The lipocalin sperm coating lizard epididymal secretory protein family: mRNA structural analysis and sequential expression during the annual cycle of the lizard, *Lacerta vivipara*

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ABSTRACT

The epididymal epithelial cells of the lizard (*Lacerta vivipara*) produce large amounts of specific proteins under androgenic control. Amongst them, a major protein family that binds to the head of spermatozoa, the lizard epididymal secretory protein (LESP) family, has been identified as a member of the lipocalin superfamily. LESPs are composed of 9 elements that present an identical molecular mass of 18 000 Da but have a large range of pHi (3·5 to 9). The structural analysis of this protein family was performed by the determination and comparison of both the aminoterminal sequence of each element and the complete sequence of three specific LESP cDNA clones. When not identical, LESP elements present randomly dispatched nucleotide and amino acid substitutions, indicating the existence of at least five LESP mRNA populations encoded by a multigenic family. We determined that these LESP genes are differentially expressed during the annual epididymal cycle.

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INTRODUCTION

In mammals, spermatozoa acquire the capacity to become motile and to fertilize ova during their transit through the epididymis (Cuasnicu *et al*. 1984, Moore & Hartman 1986). This phenomenon, called sperm maturation, is dependent on interactions between the gametes and specific epididymal secreted proteins (for reviews see Cooper 1986, Robaire & Hermo 1988). Amongst the proteins specifically synthesized by the lizard (*Lacerta vivipara*) epididymal epithelium, we identified a major protein family: the lizard epididymal secretory protein family (LESP). It is composed of 9 elements (LESP I to LESP IX) that exhibit an identical 18 kDa molecular mass, but a wide heterogeneity of pHi, ranging from 3·5 to 9 (Depeiges *et al*. 1988). These LESP elements, whose synthesis is under androgenic control (Depeiges *et al*. 1981, Courty *et al*. 1987), are secreted in the epididymal lumen where they mix and bind to the spermatozoa (Depeiges & Dufaure 1983). According to these observations and in the light of previous studies (Depeiges & Dacheux 1985), it has been proposed that the LESPs play a role in sperm maturation in the lizard epididymal fluid. The main clue as to their function has been the demonstration of significant amino acid homologies with the rat epididymal secretory protein I (ESP I) (Girotti *et al*. 1992, Morel *et al*. 1993). This lipocalin protein, which is a retinoic acid transporter in the rat epididymal fluid, participates in sperm maturation by its binding and delivery of retinoic acid to the surface of the spermatozoal membrane (Newcomer 1993). The LESP and the ESP I have also been shown to share significant amino acid homologies with proteins belonging to the widespread lipocalin superfamily, allowing us to classify the LESPs in this superfamily (Morel *et al*. 1993). Among the lipocalins, a few proteins, such as the mouse major urinary proteins (MUPs) (Shahan *et al*. 1987) or the insecticyanin of the tobacco Hornworn (Li & Riddiford 1992, Flower *et al*. 1993), form well characterized protein families. They all present an identical electrophoretic pattern where the different proteic

elements have similar molecular weights but different pHis.

During its annual sexual cycle, the lizard epididymidal cells undergo dramatic morphological, physiological and biochemical modifications (Depeiges *et al*. 1988), corresponding to successive and well characterized quiescent, maturation, active and degenerative phases. These modifications are especially important in March (stage 4) and in April (stage 5) at the commencement of the epithelial secretory activities, just before the beginning of the reproductive period that occurs in May (stage 6) (Dufaure *et al*. 1986). Here, we analyzed the sequential appearance of translatable LESP mRNAs and LESP proteins during stages 4, 5 and 6 of the reproductive cycle. Our purpose was to determine whether these different LESP proteins were encoded by a unique gene followed by post-transcriptional and post-translational modifications, or were encoded by different, related genes.

MATERIALS AND METHODS

Animals

Lizards (*Lacerta vivipara*) were collected in the Massif Central (France) at different stages of the annual cycle: March (stage 4), April (stage 5) and May (stage 6). Stage 6 corresponds to the reproductive period. Animals were killed by decapitation, the epididymides were excised, and were either frozen in liquid nitrogen and stored at −80 °C, or immediately used for short-term incubation.

Isolation of total RNA

Total RNA was purified from the epididymis using a guanidinium monothiocyanate method (Cathala *et al*. 1983). To quantify and evaluate its integrity before use, total RNA was analyzed by electrophoresis on a 2·2 M formaldehyde denaturing 1% agarose gel and visualized by UV after incubation in ethidium bromide.

