

The LIM homeobox protein mLIM3/Lhx3 induces expression of the prolactin gene by a Pit-1/GHF-1-independent pathway in corticotroph AtT20 cells

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Abstract mLIM3, a member of the LIM homeobox family, was recently demonstrated to be critical for proliferation and differentiation of the pituitary cell lineage. Using a pool of degenerate oligonucleotides we determined the DNA sequence ANNAGGAAA(T/C)GA(C/G)AA as the set preferentially recognized by mLIM3. A nearly identical sequence is found in the prolactin (*PRL*) promoter, within a 15-mer stretch from nucleotides (nts) –218 to –204 which is highly conserved between human, rat, and bovine. In order to test the hypothesis of a transcriptional effect of mLIM3 on the prolactin promoter, stable transfectants of mLIM3 cDNA in AtT20 tumor cells revealed that *PRL* mRNA expression was induced in 3 separate stable clones. Gel retardation experiments performed using nuclear extracts isolated from one of the AtT20/mLIM3 stable transfectants revealed affinity towards the 15-mer element of the *PRL* promoter. From these results, we propose that the *PRL* promoter element (nts –218 to –204) could be functional in vivo. Finally, we demonstrate that in AtT20 cells prolactin mRNA expression is not induced by the Pit-1/GHF-1 pathway and that growth hormone mRNA is not detected concomitantly with prolactin. We conclude that mLIM3 may play a key role in inducing *PRL* gene expression in lactotrophs by binding to a conserved motif close to a Pit-1/GHF-1 site within the proximal *PRL* promoter.

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Key words: mLIM3; Prolactin; AtT20 cells; Promoter; Pituitary

1. Introduction

Five distinct cell types which differ by the expression of specific trophic hormone genes emerge from the Rathke pouch during organogenesis, giving rise to the different cell populations of the anterior pituitary [1]. Progressive differentiation of the five cell lineages is dependent on the sequential induction of transcription factors such as Pit-1/GHF-1 [1–4]. The mouse homeobox-containing mLIM3 [5,6], also known as P-Lim [7] or Lhx3 [8], is a new member of this LIM-HD family and the fate of all pre-motoneurons depends on the

combined action of four LIM-HD proteins, including mLIM3 [9]. The strongest expression of mLIM3 transcript is observed in the anterior (AP) and intermediate (IP) pituitary lobes and was first detected on embryonic day 11 persisting into adulthood [5]. Within pituitary cell lines, somatolactotroph GH3 and GH4C1, as well as gonadotroph α -T3-1, express high levels of mLIM3 transcripts, while low expression is detected in the corticotroph AtT20 cells [5]. Consistent with these observations, targeted disruption of the *mLIM3* gene [10] recently revealed that homozygous mLIM3 (–/–) mice display dramatic effects on pituitary differentiation and growth. Indeed, these mice lack both the AP and IP, with all the anterior pituitary lineages being completely absent, except for some corticotrophs. Thus, mLIM3 appears to be a very critical early gene whose expression is associated with pituitary organogenesis. In addition to *mLIM3* (*Lhx3*), a closely related gene called *Lhx4* was also reported to determine the formation of the pituitary gland in mice, and together these two genes regulate proliferation and differentiation of pituitary-specific cell lineages [11].

In an effort to identify mLIM3-regulated genes expressed in the anterior pituitary, we first determined the DNA consensus sequence to which this protein binds. Next, since the proximal promoter region of the prolactin gene (*PRL*) contains this motif, we investigated the possibility that mLIM3 could regulate *PRL* expression. Corticotroph AtT20 cells which do not endogenously transcribe *PRL*, were stably transfected with mLIM3 cDNA. The resultant mLIM3-expressing cells exhibited a detectable *PRL* mRNA expression, while no *PRL* transcription is detectable in control cells. Moreover, we show that the induction of *PRL* transcription in AtT20 cells does not require Pit-1/GHF-1. These data suggest a particular role of mLIM3 in relation to *PRL* expression in lactotroph pituitary cells and constitute a step in the elucidation of the molecular basis that underlie mLIM3 action during the early stages of pituitary differentiation.

