The tissue-specific regulation of the carboxyl ester lipase gene in exocrine pancreas differs significantly between mouse and human

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The carboxyl ester lipase (*CEL*) gene is highly expressed in exocrine pancreas and expression of the human *CEL* gene is mediated by a strong tissue-specific enhancer, which is absolutely necessary for high-level expression. The mouse promoter, on the other hand, does not contain a corresponding enhancer element, but instead is totally dependent on another pancreas-specific element. This element is identified as a pancreatic transcription factor 1 (PTF 1)-binding site. The human CEL promoter also contains a putative PTF 1 element located at a position corresponding to the essential PTF 1 site in the mouse promoter. However, nucleotide changes in the human promoter 5′ flanking this PTF 1 site have created an overlapping CCAAT/enhancer-binding protein (C/EBP)-like binding motif, interfering with the binding of PTF 1. Hence, our findings provide an example of genetic divergence between species not accompanied by difference in function.

Key words: C/EBP, genetic divergence, PTF 1, transcriptional regulation.

INTRODUCTION

The proliferation and differentiation from a single stem cell to a specialized cell type involves a very intricate and fine-tuned regulation of gene expression. For each phenotype, and at each time point, a selected set of genes is needed. The genes are part of a hierarchical system in which a certain gene, by its corresponding protein, controls a gene further down the route by modulating its level of expression.

The development of the mammalian pancreas has been a useful system for studying these mechanisms. Even though pancreatic cells are believed to originate from the same ancestral cell type [1], the terminally differentiated cells of the endocrine and exocrine glands strictly express different sets of specific genes. Whereas exocrine cells synthesize and secrete digestive enzymes, endocrine cells synthesize and secrete different peptide hormones. By studying the regulation of different pancreatic genes, several important transcription factors have been identified. For example, regulation studies of genes expressed in terminally differentiated endocrine cells have identified factors such as isulin promoter factor 1 (IPF1), islet LIM/homeodomain transcription factor 1 (ISL1) and paired box protein 4 (PAX4) [2,3]. These are not only important for the expression of particular genes but have also been shown to be of importance for the development of the entire pancreas.

So far, little is known about the transcription factors that are involved in exocrine gene expression and about the factors that are involved in exocrine cell differentiation. From studies of transcriptional regulation of exocrine-specific genes the pancreas transcription factor 1 (PTF 1) [4] has been found to be necessary for exocrine expression. PTF 1 is a multiprotein complex containing three distinct subunits; p75, p64 and p48. The p75 and p64 subunits are still not characterized whereas the p48 subunit has been cloned and appears to be a new member of the bHLH (basic helix–loop–helix protein) class of transcription factors [5]. It has also been shown that p48 is important for the differentiation of exocrine cells since exocrine pancreas tissue is completely absent in p48-deficient mice [6].

However, it seems that PTF 1 alone is not sufficient to modulate the expression of individual genes because it needs to co-operate with other transcription factors. For example, efficient expression of the α-amylase II gene requires binding of both PTF 1 and hepatic nuclear factor (HNF)-3β or HNF-3γ [7]. Furthermore, from studies of the rat elastase I promoter, an exocrine-specific enhancer including three different elements (A, B and C) was identified. The factor binding to the A element is PTF 1. To the B element a complex binds containing pancreatic and duodenal homeobox transcription factor 1 (PDX1), PBX1B and MRG1 [the product of a melanocyte-specific-gene (MSG)-related gene] [8], while the factor that binds to the C element is still not identified [9]. This may reflect a need for gene-specific activator complexes for different exocrine-specific genes.

An important group of enzymes expressed in exocrine pancreas is the lipases. In contrast with the proteases, relatively little is known about the transcriptional regulation of the lipase genes. Studies of the pancreatic co-lipase gene indicate the need of PTF 1 for proper expression [10,11]. From recent studies of the human carboxyl ester lipase (*CEL*) gene, which is highly expressed in the exocrine pancreas and lactating mammary gland, a pancreas-specific enhancer was identified and characterized [12]. High-level expression of the *CEL* gene in exocrine pancreas, but not in mammary gland, is absolutely dependent on this enhancer. Characterization of the enhancer reveals two closely located *cis* elements that co-operate to give exocrine pancreas-specific function. None of these elements binds PTF 1, but instead the interacting complexes seem to be composed of unknown factors. However, the enhancer seems to co-operate with other factors binding to elements located in the proximal region of the 5′-flanking sequence.

In this study, we identify and characterize other co-operative *cis* elements in the proximal 5′-flanking region of the human *CEL* gene. Comparative studies of human and mouse *CEL* genes

Abbreviations used: PTF 1, pancreas transcription factor 1; CEL, carboxyl ester lipase; C/EBP, CCAAT/enhancer-binding protein; EMSA, electrophoretic mobility-shift assay; mDPE, mouse distal promoter element.

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reveal differences with respect to transcriptional regulation in exocrine pancreas between the species. The most essential elements do not seem to be conserved although the expression patterns are the same in the both species.

EXPERIMENTAL PROCEDURES

Cloning and reporter gene constructs

The 5′-deletion series of the human and mouse *CEL* gene promoters have been described previously [12,13]. New constructs were made mainly by restriction-enzyme digestion or PCR-based site-directed mutagenesis (Quik change™ site-directed mutagenesis kit, Stratagene) but for some of the constructs PCR amplification was necessary. The promoter sequences in these constructs were verified by sequence analysis using an automated laser fluorescence sequencer (Amersham Pharmacia Biotech).

Cell culture, transient transfections and reporter-gene assays

The rat pancreatoma cell line AR4-2J and the rat fibroblast cell line RAT 2, both obtained from the A. T. C. C., were cultured at 37 °C in a 5 % CO_2/95 % air atmosphere in Dulbecco's modified Eagle's medium containing 2 mM glutamine and 4.5 g/l glucose and supplemented with 10 % fetal calf serum and 1 % penicillin/streptomycin.

