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RNA fingerprinting using RAP-PCR identifies an EBAF homologue mRNA differentially expressed in rat oviduct

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ABSTRACT: As a step towards the identification of genes preferentially expressed in the oviduct during early rat embryo development, we isolated a cDNA fragment (Pr14) by using RNA arbitrarily primed PCR (RAP-PCR), being its expression restricted to oviduct and uterus; its mRNA is mainly expressed in oviduct during late luteal phase and early pregnancy. This fragment is 100% identical to a rat DNA sequence (Accession No. NW_047400) downstream the terminal exon of a *Rattus norvegicus* gene (Locus Link Accession No. LOC289316) similar to *ebaf* (endometrial bleeding-associated factor), a novel member of the Transforming Growth Factor superfamily. Northern analyses showed that this sequence hybridizes with 2.9 kb and 4.1 kb mRNAs in early pregnant rat oviducts. However, only the 4.1 kb mRNA was detected in the oviduct of non-pregnant rats, showing an increase from proestrus to diestrus. The expression of this oviduct-uterus specific mRNA suggests that the products of this gene may play a role in the oviductal reproductive process.

Abbreviations: bp, base pair(s); dNTP, deoxyribonucleoside triphosphate; ebaf, endometrial bleeding-associated factor; EST, expressed sequence tag; ORF, open reading frame; poly A, polyadenylation signal; RAP-PCR, RNA arbitrarily primed PCR; TGF-beta, Transforming growth factor-beta; UTR, untranslated region.

Introduction

The mammalian oviduct undergoes certain physiological and biochemical modifications to provide a suitable environment for pregnancy. These changes are partly induced by specific proteins synthesized in the oviductal epithelium in response to varying concentrations of plasma hormones, especially estrogen and progesterone (Buhi *et al*., 1997a). Different studies have disclosed temporal and regional differences in the steady-state levels of specific mRNAs and in the *de novo* protein synthesis and secretion by the oviduct (Sutton *et al*., 1984; Gandolfi *et al*., 1989; Murray, 1993; Buhi *et al*., 2000). Proteins in the oviductal fluid provide the best microenvironment for embryo development (Murray, 1995; Xu *et al*., 2001) but the way in which this is accomplished is not completely known. An oviduct-specific glycoprotein (Donnelly *et al*., 1991; Buhi *et al*., 2000), protease inhibitors (Buhi *et al*., 1997b), plasminogen activators (Jimenez-Diaz *et al*., 2002), plasminogen activator inhibitor 1 (PAI-1) and tissue inhibitors of metalloproteinases (TIMPs) (Kouba *et al*., 2000b; Gabler *et al*., 2001), cytokines (Srivastava *et al*., 1996), binding proteins (Eberhardt *et al*., 1999) and growth factors (Chegini *et al*., 1994) have been identified in

Footnotes: The nucleotide sequences of Pr1, Pr2, Pr4, Pr7, Pr10, Pr11, Pr13 and Pr14 have been submitted to the GenBank and assigned the following accession numbers: Pr1: AW862648; Pr2: AF202265; Pr4: AW862643; Pr7: AW862645; Pr10: AF202267; Pr11: AF202266; Pr13: AW862646 and Pr14: AF202268.

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mammalian oviducts. Although the role of these proteins in fertilization and embryo development remains elusive, there is growing evidence that the oviduct actively supports embryo development (Yeung *et al*., 1992; Conway-Myers, 1998; Kouba *et al*., 2000a).

Recent endeavors have been made to identify the preferentially expressed genes in the mammalian oviduct that facilitate the development of the embryo *in vivo* and/or *in vitro* (Chang *et al.,* 2000; Lee *et al.,* 2000; Lee *et al.*, 2002). It is known that gene expression of the mouse oviduct changes during the reproductive cycle (Lee *et al*., 2000) and, in addition, it has been possible to identify up-regulated genes during the early embryo preimplantation period (Lee *et al*., 2002). However, current knowledge of the majority of regulated genes that mediate the oviduct function is limited. As a step towards the identification of preferentially expressed genes in the rat oviduct, we studied the Pr14 temporal expression in normal rat oviduct during the estrous cycle and the embryo preimplantation period. The cloned sequences could reveal underlying information necessary to analyze biological processes that control the function of the oviduct during the first stages of embryonic development.

Materials and Methods

Animals

Virgin female *Rattus norvegicus* (Wistar), housed in an air-conditioned room with lights on from 7 am to 9 pm, weighing 200-250 g and exhibiting regular four-day cycles were used. In order to obtain four-day pregnant rats, animals in proestrus were mated with fertile males of the same strain. Day 1 of pregnancy was defined as the one in which spermatozoa were found in the vaginal smear of paired animals on the following morning. Female animals were killed between 9 am and 10 am on day 4 of pregnancy and the oviducts were collected.

Ovariectomized animals were obtained by surgical extraction of the ovaries through bilateral flank incisions, avoiding injury to the oviduct. Fifteen days later the animals were sacrificed and their oviducts removed.