2. Materials and methods

2.1. Antibodies and Western blotting

A chimeric GST-LIM construct was obtained by inserting the coding cDNA of mLIM3 [5] in phase with GST in a pGEX-2T expression vector (Pharmacia). An antibody against the mLIM3 protein was raised in rabbits against a synthetic peptide corresponding to amino acids 299–317 [5]. The anti-phosphothreonine antibody was kindly donated by Dr. X. Prusner (McGill University). The pGEX-LIM vector was used to prepare a fusion protein in DH5 α bacterial cells, following induction with 0.3 mM IPTG. Western blot analysis of SDS-PAGE-fractionated proteins probed for mLIM3 immunoreactivity was achieved using an ECL kit (Boehringer Mannheim).

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2.2. Amplification/selection of a consensus sequence

A degenerate double stranded 68-bp oligonucleotide was designed in order to determine the consensus binding sequence of mLIM3 [12]: 5'-GACAGTTGTTGTCTTCTACTAGCTGCAG-NNNNNNTAA-NNNNNNN-CTCGAGTGGCGGTAGTACTTCAT-3'. Both 5' (29 bp) and 3' (23 bp) ends contain non-degenerate sequences used as primers for PCR. The 16-bp core was degenerate in 13 positions, with a TAA anchor at its center. In each round of selection, GST-LIM protein was incubated with the 5'-end labeled 68-bp sequence and the oligonucleotides bound to the protein were separated from the free ones by electrophoretic mobility shift assay (EMSA) (see Section 2.3). The bands detected by autoradiography were cut, and amplified separately by PCR after extraction of the DNA from the acrylamide. PCR was carried out for 30 cycles using 1-min denaturation at 94°C, 2-min annealing at 62°C, 1-min extension at 72°C. One μ l of the amplification mixture was directly used as a template for the next round of purification by EMSA. The set of DNA obtained after the last step of purification was directly sequenced. Two parallel experiments were performed, in which either the upper band or the lower band of the shifted complex was systematically cut and its DNA amplified.

2.3. Electro-mobility shift assay (EMSA)

Three pmoles of double stranded template were labeled 30 min at 37°C with [γ - 32 P]ATP and T4 polynucleotide kinase. GST-LIM or nuclear extracts were incubated 30 min at room temperature in 18 μ l of a reaction mixture containing 10 mmol/l HEPES (pH 7.8), 1 mmol/l Na₂HPO₄ (pH 7.2), 0.1 mmol/l EDTA, 50 mmol/l KCl, 4 mmol/l MgCl₂, 4 mmol/l spermidine, 2.5% glycerol in the presence of 20 000 cpm of labeled template. The protein-DNA complexes were analyzed by non-denaturing electrophoresis through 4.5% polyacrylamide gels, run in 0.25 $\times$ Tris borate EDTA. The following double stranded oligonucleotides were used either for *PRL* promoter study: *4P/Lim*: 5'-TTCTCTCATTTCCTTTTGCTGTAATTAATCAAAA-TCTTCC-3'; *4P*: 5'-TTCTCTCATTTCCTTTTGCTG-3'; *4Pmut*: 5'-TTCTCTCATTTCCTTTTGCTGGCCCGAATCAAAATCCTTC-C-3' (mutation underlined). Nuclear extracts were prepared exactly following a protocol previously described [13].

2.4. Stable transfection of AtT20 cells with mLIM3

mLIM3 was inserted into a pRH expression vector (a gift from Dr. T. Reudelhuber, IRCM). 50 µg of pRH-mLIM3 vector and 5 µg of the Neo-resistant RccMV vector were used to transfect 10⁷ confluent AtT20 cells using lipofectin. After 48 h, the cells were selected for resistance using 0.5 mg/ml G418. Individual clones were tested for mLIM3 expression by biosynthetic analysis of [³⁵S]methionine radiolabeled cells extracted with RIPA lysis buffer [14]. Immunoprecipitates of radiolabeled proteins were resolved by SDS-PAGE on a 10% T, 2.7% C polyacrylamide gel in 0.1% SDS.

