Human testis expresses a specific poly(A)-binding protein.

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Abstract

In testis, mRNA stability and translation initiation are greatly under the control of poly(A)-binding proteins (PABP). Here, we cloned a new human testis specific PABP (PABP3) of 631 a.a. (70.1 kDa) with 92.5% identical residues to the ubiquitous PABP1. A northern blot of multiple human tissues, hybridised with PABP3 and PABP1 specific oligonucleotide probes, revealed two PABP3 mRNA (2.1 and 2.5 kb) detected only in testis, whereas PABP1 mRNA (3.2 kb) was present in all tested tissues. In human adult testis, PABP3 mRNA expression was restricted to round spermatids, whereas PABP1 was expressed in these cells, as well as in pachytene spermatocytes. PABP3 specific antibodies identified a protein of 70 kDa in human testis extracts. This protein binds poly(A) with a slightly lower affinity as compared to PABP1. The human PABP3 gene is intronless with a transcription start site 61 nt upstream from the initiation codon. A sequence of 256 bp upstream from the transcription start site drives the promoter activity of PABP3 and its tissue specific expression. The expression of PABP3 might be a way to bypass the PABP1 translational repression and to produce the amount of PABP needed for the active mRNA translation in spermatids.

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Introduction

In testis, messenger RNA (mRNA) stabilisation is of crucial importance during the different stages of differentiation from spermatogonia to spermatozoa. In fact, gene transcription is mainly active at the first steps of this process (meiosis) and most of the mRNA are stored as ribonucleoprotein particles (mRNP) and translated at later stages (1-5). The maximal levels of PABP parallel the presence of mRNP suggesting that PABP maintains the stability and the translational potentiality of long-lived mRNA.

In eukaryotic cells, mRNA and pre-mRNA associate with at least 20 different specific proteins to form mRNP (6,7). The predominant protein of this complex is a poly(A)-binding protein (PABP), which binds to the 3' poly(A) tail of mRNA (8). In mammals, the poly(A)-binding protein family comprises a nuclear isoform and several cytoplasmic forms coded by different genes. Two mouse cytoplasmic isoforms, the ubiquitous PABP1 and the testis specific PABP2, have been characterised (9,10). In human, an ubiquitous PABP (PABP1) has been cloned (11) and a cytoplasmic inducible isoform, iPABP, has been characterised following activation of T cells (12), and platelets (13). In addition, three PABP pseudogenes are present in the human genome (14-16). As initially described for *Saccharomyces cerevisiae* (15,16), mammalian cytoplasmic PABP stabilise mRNA and enhance translation in association with the eukaryotic initiation factor eIF-4G subunit (17) or the PABP-binding protein (PAIP-1) (18). The shuttle of PABP1 between the nucleus and the cytoplasm suggests an involvement of this protein in nuclear mRNP formation and cytoplasmic export (19). These key roles have been recently highlighted by the observations that PABP1 is one of the targets of enterovirus (20), coxsackievirus (21) or rotavirus (22) for host protein synthesis shut-off.

In the current study, we describe a new testis specific poly(A)-binding protein isoform (PABP3) specifically expressed in round spermatids. Its mRNA is transcribed from a retroposon under the control of a tissue specific promoter.

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Materials and Methods

The nucleotide sequences for PABP3, PABP4 mRNA, and PABP3 promoter have been deposited in GenBank database under accession numbers AF132026, AF132027, and AF315079 respectively. The other PABP sequences mentioned in this study : PABP1 mRNA, the splicing variant PABPII, iPABP mRNA, PABP1 gene, and the mouse PAPB2 mRNA have the accession numbers AH007272, Z48501, U33818, U68093 to U68105, and AF001290 respectively. All the oligonucleotides used in this study are described in table 1.

cDNA cloning

The insert of one clone (EST h08023t), susceptible to correspond to a new potential PABP (23), was used as a probe to screen a human testis library (24,25) as previously described (26).

DNA sequencing and analysis

Plasmids were prepared and sequenced as previously reported (23,27).

Northern blot analysis

Human multiple-tissue northern blots (Clontech, CA) were probed with oligonucleotides PABP1, PABP3, PABP4 and iPABP specific for each PABP (table 1). Blots were prehybridised and hybridised in ExpressHyb™ hybridisation solution (Clontech, CA) at 37°C. They were washed in 2x SSC (1x SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7); 0.1% SDS at room temperature for 15 min then in 2x SSC; 0.1% SDS at 43°C twice for one hour. The specific signals were detected using a phosphorimager STORM 840 (Molecular Dynamics, CA).

Semi-quantitative RT-PCR on human testis RNA

Total RNA from human adult testis was extracted using acid guanidium thiocyanate (28). RT-PCR were performed on 5 ng of total RNA using the Access RT-

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PCR System (Promega, WI) and two oligonucleotides (3'RT and 5'RT, this last one being labelled with 6FAM) (table 1). This set of primers frames a region with insertions or deletions in PABP3, PABP4, PABP1 and iPABP fragments which exhibited sizes of 513, 519, 528 and 555 bp respectively. RT-PCR were carried out on a Perkin-Elmer GeneAmp 9600 Thermo cycler under the following incubation conditions : 45 min of reverse transcription at 48°C, denaturation for 2 min at 94°C and 30 cycles of PCR (1 min at 94°C, 1 min at 50°C and 1 min at 72°C). The amplified products were detected on an Applied Biosystems 373A automated DNA sequencer and analysed using the GeneScan program. Under these experimental conditions, as determined by using various mixes of the four PABP RNA synthesised in vitro, the surface under each peak was proportional to the amount of specific mRNA in the assayed samples.

***In situ* hybridisation**

Human testis samples were taken during surgery for orchidectomy, fixed overnight in 10% formol, and embedded in paraffin using routine procedures. Sections of 6 µm thickness were mounted on polylysine-coated slides and stored at room temperature until used. The HPLC-purified PABP1 and PABP3 primers (table 1) were used as antisense PABP1, PABP3 probes respectively, whereas 5'RT primer was used as sense probe. These oligonucleotides were labelled using digoxigenin-11-deoxyuridine triphosphate (DIG-dUTP) Oligonucleotide Tailing kit (Roche Diagnostics, F) and kept at -20°C. Following deparaffinisation in xylene and rehydratation, sections were incubated in 50% acetic acid for 30 s, rinsed in sterile water, and digested with proteinase K (10 µg / ml) in 100 mM Tris-HCl pH 7.5, 1 mM $CaCl_2$ for 15 min at 37°C. The sections were then washed in sterile water, postfixed in 2% paraformaldehyde (PFA) for 20 min at room temperature, washed in sterile

water, dehydrated in graded ethanol baths and air-dried. Each section was prehybridised for 2 hr at 37 °C in 100 µl of hybridisation buffer (4% deionised formamide, 1x Denhardt's solution, 1.5x SSC and 330 µg / ml yeast tRNA) in a humid chamber. *In situ* hybridisations were performed for 16 hr at 37 °C in 20 µl of hybridisation buffer containing 10% dextran sulphate and 25 ng DIG-labelled oligonucleotide probe. After hybridisation, the sections were briefly rinsed in 2x SSC at room temperature, in 2x SSC for 1 hour at 37°C, and finally in 1x SSC for 30 min at room temperature. Hybridisation signals were detected as previously described (29). Sections were counterstained using methyl green in order to visualise the nuclei.

Preparation of antibodies

A 9 amino acid peptide (CAVPNQRAP) specific for PABP3 (peptide 3), corresponding to a.a. 391-398 of this protein was added with a Cys residue at its amino terminal end for the coupling to keyhole limpet hemocyanin (KLH) as immunogen (Syntem SA, F). Peptide 3 antibodies were generated by immunisation of rabbits with this antigen. A 11 a.a. peptide (CPNPVINPYQP) specific for PABP1 (peptide 1) corresponding to a.a. 393-402 of PABP1 protein sequence with an additional Cys residue at its amino terminal end was used as control in competition experiment.

Western blot analysis.

Human testis and liver tissues were homogenised in 40 mM Tris-HCl buffer pH 8.5 containing 250 mM sucrose, 1 mM EDTA, 1 mM PMSF, 1 µg / ml leupeptine. After centrifugation (13,000 g, 2 min), the supernatants were kept frozen until Bradford assay (BioRad, US). Following denaturation for 5 min at 90°C, protein extracts (70 µg / lane) were separated on 9.2% SDS-PAGE, blotted onto a PVDF

transfer membrane (Millipore, F), and probed either with the anti-peptide 3 serum (1/100), or anti-peptide 3 serum preabsorbed with peptide 3 (200 µg), or peptide 1 (200 µg). Treatment of the membrane was done according to the enhanced chemifluorescence protocol (ECF, Amersham, UK). Sheep anti-rabbit IgG, associated with alkaline phosphatase (1/10000), was used as secondary antibody. The membrane was incubated for 10 min in ECF solution, and fluorescent signals were visualised using a phosphorimager STORM 840 (Molecular Dynamics, CA).