In vitro translation assays

Ten micrograms total RNA extracted from the epididymis at different stages of the sexual cycle (stages 4, 5 and 6) were *in vitro* translated in a rabbit reticulocyte lysate cell-free system (Promega France, Charbonnieres, France), in the presence of pancreatic microsomal membranes (Promega), with 50 μCi [^{35}S]methionine (specific activity: 1000 Ci/mmol, Amersham Pharmacia Biotech, Saclay, Orsay, France) for 1 h at 30 °C.

Short term *in vitro* incubations of epididymal explants

The epididymides with epithelium at stages 4, 5 or 6 were cut into pieces. The explants were incubated for 4 h at 30 °C in 1 ml Eagle's medium without methionine (Gibco-BRL (Life Technologies), Cergy Pontoise, France) in the presence of 50 μCi/ml [^{35}S]methionine (1000 Ci/mmol, Amersham). Following incubation, fragments were washed and homogenized in a 0·12 M Tris-HCl pH=7·4 buffer, then centrifuged at 15 000 ***g***. Supernatants were collected and, when necessary, stored at −80 °C.

The assessment of the incorporated radioactivity in the proteins following *in vitro* translation or *in vitro* short-term incubation assays, was determined on filters after precipitation with 10% trichloracetic acid (TCA) by measurement with Beckmann EP 40 scintillation liquid in a Beckmann LS 6000 TA scintillation spectrophotometer.

Protein electrophoresis

Samples of [^{35}S]methionine radiolabeled proteins (400 000 d.p.m.) were analyzed by two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D SDS-PAGE) as previously described by Depeiges *et al*. (1988). Following separation, proteins were stained and fixed in gels in a 0·25% Coomassie brilliant blue/10% acetic acid/50% methanol buffer. After drying, gels were autoradiographed for 30 days with an Amersham Hyperfilm-MP (RPN 6).

Peptide sequence analysis

Total epididymal soluble proteins were separated by 2D gel electrophoresis and transferred onto Immobilon P sheet (Millipore, Saint Quentin en Yvelines, France) as previously described (Depeiges *et al*. 1988). The LESP I to LESP IX spots were sequenced for their aminoterminal extremity by the automated Edman degradation method (Denoroy, Vernaison, France).

Isolation and nucleotide sequencing of new LESP cDNA clones

A lizard epididymal cDNA library constructed in λgt11 was screened using a ^{32}P C731 LESP riboprobe (Morel *et al*. 1993). Two independent positive clones, B71 and B111 (nucleotide sequence accession numbers in Genbank are AF093791 and AF093792 respectively), were amplified and their DNA was purified according to Grossberg (1987).