The cells were transiently transfected with a mixture of 30 μl of lipofectin and 10 μg of promoter construct in serum-free medium. To correct for variations in transfection efficiency, 1 μg of a pCMV-CAT reporter plasmid was co-transfected as an internal control. After incubation for 24 h fresh medium was added, and after an additional 16 h of incubation the cells were harvested. Luciferase assay was performed using the Promega kit, with 70 μl of extract, and assayed in a luminometer (Berthold, Pforzheim, Germany). For the chloramphenicol acetyltransferase (CAT) assay, endogenous deacetylation activities was removed by heat-inactivation and then the reaction was performed using 70 μl of extract, [^{14}C]butyryl-CoA and chloramphenicol in an automatic scintillation counter (Beckman) as described previously [14]. The luciferase activities were normalized to corresponding CAT activities. Each construct was transfected on duplicate dishes and average luciferase activities were calculated and represented as means ± S.E.M. based on a minimum of three independent transfections.

Nuclear protein preparation

Preparation of nuclear extracts for electrophoretic mobility-shift assay (EMSA) were carried out as described in [15]. The extracts were aliquoted and stored at −70 °C prior to use. Protein concentrations of the extracts were determined by the method of Bradford [16] (Bio-Rad).

EMSA

The oligonucleotides used in the EMSA reactions were synthesized on a Beckman Oligo 1000. For each EMSA reaction, 25000 c.p.m. (by the method of Cerenkov [15]) of labelled double-stranded oligonucleotide were incubated in a 20-μl reaction volume containing 20 mM Hepes (pH 7.9)/50 mM KCl/10 % (v/v) glycerol/2 mM $MgCl_2$/0.5 mM EDTA/0.1 mg/ml BSA/0.5 mM dithiothreitol, with 1.5 μg of poly(dI-dC) and 4 μg of nuclear extracts. In some cases unlabelled double-stranded oligonucleotides were included in the incubation mixture as competitors. The binding reactions were incubated at room temperature for 15 min and then loaded on a gel containing 6 % polyacrylamide (29:1), 5 % glycerol, 25 mM Tris, 190 mM glycine and 1 mM EDTA. In supershift assays nuclear extract was incubated for either 15 min at room temperature or 45 min at 4 °C with a CCAAT/enhancer-binding protein (C/EBP) β-specific polyclonal antibody or a polyclonal antibody that reacts with C/EBP α, C/EBP β, C/EBP δ and C/EBP ϵ (all from Santa Cruz Biotechnology) before addition of DNA.

Northern-blot analysis

Poly(A$^+$) RNA (1 μg) from mouse and human pancreas was electrophoresed on a 1 % denaturing agarose gel and subsequently blotted on to a nylon membrane. The filter was hybridized under reduced stringency using a mouse CEL cDNA fragment as probe (nt 187–459) [17]. This region shares 87 % homology with the human sequence. Pre-hybridization and hybridization were carried out with 50 % formamide at 35 °C. Washes were performed with solutions containing 2 × SSC/0.1 % SDS (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) at 35 °C for 15 min, followed by a 15-min wash in 1 × SSC/0.1 % SDS, and finally washed in 0.1 × SSC/0.1 % SDS for 5 min. To correct for variations in the amount of mRNA in both samples, the filter was also hybridized with a human β-actin cDNA probe [18].

RESULTS

PTF 1 interacts with the enhancer in the human *CEL* gene promoter in exocrine pancreas

In a previous paper we showed that the first 850 bp of the 5′-flanking region of the human *CEL* gene is sufficient for high-level expression in exocrine pancreas [12]. The main element in mediating this expression is a strong pancreas-specific enhancer located at −672 to −637. When fused to a 62-bp minimal promoter, this enhancer alone has the capability of directing tissue-specific expression in exocrine pancreas. Earlier reported results also indicated the presence of important elements in the region between −317 and −63, since deletion of this region in the hCEL-1640Luc construct reduces the promoter activity

Table 1 Sequences of the oligonucleotides used

The sequences in bold represent PTF 1- and C/EBP-binding sites and the underlined sequence shows the potential C/EBP-binding site in the human promoter. The mutated bases are shown in bold lower-case letters.

Sequence	Description
5′-CGCCACCTTGC**CACCTG**CCTCTGC**TCCCA**GGTAAG-3′	hPTF 1:d
5′-CGCCACCTTGC**CACCTG**CCTCTGC**TCatA**GGTAAG-3′	hPTF 1:dm
5′-AAGGC**AGGGA**AAAGCA**CAGGTG**ACATG-3′	PTF 1 elastase I
5′-CTGGGATTCGCACCCAGCTTGG-3′	Unrelated in Figure 1
5′-ACC**TGGGA**AAAAATGGACCATCCCA**CACCTG**TGTGT-3′	mPTF 1:p
5′-ACC**TttGA**AAAAATGGACCATCCCA**CACCTG**TGTGT-3′	mPTF 1:pm
5′-CTCCA**TGGGA**GTTTCTGAAGAACCTT**CAGCTG**TGCAC-3′	PTF 1 α-amylase II
5′-CTGTGCCACTATGTCCTCCCCTGCCAGG-3′	Unrelated in Figures 5–7
5′-GTT**TGGGG**AAAACTGGATCTCCCTG**CACCTG**AGG-3′	hPTF 1:p
5′-CAGGGCCAGCCTGGGTTTGGGAAAAAATGG-3′	mGGTT
5′-AGTCAGTGGG**CGTTGCGCCA**CGATCT-3′	C/EBP
5′-GCACTGTGGGT**gactaGtcA**ACTGGATCTCCCTGCACCTGAGG-3′	C/EBPm
5′-AGCCCACCCATTTTTCTGAAGGTGACACTA-3′	wt
5′-AGCCC**gtgaca**TTTTCTGAAGGTGACACTA-3′	mut 1
5′-AGCCCACCCAT**ggcgga**GAAGGTGACACTA-3′	mut 2
5′-AGCCCAACCCATTTTCT**tt**AG**ccaa**CACTA-3′	mut 3