RNA preparation

Oviducts were excised from approximately eight rats in each group, frozen in liquid nitrogen, and stored at –70ºC until use. Total RNA for cDNA synthesis and Northern analysis was isolated by the Chomczynski and Sacchi method (1987). In order to prevent DNA contamination, all RNA isolated samples were DNase treated according to manufacturer specifications. Poly $(A)^+$ RNA was obtained from total RNA using the PolyATract mRNA isolation system (Promega, Madison, WI). Total RNA for RT-PCR analysis was obtained using the SV Total RNA Isolation System (Promega, Madison, WI).

cDNA synthesis and polymerase chain reaction

RAP-PCR was performed as described by Yoshida *et al*. (1994), with slight modifications. Poly $(A)^+$ RNA (10 ng) and 25 pmol of an arbitrarily chosen primer were maintained at 68ºC for 5 min in diethyl pyrocarbonate (DEPC)-treated water (10 µl) and then set on ice. A final volume of 25 µl was achieved by addition to the total RNA and specific primer mix of 15 µl of a reverse transcription reaction mixture leading to the following final concentrations of its components: 50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$; 0.5 mM of each dNTPs; 10 mM DTT; 25 units of Rnasin Ribonuclease Inhibitor (Promega) and 200 units of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV) (Promega). The first strand cDNA synthesis was carried out at 34ºC for 30 min; heat inactivation of M-MLV reverse transcriptase was made at 94ºC for 2 min. The resulting cDNA was stored at –70ºC.

Second-strand cDNA synthesis was also initiated by arbitrary priming by adding 2 µl of the first-strand cDNA to the PCR reaction mix. PCR amplification was performed in a final volume of 25 µl containing 10 mM Tris-HCl, pH 9.0; 50 mM KCl; 0.1% Triton X-100; 2.5 mM $MgCl_2$; 200 µM of each dNTP; 1 µM of the same arbitrary primer as the one used for the first-strand synthesis and 2.0 units of *Taq* DNA polymerase (Promega). After the first denaturation cycle (94ºC for 2 min), reactions were carried out for 38 cycles at 94ºC for 55 s, 34ºC for 1 min, and 72ºC for 1 min, with a 7 min extension period at 72ºC. Five arbitrary 10-mer primers were used: A1, CCCAAGGTCC; A9, CTAATGCCGT; B1, TCGAAGTCCT; B6, GTGACATGCC; B7, AGATGCAGCC.

cDNA sequencing and database analysis

Differentially amplified cDNA fragments were cut from the gel using a razor blade; the piece of agarose was placed in a microcentrifuge tube; 2 µl of the eluent were PCR amplified using the PCR protocol described

above with the corresponding primer. The amplified material was checked against the initial arbitrarily primed reaction by running a second agarose gel and the products were cloned into the pGEM-T easy vector (Promega). Cloned fragments were sequenced at The BioResource Center, Cornell University, Ithaca, NY. The sequences obtained were compared with databases from the National Center for Biotechnology Information web server (http://www.ncbi.nlm.nih.gov) using the BLAST algorithm (Altschul *et al*., 1990).

Northern blot analysis of RNA

Total oviductal RNA (10 µg) from early pregnant, ovariectomized animals and animals in proestrus, estrus and diestrus prepared as described above was denatured in the presence of glyoxal/DMSO and fractioned using 1.2% agarose gel electrophoresis (Sambrook *et al*.,1989). Ethidium bromide-stained rRNA bands were visualized (UV) to ensure that RNA degradation had not occurred and that equal amounts of RNA had been loaded into each lane (data not shown). After electrophoresis, RNA was transferred overnight to a modified charge nylon membrane (Sigma) by capillary blotting in 20 x SSC (3 M NaCl; 0.3 M sodium citrate, pH 7.0). After blotting, the membrane was baked for 2 h at 80ºC. It was then washed with 20 mM Tris-HCl, pH 8.0, at 65ºC to remove glyoxal from the RNA and prehybridized at 43ºC for 5 min in a prehybridization solution (0.25 M NaCl; 0.12 M Na_2HPO_4, pH 7.2; 1mM EDTA; 7% SDS; 50% formamide) (Ausubel *et al*., 1994). A biotin-labeled cDNA probe was added to a freshly prepared prehybridization solution (150 µl/cm^2) and the membrane was hybridized at 43ºC overnight (about 16 h). The membrane was soaked in 2 x SSC and washed at room temperature on a rotating platform in 2 x SSC/ 0.1% SDS; 0.5 x SSC/0.1% SDS and finally in high-stringency conditions at 65ºC in 0.1 x SSC/0.1% SDS for 15 min in each wash. Specific mRNAs were detected using the chemiluminescence reagent detection system, as described by BioLabs Inc. New England. Membranes were exposed to X-ray film (BioMax ML, Eastman Kodak, Rochester, NY). All the images were then captured with the Gel Doc 1000 image analyzer using the software Molecular Analyst 1.4.1 (BioRad).

Biotin-labeled probes were synthesized by PCR from cloned products (fragments Pr1, Pr2, Pr4, Pr13 and Pr14) using the T7-SP6 oligonucleotide primer set. *β-actin* mRNA was also examined to standardize for equal loading of RNA samples. Membranes were re-hybridized after removing probes by washing in 75% formamide; 0.2 x SSC and 0.5% SDS for 1 h at 65ºC.

