotide sequence CE-LPH1, centred at position −49 with respect to the transcription start site of the rat gene encoding intestinal lactase-phlorizin hydrolase, interacts in vitro with nuclear proteins present in the jejunum of suckling animals. Proteins binding to this element were also found in organs of endodermal origin that do not (or no longer) express lactase-phlorizin hydrolase, i.e. the colon, lung and the liver, but not in the brain. However, a DNA–protein interaction was hardly detected with nuclear extracts prepared from adult tissues, although typical factors binding to the Sp1 binding site were detected at the adult stage as in the sucklings. Southwestern blotting experiments conducted with nuclear extracts prepared from the tissues of suckling rats indicated that CE-LPH1 interacts with several factors in the jejunum, colon, lung and the liver. Some of these DNA-binding proteins are specifically expressed in the jejunum or in the liver, whereas others seem to be shared with the colon and the lung. Hence, the *cis*-element CE-LPH1 located in close vicinity to the pseudo-TATA-box of the intestinal lactase-phlorizin hydrolase gene promoter interacts in vitro with a family of nuclear proteins which may represent markers of the endodermal lineage predominantly expressed prior to weaning.

Key words: Lactase gene promoter; *Cis*-element; Nuclear factor; Intestine; Endoderm; Rat

1. Introduction

The intestinal mucosa of mammals has been the subject of intensive investigation at the cellular level because the epithelium of the protruding villi continuously renews and differentiates from pluripotent stem cells anchored in invaginated crypts. The process of cell differentiation is dependent on the developmental stage, on the region along the antero-posterior axis of the intestinal tract, and on the cell position along the crypt-villus axis [1]. Yet, in spite of the large number of data accumulating at the cellular level, much less is known concerning the molecular basis of intestinal cell differentiation. To approach this question, recent studies have been conducted to get a further insight into the gene expression of intestinal markers of the mature enterocytes. In particular, the promoters of the genes encoding intestinal fatty acids binding protein (Fabpi) or sucrase-isomaltase (SI) have been characterised in transgenic mice [2] and/or by cell transfection assays [3–5]. The promoters of intestinal genes expressed prominently in crypt cells or in additional tissues besides the intestine have also been recently identified [6–8].

Lactase-phlorizin hydrolase (LPH; EC 3.2.1.23–62) is the intestinal enzyme responsible for the hydrolysis of milk lactose and glycosylceramides. In rats, the control of LPH expression is complex and involves regulatory mechanisms acting at the transcriptional and posttranscriptional levels [9,10]. The 5′ region of the LPH gene has been isolated in rat, human, and pig [11–13]. The 200-bp region located immediately upstream to the transcription start site is highly conserved in these mammalian species, unlike the further upstream region. In the pig, a *cis*-element (CE-LPH1) centred at position −46 with respect to the transcription start site binds in vitro to nuclear proteins extracted from the intestine of newborn and adult animals but not from the adult liver [13]. A sequence similar to CE-LPH1 occupies nearly the same position in the upstream region of the human and rat genes. In the present study we provide evidence that this sequence in the rat LPH gene promoter interacts in vitro with nuclear proteins present in the small intestine of suckling animals but hardly detected at the adult stage. Yet, binding proteins were also found in additional tissues of endodermal origin that do not or no longer express lactase-phlorizin hydrolase.

2. Materials and methods

2.1. Oligonucleotides

Oligonucleotides were synthesized chemically (Genset, Paris, France). After hybridization, the double-stranded oligonucleotides were radiolabelled by filling in the 3′ ends with [α-^{32}P]dATP (200 TBq/mmol, Amersham, Les Ulis, France) and the three other deoxyribonucleotides in the presence of Klenow DNA polymerase. They were separated from unincorporated nucleotides by electrophoresis on a non-denaturing 20% polyacrylamide gel and recovered by elution in water. Double-stranded non-labelled oligonucleotides used for competition experiments were processed identically.