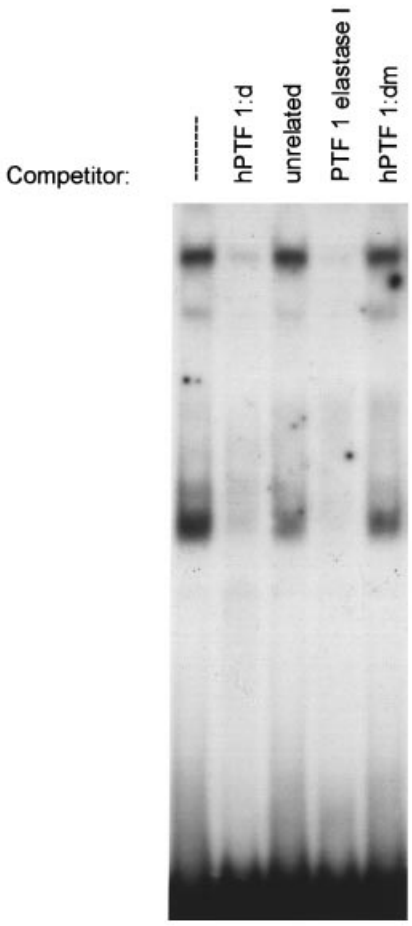

Figure 1 The interaction of PTF 1 with the human enhancer

EMSA analysis of the protein interaction to the human PTF 1 element at position −175 with 4 μg of nuclear extract from the AR4-2J cells. Unlabelled competitor oligonucleotides are in 100-fold molar excess. hPTF 1:d represents a specific competitor, 'unrelated' represents an unspecific oligonucleotide, PTF 1 elastase I represents an oligonucleotide containing the rat elastase I PTF 1 element [19], and hPTF 1:dm represents an oligonucleotide containing a mutated PTF 1 element (Table 1).

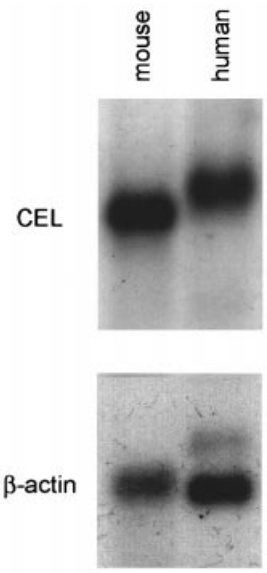

Figure 2 Northern-blot analysis of endogenous *CEL* gene expression in mouse and human pancreas

Poly(A^+) RNAs (1 μg) from mouse and human pancreas were analysed for the presence of CEL mRNA using the Northern-blot technique. The filter was hybridized with a mouse CEL cDNA probe [17] (upper panel) and a human β-actin cDNA probe [18] (lower panel) as described in the Experimental procedures section. The size difference of the CEL transcript between the two species is in agreement with earlier-reported results that have revealed that the mouse transcript is 396 bp shorter than the human transcript [17].

markedly [12]. Hence, placed in its natural context, the enhancer is dependent on the presence of other elements for exercising its effect. Since the reduction in activity for the construct hCEL-1640△156/63Luc was much less than for the construct with a deletion of the sequence between −317 and −63, we directed our search for important elements towards the region between −317 and −156.

With the purpose of identifying such response elements, the region between −317 and −156 was analysed for the presence of sequences with similarities to known *cis* elements. This revealed that there is a putative PTF 1 element, present at −175 (see Figure 3B, below) [4]. To analyse the capacity of the putative PTF 1 element to interact with nuclear proteins, an oligonucleotide, hPTF 1:d (Table 1), was made. After radiolabelling, the oligonucleotide was used in binding assays with nuclear extract from AR4-2J cells. As shown in Figure 1, the oligonucleotide shows a strong interaction. The specificity of the interaction was confirmed by the fact that it can be efficiently competed with by an excess of unlabelled oligonucleotide but not by an excess of an unrelated unlabelled oligonucleotide or an oligonucleotide containing a mutated PTF 1 site. Also, the interaction can be successfully competed with by an unlabelled oligonucleotide containing the elastase I PTF 1 element [19] (Table 1). To further confirm that this interaction involves binding of PTF 1 we labelled the elastase I oligonucleotide and used it in binding assays with nuclear extracts from AR4-2J and RAT 2 cells. The results indicate that the elastase I oligonucleotide gave the same interaction as the hPTF1:d oligonucleotide and that no interaction was detected in the RAT 2 nuclear extract (results not shown).

To determine if the PTF 1 element is responsible for the activation potential achieved from the −317/−153 region, a hCEL-839Luc construct with a mutated PTF 1 site was made. Mutation of the PTF 1 element at nt −175 reduces the promoter activity to 30% (results not shown).

As has been reported earlier there is only a slight increase in activity when extending the promoter from −156 to −317 [12], which indicates that the PTF 1 interaction alone is not capable of affecting the activity. In order to investigate whether there is an interaction between PTF 1 and the enhancer, a specific PTF 1 mutation was introduced into the constructs hCEL-322Luc and hCEL-632Luc. When these constructions were transiently transfected in AR4-2J cells there was no difference in activity between the mutated and the corresponding wild-type constructs (results not shown). These results indicate that PTF 1 requires the enhancer to be able to affect the expression. In conclusion, we have shown that PTF 1 (binding to the distal site) is involved in the regulation of the human *CEL* gene, and that the enhancer is dependent on this factor.

Expression of the mouse *CEL* gene in exocrine pancreas

As regulatory systems usually are conserved between different species we wanted to analyse whether the mouse *CEL* gene is