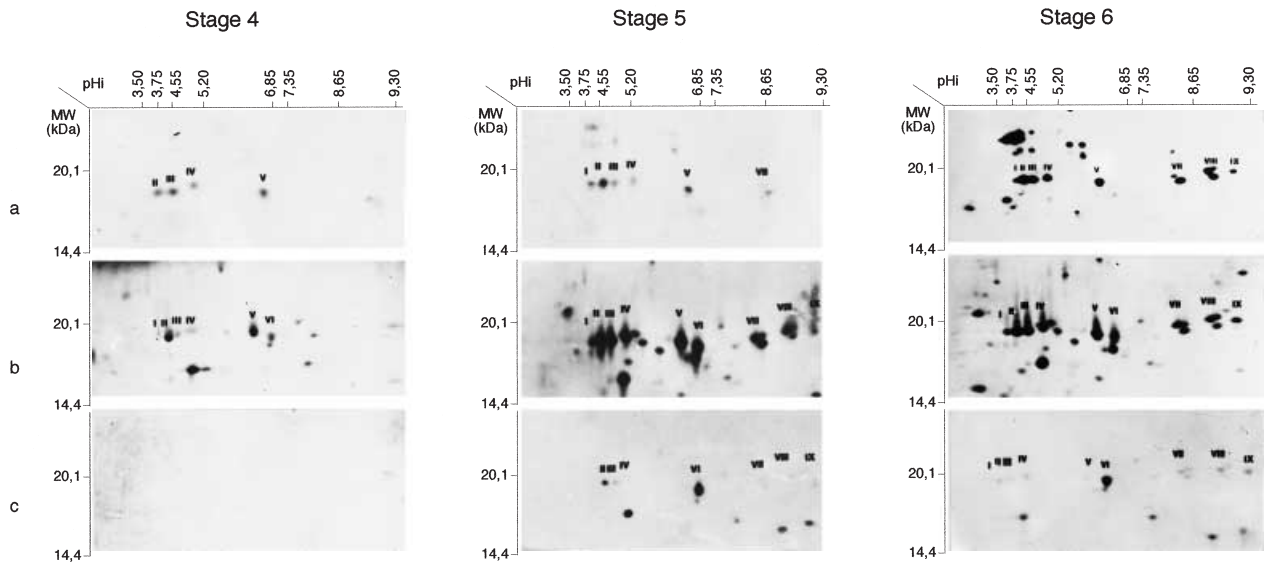

FIGURE 1. The appearence of LESP elements during 3 stages (4, 5 and 6) of the reproductive period. These stages coincide with an increase in testosterone concentrations. Three parameters were analyzed at each of the 3 stages: (a) the radiolabeled polypeptides encoded by LESP mRNAs translated *in vitro* in the presence of [^{35}S]methionine, (b) LESP elements neosynthesized *in vitro* by cultured epididymal explants in the presence of [^{35}S]methionine, and (c) the accumulation of LESP elements in these explants. ^{35}S-Labeled translation products and neosynthesized elements, as well as total accumulated soluble proteins were separated by two-dimensional gel electrophoresis. MW, molecular weight.

Inserts were subcloned in the plasmid vector pUC 19 (Boehringer Mannheim (Roche Diagnostics), Meylan, France) and sequenced using the T7 sequencing kit (Pharmacia). All reactions were carried out according to the manufacturer's instructions. Each cDNA clone was sequenced at least twice on both strands.

Reverse transcription (RT)-PCR

Two micrograms total RNA (stage 6) were denatured in the presence of 100 pmol random hexanucleotides at 65 °C for 5 min, then reverse transcribed with 200 U murine moloney leukemia virus-RT (Promega) at 37 °C according to the manufacturer's instructions. All first strand cDNAs were amplified by PCR using 100 pmol specific primers and Vent exo-DNA polymerase (Biolabs, Ozyme, Saint Quentin en Yvelines, France) following the manufacturer's instructions. Amplifications were carried out using a 5′-CGGAATTCTG TTGTTGTTTGGAATGACC-3′ primer specific to the 5′ non-translated end of the LESP IV mRNA and either a B71 3′ specific primer (5′-TTCTA CAAGCCATTGATGCG-3′) or a B111 3′ specific primer (5′-GTGGACAGGGGCATTGATCT-3′).

All sequence data were analyzed using the BISANCE program (Dessen *et al*. 1990) at Infobiogen in Paris.

RESULTS

Evolution of LESPs expression during the reproductive period

The appearance of translatable LESP mRNAs and the corresponding proteins were studied from the beginning of the epididymal cells secretory activity, from stages 4 to 6 of the annual cycle (reproductive period). Three parameters were analyzed using 2D SDS-PAGE (Fig. 1): (a) the presence of translatable LESP mRNA, as determined by *in vitro* translation assays using total epididymal RNA in the presence of [^{35}S]methionine; (b) the presence of newly synthesized LESP elements following *in vitro* neosynthesis assays, using epididymal explants in the presence of [^{35}S]methionine; (c) the presence of accumulated LESP elements in the epididymal tissue and fluid, as estimated by a Coomassie brilliant blue coloration of total soluble proteins.