3. Results

3.1. Determination of the DNA consensus sequence recognized by mLIM3

Western blot analysis of the GST-LIM fusion protein purified on a glutathione-agarose column using an antiserum raised against a C-terminal sequence of GST-LIM is shown in Fig. 1A. Two main bands were observed with the major form migrating with an apparent mass of 75 kDa, as expected for the fusion protein, while the upper one (83 kDa) could be a post-translationally modified form, possibly involving a phosphorylation event (Girardin et al., unpublished data).

In order to identify the preferred DNA sequence recognized by mLIM3 a double stranded 68-bp oligonucleotide was designed (see Section 2), in which the central core was degenerate whereas the ends contained specific sequences allowing amplification by PCR. Since in most of the reported cases homeodomains recognize a motif containing a TAA sequence [12], a TAA anchor was placed at the center of the degenerate 16-mer core. We used this oligonucleotide in a selection/amplification experiment as described [15], except that after PCR

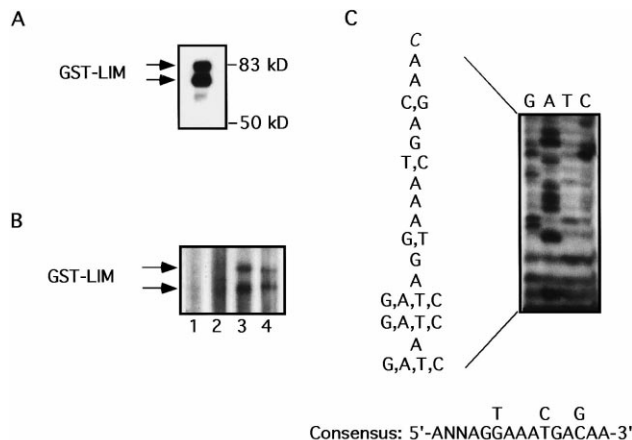


Fig. 1. Determination of the DNA consensus sequence recognized by mLIM3. A: Analysis of the two prokaryotic GST-fusion proteins by Western blotting using an anti GST-LIM antibody. B: A 68-bp partially degenerated double stranded oligonucleotide was designed and used as a probe in a purification/selection assay carried out by EMSA. The migration positions of the two DNA-containing bands representing specific interactions with GST-LIM are indicated for each of the four rounds of PCR amplification. C: Sequence autoradiogram of the mixture of 68-bp oligonucleotides obtained after 4 rounds of purification of the upper DNA band. The oligonucleotide of 29 bases was used as a radioactive primer in a PCR-driven sequencing. At each position, the base most frequently present is indicated. The last C nucleotide in the 3' end is the first base outside of the degenerate region in the 23-bp conserved motif.

amplification the selection was achieved by EMSA. Following 4 rounds of amplification/selection, we extracted and purified two enriched bands containing pools of DNA sequences interacting with GST-LIM, representing differentially migrating complexes (Fig. 1B). The increase in specificity was gauged at each step by the progressive loss of competition with the unlabeled degenerate 68-bp oligonucleotide (data not shown). Moreover, these two DNA pools interact specifically with the GST-LIM protein by the degenerate core of the probe, since no interaction could be detected using the double-stranded non-degenerate ends (23 bp and 29 bp) as individual probes for EMSA (not shown).

The two eluted pairs of DNA pools obtained after the fourth round of amplification (Fig. 1B) were used separately as templates for a PCR-driven manual sequencing. The DNA sequence corresponding to the upper pool is shown in Fig. 1C. A similar result was obtained using the lower pool (data not shown). Because of the presence of the TAA anchor, the consensus sequence is directly visualized by comparing the intensities of the 4 bases for each position around the TAA core (Fig. 1C). Surprisingly, approximately half of the sequences harbor a G instead of a T in the TAA core. This observation is not an artifact of the PCR-driven sequencing since analysis of 12 individual clones revealed that half of them contain a GAA in their middle (data not shown). Two explanations can be provided for this observation: the oligonucleotide synthesis was not perfect and in the pool some G bases were added instead of T; alternatively, a G mutation might have occurred during the PCR reactions. In each case, however, the ratio G/T would be expected to be very low, in contrast to the observed ratio of 1 which indicates that the selection steps favored GAA far more than TAA. Interestingly, some sequence positions preceding the G/TAA core exhibit similar intensities