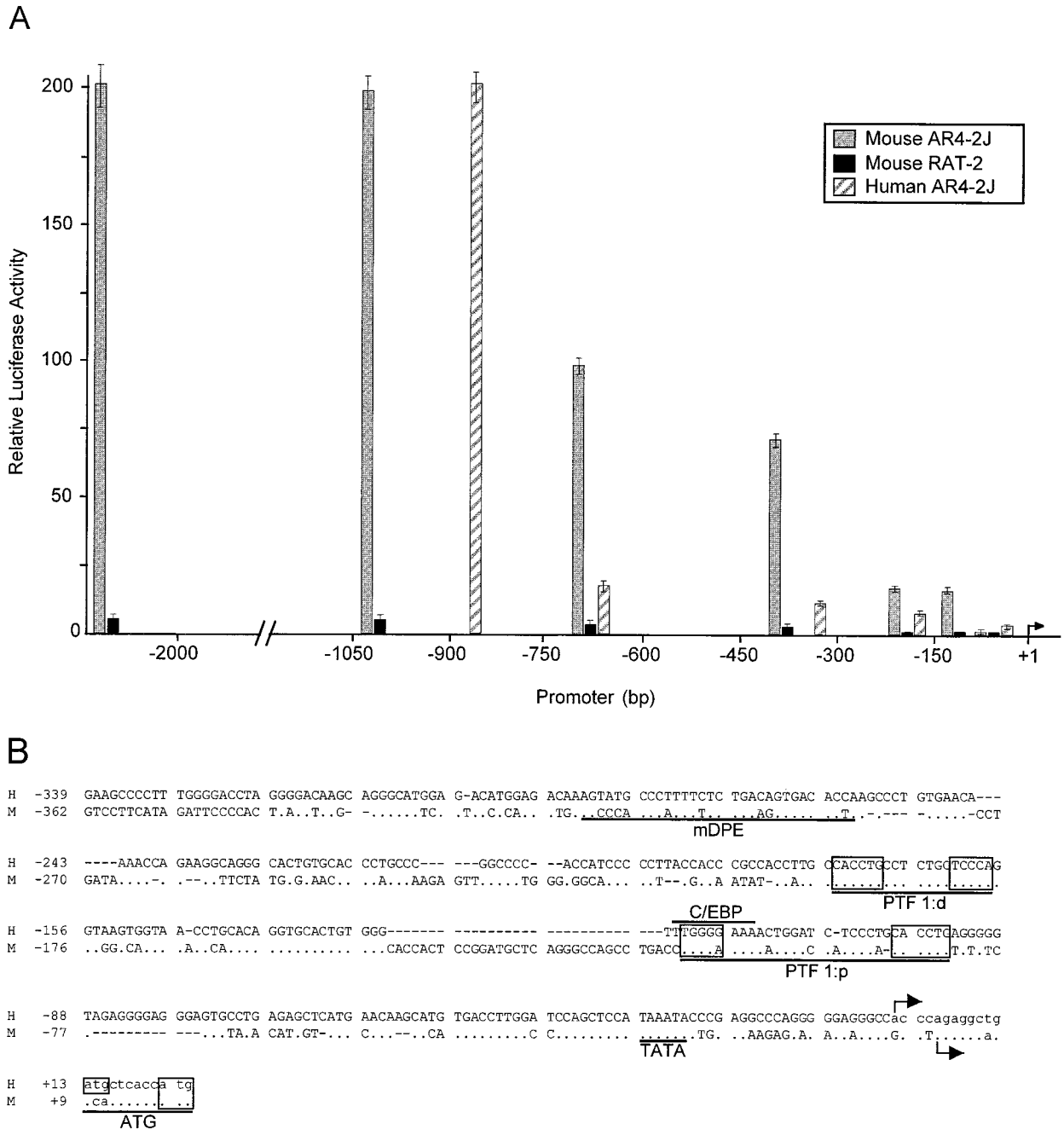

Figure 3 Reporter-gene analysis of a 5′-deletion series of the mouse *CEL* gene promoter

(**A**) A set of 5′-deletion constructs of the mouse CEL promoter, extending from +17 to −2158 relative to the transcription-initiation site, was transiently transfected into AR4-2J and RAT 2 cells. Some of the human CEL promoter constructs [12] are shown as comparison. Bars represent luciferase activities which are expressed in arbitrary units relative to the activity produced by the UMS-Luc construct, which is the pGL2-basic vector (Promega) with an upstream mouse sequence (UMS) inserted [12]. The UMS-Luc construct was adjusted to the value of 1. The *x* axis indicates the nucleotide position of the promoter sequence relative to the transcription-initiation site, which is indicated by an arrow. (**B**) Sequence comparison of the proximal promoter from the human and mouse *CEL* genes. The proximal 339 bp of the human (H) and the proximal 362 bp of the mouse (M) *CEL* gene 5′-flanking regions. Nucleotide positions are relative the transcription start site and are shown to the left. Dots represent identical nucleotides, whereas a gap is represented by a dash. The transcription start sites are indicated by arrows. The translation start site, the TATA box and the putative motifs are underlined, except for the C/EBP site which is marked above the sequence. The putative A and B boxes of the PTF 1-binding elements are boxed. The PTF 1:p and PTF 1:d indicate the binding sites in both promoters while C/EBP and mDPE (mouse distal promoter element) indicate the binding sites only in the human and mouse promoters, respectively.

regulated by the same factors as the human gene. The 5′-flanking region of the mouse *CEL* gene has recently been sequenced [13]. When performing sequence comparisons between the two promoters it seems that the strong pancreas-specific human enhancer is not present at the corresponding region in the mouse promoter. This raises the question of whether the pancreatic expression of the mouse *CEL* gene is lower than the corresponding expression of the human *CEL* gene. To compare the endogenous expression level of the *CEL* gene in exocrine pancreas between mouse and human a Northern blot was performed. Poly(A^+) RNAs from

mouse and human pancreas were prepared and, after blotting, the filter was hybridized with reduced stringency using a mouse CEL cDNA fragment as a probe. The result reveals that the expression levels of the *CEL* gene in the two species are approximately comparable (Figure 2). This suggests that the two promoters have a similar activity, even though that of the mouse is missing the strong enhancer found in the human promoter. Hence, other elements must be present in the mouse promoter that compensate for the element corresponding to the human enhancer.

The proximal 375 bp of the mouse 5′-flanking region is sufficient for high-level tissue-specific expression

In order to identify the essential regulatory elements for the regulation of the mouse *CEL* gene, a mouse promoter construct containing 2158 bp of the mouse promoter fused to the luciferase reporter gene (mCEL-2158Luc) [13], was analysed by transient transfection in the cell line AR4-2J. As shown in Figure 3(A), the mCEL-2158Luc construct exhibits strong activity, approx. 160 times higher than the activity of the core promoter construct mCEL-72Luc. This is of similar magnitude to the human construct, hCEL-839Luc. These results correlate with the Northern-blot results showing similar expression levels in mouse and human, and they indicate that the most important elements for the pancreatic expression of the mouse *CEL* gene are present within this fragment. Furthermore, the transcriptional activity of the mouse *CEL* gene promoter seems to be tissue-specific since almost no promoter activity was achieved from transfection into the RAT 2 cells.

To further investigate the regions of importance for the promoter activity a series of 5′-promoter deletion constructs was analysed. As can be seen in Figure 3(A), progressive deletions of the region from −2158 to −375 result in a stepwise decrease in activity. Further deletion, down to −178, leads to a 4-fold decrease in activity, which indicates the presence of important elements. Furthermore, deletion down to −73 results in the loss of virtually all activity. Hence, the proximal 375 bp of the mouse 5′-flanking region is sufficient for high-level pancreas-specific expression and the region between −375 and −73 appears to contain essential elements for this activity.