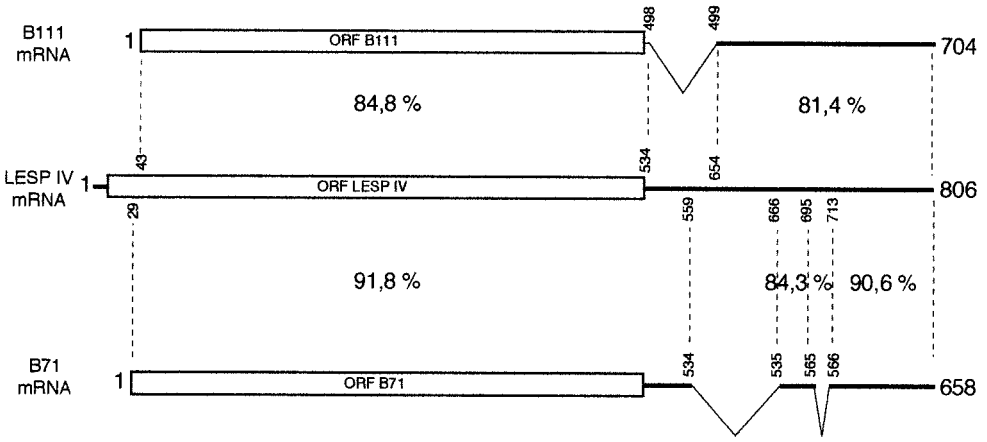

FIGURE 2. Schematic representation of the sequence homologies shared by the B71 and B111 cDNAs and the C731-LESP IV mRNA.

At the beginning of the reproductive period (stage 4), only acidic LESP elements are detectable (Fig. 1, left panel a,b). No element accumulated in the tissue at this period (Fig. 1, left panel c). At stage 5, the most basic LESP elements (VII to IX) are now expressed (Fig. 1, middle panel a,b). Elements I, II, III and IV, which were already synthesized at the previous stage are now present at a sufficient level in the tissue to be detected by coloration (Fig. 1, middle panel c). At stage 6, the mRNAs encoding for all LESP elements except LESP VI, are present (Fig. 1, right panel a). All elements are neosynthesized and accumulated (Fig. 1, right panel b,c). Interestingly, elements V and VI present a particular behavior: LESP V was only detected as an *in vitro* translational product (Fig. 1a) while LESP VI was not. Conversely, LESP VI was only detected when assays were carried out using epididymal tissue (Fig.1, middle and right panels c).

Isolation and characterization of LESP cDNA clones

One hundred thousand clones of the previously constructed lizard epididymal cDNA library were screened with the C731-LESP IV RNA probe and 20 positive cDNA clones were selected. Taking into account their restriction patterns, we could establish the existence of three distinct cDNA populations: the first corresponding to LESP IV cDNAs, and the second and third populations corresponding to new LESP mRNAs represented by the B71 and B111 cDNAs. As they were incomplete at their 5′ ends, RT-PCR was carried out using B71 and B111 specific primers to generate full length cDNAs.

The coding regions are very similar, and when aligned they present an average homology of 86 to 92%. The resulting 10% mismatches correspond to nucleotide substitutions randomly distributed on the length of the analyzed sequences. The 3′ non-coding regions present sites of punctual nucleotide substitutions on the overlapping fragments, but also vary widely in size. In fact, large fragments of the LESP IV mRNA non-coding regions are absent on the B71 or on the B111 cDNAs (Fig. 2), indicating that the non-coding regions are more divergent than the open reading frame (ORF) sequences.

The deduced primary amino acid sequences have also been compared (Fig. 3). As observed at the nucleotide level with the corresponding cDNAs, the three sequences are highly homologous. They present 70 to 80% identity, and when conservative substitutions are taken into account, this rises to 76% to 91% identity. Furthermore, as for the cDNAs, the substituted amino acids are randomly distributed along the sequences.

The different elements of the LESP family, except LESP I, have been purified on two-dimensional electrophoresis gels, and their NH_2 terminal extremities sequenced (Fig. 4). Two main features can be pointed out. First, on the basis of amino acid sequence identities, the comparative multialignment allowed us to define 5 groups of LESP elements. Group 1 is composed of LESP II and LESP III and group 2 contains only LESP IV. Groups 3 and 4 are composed respectively of LESP V and VI, and of LESP VII and VIII, while group 5 contains only LESP IX. According to this classification, elements belonging to the same group show identical aminoterminal sequences. When elements belong to different groups, they present 1

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                  10        20        30        40        50        60
LESPIV    DIPVVPNFDAQKTVGKWHPIGMASKLPEVPEYEQKISPMDHMVELTDGDMKLTANYM-DG  59
ProtB71   DIPVVPNFDPQKTVGKWHPIGMASKLPELTPYEQKISPMDHIVEVIDGDMKLTANYMSDG  60
ProtB111  DIPVVANFDTPKTVGKWHPIGMASKLPELTPYEQKISPMDHIVEVTDGDMKLTANYMSDG  60
Cons.     DIPVV+NFD.+KTVGKWHPIGMASKLPE+++YEQKISPMDH+VE++DGDMKLTANYM+DG