for each of the 4 bases, suggesting that these positions are not critical for binding. Although the sequence following the G/TAA is relatively easy to deduce, in some positions we observed an excess of two bases relative to the two others. Consistent with this analysis, although we could read a AN-NAG(G/T)AAA(T/C)GA(C/G)AA sequence (Fig. 1C), the above arguments led us to propose the following 15-mer DNA consensus sequence: ANNAGGAAA(T/C)GA(C/G)AA. Alignment of the consensus sequence presented in Fig. 1C with those found in the EMBL gene bank revealed an identical 14-mer sequence within the 5' end of the prolactin promoter (nts -205 to -218), in a highly conserved segment of the rat, human and bovine sequences, suggestive of a selective pressure towards this small stretch of the *PRL* promoter [16]. We did not find such a homologous sequence in other pituitary promoters.

3.2. *mLIM3* induces endogenous *PRL* expression in AtT20 cells

In order to test the hypothesis of a transcriptional effect of *mLIM3* on the prolactin promoter, we generated stable transfectants in the corticotroph AtT20 cells, which have been chosen on the basis of: (i) their low endogenous expression of *mLIM3* [5]; (ii) the absence of endogenous *PRL* expression; and (iii) the fact that in *mLIM3* knockout mice some corticotroph cells survived [10]. Positive clones resistant to G418 were tested for *mLIM3* expression following an [³⁵S]methionine pulse of 4 h and immunoprecipitation using the anti-*mLIM3* antiserum as compared to a control normal rabbit serum (NRS). Using such a selection, we were able to isolate three stable clones which overexpress *mLIM3*, with clones 1 and 6 exhibiting the highest expression (Fig. 2), and clone 2 being a lower expressor (not shown).

Northern blot analysis was used to measure the expression of several genes in transfected AtT20 cells overexpressing *mLIM3* as compared to control AtT20 cells transfected with the vector alone (Fig. 3). First, the expression of *POMC* was examined, since AtT20 cells endogenously express this precursor. We note that both *mLIM3*-transfected cells and control cells express similar levels of *POMC* mRNA (Fig. 3A), suggesting that *mLIM3* has no significant effect on the expression of *POMC*. Second, based on the reported in vitro interaction of *mLIM3* with the promoter of the α -subunit of LH, FSH and TSH [7], we tested the effect of the stable transfection on the expression of the α -subunit (α -GSU) in AtT20 cells. Using a rat pituitary extract as a positive control for the probe, the

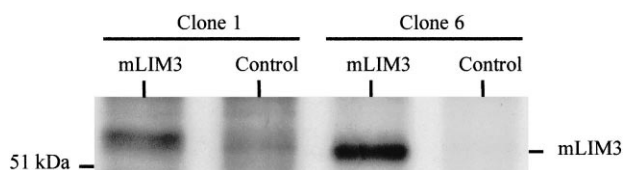


Fig. 2. AtT20/*mLIM3* stable transfectant analysis on three separate clones. After stable transfection of *mLIM3* in AtT20 cells, three separate clones (1, 2 and 6) were analyzed for the presence of the *mLIM3* protein by immunoprecipitation of the [³⁵S]methionine labeled cellular proteins obtained following a 4-h pulse. While we obtained immunoreactive *mLIM3* with clones 1, 2 and 6, we only show the data with clones 1 and 6 which express the highest level of *mLIM3*. As control for the specificity of the immunoprecipitation we also show the results obtained using normal rabbit serum (NRS). The specific *mLIM3* band is indicated by an arrow.