A PTF 1-binding element located at −111 is crucial for expression of the mouse *CEL* gene

Sequence comparison between the first 350 bp of the mouse and the human *CEL* gene promoters reveals that this region is highly conserved between the two species (Figure 3B). Despite this, the two regions have remarkably different activation potential. To examine whether nucleotide differences between the promoters are responsible for this difference, chimaeric mouse–human reporter constructs, called Swaps A–F (Figure 4A), were made. The constructs were transiently transfected into AR4-2J cells and as can be seen in Figure 4(B) the activation potential is 25-fold higher for Swap A compared with Swap B. The nucleotide differences between the two constructs are that the mouse sequence −178 to −73 in Swap A has been replaced by the human sequence −152 to −63 in Swap B. The higher activation potential of the mouse region between −178 and −73 compared with the corresponding region in the human promoter is also confirmed by Swap D and Swap E.

The results obtained from transfection of the 5′-deletion series and the different chimaeric constructs indicate that the region between −178 and −73 in the mouse *CEL* gene promoter contains elements that are essential for activity. A computer analysis revealed that there is a putative PTF 1-binding element located at position −111 in this region (Figure 3B). To investigate whether this site interacts with PTF 1, an oligonucleotide mPTF 1:p (Table 1) extending from −114 to −79 in the mouse promoter was used in an EMSA. Results from this assay show that there is a specific binding to the PTF 1 site (Figure 5A). The specificity of this interaction was demonstrated by the ability to efficiently compete for the interaction using a 100-fold molar excess of a specific competitor, whereas an unspecific oligonucleotide or an oligonucleotide containing a mutated PTF 1 site was unable to compete. Furthermore, the interaction could also be efficiently competed with by an oligonucleotide containing the α-amylase II or the elastase I PTF 1 elements [19], which clearly demonstrates the presence of PTF 1 interactions.

To determine whether binding of PTF 1 to this site is responsible for the increased activity observed in this region, a 2-bp mutation was introduced into the PTF 1 site in the construct mCEL-375 Luc. This mutation almost entirely abolishes the activity. Furthermore, in order to have high affinity for the PTF 1 factor, the A and B boxes should be separated by approximately integral turns of B-form DNA. Therefore we made a construct in which three bases between the A and B boxes were deleted. This deletion resulted in loss of virtually all activity. To rule out that this was not due to the three bases themselves, a new construct was made in which the bases were not deleted but merely mutated. These mutations did not affect the activity at all, confirming that the spacing between the A and B boxes is important for appropriate function of the site (results not shown).

As shown in Figure 3(B) this proximal PTF 1 element is conserved in the human promoter; hence it is quite surprising that it does not have the same capability to activate transcription as that in mouse. Comparison of the mouse and human elements shows that they differ at six nucleotides in the motif (Figure 5B). To analyse whether these differences affect the capability to interact with PTF 1, an EMSA was performed. As shown in Figure 5(B), PTF 1 binds with significantly higher affinity to the mouse element compared with that of the human, which might explain the difference in activation potential. To test whether these nucleotide differences between the promoters also are responsible for the difference in activation potential, a construct in which the human PTF 1 element was introduced into the mouse sequence was made. However, the result from this trial revealed that when the human PTF 1 element is placed in the mouse promoter it functions just as well as the mouse PTF 1 element (results not shown). This gives rise to the question of whether there are other nucleotide differences in this region that affect the binding of PTF 1 to the human element. When comparing the sequence between the two species we found that there has been a deletion of 30 bp in the human promoter in the flanking region just 5′ of the PTF 1 element (Figure 3B). Another difference is that the human sequence contains two Ts just in front of the PTF 1 site, whereas at the corresponding positions there are two Cs in the mouse sequence. In order to investigate whether these differences have something to do with the capability of PTF 1 to interact, we made a mutated mouse construct in which the mouse sequence between −114 and −112 (ACC) was replaced with GGTT. Introduction of the GGTT sequence in the construct mCEL-375Luc resulted in a reduction of the promoter activity to 25% (results not shown).

As can be seen in Figure 5(B) there is another, stronger interaction with the human PTF 1 oligonucleotide that is not observed for the corresponding mouse PTF 1 oligonucleotide. To examine if this shift corresponds to interaction of a factor with the 5′ end of the hPTF 1:p oligonucleotide an EMSA was performed. Since the shift could be efficiently competed with by