Three pairs of oligonucleotides were synthesized from the rat genomic DNA region similar to the pig *cis*-element CE-LPH1. Their sequences are the following:

Lcel1a: 5′-ATGTTTTCAAGCCTTGGCTGT-3′
Lcel1b: 5′-TGAGCACAGCCAAGGCTTGA-3′
Scel1a: 5′-TTTTCAAGCCTTGGCT-3′
Scel1b: 5′-AGCCAAGGCTTG-3′
Dcel1a: 5′-ATGTTGGCTGT-3′
Dcel1b: 5′-TGAGCACAGCCAA-3′

The double stranded oligonucleotide Lcel1a/b covers 26bp of the rat genomic sequence centred at position −49 with respect to the LPH gene transcription start site. Scel1a/b is smaller and corresponds to 16 bp of the rat sequence centred at position −49. Dcel1a/b is a mutated form of Lcel1a/b with a 10bp deletion at the centre. The double-stranded Scel1a/b and Dcel1a/b have the same size.

Oligonucleotides carrying the Sp1 binding site (OLGSp1a/b) were also produced [13].

2.2. Nuclear extracts

Nuclear proteins from the proximal jejunum, colon, lung, liver and the brain were prepared as described by Roy et al. [14] from pools of

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twenty 10-day-old suckling rats and two 3-month-old adults. Briefly, the samples were homogenized in the NE1 solution containing 250 mM sucrose, 140 mM NaCl, 25 mM KCl, 15 mM Tris-HCl pH 7.9, 2 mM $MgCl_2$, 2 mM EDTA, 1 mM DTT, 0.5 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine and 0.4 mM PMSF. Nonidet P40 was added to 0.5% and the solution was centrifuged at $1,000 \times g$. The pellet containing nuclei was washed with NE1 until it was blood- and mucus-depleted. It was resuspended in NE2 (NE1 supplemented with 350 mM KCl), homogenized and clarified by a centrifugation at $12,000 \times g$ followed by an ultracentrifugation at $180,000 \times g$. The supernatant was dialysed overnight against the NE3 solution (50 mM KCl, 20 mM Phosphate buffer pH7.4, 4 mM $MgCl_2$, 1 mM β-mercaptoethanol, 20% glycerol) and stored at −80°C. Protein concentration was determined according to Bradford [15].

2.3. Electrophoretic mobility shift assays (EMSA)

For EMSA, 10 μg of nuclear extracts were incubated for 30 min at 25°C with 10,000 cpm of the radiolabelled double-stranded oligonucleotides in a binding buffer containing 50 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES pH 7.9, 10% glycerol and 1 μg/ml of poly(dI–dC). When stated, specific or non-specific competitors were added. 5% polyacrylamide gels in TAE buffer (6.7 mM Tris base, 3.3 mM Na Acetate, 1 mM EDTA) were pre-run for 30 min at 180 V, and the DNA–protein samples were then loaded on the gels for 2 h of electrophoresis at 4° C. Gels were exposed to autoradiography.

2.4. Southwestern blotting

The nuclear proteins (50 μg) were incubated for 30 min at room temperature in 2.5% SDS, 2.5 mM Tris-HCl pH 6.8, 50 mM DTT and 10% Glycerol, and loaded for electrophoresis on 15% polyacrylamide, 0.1% SDS gels. The electrophoresis buffer was 25 mM Tris base, 190 mM glycine, 0.1% SDS. Proteins were electrotransferred for 2 h to nitrocellulose filters in the electrophoresis buffer supplemented with 20% methanol. The filters were successively placed for 10 min in 6 M, 3 M, 1.5 M, 0.75 M, 0.37 M and 0.18 M guanidinium chloride, washed twice in the binding buffer containing 50 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES pH 7.9, 10% glycerol, placed for 1 h in binding buffer containing 1 μg/ml of poly(dI–dC) and supplemented with 5% skimmed milk, and then incubated overnight with the radiolabelled double-stranded oligonucleotides (10^6 cpm/ml). The filters were washed twice 10 min in binding buffer and exposed to autoradiography.