Protein synthesis and RNA binding assays.

The open reading frames (ORF) of PABP1 and PABP3 were amplified using the 5'ORF and 3'ORF primers (table 1), iPABP was amplified using 5'iORF and 3'iORF primers. Each PCR product was cloned in both orientations into the pTargeT^{TM} vector (Promega, WI). The inserts of the plasmids, linearised with KpnI restriction enzyme, were transcribed and translated using the Linked *in vitro* T7 Transcription/Translation-radioactive kit (Roche Diagnostics, F) in the presence of [^{35}S] methionine (Amersham, UK) (1000 Ci/mmole). One tenth of the translation reaction was analysed on a (10%) acrylamide (29:1) denaturing gel. The specific signals from dried gels were detected using a phosphorimager STORM 840 (Molecular Dynamics, CA).

Binding of *in vitro* translated PABP to RNA homopolymers coupled to Sepharose was performed as described by others (12,30,31), with the following changes. For each binding experiment, poly(A)-Sepharose (50 ng poly(A) per reaction), was equilibrated in 500 µl of KCl (0.1 M) binding buffer (10 mM Tris pH 7.4, 2.5 mM $MgCl_2$, 0.1 M KCl and 0.5% Triton X100). The standard binding reaction was performed for one hour at 4°C with 3 µl of the *in vitro* translated products in a final volume of 80 µl KCl (0.1 to 2 M) binding buffer, containing 20 units of RNasin

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(Promega, CA), 20 µg yeast tRNA (Sigma-Aldrich, F) and 8 µg poly(dI-dC) (Pharmacia Biotech, S) as non specific competitors. In competition experiments, a 30 min pre-incubation with 5 µg of HPLC purified oligonucleotides, polyA[40], polyU[40], polyC[40] or polyG[40] (Genset SA, F), was performed before poly(A)-Sepharose addition to the binding reaction (KCl 1 M). The poly(A)-Sepharose was then washed two times with 500 µl of KCl (0.1 to 2 M) binding buffer, resuspended in SDS sample buffer (50 mM Tris pH 6.8, 100 mM DTT, 2% SDS and 10% glycerol), boiled for 3 min and loaded on a 10% acrylamide (29:1) denaturing gel. The dried gels were exposed overnight on a phosphor screen and the signal detected on a phosphorimager STORM 840 (Molecular Dynamics, CA).

Gene structure analysis

Standard PCR reactions were carried out on a Perkin-Elmer GeneAmp 9600 Thermo cycler using the Advantage cDNA PCR Kit (Clontech, CA) in 50 µl containing either high molecular weight human DNA (60 ng) from blood peripheral leukocyte cells, or PABP1 and PABP3 cDNA (1 ng) as positive controls. These DNA sequences were amplified with 5'ORF/3'ORF (PABP3), or 5'PCR/3'ORF (PABP1), or T7 (Promega, WI)/3'ORF primers. This last set of primers, which is able to amplify PABP sequence cloned in plasmids, was used to check that the genomic DNA was not contaminated with PABP containing plasmids. The PCR cycles for PABP3 started with the DNA-polymerase activation step (94°C, 1 min) followed by 10 cycles of « touchdown » from 60°C to 50°C (32), 30 cycles of PCR (5 sec at 94°C, 10 sec at 50°C and 3 min at 72°C) and a final extension for 10 min at 72°C. The PCR for PABP1 were performed under the following incubation conditions : 94°C 1 min, followed by 35 cycles of PCR including 2 steps (95°C 15 sec and 68°C for 22 min). The amplified products were loaded on a 1 % agarose gel in TBE buffer (90 mM Tris-

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borate pH 8, 1 mM EDTA) for electrophoresis. The gel was then blotted on a Hybond N^+ membrane (Amersham, UK) and probed with the specific PABP1 and PABP3 primers as described for the northern blot.

Cloning of the 5' region of PAPB3 mRNA.

5'RACE cloning was carried out on one µg of human testis mRNA (Clontech, CA) using the SMART RACE cloning Kit (Clontech, CA) and the RACE primer (table 1). The resulting products were subcloned into the pCR4-TOPO vector using the TOPO-TA Cloning kit (Invitrogen, ND). Positive clones were sequenced as mentioned for PABP3 cDNA.

PABP3 promoter cloning and sequencing.

The PABP3 gene was isolated from the Single Human BAC Library (Research Genetics, AL) according to supplier's protocols. Briefly, two PABP3 specific oligonucleotides (5'BAC and 3'BAC) were used to amplify a fragment which was detected by the oligonucleotide BAC probe. PCR reactions were performed as previously described (33) on 1 µl DNA from the "Superpool" plate (PCR step 1), then on 2 µl DNA from the "Plate pool" plates (PCR step 2). The final screening was achieved by hybridisation of the selected "384 Colony BAC DNA membranes" with the PABP3 [^{32}P] α−dCTP labelled insert, as probe. DNA from the positive BAC clones was extracted and purified using the QIAGEN Large-Construct Kit (Qiagen, CA). PABP3 putative promoter sequence was obtained directly on 2 µg of SphI digested BAC DNA using the dye terminator sequencing kit (Applied Biosystems, CA) and oligonucleotides RACE, Seq1, Seq2 and Seq3 designed from the sequences of PABP3, 5'-RACE products, and the results of RACE and Seq1 sequence reactions, respectively. The PABP3 putative promoter sequence was analysed using

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MatInspector program (core similarity = 1, and matrix similarity > 0.87) (34) in order to identify consensus binding sites for transcription factors.

S1 nuclease mapping analysis.

A 737 bp genomic DNA fragment was amplified from 10 ng BAC DNA using oligonucleotides 5'PROM and RACE (table 1), as 5' and 3' primers respectively, under conditions previously described (33). This PCR fragment was gel-purified using QIAEX II (Qiagen, CA), and then used as matrix for the S1 mapping probe synthesis. Briefly, the 6FAM labelled RACE antisense primer (Genset SA, F) was used to generate a 5'-labelled single-stranded DNA fragment using 50 ng of the 737 bp genomic fragment. The probe was checked on agarose gel, purified using QIAEX II kit, and quantified by ethidium bromide staining. PolyA$^+$ RNA from either testis or liver (2 µg) (Clontech SA, F) was hybridised to the end-labelled probe (50 ng) in 80% formamide, 100 mM sodium citrate, 300 mM sodium acetate and 1 mM EDTA, pH 6.5, at 37°C overnight. S1 nuclease (1U / µl) (Life technologies, SARL, F) was then added to the digestion buffer (50 mM sodium acetate, 200 mM sodium chloride, 1 mM zinc chloride, 0.5% glycerol) for 30 min at 37°C. As a control, the probe was also hybridised to 10 ng PABP3 synthetic RNA produced using the Riboprobe Gemini II kit (Promega, WI) and the corresponding PABP3 plasmid as template, according to the supplier's protocol. Protected fragments were detected on an Applied Biosystems 373A automated DNA sequencer and analysed using the GeneScan program.

Plasmid constructs for promoter analysis.

The PCR products corresponding to different regions of the promoter were subcloned upstream from the Enhanced Green Fluorescent Protein (EGFP) reporter gene in the pEGFP-1 vector (Clontech, CA) under conditions previously described (33). These inserts were generated by PCR reaction on 10 ng of BAC DNA using

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Advantage cDNA PCR Kit (Clontech, CA). The four oligonucleotide couples used for PCR were 5'PROM/3'PROM, 5'PROM1/3'PROM, 5'PROM2/3'PROM, and 5'PROM3/3'PROM in order to amplify fragments PC (528 bp), P1 (473 bp), P2 (286 bp) and P3 (160 bp) respectively. The resulting products were digested with Bgl II/Hind III (see table 1), and then subcloned into the promoter-less pEGFP-1 reporter vector. Plasmids were purified using the Maxiprep reagent system (Qiagen, CA), and verified by sequence analysis. Plasmid CMV/pEGFP-C1 (Clontech, CA), which contains the human cytomegalovirus (CMV) promoter, was used as positive control in transfection experiments.

Cell culture and transfection assays.