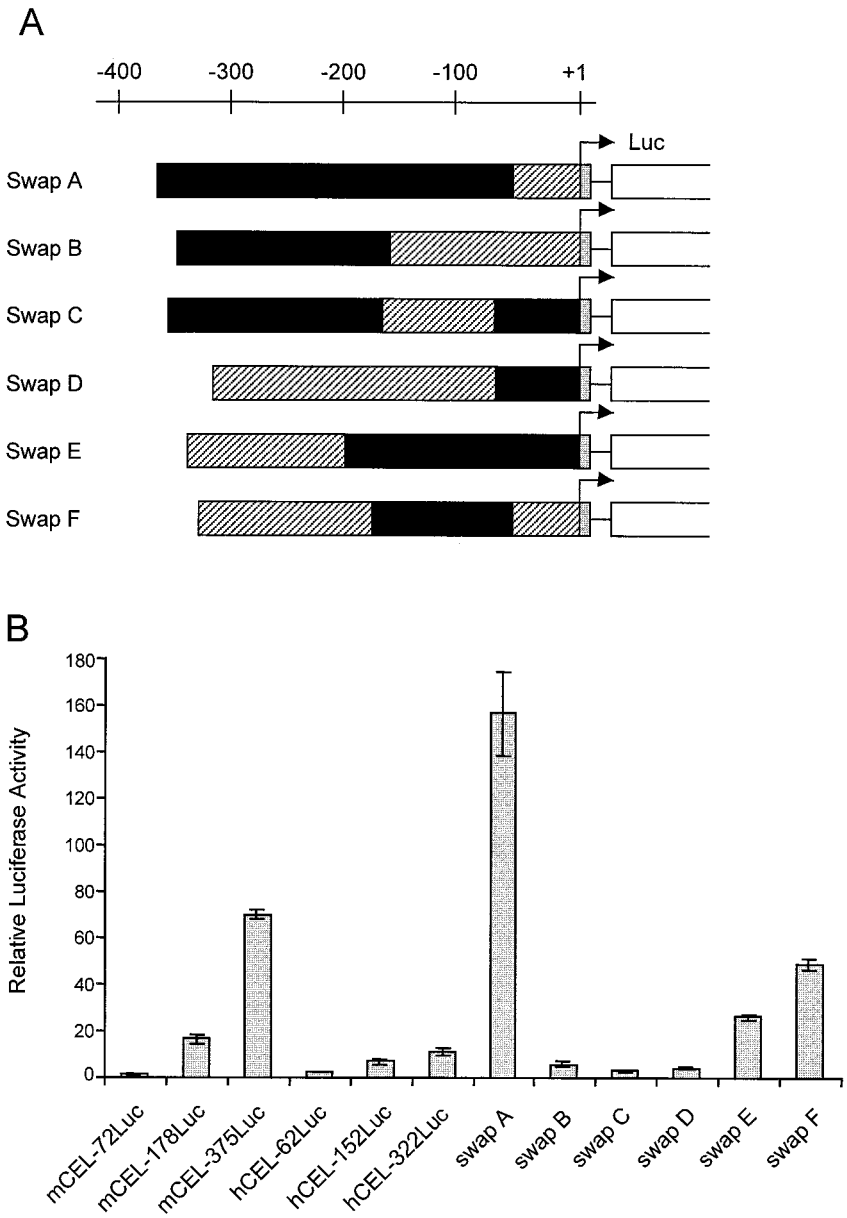

Figure 4 The region between −178 and −73 in the mouse *CEL* gene promoter contains elements that are essential for high-level expression

(**A**) Design of the mouse–human hybrid reporter-gene constructs, Swaps A–F. The mouse gene promoter is shown in black whereas the human gene promoter is represented by the hatched area. In Swap A the mouse promoter region between −375 and −73 was fused to the −62 fragment of the human promoter. Swap B consists of the −152 region of the human promoter fused to the −375/−179 fragment of the mouse promoter. Swap C was generated by replacing the region between −178 and −73 in the mouse promoter with the −152/−63 region of the human promoter. In Swap D the human region between −322 and −63 was fused to the −72 fragment of the mouse promoter. Swap E consists of the −178 region of the mouse promoter fused to the −322/−153 fragment of the human promoter. Construct F was generated by replacing the region between −152 and −63 in the human promoter with the −178/−73 region of the mouse promoter. (**B**) The different hybrid constructs, Swaps A–F, were analysed by transient transfection in AR4-2J cells as indicated. Three of the mouse and three of the human deletion constructs are included for comparison. Bars represent luciferase activities that are expressed in arbitrary units relative to the activity produced by the UMS-Luc construct [12], which was adjusted to the value of 1.

a mouse oligonucleotide in which GGTT had been introduced it was concluded that these bases are important for this interaction (Figure 6A).

The conclusion from the transfection assay and EMSA is that the bases flanking the PTF 1 site in the 5′ direction abolish binding of PTF 1 to the human promoter. This is clarified further by the fact that when a specific PTF 1 mutation was introduced in the human promoter construct hCEL-839Luc, the promoter activity was not reduced compared with the wild-type construct (results not shown).

Binding of a protein to a C/EBP-like motif prevents PTF 1 binding to the human promoter

When comparing the human sequence of the CEL sequence between −125 and −92 with known *cis* elements, besides the

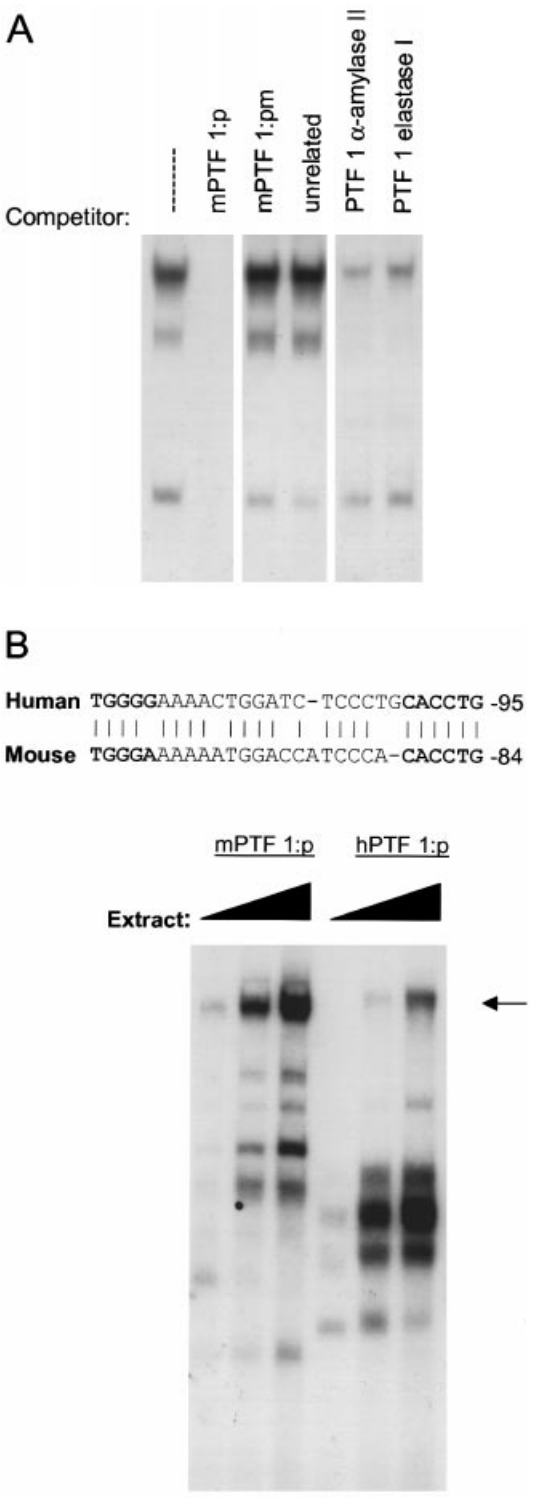

Figure 5 EMSA analysis of the mouse and the human proximal PTF 1-binding element

(**A**) EMSA analysis of the protein interactions to the proximal mouse PTF 1 element at — 111, with 4 μg of nuclear extract from AR4-2J cells. Unlabelled competitor oligonucleotides (Table 1) were in 100-fold molar excess. (**B**) Sequence comparison of the human and mouse proximal PTF 1 elements. EMSA analysis is shown of the protein interactions with the mPTF 1 :p and the hPTF 1 :p oligonucleotides with increasing amounts (1, 4 and 16 μg) of nuclear extract from AR4-2J cells. The arrow indicates the PTF 1 interaction.