Semiquantitative RT-PCR

Total RNA (1 µg) from early pregnant and ovariectomized rat oviducts, and from uterus, ovary, kidney, muscle, intestine and liver from early pregnant rats, was reverse transcribed using oligo dT and M-MLV Reverse Transcriptase (Promega) in a 25 µl volume reaction. Reverse Transciptase was inactivated at 94ºC for 2 min and the RT sample was diluted to 50 µl with water; 1 µl of each sample was analyzed by PCR for Pr2, Pr4, Pr7, Pr10, Pr11 and Pr14 fragments with specific primer pairs. Primers and expected product sizes are as follows: Pr2-A (5'-CCTGCACTCTGGTTCAGTTCC -3') and Pr2-B (5'-CTGCGACACAATGCTATACTGG -3'), 417 bp product; Pr4-A (5'- TCTGTAGAATGAATCCCTGCAA -3') and Pr4-B (5'-TTGTGTCCAGCAAAGAATGC -3'), 397 bp product; Pr7-A (5' CCTGAGACCCATGAAGTGGT -3') and Pr7-B (5'-GCTCTCCTGGTCCATCTCAC -3'), 350 bp product; Pr10-A (5'- CGGGACATAATAGAAGCAGTGG -3') and Pr10-B (5'- AGCATAAGTCAGTGGCTACCG -3'), 394 bp product; Pr11-A (5'- TTCAGCCACGAGGAAGAACT -3') and Pr11-B (5'- CCCTGGACTTCCTGTTCAAG -3'), 349 bp product; Pr14-A (5'- TTACAAAGAGCTGCAGAGGGC -3') and Pr14-B (5'- CACGTCACTCCAGTAGGACACC -3'), 349 bp product. The *β-actin* primer sequences target a region of the *β-actin* cDNA near the 5' end of the molecule and result in an amplified fragment of 243 bp. The sequences are: 5'- CGTGGGCCGCCCTAGGCACCA -3' for the upstream primer, and 5'- TTGGCCTTAGGGTTCAGGGGGG -3' for the downstream primer; the primer pair spans an intron, which helps distinguish mRNA reverse-transcribed to cDNA from contaminating genomic DNA, which would generate a second PCR product larger than the one expected (Nudel *et al*., 1983; Watson *et al*., 1992).

β-actin amplification was as follows: 94ºC for 1 min; 19 cycles of 94ºC for 30 s, 58ºC for 30 s, and 72ºC for 1 min; with a 5 min extension period at 72ºC. The number of cycles for the other PCR reactions were: 28 for Pr2; 34 for Pr4; 32 for Pr7; 33 for Pr10; 34 for Pr11; 23 for Pr14; annealing temperatures were: 58ºC for Pr2, Pr10 and Pr14; 51ºC for Pr4; 52ºC for Pr7 and Pr11. Annealing temperatures were chosen on the basis of the specific primer sequences and amplification efficiency; the number of cycles for each primer set was chosen so that the amplified product would be in the linear range of amplification (not shown). RT-PCR samples were analyzed by 2% ethidium bromide-stained agarose gel electrophoresis.

RT-PCR assay for the identification of the Pr14 template strand

Two cDNA reactions were performed, one with primer Pr14-A and the other with primer Pr14-B. Total RNA (1 µg) from early pregnant rat oviduct and 25 pmol of each one of the two specific primers were maintained at 70ºC for 5 min in 10 µl of DEPC treated water and then put on ice. 15 µl of a reverse transcription reaction mixture to give a 25 µl final volume and concentration of the following components: 50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$; 0.5 mM of each dNTP; 10 mM DTT, 25 units of Rnasin Ribonuclease Inhibitor (Promega) and 200 units of M-MLV Reverse Transcriptase (Promega) was added to total RNA and the specific primer mix. First-strand cDNA synthesis was carried out at 42ºC for 60 min; Reverse Transcriptase was heat inactivated at 94ºC for 2 min and the RT sample was diluted with water to 50 µl. The resulting reverse transcribed products were stored at –70ºC.

Each cDNA reaction product (1 µl) was PCR amplified with both Pr14-A and Pr14-B specific primers as follows: after the first denaturation cycle at 94ºC for 1 min, PCR reactions consisted of 25 cycles of 94ºC for 30 s, 58ºC for 30 s, and 72ºC for 50 s, with a 7 min extension period at 72ºC. RT-PCR samples were analyzed using 2% ethidium bromide-stained agarose gel electrophoresis.

Results

Detection of oviduct-specific transcripts by RNA fingerprinting

First-strand cDNA was synthesized with M-MLV Reverse Transcriptase using an arbitrarily selected primer (10-mer) as described in Materials and Methods. Reverse-transcribed cDNA fragments from poly $(A)^+$ RNA isolated from pregnant and ovariectomized rat oviducts were used as templates for PCR amplification, with the same primer used for first-strand synthesis. To establish the most effective fingerprinting conditions, different assay conditions were examined,