The human NTERA-2/D1 cell line (NTERA-2/clone D1, a human pluripotent embryonic carcinoma cell line derived from a lung metastasis of a testicular teratocarcinoma) was obtained from the American Type Culture Collection (number CRL 1973, ATCC, Biovalley, F). NTERA-2/D1, and HeLa cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies SARL, F) containing 10% (v/v) foetal calf serum (LifeTechnologies, F), penicillin/streptomycin, and 2 mM glutamine with a pCO_2 of 10% (NTERA-2 cells) or 5% (HeLa cells) at 37 °C in humidified air. Cells were transfected using the LipofectAMINE$^+$ protocol (Life Technologies SARL, F). Briefly, HeLa, or NTERA-2/D1 cells, at 60% and 90% confluence respectively, were washed twice with serum-free medium. They were transfected with 400 ng of either CMV/pEGFP-C1 plasmid (Clontech, CA) as positive control, or PABP3/pEGFP-1 constructs with 4 µl of LipofectAMINE and 1 µl of PLUS reagent (Life Technologies, F) in 200 µl of serum-free medium. After 3 h of incubation, the medium was replaced with 500 µl of medium supplemented with 10% serum, and cells were harvested after 48 h of culture by trypsin/EDTA digestion

and resuspended in PBS containing 0.5 µg/ml propidium iodide (PI). Enhanced Green Fluorescent Protein (EGFP) fluorescence was detected by a FACS (Coulter EPICS XL-MCL, Beckman Coulter, CH) flow cytometer equipped with an argon laser emitting at 488 nm. The FL1 emission channel (normally used to detect FITC) was used to monitor EGFP fluorescence; the FL3 channel was used to identify dead or dying cells with PI red fluorescence.

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Results

Isolation and sequence of PABP3 and PABP4.

Recently, we identified a large series of human testis EST (23). One of them (EST h08023t), corresponding to a new PABP isoform, was used as probe in order to screen a human testis cDNA library. Seven out of 10 positive clones corresponded to PABP1, and three of them revealed two new PABP sequences, PABP3 and PABP4. PABP3 cDNA (2397 nucleotides) exhibits an open reading frame (ORF) of 1893 nucleotides susceptible to encode a 631 a.a. protein (figure 1). The initiation codon (ATG), at position 24, is flanked by the Kozak consensus sequence favourable for translation initiation (35,36). Three polyadenylation consensus signal sequences (AATAAA) are detected in the 3' untranslated region at position 2015, 2035 and 2373. PABP3 exhibits four RNA binding domains (RBD) each of them containing the two ribonucleoprotein consensus motives (RNP1 and RNP2). Alignment of PABP3 and PABP1 amino acid sequences reveals 92.5% identical residues (data not shown). The major differences between these two species are a deletion of six amino acids (PVINPY), in the PABP3 sequence, corresponding to the position 395 to 400 of PABP1, and four amino acid substitutions in RBD I, II and III at position 52, 53, 102 and 191.

The sequence of the PABP4 cDNA (Acc n°: AF132027) reveals two ORF corresponding to incomplete PABP proteins (data not shown). The first one starts at nucleotide 81, with an initiation codon in a favourable consensus sequence for translation initiation (35,36). It codes for a peptide of 56 amino acids which contains the RNP2 of the RBD1. The second ORF (269 amino acids) starts at nucleotide 548 with an ATG in a poor context for initiation of translation. It might code for a protein of 269 amino acids containing only one and a half RBD. In the 3'

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untranslated region, an imperfect polyadenylation consensus signal sequence (AATTAA) (37,38) is located 52 bases upstream from the poly(A) tail.

Analysis of the PABP mRNA expression in human tissues.

Since the nucleotide sequences of the different PABP are very well conserved, we designed oligonucleotides specific for PABP1, iPABP, PABP3 and PABP4 (named PABP1, iPABP, PABP3 and PABP4 respectively) (table 1) in order to differentiate the expression of their respective mRNA. The specificity of these oligonucleotides was checked by hybridisation to *in vitro* transcribed RNA of the four PABP isoforms (data not shown). Hybridisation of the PABP1 specific probe to a multiple-tissue northern blot revealed one major band at 3.2 kb corresponding to the PABP1 mRNA in all the tissues tested (figure 2A). In contrast, hybridisation to PABP3 revealed two weak but distinct bands at 2.1 kb and 2.5 kb only in human testis. Oligonucleotides specific for PABP4 and iPABP did not revealed any specific signal (data not shown).

Due to the weakness of the signal detected on northern blot, a more accurate quantification of PABP mRNA isoforms expressed in human testis was achieved using semi-quantitative RT-PCR on total RNA. To that end we designed a couple of specific primers, 5'RT and 3'RT which was able to amplify fragments of different sizes for the four human PABP in the same reaction sample (see Material and Methods). A characteristic electrophoretic profile of the amplified products is presented in figure 2B. As expected, PABP1 and PABP3 are the two major PABP mRNA isoforms expressed in human testis. Based on 11 independent experiments, the amount of PABP3 mRNA represents 18.8 ± 5.2 % of PABP1 messenger level, whereas PABP4 and iPABP mRNA expression represent only 1.77 ± 0.57 % and 5.5 ± 0.06 % of those of PABP1 respectively. These results are in agreement with the northern blot analysis in which these two later mRNA were undetectable.

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The PABP1 and PABP3 mRNA were localised by *in situ* hybridisation on human adult testis sections using PABP1 and PABP3 specific oligonucleotide probes, respectively. PABP1 mRNA was strongly expressed in round spermatids and to a lower extend in pachytene spermatocytes (figure 3 A and B). In contrast, PABP3 mRNA was only detected in round spermatids and to a lower level as compared to PABP1 (24 h revelation as compared to 6 h for PABP1) (figure 3 C and D). No signal was observed with the control sense oligonucleotide 5'RT (figure 3 E and F).

Western blot analysis of PABP3

In order to demonstrate the presence of PABP3 in testis, we raised antibodies against the oligopeptide 3 : CAVPNQRAP (a.a. 391-398 of PABP3) elongated with a Cys at the amino terminal end. This sequence is specific for PABP3 as compared to PABP1, since six amino acids (PVINPY) are inserted in PABP1 at position 391. These antibodies were used to detect PAPB3 on a western blot of testis and liver extracts. Liver was used as a negative control, since no PABP3 mRNA was detected in this tissue (figure 2). As shown in figure 4, these antibodies recognised a band in testis at 70 kDa but no signal in liver extracts. This band was competed out by an excess of peptide 3 (used to raise the antibodies) whereas an excess of peptide 1 (specific for PABP1) did not altered the specific signal. This analysis clearly established the presence of PABP3 in human testis.

PABP synthesis and binding to RNA homopolymers.

In order to demonstrate the functionality of PABP3, we compared its ability to bind RNA homopolymers with that of PABP1 and iPABP. We synthesised mRNA corresponding to PABP1, iPABP and PABP3 and to the luciferase control. These mRNA were translated in the presence of [^{35}S] methionine and the labelled proteins were analysed on acrylamide-SDS gel (data not shown). For each sense RNA, we

observed a band corresponding to a protein at the expected size from the length of the different ORFs (PABP1: 70.3 kDa, iPABP: 72.4 kDa, PABP3: 70.1 kDa). No labelled protein was detected when antisense RNA were used as template.

In a second step, we determined whether these proteins were able to bind poly(A)-Sepharose. In order to estimate the relative affinities of each PABP for the polymer, each of the three PABP was incubated in the presence of poly(A)-Sepharose in a buffer containing increasing KCl concentrations of 0.1 M, 0.5 M, 1 M and 2 M (figure 5A). PABP3 exhibited an optimal binding at 0.5 to 1 M KCl and a strong decrease in affinity at 2 M KCl. iPABP exhibited it maximal binding activity at 0.1 and 0.5 M KCl and a sharp decrease at 1 and 2 M Kcl. The binding of PABP1 was optimal at 1M KCl and it was only slightly affected at 2 M KCl (figure 5A). The decrease of the binding of PABP3 and PABP1 to poly(A) homopolymer in the presence of 1 M and 2 M KCl was evaluated to 78 and 32 % respectively (figure 5B). The binding properties of PABP1 and iPABP were compatible with the results obtained by other groups (12,30,31). The three PABP were also incubated with poly(A)-Sepharose in 1M KCl binding buffer in the presence of 100x molar excess of ribohomopolymers (poly(U), poly(A), poly(G) and poly(C)) (figure 5A). The binding competition of PABP to poly(A)-Sepharose with purified soluble poly(U) appears slightly more efficient for PABP3 and iPABP than for PABP1. For each PABP, an increased binding to poly(A)-Sepharose was observed in presence of poly(G). Under these experimental conditions, we did not observed any non specific binding of luciferase to poly(A)-Sepharose or of PABP to Sepharose 4B (data not shown).

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Genomic analysis of PABP3 and PABP4 sequences.