PTF 1 element, similarity to a binding site for the C/EBP family was found. An EMSA revealed that the strong shift observed in Figure 5(B) corresponds to a protein binding to the C/EBP motif

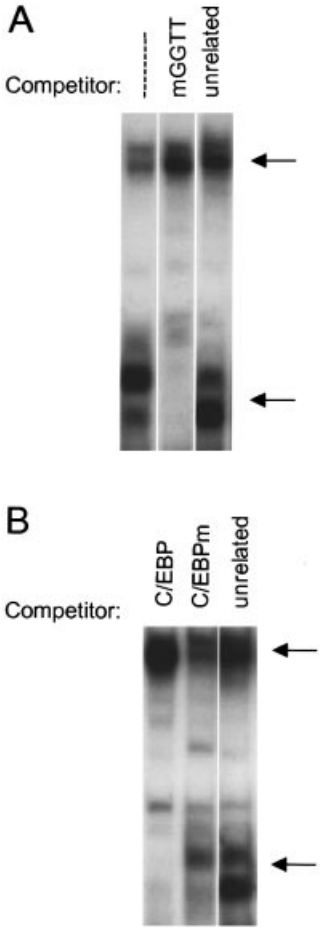

Figure 6 A protein binding to a C/EBP-like motif prevents binding of PTF 1 to the proximal PTF 1-binding site in the human promoter

(**A**) EMSA analysis of the protein interaction to the hPTF 1 :p oligonucleotide. The reaction was carried out in the presence or absence of competitors (Table 1), as indicated at the top of each lane. The upper arrow indicates the PTF 1 interaction while the lower arrow indicates an interaction with the 5′ end of the hPTF 1 :p oligonucleotide. (**B**) EMSA analysis using the hPTF 1 :p oligonucleotide as a probe. C/EBP, C/EBPm or an unrelated oligonucleotide were included in the binding reactions, as indicated. The upper arrow indicates the PTF 1 interaction while the lower arrow indicates the interaction to the C/EBP-like motif. All competitors were in 100-fold molar excess.

since an oligonucleotide containing a C/EBP site derived from the C/EBP α promoter [20] competed successfully for the protein–DNA interaction, whereas an oligonucleotide containing a mutated C/EBP site was unable to compete (Figure 6B, Table 1). However, supershift experiments with either a C/EBP β-specific antiserum or an antiserum that interacts with several members of the C/EBP family, α, β, δ and ϵ, showed that none of the antisera recognized the protein interacting with the hPTF 1 :p oligonucleotide.

Since the C/EBP-like motif overlaps with the p64-binding site (TGGGG) of the PTF 1 element, we proceeded to investigate whether PTF 1 and the other interacting protein competed for binding to the element. As can be seen in Figure 6(B) the binding activity of PTF 1 increased when the oligonucleotide including the C/EBP-like motif was added as competitor and the reverse was observed when the mutated C/EBP oligonucleotide was added as competitor to the binding reaction, showing that there is competition between them, but that the affinity for the protein binding to the C/EBP-like motif is higher.

To find out if binding of the protein to the C/EBP-like motif affects the expression of the human *CEL* gene, the C/EBP mutation was introduced in the hCEL-839Luc construct. This resulted in a 65 % reduction of the activity. In order to investigate whether the protein also interacts with the enhancer in a similar way as PTF 1, we introduced the corresponding mutation into the hCEL-322Luc and hCEL-632Luc constructs. The activity of the mutated hCEL-322Luc construct was the same as for the wild-type construct, while the mutated hCEL-632Luc construct displayed a slight decrease (results not shown). We therefore concluded that a protein binding to the C/EBP-like motif is indeed interacting with the human enhancer.

A more distal PTF 1 site and an unknown element requires binding of PTF 1 at −111 in the mouse promoter to affect the expression

After concluding that the most important factors involved in the regulation of the human and mouse *CEL* genes are different, we wanted to investigate whether there are further differences in the regulation of the two genes. Analysis of the 5′-deletion series of the mouse *CEL* gene promoter suggests that the region between −375 and −179 includes *cis*-acting elements that are important for high expression. When comparing the activity of the mCEL-375Luc construct with that of the Swap E construct, the activation potential for Swap E was approx. 2-fold lower than that of the mCEL-375Luc construct (Figure 4B). This suggests that the element involved in the regulation of the mouse *CEL* gene is not present in the corresponding region of the human *CEL* gene promoter. Furthermore, when comparing the activity of Swap E with the mCEL-178Luc construct, the activity for Swap E is 1.5 times higher. This is in agreement with the transfection results obtained for the hCEL-322Luc construct compared with the hCEL-152Luc construct, which indicate that the human promoter region between −322 and −153 includes elements that by themselves increase the expression 1.5 times. As mentioned earlier, this increase in activity is not due to the PTF 1 site located at −175 since PTF 1 needs interaction with the enhancer to be able to affect the expression.

Another interesting observation is that it seems that the elements of importance in the mouse *CEL* gene promoter between −375 and −179 require binding of PTF 1 to the element located at −111 for effect. This suggestion is based on the results obtained for the Swap B and the hCEL-152Luc constructs (Figure 4B). The activation potentials for these two constructs are comparable. If the elements important for mouse *CEL* gene expression had been able to activate the expression by themselves, the activity of Swap B would have been 3–4 times higher than the activity for the hCEL-152Luc construct. Hence, the conclusion from transfection assays is that we have to search for *cis* elements in the mouse promoter between −375 and −179, which are not present in the human promoter.