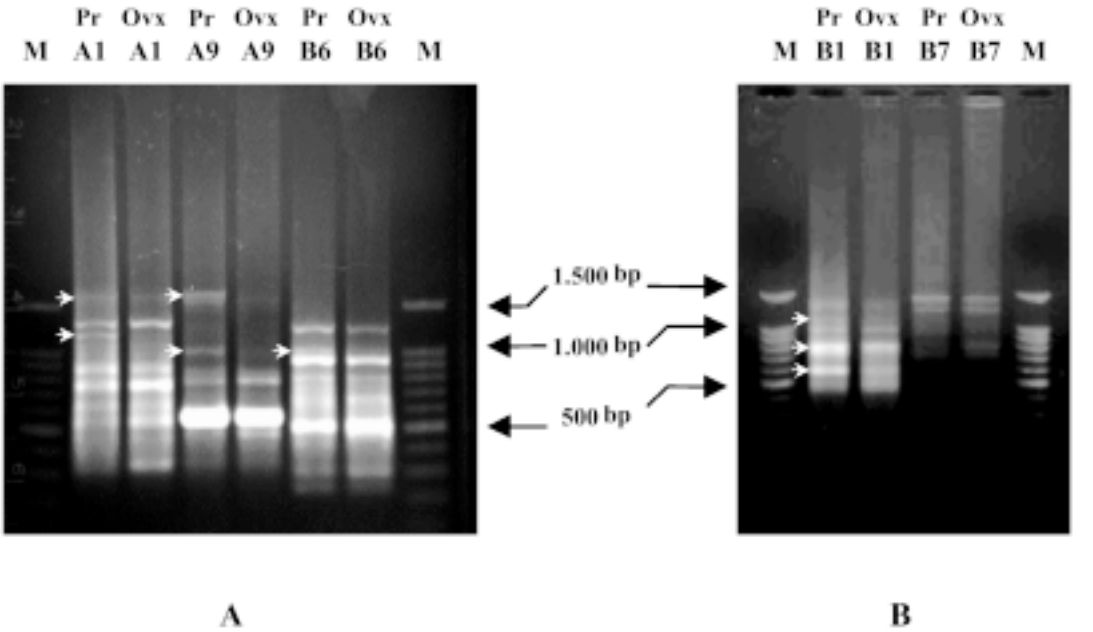

FIGURE 1. Representative profiles of RAP-PCR of RNA from early pregnant (Pr) and ovariectomized (Ovx) rat oviduct, subjected to 1.0 % (w/v) agarose gel electrophoresis. Five primers were used for PCR reactions. In A and B, these primers are indicated as A1, A9, B1, B6 and B7 (see Materials and Methods), at the top of the profiles. Arrows indicate the differentially amplified cDNA fragments. Positions and sizes of the components of a 100 bp DNA ladder (Promega) are indicated in the first and last lines of each profile.

including magnesium and cDNA concentrations and annealing temperature. The optimum magnesium concentration was 2.5 mM. The resulting fingerprint patterns were highly reproducible using agarose gel electrophoresis followed by simple ethidium bromide staining (Fig. 1). We recovered from gels twelve specific PCR fragments differentially amplified. Lengths of Pr fragments were 500-1,500 bp. In this paper, we describe the analysis of eight of the differentially amplified fragments that were present in all the experiments carried out (Pr1, Pr2, Pr4, Pr7, Pr10, Pr11, Pr13 and Pr14) that were cloned and sequenced as indicated in Materials and Methods.

Oviductal mRNA analysis by Northern blot hybridization and RT-PCR

Northern blot and RT-PCR analyses were used to verify that differentially displayed cloned cDNA fragments were differentially expressed in early pregnant and ovariectomized rat oviducts. Pr1, Pr2, Pr4, Pr13 and Pr14 fragments were selected for Northern blot hybridization assays. Biotinylated probes were generated by PCR from the cloned product. The mRNA of *β-actin* was determined in order to standardize the equal loading of RNA samples. The Pr1 and Pr13 probes detected 0.96 kb and 2.4 kb mRNAs respectively in both early