Recently, Kleene et al. (39) reported that the mouse testis PABP2 is encoded by an intronless gene. In order to determine whether the human testis specific PABP3 gene, might also correspond to an intronless gene, we amplified the PABP1 and PABP3 genomic sequences using the set of oligonucleotides designed for the cloning of the ORFs. The specific fragments were revealed by hybridisation with oligonucleotides specific for PABP1 and PABP3 (figure 6). We obtained a 23 kb genomic PABP1 fragment as expected from the human PABP1 gene sequence. The same set of oligonucleotides amplified a 2 kb band on the PABP1 plasmid. In contrast, a fragment of 1.9 kb was amplified from genomic DNA or plasmid cDNA using PAPBP3 specific oligonucleotide, a result which is compatible with the presence of an intronless gene. The lack of amplified fragment on genomic DNA using T7 and 3'ORF primers, indicated that the signal cannot arise from genomic DNA contamination by a plasmid. The recent availability of the working draft for the human chromosome 13 (acc. n°: AL359757.4), on which the PABP3 gene is located (33), confirmed that this gene is intronless. We also determined that the PABP4 pseudogene is an intronless (data not shown)

5' end cloning of the PABP3 mRNA

In order to determine whether the PABP3 cDNA corresponded to a full length mRNA, the oligonucleotide RACE (table 1) was hybridised to human testis poly(A) mRNA and we cloned the extension products. The sequence of 25 clones revealed four potential initiation start sites located 26, 40, 42 and 58 bp upstream from the initiator ATG, extending the PABP3 cDNA of a maximum of 35 nt (figure 7). Therefore, the 5'UTR of PABP3 is expected to be very short and it should be noted that is does not contain any poly(A) stretches as described for PABP1 (14).

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Isolation and characterisation of promoter region of PABP3 gene

Screening of a human genomic library for the PABP3 gene resulted in the isolation of two positive BAC clones. They contain a 100 kb and 150 kb insert respectively, each of them including a 3.5 kb Sph1 fragment that hybridised to the oligonucleotide Seq1 specific to the extremity of the 5' UTR of the PAPB3 cDNA. This fragment rearranged systematically during subcloning. Therefore, we sequenced the BAC insert as described under « Materials and Methods » up to 556 bp upstream from the initiator ATG. This fragment was cloned by PCR and its sequence is reported on figure 7. Alignment of the PABP3 cDNA with this genomic fragment showed that 23 nt of its 5'-UTR part, and the 35 further nt determined by primer extension were contiguous on the gene and lie within a single exon.

S1 nuclease protection analysis of this genomic DNA fragment by hybridisation to poly(A) mRNA from human testis resulted in the protection of a 239 bp fragment (figure 8). The 5' end of this fragment maps exactly at the 5' end of the longest PABP3 cDNA obtained by primer extension of the PABP3 mRNA located 58 nt upstream of the initiation codon. As expected, no DNA fragment was protected by hybridisation of the probe with liver poly(A) mRNA, which does not contain PABP3 mRNA. The specificity of this S1 nuclease experiment was also confirmed by the protection obtained by hybridisation to a synthetic PABP3 mRNA (240 bp) and not to a PABP1 mRNA (data not shown). This experiment identifies the start site of PABP3 mRNA and therefore the promoter region of this gene. As shown in figure 7, this region revealed several consensus sequences : AP4 (-456, -241, -216, -205, -202, -125, -108) ; HNF3ß (-406, -391, -326); GFI1 (-281, -267) ; NF1 (-227, -220) ; AP2 (-219, -131) ; and MYB (-83). Other consensus sequences are mentioned in the legend of figure 7. The CpG residues represent 21.6 % of the nucleotides between base 1 to -256

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of the promoter and 0.8 % further upstream. The alignment of the human PABP3 promoter, with the human PABP1 and the mouse PABP2 promoter, revealed that the first 277 bp of the promoter of the PAPB3 gene exhibit 91.4 % identity with the 5' untranslated region of the PABP1 mRNA and that the promoter of the human testis PABP3 has an initiation start site located 100 bp downstream as compared to the putative start site of the mouse PABP2 gene (figure 7).

Promoter activity of the genomic sequence flanking the PABP3 mRNA start site

The promoter activity of the 498 bp sequence flanking the PABP3 mRNA start site was assessed by its ability to drive the expression of enhanced green fluorescent protein (EGFP) reporter gene in transiently transfected NTERA-2 cells which expresses endogenous PABP3 mRNA as demonstrated by RT-PCR (data not shown). The shortest construct (–130, +1) drove a low but significant activity in NTERA-2 cells which represented 1.42 ± 1.02 % of the activity driven by the CMV promoter in the same cells (figure 9). Extension of this construct up to residue -256 resulted in a 14 fold increase (19.87 ± 3.99 %) of EGFP fluorescence in the NTERA-2 cells, the activity of the construct dropped to 6.43 ± 1.8 % of the control when it was extended to -443 bp, whereas a further extension of the sequence up to -498 bp induced an increase of EGFP fluorescence up to 12.2 % ± 0.77 % of the activity driven by the CMV promoter. As a negative control, we used HeLa cells which do not expressed PABP3 mRNA as checked by RT-PCR (data not shown). The transfection of these cells with EGFP under the control of CMV promoter exhibited 4 times more activity than in NTERA-2 cells. Following the transfection of the different PABP3 promoter construct in HeLa cells, only the P2 construct exhibited a very weak promoter activity (2.42 ± 0.44 % of the control). These data demonstrate that the -498

nucleotides sequence upstream from the transcription start site of the PAPB3 mRNA, contains all the elements necessary to a cell specific expression of the PABP3 promoter.

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Discussion

We characterised two novel PABP mRNA (PABP3 and PABP4), isolated from human testis, which exhibit strong similarities with the human PABP1. Sequence analysis of PABP4 mRNA reveals an ORF coding for a truncated protein of 56 amino-acids which contains only half of the RBDI (RNP2) unable to bind to ribohomopolymers. Therefore PABP4 gene which encodes a non functional protein should be considered as a pseudogene.

More interestingly, we identified a new functional PABP in human testis (PABP3) which is under the control of a cell specific promoter. PABP3 is highly similar to the ubiquitous human PABP1. These two proteins differ mainly by four amino acid changes in the RBDs, and by a deletion of 18 nucleotides in PABP3 as compared to PABP1, which eliminates the PVINPY sequence in PABP3. This motif is located immediately upstream from the proline-rich region involved in protein-protein interactions (18). Therefore, PABP3 might display specific properties in its interaction with other proteins such as translation initiation factors or viral proteins.

Northern blot revealed two testis specific PABP3 mRNA (2.5 and 2.1 kb). Three polyadenylation signals are present in the PAPB3 cDNA sequence located at positions 2015, 2035 and 2373 respectively. The mRNA that we cloned was polyadenylated at the last site, but we cloned a shorter PABP3 cDNA which used the first polyadenylation signal located 358 bp upstream. Therefore, it is very likely that the 2.1 and the 2.5 kb PABP3 mRNA species are generated by alternative polyadenylation. A similar situation exists for PABP1 mRNA which contains 2 polyadenylation signals at nucleotide 2507 and 2823 respectively (11). In fact, out of 270 PABP1 EST in dbest database, 90 were generated from transcripts polyadenylated

at the upstream site, and 180 were issued from transcripts polyadenylated at the downstream site.

Due to high similarities among the human PABP species, we analysed PABP expression using specific oligonucleotides for the detection of PABP specific transcripts by northern blotting and by semi-quantitative RT-PCR. PABP1 mRNA is the major transcript in human testis, as well as in other tissues, whereas PABP3 expression appears to be testis specific where it represents 18% of PABP mRNA, and no signal was observed for PABP4 and iPABP. By using semi-quantitative RT-PCR iPABP mRNA level represented less than 7% of the PABP1 mRNA level in human testis. This result differs from those of Yang et al. (12) who reported that both PABP1 and iPABP mRNA were expressed at similar levels in all human tissues tested including testis. This discrepancy might result from a cross hybridisation between the PABP probes, since they used a cDNA probe.

We demonstrated that PABP1 and PABP3 are expressed in spermatogenic cells at different differentiation stages. The human PABP1 is expressed in pachytene spermatocytes and round spermatids whereas the specific testis isoform expression appears later in spermatogenesis and is restricted to round spermatids. The distribution of PAPB mRNA in human testis differs slightly from the PABP mRNA expression reported in mouse testis where the PABP1 and the testis specific PABP2 mRNA colocalised in pachytene spermatocytes and round spermatids (10).

Human PABP3 binds to poly(A) sequences with a lower affinity than PABP1. It can be related to the amino acid substitutions detected in the different RBD ; however we did not observed any of the tyrosine substitutions, described in mouse testis PABP (10) and reported to decrease the binding to poly (A). As for other PABP (10,12,30,31,40), poly(A) binding to PABP3 is poorly affected by poly(C) and poly(G),

whereas poly(G) enhances its poly(A) binding activity. This might result from a poly(G) binding site on PABP, located on RBDs III and/or IV and distinct from the poly(A) sites (30). The binding of poly(G) would promote conformational changes of the protein leading to an increased affinity for poly(A) sites of the two first RBDs.