The search for known nuclear-factor-binding sites within this region identified several putative elements. Two motifs with homology with the elastase I B-element present at −340 and −271 [21], a putative C/EBP element located at −301 [20] and a PTF 1 motif present at −225 [4], were found. We verified by EMSA that B-element-binding proteins interact with the elements located at −340 and −271, but mutation of these sites in the mCEL-375Luc construct did not affect the expression at all (results not shown). Neither did mutation in the PTF 1 site at −225 (results not shown). In an attempt to identify the sequence necessary for the interaction with the putative C/EBP motif, a series of mutated oligonucleotides was constructed and used as unlabelled competitors to the radiolabelled wild-type oligonucleotide (wt, Table 1) in an EMSA. This revealed that the formation of the complexes could be competed with by an excess of all oligonucleotides except for the oligonucleotide containing a mutation in the region −299 to −294. However, an oligonucleotide containing a C/EBP site was unable to compete (Figure 7) and when the bases between −299 and −294 were mutated in the mCEL-375Luc construct the promoter activity was not affected either (results not shown).

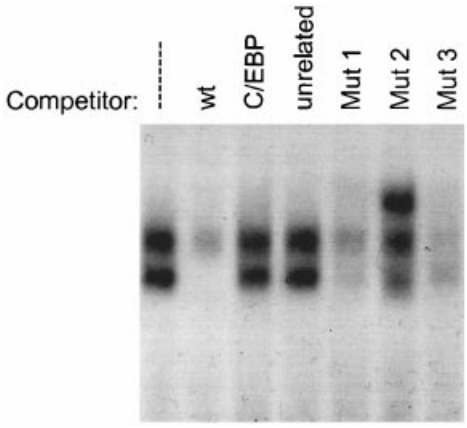

Figure 7 Analysis of the mouse *CEL* gene promoter between −310 and −281

EMSA analysis of the protein interactions to the −310/−281 region of the mouse promoter with 4 μg of nuclear extract from AR4-2J cells. A 100-fold molar excess of various competitor oligonucleotides (Table 1) was added to the reaction mixtures, as indicated.

Even though earlier findings have revealed that different elements in this part of the promoter must be involved in the regulation of the gene in the two species it cannot be excluded that one of two or more elements is conserved in both promoters. Therefore we made constructs in which we mutated the conserved PTF 1 site located at −195 in the mouse promoter together with either of the elements mentioned above. The only construct that affected the expression was the one in which both the PTF 1 element and the element located around bases −299 and −294, hereafter referred to as mouse distal promoter element (mDPE), were mutated. The activity of this mutant was only 30 % compared with the wild-type construct (results not shown). It should also be mentioned that mutation of the PTF 1 site at −195 alone did not affect the activity at all.

In conclusion, we have found that the mDPE and the PTF 1-binding element at −195 are important for the expression of the mouse *CEL* gene, but to be able to affect the expression they require PTF 1 binding to the element located at −111.

DISCUSSION

Several types of genetic difference between rodent and human accompanied by functional divergence have been described. For example, the evolutionary silencing of the human elastase I gene appears to be due to mutations in the 5′-flanking sequence that inactivate crucial enhancer and promoter elements important for the expression of the corresponding rat homologue [22]. Furthermore, the glycoprotein hormone α-subunit provides an example of divergent expression pattern in human and rodent due to nucleotide sequences of the 5′-upstream flanking regions [23]. Similarly, the myosin VIIA gene is expressed in both retinal pigmentary epithelium cells and photoreceptor cells in human

fetus, whereas it is confined to the retinal pigmentary epithelium in mice [24].

However, it is increasingly apparent that some of the genetic differences are not accompanied by changes in function. An example is the study of the production of the growth-hormone-binding protein and the growth-hormone receptor. These proteins are conserved, but they arise by alternative splicing in mice and by proteolytic cleavage in humans [25,26]. The *CEL* gene is another example of functional conservation in spite of genetic divergence. Even though the *CEL* gene is expressed at comparable levels in exocrine pancreas in both mouse and human the most important elements in the two promoters are different. In the human promoter there is a strong pancreas-specific enhancer which, together with PTF 1 and a protein binding to a C/EBP-like motif, is responsible for high-level expression. On the other hand, in the mouse promoter there is a PTF 1-binding site which is essential for expression, and the effect of PTF 1 binding to this site is augmented by a more distal PTF 1 interaction and by a factor binding to the mDPE. Mutation analysis shows that the effect of the distal PTF 1 and mDPE interactions is only observed when both sites are mutated. This is in direct contrast with the function of the rat elastase EI enhancer, in which mutation of any of the three elements, A, B or C, abolishes the activity of a reporter construct in transfected cells [27]. These results may reflect a situation where single genes, or groups of genes, expressed in different species are regulated by a specific set of factors, even though they are expressed in the same tissue.

The human CEL promoter has a potential PTF 1 site in a corresponding position to the essential PTF 1 site in the mouse promoter, and results from our study indicate that the human site, placed in the context of the mouse sequence, functions just as well as the mouse site. However, nucleotide differences in the 5′-flanking region of the human site almost abolish binding of PTF 1. The search for known nuclear-factor-binding sites within this region of the human CEL promoter identified a potential binding site for C/EBP (TKNNGNAAK) overlapping the p64-binding site (TGGGG) of the PTF 1 motif. It was revealed by EMSA that a protein binding to the C/EBP-like motif interacts with this site and thereby prevents PTF 1 from binding. Furthermore, by introducing GGTT in front of the mouse PTF 1 site in the mCEL-375Luc construct and thereby creating a C/EBP site, a reduction of the luciferase activity by 75 % was achieved, indicating that binding of PTF 1 is prevented.

It is known that modification of the regulatory control of gene expression can be a result of gene duplication, since the duplicated gene thereby acquired new specific functions, either retaining or loosing ancestral properties. The functional *CEL* gene in human has arisen following gene duplication. The original gene has during evolution been inactivated and become a pseudogene [28]. The inactivation of an original gene is unusual and the selection pressure involved is difficult to understand since neither tissue specificity nor enzyme function seems to have become changed. This gene duplication has not occurred in mouse because it was found to be restricted to higher primates only [29], and could explain the differences observed between mouse and human. We would now like to suggest that such differences have resulted in the formation of a new exocrine-specific complex in the human gene, compensating for the fact that PTF 1 is prevented from binding to the proximal site in the human promoter. Future studies involving transgenic animals will be designed to further evaluate the effects of the two essential regions in the different species.

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