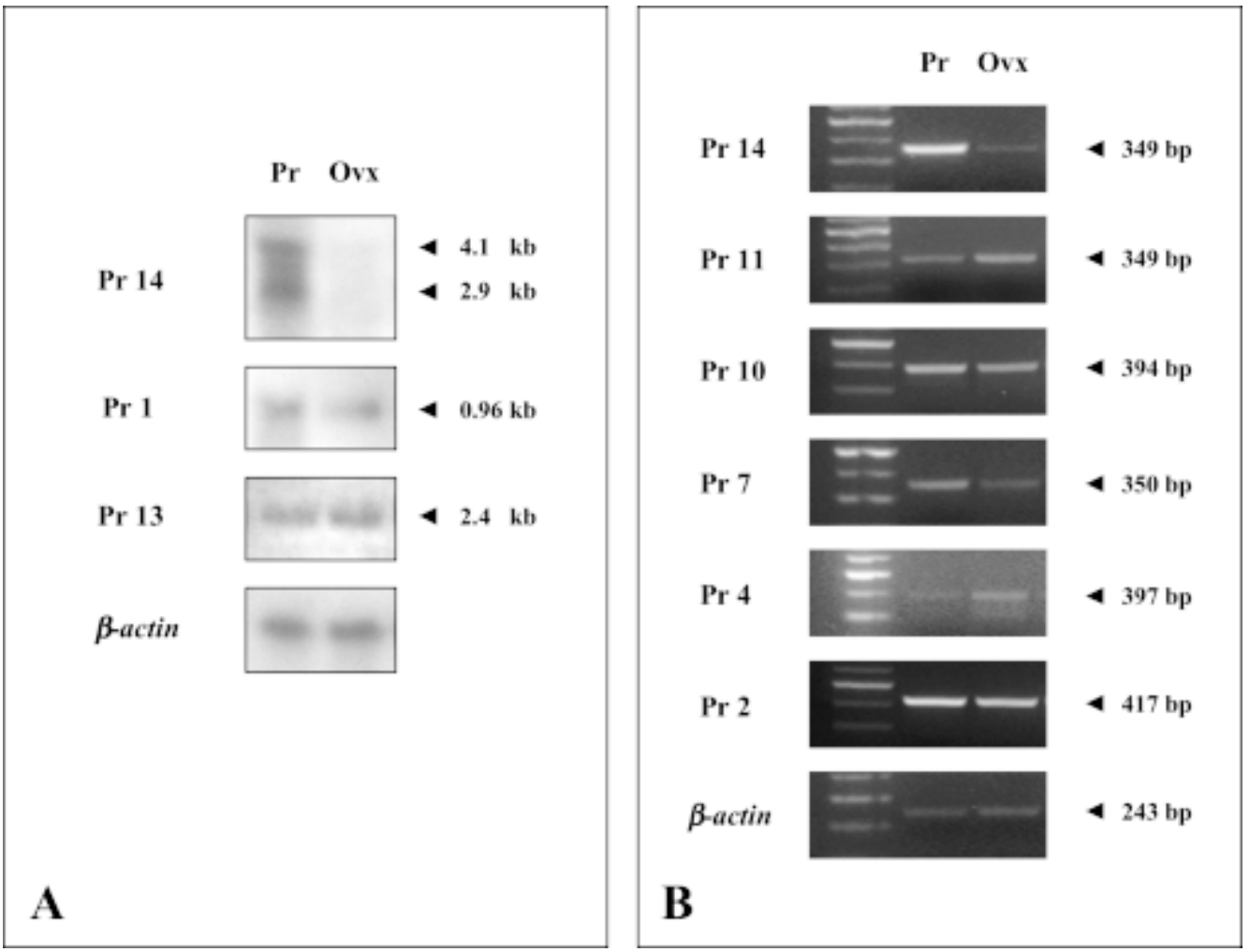

FIGURE 2. Verification of the expression of isolated cDNAs from RAP-PCR. A: Northern blot analysis of total oviductal RNA (10 mg) from early pregnant (Pr) and ovariectomized (Ovx) rats. The membrane was hybridized with biotin-labeled cDNA probes from clones Pr14, Pr1 and Pr13. mRNA sizes were determined by comparison with an RNA ladder (Promega). B: Semiquantitative RT-PCR of Pr2, Pr4, Pr7, Pr10, Pr11 and Pr14 from Pr and Ovx RNA samples as described in Materials and Methods. RNA samples in A and B were normalized using the house-keeping *β-actin* gene.

pregnant and ovariectomized rat oviducts. In early pregnant oviducts, two hybridization signals of about 2.9 kb and 4.1 kb were obtained with the Pr14 probe; both signals were absent in ovariectomized oviducts (Fig. 2A). No hybridization signals were detected in samples from either early pregnant or ovariectomized oviducts when the Pr2 and Pr4 probes were used (not shown). On the contrary, when reevaluating the expression of these fragments by RT-PCR, a sensitive technique capable of detecting small amounts of transcript, it was possible to detect mRNA fragments amplified by specific Pr2 and Pr4 primers. On the other hand, coincidentally with Northern blot, the differential expression of Pr14 in oviducts from early pregnant rats was confirmed by RT-PCR (Fig. 2B). The expression of other fragments (Pr7, Pr10, Pr11) in oviducts from early pregnant and ovariectomized animals was also evaluated by this technique. Neither early pregnant nor ovariectomized oviducts showed significant differences in the expression levels of these fragments.

In each cDNA sample, *β-actin* expression (using primers described in Materials and Methods) was used as an internal control and as a means of detecting contaminating genomic DNA. No amplified *β-actin* genomic DNA product was observed in any of the RT-PCR assays (Fig. 2B).

Pr14 expression in normal cycling rat oviducts and other organs

Pr14 expression was also evaluated by Northern hybridization in rat oviducts at different stages of the sexual cycle. 4.1 kb mRNA was detected during diestrus but with lower expression levels than the one observed in oviducts from pregnant rats when compared with the actin mRNA levels; the 2.9 kb hybridization signal was undetectable in the cycling rat oviducts (Fig. 3A).

The expression pattern of Pr14 in different organs of early pregnant rats was studied by RT-PCR using the specific pair of primers for Pr14. The specific 349 bp