Human testis specific PABP3 and PABP4 mRNA are transcribed from intronless genes which are very likely to correspond to retroposons. The expression of functional retroposons is a common phenomenon in meiotic and haploid spermatogenic cells as pointed out by Kleene et al. (39). The gene tree of 21 PABP sequences from various species (data not shown), revealed that the human PABP3 and PABP4 genes appeared independently of the mouse testis specific retroposon PABP2. These genes are likely to derive from PABP1 which exhibits 94% and 87% identity with the human PABP3 and PABP4 sequences, respectively. Based on these similarities, PABP4 might have appeared before PABP3.

We identified the transcription start site on the PABP3 gene by 5' RACE and S1 mapping. Several constructs of genomic region with this start site were associated to an EGFP reporter gene and transfected in NTERA-2 and HeLa cells. From these experiments, it appears that the PABP3 promoter is tissue specific since it was not functional in HeLa cells, although these cells expressed the control CMV/pEGFP construct at a very high level. The region (-130, +1) is sufficient to drive a low, but significant activity in NTERA-2 cells, but not in HeLa cells. Interestingly, a myb binding site, susceptible to bind A-myb, possibly involved in the proliferation and differentiation of spermatogonia (41-43), is located in this sequence. The activation of this myb element could participate in the tissue specific expression of the PABP3 promoter as it has been suggested for other testis specific genes such as PGK-2 (44) and Hsp70-2 (45). An extension of the promoter sequence up to the nucleotide 256

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upstream from the initiation start sites strongly induces the promoter activity up to 20% of the activity of the control CMV / pEGFP. This region contains four AP4, two AP2 and one NF1 binding sequences. The binding of the ubiquitous factors on this region could depend on the presence of other interacting testis specific factors as proposed for the mouse Tcp-10bt gene (46). A further extension of the promoter sequence up to -446, inhibits the promoter activity. This region contains a well conserved site for Gf-1 (47), a transcriptional repressor described in a large series of genes encoding various cytokines and regulators of cell proliferation and differentiation. Therefore, it is tempting to speculate that the decrease in promoter activity, of the longest construct might result from an interaction with the Gfi-1 factor. Finally, it should be pointed out that the PABP3 gene is transcribed from a CpG-rich promoter. Several germ line specific genes with CpG-rich promoter such as MAGE-AI or LAGE-I are largely unmethylated in male germ cells and methylated in somatic tissues (48). Such a lack of methylation in testis would be sufficient to explain the testis specificity of the PABP3 promoter.

Interestingly, the 256 base pairs upstream from the initiation start site of the PABP3 gene which displays the highest promoter activity, exhibits more than 91% identical nucleotides with the 5'UTR of the PABP1 mRNA. This indicates that the 5'UTR region of this PAPB1 mRNA contains a potential promoter sequence for a testis specific expression. Until now, there is no evidence that this region function as an alternative promoter for the PABP 1 gene. Thus, it is likely that the region upstream from the 256 base pairs of the PABP3 promoter plays a crucial role in the commitment of this promoter in vivo. A mouse testis specific PABP2 gene has also been described but the promoter functionality and specificity of this gene has not yet been analysed. Its transcription start site would correspond to position -108 of the

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PABP3 promoter, therefore lacking the region where we observed the myb sequence. As for the PABP3 promoter, it exhibits AP2 binding sites susceptible to direct transcription in spermatogenic cells (49).

The presence of a functional PABP3 isoform in spermatogenic cells might contribute to testis specific regulations of mRNA homeostasis. In meiotic and early haploid cells, stages at which many mRNA are synthesised, PABP mRNA are present at a high level (50). Nevertheless, most of the testicular PABP mRNA are not engaged in polysomes indicating that translation of PABP mRNA is strongly repressed at this stages (10,51). However this repression might be specific for PABP1 which contains an A-rich sequence in its 5' untranslated region allowing the binding of PABP and blocking the translation of its own mRNA (52-54). Clearly the human PABP3 does not contain any A-rich sequence in its 5'UTR. Therefore, PAPB3 might represent the major functional species associated to polysomes in round spermatids. The expression of this testis specific PABP mRNA might be a way for the cell to escape the negative feedback control and to produce the amount of PABP protein needed for the active mRNA synthesis phase of spermatogenesis.

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Legend to figures

Figure 1: **cDNA and predicted amino acid sequences of PABP3.**

Lower-case letters indicate the non-coding regions. The upper-case letters correspond to the detected open reading frame. White and shaded boxes frame the consensus RNP2 and RNP1 sequences, respectively. The bold letters, in each RNP consensus, highlight the conserved amino acid residues as compared to similar regions in the PABP1 sequence. The 3 polyadenylation signal sequences (AATAAA) are underlined. The vertical bar represents the site of deletion of six amino acids (PVINPY) in PABP3 as compared to PABP1.

Figure 2: **Northern blot analysis of PABP3 expression using specific oligonucleotide probes.**

A) A multiple tissue blot was sequentially hybridised with the oligonucleotides PABP1 and PABP3, exposed to phosphor screen for 16h and 48h respectively and analysed on a phosphorimager STORM 840 (Molecular Dynamics, CA). PBL: peripheral blood leukocytes. The size of the markers is indicated on the left. B) Oligonucleotide 3'RT and 5'RT were used in RT-PCR on 5 ng of total RNA from human adult normal testis. The amplified product were migrated on a sequencing gel and analysed using the GeneScan program as described under « Material and Methods ». The surface under each peak reflects the amount of each PABP mRNA.

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Figure 3: **Localisation of PABP1 and PABP3 mRNA in human adult testis sections**.

Paraffin-embedded human testis sections were hybridised with the PABP1 specific antisense oligonucleotide probe (PABP1) (A and B), the PABP3 specific antisense oligonucleotide probe (PABP3) (C and D), or the common sense oligonucleotide probe (5'RT) (E and F), and counterstained with methyl green. Original magnifications 200x (A, C, and E), 630x (B, D, and F). Signals were confined to the seminiferous tubules in germinal cells (A-D). After 6 hours of detection, the PABP1 mRNA specific signal appeared in pachytene spermatocytes (arrowheads) and round spermatids (arrows) (A and B). The PABP3 mRNA signal appeared after 24 hours of reaction only in round spermatids (arrows) (C and D). No signal was observed with the control 5'RT probe after 24 hours of detection (E and F).

Figure 4 : **Detection of PABP3 by western blot analysis**

Western blot of human testis protein extracts incubated with serum raised against a 9 amino-acid specific PABP3 peptide (peptide 3). A human liver extract was used as negative control. In competition experiments the serum was preabsorbed with peptide 1 or peptide 3.

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Figure 5: **RNA-binding assays of PABP to poly(A)-Sepharose.**

A) Binding of in vitro [^{35}S]methionine-labeled PABP proteins to poly(A)-Sepharose was performed in the presence of 0.25 mg / ml and 0.1 mg / ml of poly(dI-dC) as non specific competitors. In the four first lanes on the left, the KCl concentration of the binding buffer varied from 0.1 to 2 M. In the four lanes on the right, binding reactions were carried out in 1 M KCl and in presence of 100 fold excess of the indicated competitors. B) Analysis of 5 independent binding experiments of PABP1 and PABP3 to poly(A)-Sepharose in the presence of KCl 1 or 2 M. * and ** indicates $P<0.05$ and $P<0.001$ respectively.

Figure 6: **PCR analysis of PABP1 and PABP3 genomic sequences.**

PCR amplification reactions of human genomic DNA (DNA) were performed using a 3' primer (3'ORF) common for PABP1 and PABP3 and the 5' primer (5'ORF) specific for PABP1 and PABP3 or a T7 sequence specific primer which generates a fragment from a plasmid (plasmid). The lanes ORF contain samples amplified with oligonucleotides 3'ORF and 5'ORF, the lanes T7 contain the samples amplified with oligonucleotides 3'ORF and T7.

Figure 7 : **Sequence of the human PABP3 promoter.**

Grey boxes represent the location of potential binding sites for transcription factors identified using the program MatInspector (core similarity = 1 and matrix similarity > 0.87). Other motives meeting with the above criteria are not shown : BRN2, CETS1P54, E47, FREAC7, IK2, LMO2COM, SRY, NF1, ROX and XFD2. The transcription start site identified by S1 mapping and corresponding to the 5'end of the longest RACE product, is indicated by an arrow at position +1. Asterisks indicate

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the 5'end of cDNA sequences obtained by 5'RACE. The translation start site is in bold face and the CpG are underlined. Brackets indicate the 5'end of the four constructs used in the transfection experiments, respectively PC, P1, P2 and P3. The black arrowhead indicates the relative position of the transcription start site in the mouse testis specific PABP2 gene (Acc. n°: AF001290). The underlined sequence exhibit 91 % identical nucleotide residues with the 5'UTR of human PABP1 (Acc n°: AH007272).