3. Results

We have shown by cell transfection experiments that the 230-bp genomic region upstream to the transcription start site of the rat LPH gene triggers the expression of a reporter gene in a human colon cancer cell line (in preparation). Therefore, this region is supposed to cover a part of the rat LPH gene promoter. Fig. 1 illustrates the sequence alignment of the proximal promoter of the pig, human and rat LPH genes. In pigs, the nucleotide sequence centred at position −46 has been identified as a *cis*-element (CE-LPH1) that interacts in vitro with intestinal nuclear protein(s) [13]. An identical sequence occupies the same position in human, and a similar sequence is found around position −49 in the rat. We asked whether this rat sequence also interacts in vitro with nuclear factors.

The double-stranded oligonucleotides Scel1a/b and Lcel1a/b cover 16 bp and 26 bp, respectively, of the sequence centred at position −49 in the rat LPH gene promoter. After radiolabelling by filling in the 3′ ends, each of these oligonucleotides was incubated in solution with nuclear extracts prepared from the proximal jejunum, colon, liver, lung and the brain of 10-day-old suckling rats. EMSA was performed to display a possible DNA–protein interaction. Identical results were obtained with the short (Scel1a/b) and with the long (Lcel1a/b) oligonucleotides. As shown in Fig. 2A,B, nuclear factor(s) binding in vitro to the labelled double-stranded oligonucleotide and forming a DNA–protein complex of low mobility were present in the jejunum, colon, liver and the lung. However no binding factor was detected in brain extracts. In contrast with Lcel1a/b and Scel1a/b, the double-stranded oligonucleotide Dcel1a/b corresponding to a 10-bp-deleted form of Lcel1a/b did not associate with nuclear proteins, as shown with samples prepared from the jejunum (Fig. 2B). The specificity of the DNA–protein interaction between Lcel1a/b and nuclear factor(s) was addressed using specific and non-specific competitors. As illustrated with nuclear extracts prepared from the jejunum and from the liver, the labelled oligonucleotide Lcel1a/b was competed out with a 50× molar excess of non-labelled oligonucleotide, whereas the DNA–protein interaction was not altered by a 500× molar excess of a non-specific competitor corresponding to the double-stranded oligonucleotide carrying the Sp1 binding site (Fig. 3, respectively lanes LC and NSC). In addition, the DNA–protein interaction between Lcel1a/b and nuclear factor(s) present in the jejunum was competed out by a 50× molar excess of the small Scel1a/b oligonucleotide whereas no competition was obvious with the deleted form of oligonucleotide: Dcel1a/b (Fig. 3, respectively lanes SC and DC). These data demonstrate the specificity of the binding between Lcel1a/b and nuclear protein(s) present in several organs including the jejunum, colon, liver and the lung, but absent in the brain of suckling rats.

As shown in Fig. 2A, the quantity of oligonucleotide incorporated in the DNA–protein complex was different in each organ of the suckling animals. Indeed, the highest level appeared in the liver and a decreasing amount was observed in the jejunum, in the lung and in the colon. In contrast with the situation in suckling rats, a DNA–protein complex of low mobility was hardly detected when EMSA was carried out with Lcel1a/b and nuclear proteins extracted from the jejunum and liver of adult animals (Fig. 2C). The quality of the nuclear extracts prepared from the jejunum and the liver of sucklings and adults was assessed by EMSA using the double-stranded oligonucleotide carrying the Sp1 binding-site as a nucleotide probe. Typically, three complexes of low mobility were detected in these tissues at both developmental stages (Fig. 2D).