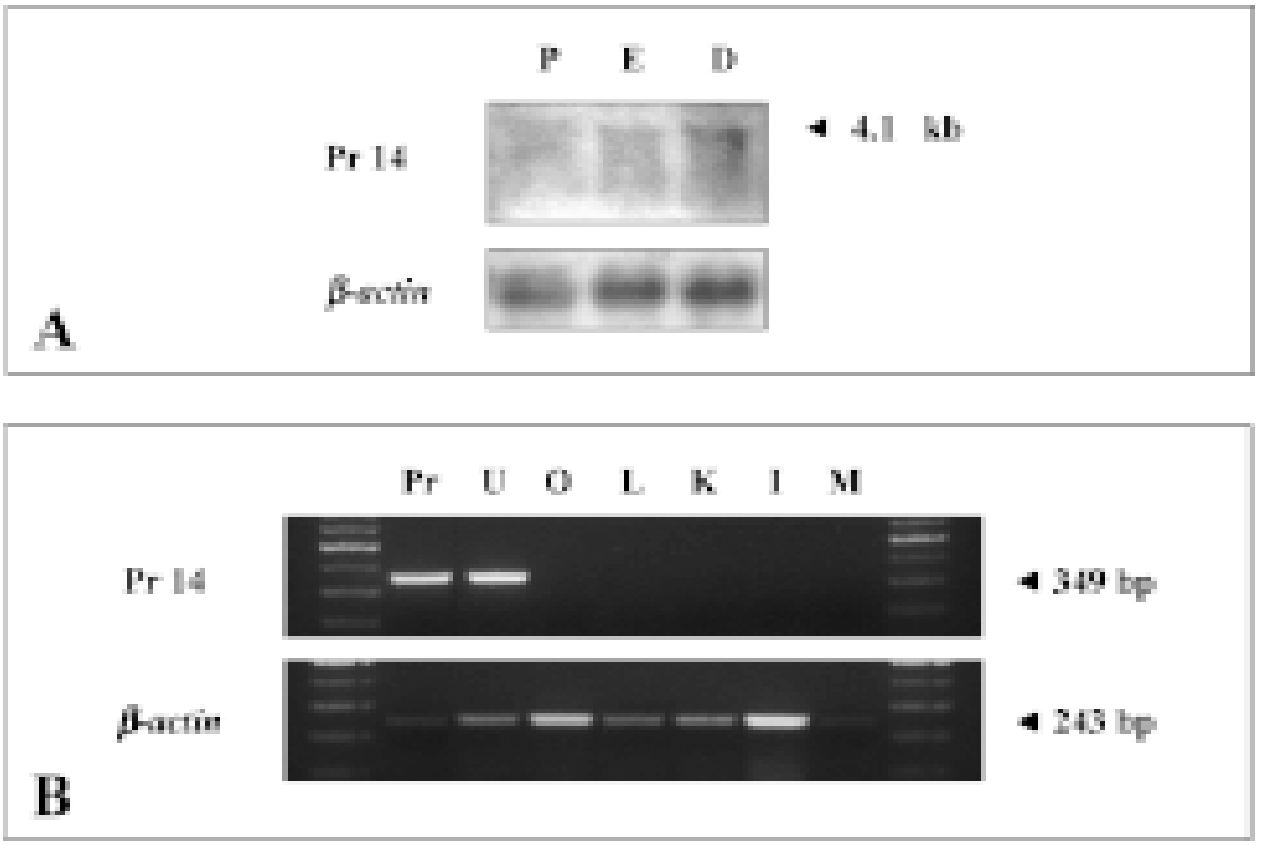

FIGURE 3. Pattern of expression of Pr14. A: Northern blot analysis of total oviductal RNA (10 μg) from normal cycling rats in proestrus (P), estrus (E), and diestrus (D). The membrane was hybridized with biotin-labeled cDNA probe from Pr14. B: semi-quantitative RT-PCR of Pr14 clone in early pregnant rat oviducts (Pr), rat uterus (U), ovary (O), liver (L), kidney (K), intestine (I) and muscle (M). Rat tissue total RNA was subjected to RT-PCR, as described in Materials and Methods. RNA samples were normalized and sizes were determined, as indicated in figure 2.

RT-PCR product was detected in uterus, but not in ovary, kidney, muscle, intestine or liver (Fig. 3B).

Identification of the Pr14 template strand

Since Pr14 is a RAP-PCR product that contains terminal repeats and we were not able to confirm the presence of a functional ORF, the knowledge of the template strand of Pr14 was of great interest for us. In order to determine the sense strand of the Pr14 clone, we designed a rapid RT-PCR based approach (Fig. 4A). Two cDNA reactions using total RNA from early pregnant rat oviduct as a template were performed, one with primer Pr14-A and the other with Pr14-B. PCR amplification of each cDNA product was performed using both Pr14-A and Pr14-B. In theory, only one of the two amplification reactions should generate a RT-PCR product of the expected size. Results are shown in Fig. 4B. The specific product was observed only in the cDNA obtained with primer Pr14-A, the antisense primer, which allowed the identification of the polarity of the natural RNA. This RT-PCR approach could be useful for the rapid identification of the sense strand of any uncharacterized Expressed Sequence Tag (EST) instead of the time consuming classical Northern blotting experiment with single strand probes generated from the cloned RAP-PCR product.

Analysis of differentially expressed cDNA sequences

BLAST algorithms were employed to search the cDNA sequences of the cloned fragments for known genes and EST similarities in the GenBank/EMBL database.

Pr1 showed identity with the mouse ATPase subunit 6 mRNA (Accession No. AF093677). The Pr2 sequence is similar to the 4,614 bp *Homo sapiens* mRNA for KIAA1380 protein (Accession No. AB037801). Pr4 and Pr7 showed no matches with any of the database entries. The Pr10 fragment was homologous to a 4,039 bp cDNA of the human KIAA1585 protein (Accession No. AB046805). The sequence of Pr11 matched a *Rattus norvegicus* mRNA coding for the centrosomal protein CG-NAP (Accession No. AB071391). The cDNA