Figure 8 : **S1 mapping of PAPB3 promoter on human testis and liver mRNA**

I) S1 nuclease protection assay. A 737 bp gel-purified 6FAM-labeled RACE antisense probe containing potential transcription start sites identified by mRNA primer extension analysis was hybridised to 2µg of polyA$^+$ RNA from either testis (A), or liver (B). As positive control, the S1 probe was also hybridised to 10 ng of PABP3 synthetic RNA (Ac n°: AF132026) lacking the nucleotide 1 to 36 (see figure 7) (C). The probe undigested or digested by S1 in the absence of RNA is shown in D and E, respectively. The arrow indicates the DNA fragment protected by hybridisation to poly(A$^+$) mRNA. The grey peaks in panel A correspond to ROX-1000 DNA standards (Perkin Elmer, CA). The 6FAM relative fluorescence intensity (arbitrary unit) is reported on the left. II) The position of the single strand PABP3 DNA probe with respect to the endogenous and synthetic PABP3 mRNA, as well as the protected fragment is diagrammed at the bottom. Numeration is relative to the transcription start site.

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Figure 9 : **Expression of EGFP protein in cells transiently transfected with EGFP under the control of PABP3 promoter sequences**

PABP3 genomic sequences extending from the nt -498, up to +30 were amplified by PCR, as described under Materials and Methods, and then inserted into a promoter-less EGFP vector (pEGFP-1). These constructs were transiently transfected into HeLa (black boxes) or NTERA-2 cells (grey boxes). Number of counted cells was identical between each transfection assay. EGFP fluorescence driven by each construct was normalised to that obtained with the CMV promoter/pEGFP-C1 vector, (fluorescence of 100%). Results are the means of triplicates obtained in two independent experiments. CMV corresponds to the controls construct of the CMV promoter upstream from EGFP. PC, P1, P2 and P3 refer to the different PABP3 promoter constructs upstream from the EGFP reporter gene as indicated on the left.

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Tables

Name	Sequences	Position (nt)	PABP type and Acc. N°
PABP1	5' CAGTAGCTGCAGCGGCTG 3'	2012 to 1995	PABP1 AH007272
PABP3	5 'GTCCTTCGTATGCTGGAAG 3'	215 to 197	PABP3 AF132026
PABP4	5' GGCAGGTCCAACTTTGG 3'	670 to 654	PABP4 AF132027
iPABP	5' GGACCTCAACACCAAGGATTAC 3'	2191 to 2170	iPABP U33818
3'RT	5' CCAACATGGAAGCAGTC 3'	2156 to 2140	PABP1 AH007272
5'RT	5' GGCTCACCTCACTAACCAGTATATGCA 3'	1630 to 1656	PABP1 AH007272
5'ORF	5' CCCTGCGGGCAGCCGTGCCG 3'	484 to 503	PABP1 AH007272
3'ORF	5' TTTAAACAGTTGGAACACCGGTGGC 3'	2417 to 2393	PABP1 AH007272
5'iORF	5' GGGGCGGGGAGATGAACGCTGCG 3'	143 to 165	iPABP U33818
3'iORF	5' CATAAGGGGTTATTTGGCTTTTGAATCGG 3'	2128 to 2100	iPABP U33818
5'PCR	5' ATGAACCCCAGTGCCCCCAGCTACCCCAT 3'	506 to 534	PABP1 AH007272
RACE	5' GCTGGAAGTTCACATACGCGTAGTTGG 3'	204 to 178	PABP3 AF132026
5'BAC	5' GAGAATGAACCCCAGCA 3'	20 to 36	PABP3 AF132026
3'BAC	5' GTTCACATACGCGTAGTTGG 3'	197 to 178	PABP3 AF132026
BACprobe	5' ATGCTCTACGAGAAGTTCAGCC 3'	99 to 120	PABP3 AF132026
seq1	5' AGCCGCGACCTTTCCGTTACAGGAGTAGAG 3'	30 to +1	prom. AF315079
seq2	5' GGCTCGGGCTCAGCTGCTTCAC 3'	-253 to -232	prom. AF315079
seq3	5' CTCGTCTCTACTAAAATTACAAAAAATTAGCTG 3'	-467 to -433	prom. AF315079
5'PROM	5' atctagatctAGTAGCTGGGATTACAGGTGAGCACAT CAAG3'	-468 to -498	prom. AF315079
5'PROM1	5' atctagatctGTAGAGACGAGGTAAAAAGTAGAAAAT ATTTTATAAAAATATATTT 3'	-398 to -443	prom. AF315079
5'PROM2	5' atctagatctCCGGTGAAGCAGCTGAGCCCGAG 3'	-234 to -256	prom. AF315079
5'PROM3	5' atctagatctCGGCAGCCAGCGGCAGCG 3'	-113 to -130	prom. AF315079
3'PROM	5' gaattcgaagcttAGCCGCGACCTTTCCGTT-ACAGGAGTAGAG 3'	30 to +1	prom. AF315079

Table 1. Primers used in this study as detailed under Materials and Methods. Definitions are as follows : « Name » : the name of the oligonucleotide primer ; "Sequences" : sequence from 5' end to 3' end of each oligonucleotide ; « Position » : position in nt of the oligonucleotide on each specific sequence ; these positions refer to sequences and Genbank accession numbers specified in the last column : « PABP type and Acc. N° » . The different 5'PROM and 3'PROM oligonucleotides have been added with BglII and HindIII restriction site (low cap) at their 5'end sequence.

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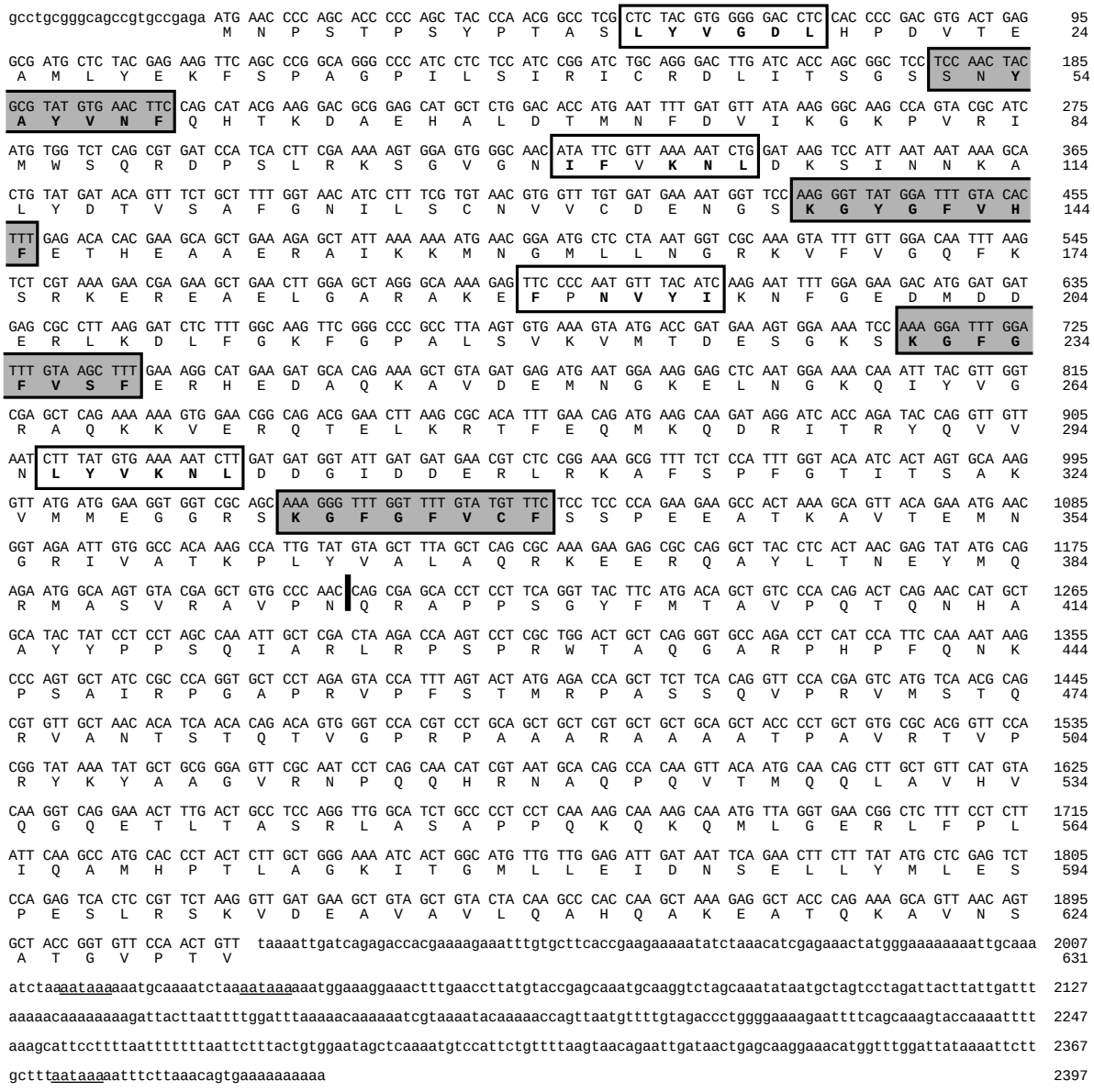