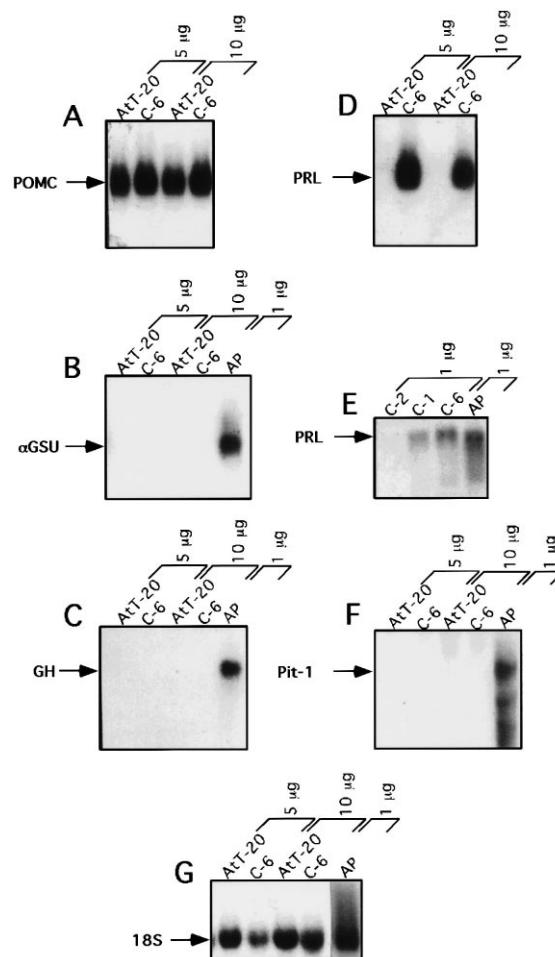


Fig. 3. The *PRL* gene is induced in AtT20/*mLIM3* cells. Total RNA extracts of AtT20 or AtT20/*mLIM3* clone 6 (C-6) were used for Northern blotting experiments using different cRNA probes and distinct but equivalent blots in each case (A–D, F and G). One μ g of total RNA extracts from adult rat anterior pituitary (AP) were loaded as a positive control for the probe. The *PRL* gene induction is also observed using total RNA extracts of two other AtT20/*mLIM3* clones (C-1 and C-2) (E). Autoradiograms were quantified by densitometry and normalized for loading control with an 18S probe (G).

data did not reveal an induction of the α -subunit mRNA in our clones (Fig. 3B). Similarly, no induction of GH was observed in the *mLIM3*-transfectants (Fig. 3C), even though expression of *mLIM3* is detected in GH-containing cells of the mouse pituitary [5].

Using a *PRL* probe we were able to detect a strong induction of mRNA expression in the highest expressor of *mLIM3* (clone 6 (C-6), see Fig. 2), whereas no *PRL* could be detected in control cells (Fig. 3D). However, we note that the level of induced *PRL* is lower than that observed using equivalent amounts of total RNA isolated from mouse anterior pituitary (Fig. 3E). This may explain our inability to detect the *PRL* protein from AtT20/*mLIM3* cells extracts using a radioimmunoassay ranging in sensitivity from 5 to 40 ng/ml (not shown). In order to assess whether this activation results from a direct interaction with the *PRL* promoter or from an indirect cascade, we tested the possible expression of Pit-1/GHF-1 in the transfected cells, using a rat pituitary extract as a positive control (Fig. 3F). No induction could be detected, even with

much longer exposure of the film, suggesting that if the mLIM3 induction on the *PRL* promoter is indirect, it does not act through the Pit-1/GHF-1 pathway.

3.3. mLIM3 binds in vitro to a sequence of the *PRL* promoter

We noted that the *PRL* region suggested to interact with mLIM3 is within 10 bp 5' to a Pit-1/GHF-1 binding site, known as the proximal 4P site [16]. Interestingly, Bach et al. have shown that mLIM3 is able to bind directly Pit-1/GHF-1 by its LIM domains [7], suggesting that Pit-1/GHF-1 could be a co-factor necessary for the suppression of the inhibitory effect of the LIM domains of mLIM3, in a model similar to that reported for other LIM family members [17].

In order to confirm that this *PRL* promoter sequence binds to native mLIM3, an EMSA was done using an rPRL oligonucleotide (nts -181 to -222) encompassing the rat Pit-1/GHF-1 4P sequence [16] and the proposed mLIM3 site [4P/Lim]. Thus, addition of increasing amounts of nuclear extracts from either AtT20 or AtT20/mLIM3 (C-6) cells to ³²P-labeled double stranded 4P/LIM revealed a complex which is especially evident in AtT20/mLIM3 (Fig. 4A, lane 9). In contrast, only a very weak complex was observed when equivalent amounts of nuclear extracts obtained from control AtT20 cells were used (Fig. 4A, lane 5). The very low endogenous level of mLIM3 expression in AtT20 cells [5] is likely to be responsible for this interaction. In order to confirm the presence of mLIM3 in the low mobility complex observed, a