Southwestern blotting experiments were performed to identify the nuclear factor(s) that interact in vitro with Lcel1a/b. For this goal, the nuclear extracts prepared from the jejunum, colon, liver, lung and the brain of 10-day-old suckling rats were fractionated by polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. They were incubated with the labelled double-stranded oligonucleotide and the protein bands interacting with the probe were revealed by autoradiography

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                                                        *
TTTACAA-CCTCAGTTGTAGTTATAAAGTAAGTATTCC----ACATACCTCCTAACAG  PIG
TTTACAA-CCTCAGTTGCAGTTATAAAGTAAGGGTTCC----ACATACCTCCTAACAG  HUMAN
TTTTCAAGCCTTGGCTGTGCTCATAAAGTTAGGATTCCTTCCACATG-CTTCTAACAG  RAT
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Fig. 1. Sequence alignment of the proximal promoter region of the pig, human and rat LPH genes. The transcription start site is labelled with an asterisk and the TATA-box is dotted. The bar shows the *cis*-element CE-LPH1 identified in the pig.

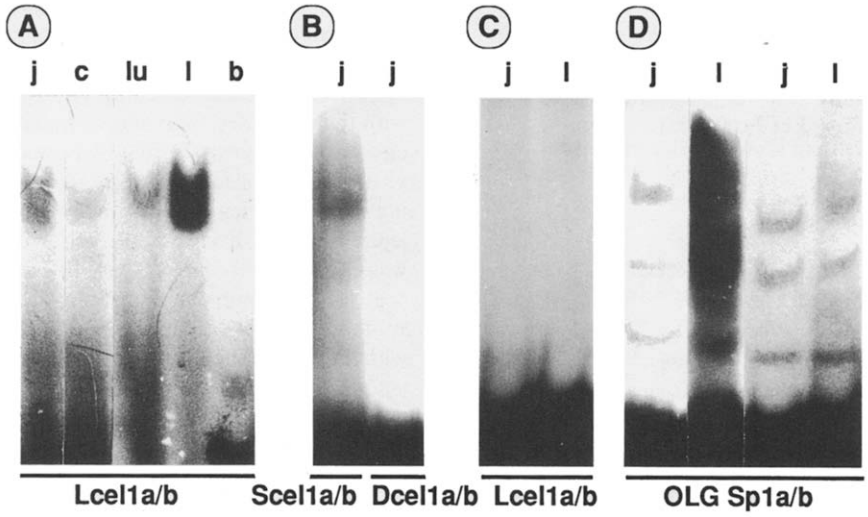

Fig. 2. (A) EMSA performed with the double-stranded oligonucleotide Lcel1a/b and nuclear extracts prepared from the jejunum (j), colon (c), lung (lu), liver (l) and the brain (b) of suckling rats. (B) EMSA carried out with jejunal extracts of suckling animals and the oligonucleotides Scel1a/b or Dcel1a/b, respectively. (C) EMSA with Lcel1a/b and nuclear extracts from the jejunum and liver of adult rats. (D) EMSA with the double-stranded oligonucleotide carrying the Sp1 binding site (OLGSp1a/b) and nuclear extracts from the jejunum and from the liver of suckling (left lanes) and adult animals (right lanes).

(Fig. 4A). Consistent with the results obtained by EMSA, Lcel1a/b interacted with nuclear proteins present in the jejunum, colon, liver and the lung of suckling animals but not in the brain. Noteworthy, the jejunum, colon, liver and the lung exhibited several binding proteins with different molecular masses in each organ. A major band of ~30 kDa and a minor band of ~22 kDa were revealed in the jejunum. In the colon and in the lung, two bands of ~30 kDa and ~35 kDa were found. The liver exhibited three binding proteins: two prominent molecular species of ~35 kDa and ~55 kDa, and a minor species of ~30 kDa. No binding protein of higher molecular mass was detected when electrophoresis was carried out using 7% instead of the 15% polyacrylamide gels (not shown). As shown in Fig. 4B, the formation of DNA–protein complexes was largely prevented when the labelled oligonucleotide Lcel1a/b was competed out with a 50× molar excess of non-labelled oligonucleotide. No specific binding was detected with the oligonucleotide pair Dcel1a/b (data not shown).