                  70        80        90       100       110       120
LESPIV    VCKEATAMLKHTDKPGVFKFTGGEIRMMDIDYEKYLIMYMKKS-TFEAMYLSARGSDVGD 118
ProtB71   VSKEATAMLKHTDKPGVFKFTDGEVHVLDVDFEKYIMLYVKKS-SHEALFLSARGPDVED 119
ProtB111  VCKTSVLVLKHTDKPGVFKVPDGEVHVIKMLILKSISFFTSKKPTHEALFLSARGSTGGD 120
Cons.     V+K+++++LKHTDKPGVFK+++GE+++.+.+.+K++..+.+K+.++EA++LSARG++++D

                  130       140       150
LESPIV    DIKEKFKKLVLEQNFPEAHIKYFNAEQCTPTAA 151
ProtB71   DIKEKFKKLVLEQSFPEANIKYFNAEQCTPTAA 152
ProtB111  DIKAKFKKLVWEQIILEAHIKYLNVEQCTPKAG 153
Cons.     DIK+KFKKLV+EQ.++EA+IKY+N+EQCTP+A+
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FIGURE 3. Alignment of the B71 and B111 ORF amino acid-deduced sequences with the LESP IV sequence. Identical amino acids are noted in the consensus sequence (*Cons.*). The numbering of the LESP IV amino acids corresponds to the complete protein numbering.

to 3 amino acid substitutions with respect to the LESP IV element, which corresponds to the C731 encoded polypeptide (Morel *et al*. 1993).

Secondly, this classification is supported by the LESP cDNA analysis as we identified B71 as the mRNA able to code for LESP II and/or LESP III proteins, and B111 as the mRNA able to code for LESP V and/or VI proteins.

DISCUSSION

Following an androgenic stimulation, the epididymal epithelial cells of the lizard, *Lacerta vivipara*, have been shown specifically to synthesize and to secrete large amounts of soluble proteins in the seminal fluid. Amongst these proteins, Depeiges *et al*. (1987) have characterized the LESP protein family, a lipocalin-related protein family (Morel *et al*. 1993). When analyzed on 2D SDS-PAGE, the LESP family appears to be composed of 9 immunorelated elements of identical molecular mass (18 kDa), but with different pHi (3·5 to 9) (Depeiges *et al*. 1988). Several hypotheses can be proposed to explain such a diversity in the LESP family, with the extremes being: (i) 9 genes code for the 9 LESP proteins, and (ii) a unique gene codes for the whole protein family, the proteic diversity being the result of post-transcriptional and/or post-translational events.

1	LESP-II	-	I	P	V	V	P	N	F	D	**P**	Q	K	T	-	-	..
	LESP-III	D	I	P	V	V	P	N	F	D	**P**	Q	K	T	V	G	..
2	LESP-IV	D	I	P	V	V	P	N	F	D	**A**	Q	K	T	V	G	..
3	LESP-V	D	I	P	V	V	**A**	N	F	D	-	**P**	K	-	V	-	..
	LESP-VI	-	I	P	V	V	**A**	N	F	D	**T**	**P**	K	T	-	-	..
4	LESP-VIIa	**K**	I	P	V	V	**P**	N	F	D	**P**	**Q**	-	-	V	G	..
	LESP-VIIb	**K**	I	P	V	V	P	N	F	D	**P**	Q	K	-	V	G	..
	LESP-VIII	**K**	I	P	V	V	P	N	F	D	**P**	Q	-	-	-	-	..
5	LESP-IX	**V**	I	P	V	V	P	N	F	D	**P**	Q	K	T	V	G	..

FIGURE 4. Amino acid alignment of the NH_2 terminus sequences of the mature LESP elements. On the basis of sequence homologies, 5 groups of LESP elements were defined. The boxed amino acid clusters correspond to sequences that are identical to the LESP IV sequence, which is our reference sequence. -, indicates undetermined amino acids. Heavy type and underlined characters represent amino acids that differ from those of the LESP IV sequence.

In order to understand the structural organization of the LESP family and to test these different hypotheses, we determined the structure of the LESP family at the protein and mRNA levels, and analyzed the expression of these different proteins and mRNAs during the annual cycle.