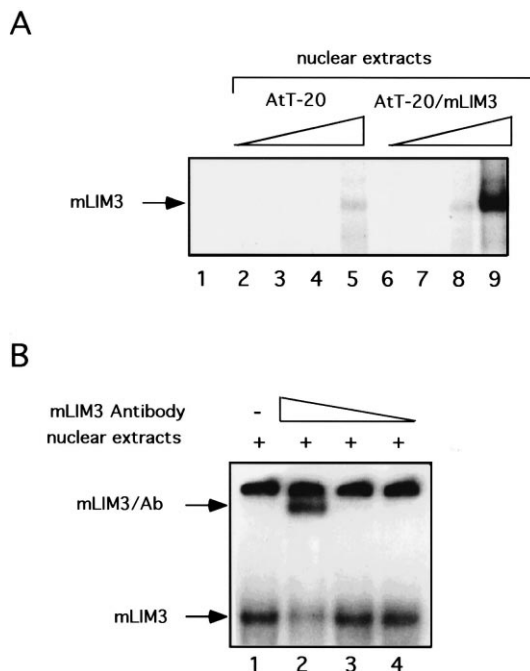


Fig. 4. The GST-LIM protein specifically interacts with the *PRL* promoter in vitro. A: [4P/Lim] was used as a probe either without protein extract (lane 1); with increasing amounts (10, 50, 100 pg or 1 ng) of AtT20 nuclear extracts (lanes 2–5); or with increasing amounts (10, 50, 100 pg or 1 ng) of AtT20/mLIM3 nuclear extracts (lanes 6–9). B: mLIM3 antibody was added at different dilutions to a mixture of ³²P-labeled [4P/Lim] probe and 1 ng of AtT20/mLIM3 nuclear extracts. The migration position of the complex (mLIM3/Ab) on the DNA probe is indicated. Final dilutions of the antibody are 1:100, 1:1000, 1:5000. The upper non-specific band observed in all lanes represents precipitated viscous DNA in each well.

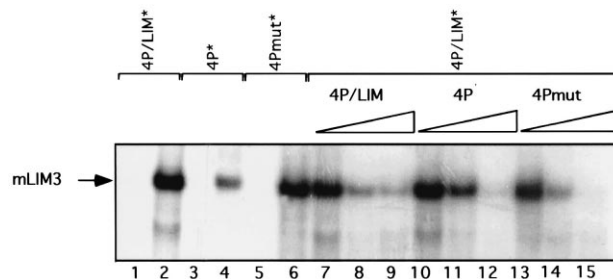


Fig. 5. The 4P site is not involved in mLIM3 binding to the 4P/LIM element. Three sets of radioactive probes were incubated in the absence (lanes 1, 3, 5) or presence (lanes 2, 4, 6) of a combination of 10 pg of GST-LIM and 1 ng of AtT20/mLIM3 nuclear extracts. In lanes 7–15, the *Lim/4P* radioactive probe was competed with an excess (10-, 20-, 100-fold) of each of the three non-radioactive oligonucleotides 4P/LIM, 4P, and 4Pmut.

supershift experiment was achieved using the mLIM3 antibody (Fig. 4B). Noticeably, at the 1:100 dilution of the mLIM3 antibody most of the complex was shifted close to the top of the gel, indicating that mLIM3 was indeed present in the complex.

Demonstration that the *PRL* 4P site, located 10 bp 3' of the mLIM3 site, was not involved in the observed DNA binding, was achieved by the use of two other double stranded oligonucleotides, i.e. a mutated *PRL* 4P Pit-1 binding site [4Pmut], and one in which this site is deleted [4P]. The two oligonucleotides gave the same result as the original [4P/LIM], revealing that in vitro the interaction of mLIM3 on the *PRL* promoter does not require the 4P site (Fig. 5).