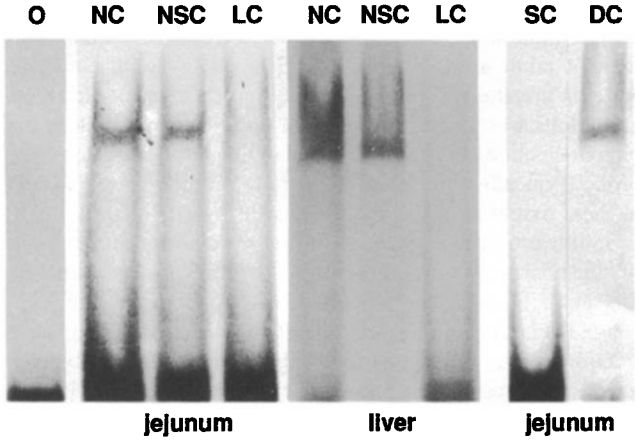

Fig. 3. EMSA was conducted with the labelled oligonucleotide Lcel1a/b and nuclear extracts from the jejunum and the liver of suckling rats, without competitor (lanes NC), with a non-specific competitor (a 500× molar excess of OLGSp1a/b, lanes NSC), and with a specific competitor (a 50× molar excess of non-labelled Lcel1a/b, lanes SC). Lanes SC and DC correspond to EMSA carried out with jejunal nuclear extracts using the labelled oligonucleotide Lcel1a/b, in the presence of a 50× molar excess of non-labelled Scel1a/b and Dcel1a/b, respectively. O shows the labelled oligonucleotide Lcel1a/b without nuclear extract.

4. Discussion

The comparative analysis of *cis*-elements and *trans*-acting factors involved in the control of expression of intestinal genes in different mammalian species may help to understand the key molecular mechanisms that govern cell differentiation in the intestinal epithelium. In this study, oligonucleotides corresponding to the region centred at position −49 in the rat LPH gene promoter have been examined because of a sequence similarity to the *cis*-element CE-LPH1 identified in the pig LPH gene promoter [13]. EMSA and southwestern blotting experiments indicate that this rat promoter sequence interacts in vitro with intestinal nuclear proteins present in suckling animals. Hence, it should represent the structural and functional homologue to the pig *cis*-element CE-LPH1.

The *cis*-element CE-LPH1 present in the pig LPH gene promoter binds to nuclear factor(s) expected to be 15 times more abundant in the intestine of suckling animals than in adults, and absent in the adult liver [13]. The rat homologous element also interacts with nuclear proteins present in the suckling intestine, and binding proteins were hardly detected in the adult intestine and liver. The Southwestern blotting experiments reported here provide further information concerning the nuclear factors that recognize CE-LPH1 in vitro. (i) Two factors present in a different proportion and/or exhibiting a different affinity to the oligonucleotide are found in the intestine. (ii) Binding proteins are also present in sucklings' organs that do not express LPH, i.e. the liver and the lung, or in the colon which does no longer synthesize this enzyme in 10-day-old animals. In this regard, it

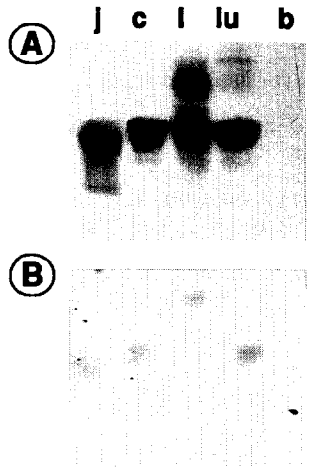

Fig. 4. (A) Southwestern blotting experiments carried out with the labelled double-stranded oligonucleotide Lcel1a/b and nuclear proteins extracted from the proximal jejunum (j), colon (c), liver (l), lung (lu) and the brain (b) of suckling rats. (B) Similar experiment as in (A) except that the nitrocellulose filter was incubated with a mixture of labelled and non-labelled oligonucleotide at a ratio of 1:50.