All the LESP NH_2 terminal sequences were obtained (Fig. 4). On the basis of these sequences 5 groups were defined. In order to identify the mRNAs that code for the different groups of LESPs, a lizard epididymal cDNA library (Morel *et al*. 1991) was screened. Amongst the selected clones, we distinguished 3 different cDNA populations represented by LESP IV, B71 and B111 cDNAs. The newly identified B71 and B111 cDNAs can potentially code for the group of LESP II-III and LESP V-VI respectively. A comparative nucleotide sequence analysis revealed that these 3 clones are 90% homologous. Within the open reading frames, the 10% divergence corresponds to punctual nucleotides substitutions randomly distributed along the sequences. The 3′ non-coding regions present greater rearrangements with the lack of 10 to 100 nucleotide fragments on B71 and B111 sequences in comparison to the LESP IV cDNA (Fig. 3). Nevertheless, the punctual nucleotides substitutions remain the main source of divergence between the 3 clones, such that LESP IV, B71 and B111 cDNAs could correspond to 3 mRNAs encoded by 3 distinct genes and not to a splicing

process of a unique pre-mRNA. Together with the diversity of mRNA populations, post-translational processes are involved, adding diversity to the family. This is sustained by previous experiments (Ravet *et al*. 1991) showing that even if no element is sulfated, LESP V is phosphorylated and all of them are slightly glycosylated (Depeiges *et al*. 1987).

The particularity of the lizard sexual cycle allowed us to study the expression of the LESP genes during the 3 stages of the reproductive period. From March to May (stages 4, 5 and 6), the lizard cycle is characterized by a spectacular increase in the plasma and intra-epididymal testosterone concentrations (Dufaure *et al*. 1986) correlated with an increase in the synthesis and secretory activities of the epididymal epithelial cells. At each of these 3 stages, we looked for the LESP mRNA translatability *in vitro*, LESP neosynthesis and accumulation in the tissue (Fig. 1a and b). At stage 4, only acidic translation products and acidic neosynthesized proteins are present. On the other hand, both acidic and basic proteins are strongly represented at stage 6. This could support the existence of several LESP messenger RNA populations, the expression of which is stage-related. It is likely that LESP gene expression is differentially induced, being correlated to the rate of intra-epididymal testosterone concentrations and/or to the presence of different luminal and/or plasmatic factors (glucocorticoids, transcriptional activators, ...). Furthermore, the behavior of elements V and VI is noteworthy - whatever the stage analyzed, no translation product corresponding to LESP VI could be detected, while LESP V is not, or is only slightly, accumulated. As they possess identical aminoterminal sequences (Fig. 4), these 2 elements could be encoded by a single mRNA species corresponding to the B111 sequence. Thus, LESP V could correspond to the primary translation product of this mRNA, and LESP VI to the complete mature form accumulated in the organ. As LESP V is the most acidic, and because it has been shown to be the only phosphorylated LESP element (Ravet *et al*. 1991), we hypothesize that LESP V is phosphorylated as it is synthesized, and the post-translational maturation of this element into LESP VI is the result of the excision of the phosphate group.

LESPs have been shown to belong to the lipocalin protein superfamily (Morel *et al*. 1993), amongst which the MUPs are well characterized. The MUPs form a multihormonally regulated protein family (Knopf *et al*. 1983) whose members, as for the LESP elements, present an identical molecular weight, and a diversity of pHi (Finlayson *et al*. 1968, Szoka *et al*. 1980). The structural analysis of this family revealed that it is multigenic (Hastie *et al*. 1979, Bishop *et al*. 1982) and that the corresponding mRNAs differ from each other by punctual nucleotide substitutions (Shahan *et al*. 1987). Furthermore, in the liver, two main populations of MUP mRNAs exhibit two different sizes that result from a differential splicing of the 7th exon (Clark *et al*. 1984). As this occurs in the non-coding regions, the encoded proteins present a similar size, and their differences only result from punctual mutations in the ORF. All of these characteristics of the MUPs are shared by the LESP family, and even though we need to complete the work at the genomic level, we can assume that LESPs form a multigenic family, as do the MUPs.

In conclusion, both the physiological and molecular analyses allowed us to determine a first structural image of the LESP family organization. Hence, by analogy with the MUPs family structure, these analyses led us to conclude that LESP is a multigenic family with at least 5 distinct genes and mRNA populations. Nevertheless, the diversity of the family results not only from the gene and mRNA diversity but also from post-translational maturations. The expression of this multigenic family is under multihormonal control (Courty 1991), as has been described for MUPs regulation (Knopf *et al*. 1983). Moreover, the present analysis points out the existence of differentially expressed LESP genes.

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