4. Discussion

The aim of this study was to investigate how mLIM3 could mediate its effect during pituitary organogenesis, since this protein has been shown to be crucial for the development and differentiation of this tissue [10,11]. This is especially needed since the study of *mLIM3* null animals revealed that the elaboration of a definitive Rathke's pouch is directed by either *mLIM3* (*Lhx3*) or *Lhx4*, and that subsequent organ fate commitment is regulated solely by *mLIM3*, an event not replaceable by *Lhx4* [11]. In accordance with our detection of mLIM3 in both the nucleus and cytosol of vaccinia virus infected cells (Marcinkiewicz, M. and Seidah, N.G., unpublished data) it was quite plausible that this protein is indeed a transcription factor. Accordingly, it was necessary to characterize the direct DNA binding activity of mLIM3, and to identify the DNA consensus sequence necessary for its optimum binding.

Investigation of the DNA sequence recognized by mLIM3 led to the determination of a 15-bp consensus sequence (Fig. 1C). It is noteworthy that the first 14 nucleotides of the deduced 15-mer consensus sequence found for optimal mLIM3-DNA binding are: (i) located on the proximal *PRL* promoter, (ii) highly conserved between rat, human and bovine, and (iii) 10 bp away from the 5' end of the fully conserved *PRL* 4P site known to bind Pit-1/GHF-1 [16]. Several groups have reported in vitro protection studies of this region with various nuclear extracts, including those from a GC lactotroph/somatotroph cell line which expresses mLIM3 [7]. It was recently proposed that the (nts -217 to -209) element was necessary

for Ets-1 mediated activation of the *PRL* gene by the Ras/Raf pathway [18]. Since this activation depends on Pit-1/GHF-1, it was proposed that the (nts –217 to –190) element encompassing the Pit-1/GHF-1 site is a functional Ras response element. Could mLIM3 and Ets-1 compete for the same site on the *PRL* promoter, either during development or in the adult pituitary? An answer to this question must await future studies demonstrating the co-localization of Ets-1 and mLIM3 during embryogenesis and the proof that Ets1 binds to the 14-mer recognized by mLIM3. As the results of mLIM3 null mice [10,11] suggest that this protein controls the expression of several pituitary genes, we were surprised to find the 14-mer consensus sequence only in the *PRL* promoter and not in other mammalian gene regulatory elements. Therefore, it is possible that a shorter minimal DNA-binding consensus sequence lies within the 14-mer that we found. Indeed, Bach et al. [19] recently defined an mLIM3 DNA-binding site in the α -GSU promoter region, and described the association on DNA of mLIM3 with Otx family members. This element contains the same *GAAAT* sequence that we found in the center of our consensus sequence. We propose that the 15-mer consensus sequence is preferred if mLIM3 associates alone on DNA, although a shorter sequence could be sufficient if the DNA-binding specificity is enhanced by a co-factor such as Otx.

Considering the partner-induced-binding hypothesis, and consistent with the results of Bach et al. [7], showing that mLIM3 and Pit-1/GHF-1 directly interact through a LIM-POU domains contact, we tested the hypothesis that Pit-1/GHF-1 could be the partner in mLIM3 binding to the *PRL* promoter in cell lines. For this purpose, we selected AtT20 cells which endogenously do not express Pit-1/GHF-1 [2], and obtained three stable transfectants expressing variable levels of mLIM3 protein. Northern blots of these stable transfectants revealed the absence of Pit-1/GHF-1 mRNA, thus eliminating the possibility that mLIM3 could induce the transcription of this factor. The ultimate demonstration that Pit-1/GHF-1 is not involved in mLIM3-induced *PRL* transcription came from the use of two double stranded oligonucleotides, *4Pmut* and *4P* which carry a mutation in or a deletion of the *4P* site, respectively. None of these modifications altered the DNA-binding affinity of mLIM3 (Fig. 5). It must be emphasized that our experiments cannot exclude that mLIM3 could interact on the *PRL* promoter in association with other proteins, as was proposed for other members of the LIM family [20]. The present results do not contradict the model proposed by Bach et al. [7] of a direct interaction between mLIM3 and Pit-1/GHF-1 which could occur in pituitary somatolactotrophs, but rather suggest an alternate combination of proteins in a cell line that does not express Pit-1/GHF-1. It must be noted that in our stable transfectants, even though the mRNA levels of *PRL* are easily detectable, they are lower than those found in the anterior pituitary (AP) extracts (Fig. 3E). This may provide an explanation for our inability to detect the *PRL* protein in our stable transfectants by a specific radioimmunoassay sensitive between 5 and 40 ng/ml. Furthermore, since Pit-1/GHF-1 not only activates the *PRL* gene but also enhances the positioning of the transcription initiation complex at the correct start site [21–23], its absence from AtT20 cells (Fig. 3F) may also contribute to the non-translatability of the *PRL* mRNA in these cells.