should be emphasised that all these organs share a common embryonic origin, as they derive from the endoderm, whereas the brain of neurectectodermal origin does not express CE-LPH1-binding factors. (iii) Some factors seem to be expressed in several organs (the 30 kDa and the 35 kDa proteins), whereas others are restricted to a single organ (the 22 kDa protein in the intestine and the 55 kDa protein in the liver). Altogether, these data suggest that nuclear proteins binding in vitro to CE-LPH1 may be considered as markers of the endodermal embryonic origin, which are predominantly expressed prior to weaning. Furthermore, they indicate on the one hand that there may be a competition between several nuclear factors to bind CE-LPH1 within the cells, and on the other hand that CE-LPH1-like *cis*-elements could exist in promoters others than that of the LPH gene, in particular in genes transcribed in endodermal tissues. A search in the EMBL data bank showed that an nucleotide sequence exhibiting some similarity to the rat CE-LPH1 *cis*-element is present in the 5′ upstream region of the rat α1-acid glycoprotein gene and in the human *N-myc* gene promoter [16,17].

The molecular basis of the ontogenic decline of lactase activity at weaning when the animals adapt from the suckling to the adult condition has been the subject of many investigations during the last years. Studies of enzyme activity, mRNA accumulation and gene transcription in different regions of the intestinal tract have shown that the expression of LPH in rats is regulated by a combination of transcriptional, posttranscriptional and posttranslational mechanisms, leading to an excess of mRNA over the enzyme at the adult stage in a large part of the small intestine (with the exception of the very proximal duodenum and of the distal ileum) [9,18,19]. The co-variation of the amount of CE-LPH1-binding factor(s) and lactase activity in adult pigs versus suckling animals [13] as well as the fact that a 1-kb segment of the LPH gene promoter directs postweaning decline of expression in transgenic mice [20] were interpreted as an indication that the ontogenic decline of lactase is primarily controlled at the transcriptional level. However, the maintenance of a high level of LPH mRNA in adult animals despite the reduced enzyme activity has been reported in pigs, as in rats [21,22]. The data obtained in the present study in rats suggest that more than one nuclear factor may interact with CE-LPH1 in the intestine, and that CE-LPH1-binding factors are present in tissues that do not express lactase. Hence, we conclude that the transcriptional status of the LPH gene during development may not be exclusively fixed by the amount of nuclear factor interacting with CE-LPH1. In the Fabpi gene, for instance, the rate of transcription depends on *cis*-elements located in closed vicinity to the TATA-box, in combination with far upstream regulatory elements which sustained or suppress the transcription in distinct parts of the intestine, at specific developmental stages [2].

The proximal promoter region (up to the position −100 to −200 with respect to the transcription start site) of the intestinal genes encoding SI and Fabpi is sufficient to trigger the expression of a reporter gene in cell transfection experiments [3–5]. The 142 bp upstream region of the pig LPH gene [13] and the 230 bp upstream region of the rat gene (submitted) also promotes the expression of a reporter gene in transfected colonic cells. Noteworthy, the *cis*-elements identified so far for all these intestinal genes expressed in the differentiated enterocytes do not share obvious sequence similarity, suggesting that multiple and distinct molecular paths are required to control the transcription of the marker genes, the expression of which defines the coordinated differentiation of the intestinal epithelial cells. The definitive characterization of intestinal nuclear factors may help to get more insight into the differentiation process of the enterocytes, and it could also account for recent puzzling observations. Indeed, the *cis*-element SIF1 of the SI gene promoter and CE-LPH1 may bind to a similar factor [23]. SIF1 has been shown to interact with the homeoprotein CDX3 [24], while CDX3, in contrast with CE-LPH1 binding proteins, is expressed at a high level in the adult intestine [24,25].

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