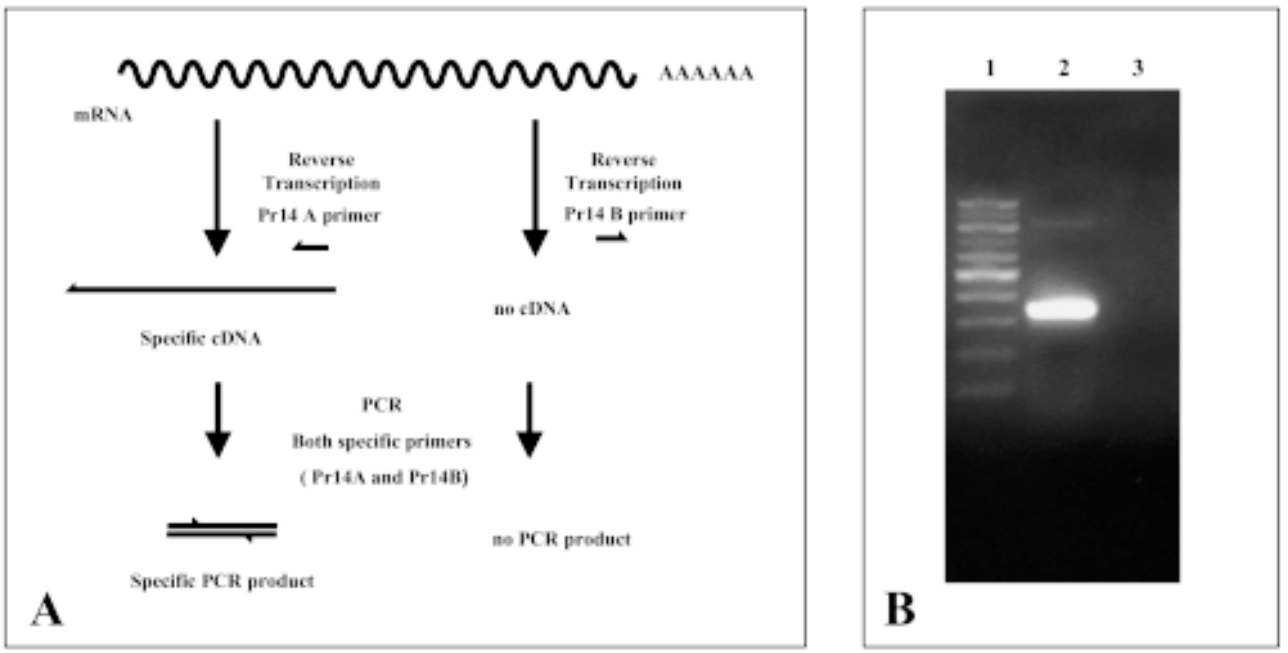

FIGURE 4. Template strand identification of Pr14. A, Schematic diagram of the RT-PCR approach. B, Agarose gel electrophoresis of amplified cDNA from pregnant rat oviduct total RNA. Positions and sizes of markers are indicated in Line 1. Line 2 depicts the PCR amplification of reverse transcription reaction products, using primer Pr14-A: 5' TTACAAAGAGCTGCAGAGGGC-3'. Line 3: PCR amplification of reverse transcription reaction products, using primer Pr14-B: 5'-CACGTCACTCCAGTAGGACACC-3.

sequence of Pr13 showed homology with the 2,073-nucleotide mRNA of the rat heat-shock cognate protein 70 (Hsc70).

Sequence homology searches and comparisons of the Pr14 sequence against the GenBank/EMBL databases found an identical sequence in the Rat genomic BLAST page (http://www.ncbi.nlm.nih.gov/genome/seq/RnBlast.html), showing that Pr14 has a 100% identity to a 616 bp of the *Rattus norvegicus* chromosome 13 (Accession No. NW_047400). Data obtained from this sequence from the GenBank predicted a *Rattus norvegicus* gene between nucleotides 1,462,207 and 1,467,028 with four exons and a coding deducted sequence (CDS) of 1,101 bp coding for a 366 amino acids protein similar to the human endometrial bleeding-associated factor (Accession No. AAB53269).

Our results indicate that the Pr14 sequence is downstream the terminal exon between nucleotides 1,467,040 and 1,467,655 and upstream potential poly A signals at nucleotides 1,468,056; 1,468,060; 1,468,268; 1,469,985 and 1,470,372 of the NW_047400 sequence. These poly A signals are at 1,028; 1,032; 1,240; 2,957 and 3,344 nucleotides from the end of the CDS of the terminal exon.

Discussion

RNA fingerprinting using arbitrarily primed PCR (RAP-PCR) (Welsh *et al*., 1992) and differential display reverse transcription PCR (DDRT-PCR) (Liang and Pardee, 1992) allow the semiquantitative simultaneous comparison of the abundance of several hundred randomly sampled RNAs. RNA fingerprinting has been used to identify transcripts that are aberrantly regulated in human tumors (Liang *et al*., 1992), differentially expressed in neurons of different culture age (Li *et al*., 2002), in human common congenital heart defects (Sun *et al*., 2002), and in the identification of genes that are up regulated in rheumatoid synovial fibroblasts (Kullmann *et al*., 1999).

As demonstrated in this report, RAP fingerprinting can be used to identify transcripts that are differentially expressed in the oviduct of early pregnant rats. It allows the direct comparison of gene expression with the oviduct of ovariectomized rats used as an internal standard. Several fingerprint products from putative differentially expressed messages in the pregnant oviduct were detected. Although RAP-PCR is a rapid and convenient method for identifying differentially expressed genes, it generates a high level of false positives and is biased for high copy-number mRNA (Bertioli *et al.,* 1995). The pattern of amplified products from the oviduct was obtained with high reproducibility in independent PCR experiments. Seven fingerprint PCR products (Pr1, Pr2, Pr4, Pr7, Pr10, Pr11 and Pr13) were detected in both early pregnant and ovariectomized oviducts. Pr1, Pr11 and Pr13 sequences were identical to known rat genes, but Pr2 and Pr10 share a high similarity with two human mRNA sequences coding for proteins with unknown functions not yet described in rats: *Homo sapiens* KIAA1380 mRNA (Accession No. AB037801) and *Homo sapiens* KIAA1585 mRNA (Accession No. AB046805) respectively. Although the Pr2 transcript was amplified by RT-PCR, it was not detectable in Northern blots using total RNA, indicating that this fragment might correspond to a low abundance gene type. The identities of Pr4 and of Pr7 are as yet unknown, since they showed no similarity to GenBank entries, although the presence of numerous STOP codons suggests that these fragments probably belong to a UTR region of mRNAs.