Figure 1

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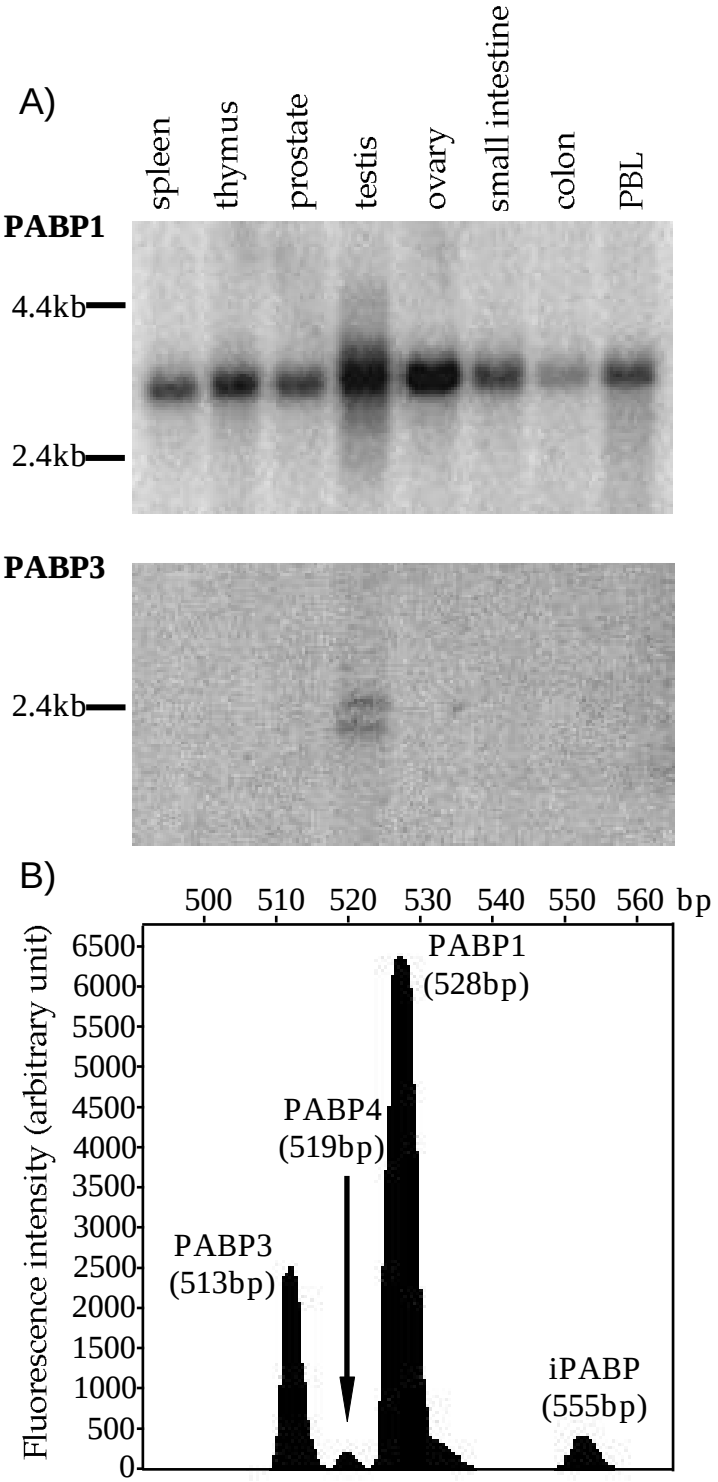

Figure 2

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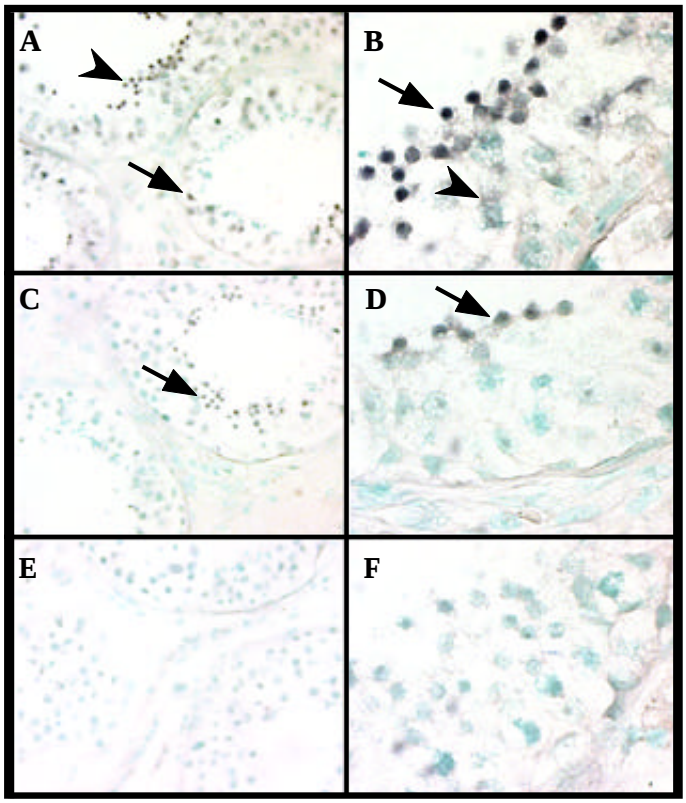

Figure 3

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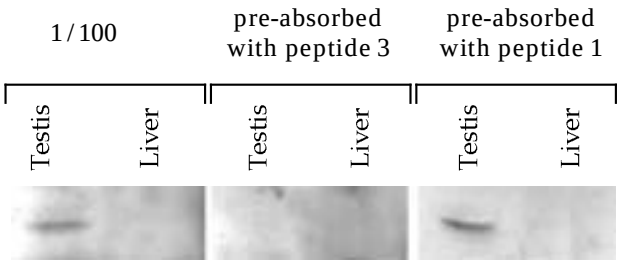

Figure 4

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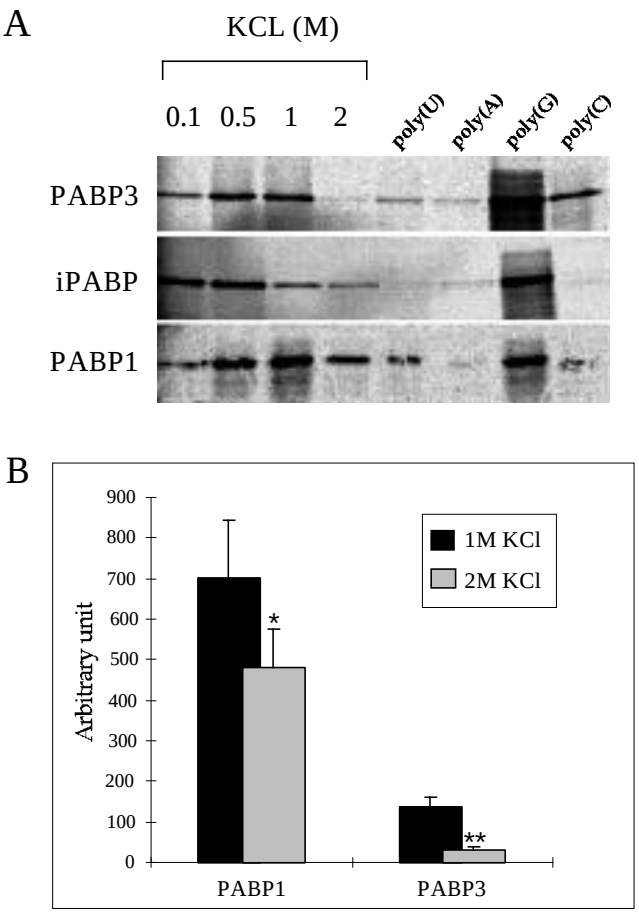