Interestingly, Agulnick et al. [24] recently isolated a new

LIM-domains binding protein, Ldb1, demonstrating a specificity for LIM-HD or LMO proteins, especially for mLIM3. Independently, another group reported the characterization of two mLIM3-interacting proteins, CLIM-1 and CLIM-2 (identified as Ldb1) which recognize the LIM domain of mLIM3 [19]. The spatial and temporal expression patterns of CLIM-1 and CLIM-2 precisely overlap those of mLIM3 and P-OTX/Ptx1, another protein of the Otx class of transcription factors detected very early during pituitary organogenesis [19]. Moreover, Bach et al. demonstrated the involvement of CLIM-1 and CLIM-2 as *trans*-activating factors in the synergistic activation of the α -GSU promoter by mLIM3 and P-OTX/Ptx1. It is noteworthy that P-OTX/Ptx1 is expressed in AtT20 cells [19], and therefore could account for the observed induction of the *PRL* gene transcription.

The effect described here on the *PRL* promoter accounts for one of the expected multiple activities of mLIM3 in the anterior pituitary. Indeed, analysis of homozygous *mLIM3* (–/–) mice revealed dramatically impaired growth and differentiation in the Rathke's pouch [10,11]. Our data show that in the adult mouse anterior pituitary, mLIM3 mRNA expression can be detected within the five endocrine cell types [5], suggesting that mLIM3 can participate in the differentiation process and/or maintenance of the differentiated states of the five cell lineages of the anterior pituitary, in agreement with the genetic knockout data [11]. The results presented here show that a stable transfection of mLIM3 in a corticotroph pituitary cell line that expresses very low endogenous levels of mLIM3 [5] is sufficient to induce *PRL* mRNA expression. Since AtT20 cells do not express *PRL* (Fig. 3D), we presume that the level of endogenous mLIM3 is too low to allow for *PRL* expression, and only upon overexpression conditions can we observe the induction of *PRL* mRNA. In contrast, high levels of mLIM3 mRNA in the gonadotroph cells α T3 [5] are not sufficient to induce *PRL* transcription. This suggests that one or more partner(s) of mLIM3 in AtT20 cells (Fig. 4) are absent from α T3 cells. Alternatively, it is possible that the lack of Pit-1/GHF-1 in AtT20 cells displaces the *PRL* transcription starting point from the +1 position to the alternate –27 position, since it has been reported that Pit-1/GHF-1 enhance the positioning of the transcription initiation complex at position +1 [21–23]. The –27 starting point represents a non-pituitary site for *PRL* initiation of transcription. This different transcript could have altered stability or be less efficiently translated, explaining why we cannot detect *PRL* in our experiments.

The complex comprising mLIM3 and its putative partner is likely to be centered around the *4P/LIM* site (nts –181 to –222) on the *PRL* promoter. Computer data bank searches did not detect such a site elsewhere even within the evolutionary closely related *GH* gene. Crenshaw et al. [25] showed that the proximal *PRL* promoter (which contains the nts –181 to –222) is sufficient to restrict *PRL* expression to the lactotroph subset. What may then be the factor(s) involved in this cell-specific expression of *PRL*? Since the *PRL* and *GH* proximal promoters contain 4 and 3 Pit-1/GHF-1 sites, respectively, the single transcription factor Pit-1/GHF-1 which is expressed in both somatotrophs and lactotrophs [2,3] may not be sufficient to induce a cell-restricted *GH* and *PRL* expression. It is therefore possible that in combination with an undefined partner(s), mLIM3 is critical for the expression of *PRL* mRNA in cells where the *GH* gene is silent.

In conclusion, the data presented in this work open up new avenues in our understanding of the complex interplay between transcription factors involved in the regulation of the cell-specific expression of anterior pituitary hormones, and suggest a functional role for mLIM3 in the induction of *PRL* transcription.

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