Two transcripts of about 2.9 kb and 4.1 kb detected by Northern hybridization as being differentially expressed in early pregnant rat oviducts at the pre-implantational stage were identified with the Pr14 probe.

FIGURE 5. Schematic representation (not drawn to scale) of the *ebaf* gene from the unordered rat chromosome 13 sequence (Accession No. NW_047400). Empty hollow rectangles indicate the coding deducted sequences (CDS). The full rectangle represents the Pr14 sequence. Triangles indicate potential poly A signals. Numbers indicate nucleotide positions of the beginning and the end of each CDS, Pr14 sequence, and potential poly A sites positions. Nucleotide 1 corresponds to START codon; nucleotide 4,464 corresponds to STOP codon.

Further analyses during the estrous cycle showed that the 2.9 kb mRNA was virtually absent in cycling non-pregnant rat oviducts. Although the 4.1 kb mRNA was observed in cycling non-pregnant rat oviducts, the hybridization signal was weaker than in early pregnant ones. The 4.1 kb mRNA expression levels detected during proestrus gradually increased through estrus to diestrus, suggesting that this cDNA possibly corresponds to a gene expressed under steroids hormone influence.

Pr14 sequence comparison with the Rat Genomic database showed a 100% identity to the *Rattus norvegicus* chromosome 13 (Accession No. NW_047400) harboring a predicted four exon gene coding for a 366 amino acids protein similar to the human ebaf (AAB53269), a secreted protein member of the TGF-beta superfamily with two conserved domains: the TGF-beta propeptide, also known as latency associated peptide (LAP) in TGF-beta, and the TGF-beta domain, which is a multifunctional peptide that controls proliferation, differentiation, and other functions in many cell types.

As a first step towards the characterization of the Pr14 clone, we developed a rapid RT-PCR method using sense and antisense primers in the first-step cDNA synthesis, an approach that proved effective in the identification of the sense strand of the Pr14 clone. Results suggest that the Pr14 sequence is downstream the terminal exon of the rat *ebaf* homolog gene and belongs to its 3' UTR. Potential poly A signals are downstream the Pr14 sequence at 1,028; 1,032; 1,240; 2,957 and 3,344 nucleotides from the end of the CDS of the terminal exon; taking into account that the predicted CDS of this gene is 1,101 bp in length, the sizes of the mRNAs could be 2,129; 2,133; 2,341; 4,058 and 4,445 nucleotides respectively. Interestingly, the sizes of the two transcripts of about 2.9 kb and 4.1 kb detected by Northern hybridization are in agreement with some of those calculated from the genomic sequence. As Pr14 is upstream to all the potential poly A signals, we hypothesized that the 2.9 kb and 4.1 kb transcripts could be originated by an alternative poly A site recognition mechanism (Cudry *et al*., 1999; Qu *et al*., 2002). In our scheme, the poly A site nearest to the end of the CDS of the terminal exon could be well read during early pregnancy (producing the 2.9 kb mRNA) but could also be skipped during the non pregnant stage (producing preferentially the 4.1 kb mRNA), although in low quantities when compared with the early pregnant stage. It is important to note that up to three mRNA species of the human *ebaf* in endometria have been detected (Tabibzadeh, *et al*. 2000). When checking the GenBank nucleotide sequence of the *ebaf* (Accession No. U81523), we found potential poly A sites that could generate the ebaf mRNA species reported in human. At present, it is known that certain UTRs of eukaryotic mRNAs play an important role in gene regulation and expression (Chen and Shyu, 1995; Qi and Pekala, 1999). The 3' UTR of the rat mRNA harboring the Pr14 sequence could be involved in the regulation of the levels of this mRNA. The human *ebaf* expression in endometria has been reported to be confined to both the late secretory phase and during endometrial bleeding; the expression of this gene was absent in a host of normal tissues including lung, kidney, ovary, liver, colon, stomach, breast, lymph node, spleen and fallopian tube (Kothapalli *et al*., 1997). However, a weak expression of the 2.1 kb variant mRNA was observed in rectal, ovarian, and testicular tissues and the 2.1 and 2.5 kb mRNAs were observed in the pancreatic tissue (Tabibzadeh *et al*., 1997). It has also been reported that ebaf stimulates the production of human matrix metalloproteinases (MMPs) during menstruation and that progesterone controls both the expression of *ebaf* and its effect on these MMPs when combined with estradiol (Cornet *et al*., 2002).

To the best of our knowledge, this is the first report of *ebaf* expression in the oviduct. Taking into account that bovine ovarian granulosa- and theca-cells, oocyte-cumulus complex and oviductal epithelium synthesize growth factors and extra-cellular matrix-components such as matrix MMPs (Einspanier *et al*., 1999; Gabler *et al*., 2001) and that the expression pattern of Pr14 by RT-PCR has shown that Pr14 is expressed in the rat oviduct and uterus but in no other organ, this expression being hormonally regulated apparently by ovarian steroids, it seems probable that the corresponding gene should have a function in the oviductal reproductive process.

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