Figure 5

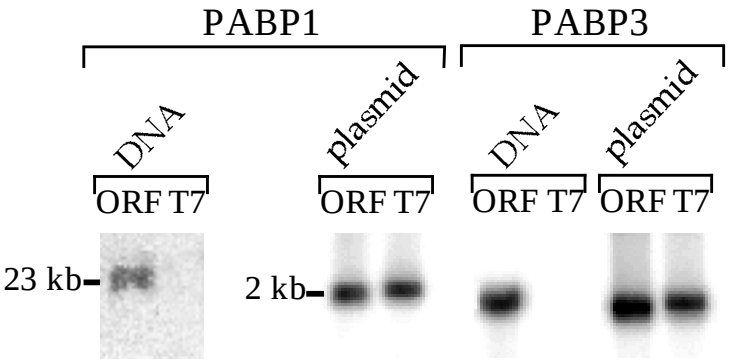

Figure 6

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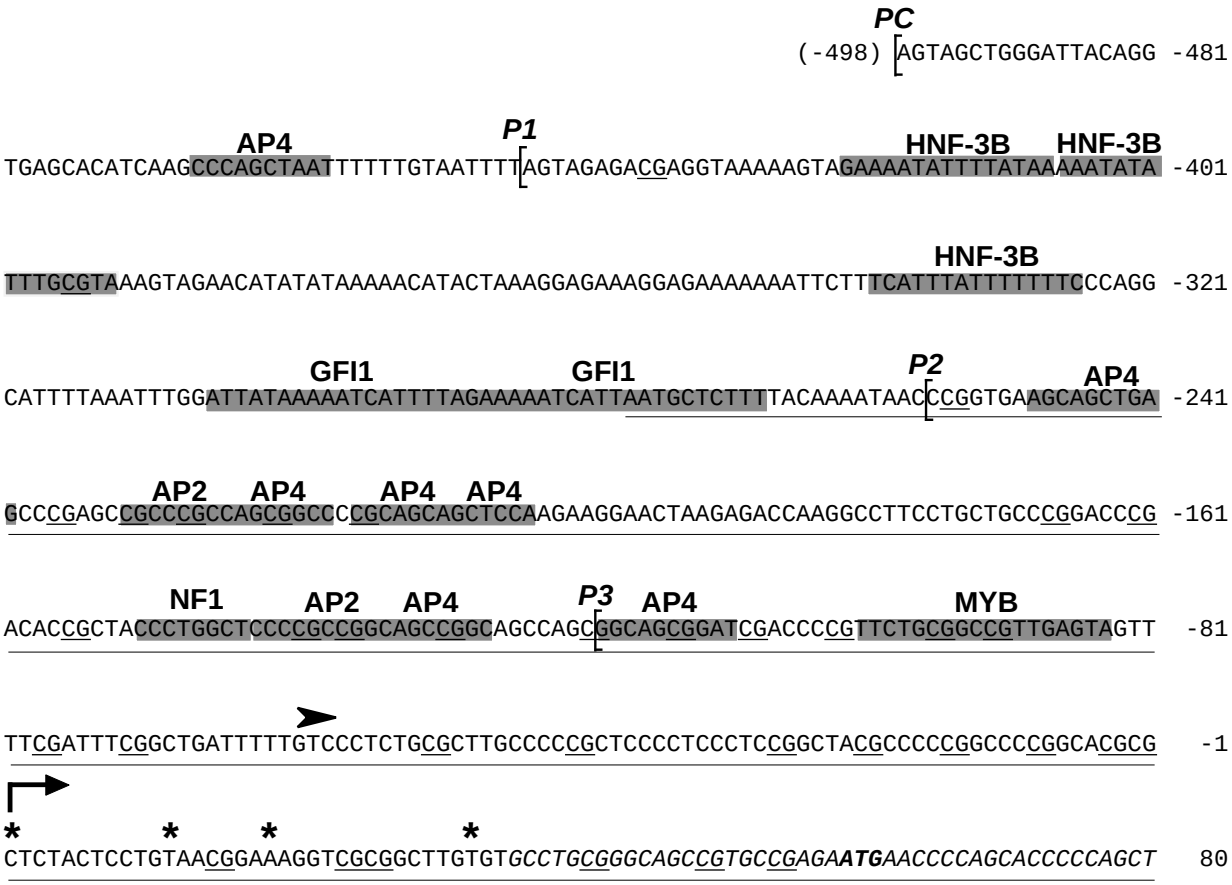

Figure 7

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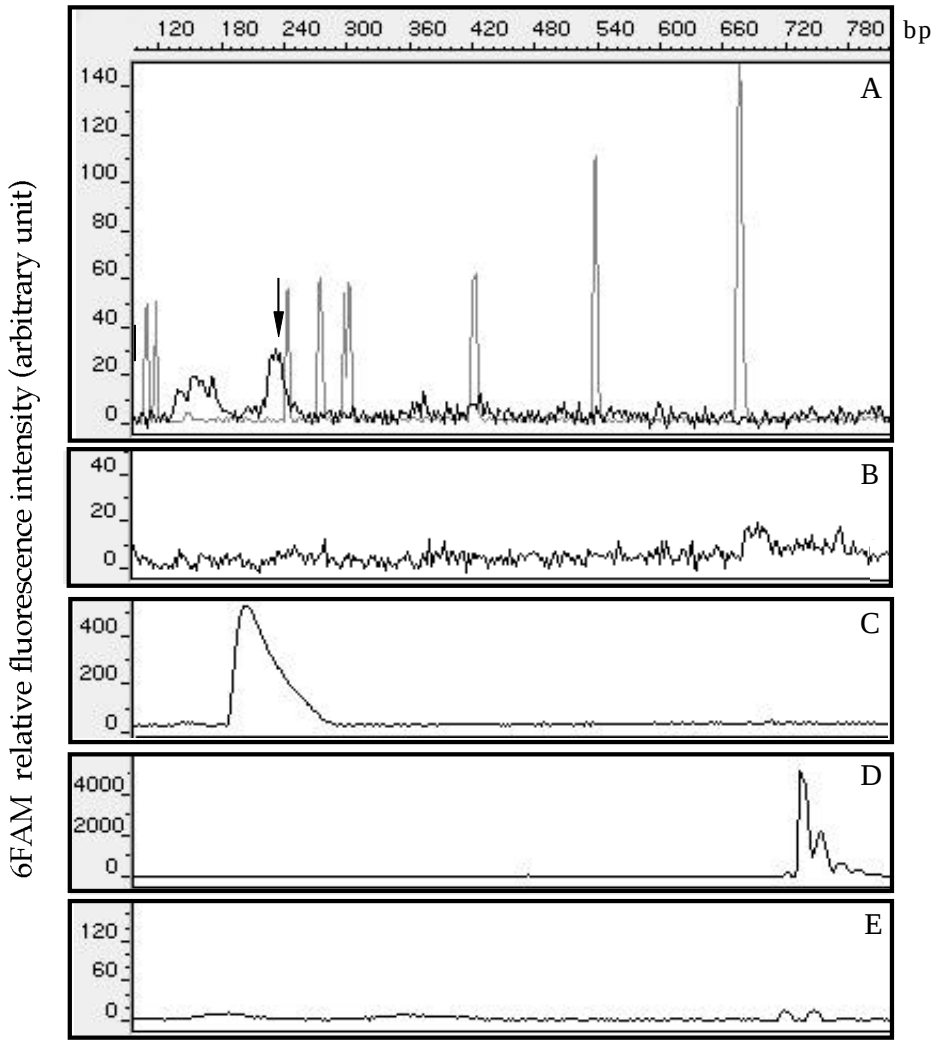
I



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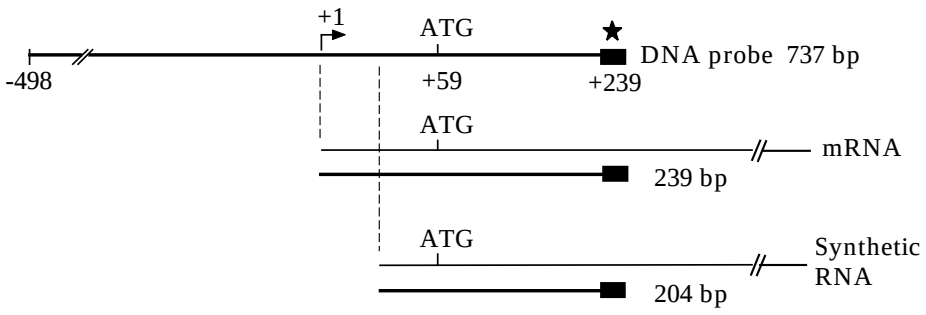

Figure 8

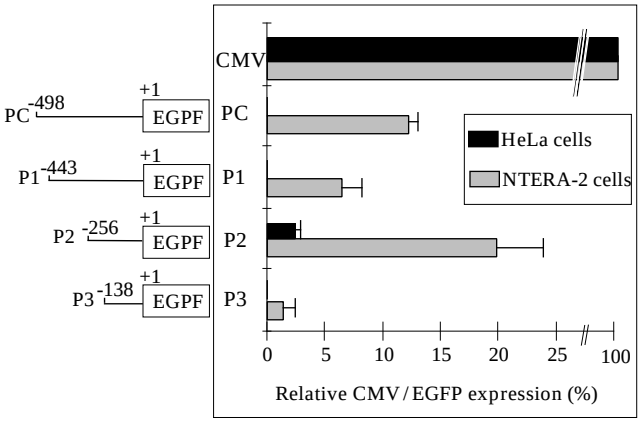

Figure 9

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