pmg-1, a novel gene specifically expressed during the invasive growth phase of the mammary gland at puberty

Franziska Kuhn, Anne-Catherine Andres*

Department of Clinical Research, University of Berne, Tiefenaustrasse 120, CH-3004 Berne, Switzerland

Received 2 June 1997

Abstract The acquisition of invasive properties is a crucial event during carcinogenesis, determining the clinical outcome. The mammary gland at puberty provides an ideal model for investigating the induction and control of invasive growth. During this growth phase, the mammary epithelium participates in a normal, hormonally controlled invasive penetration into the stroma. We have applied the differential display method to search for genes specifically activated during this developmental stage. We have identified and molecularly characterized a novel *p*ubertal *m*ammary *g*land specific gene, *pmg*-1. This gene is conserved in mammals and encodes a protein of 19.9 kDa. Northern blotting and in situ hybridization revealed that *pmg*-1 expression was exquisitely restricted to the epithelium at early puberty. To our knowledge this represents the first isolation of a gene specifically associated with the induction of mammary epithelial invasiveness at puberty.

Key words: Mammary epithelial cell; Differential display; Puberty; Carcinogenesis

1. Introduction

The clinical outcome of malignant disease is determined not only by the primary tumor itself, but rather by its potential to invade and to metastasize. The acquirement of the invasive potential is thus a key event during carcinogenesis and a better understanding of the molecular events involved will lead to new therapeutic possibilities. The mammary gland at puberty provides an excellent biological system to investigate the molecular mechanisms controlling invasive growth. During this growth phase, the proliferating epithelium participates in a hormonally controlled, limited invasive penetration of the surrounding stroma giving rise to the characteristic ductal tree of the mature mammary gland [1]. It is striking that the two characteristics of pubertal growth, proliferation and invasion, are also the main characteristics of malignant growth. Indeed, we have found close parallels in tissue specificity of proliferation and matrix metalloproteinase expression between normal pubertal growth and the development of invasive metastasizing mammary tumours [2]. Furthermore, the relevance of pubertal development for mammary carcinogenesis is supported by epidemiological and experimental studies which have identified the exposure to estrogen as a main risk factor and the developmental processes induced at puberty as prerequisites for malignant development [3,4].

The molecular mechanism(s) ensuring correct control of the pubertal epithelial proliferation, stromal invasion, pattern formation and finally growth arrest are poorly understood. Growth inhibitory effects of TGFβ-1, -2 and -3 on ductal morphogenesis in the pubertal mammary gland have been demonstrated [5,6]. In contrast, growth promoting effects of EGF and TGFα on the ductal outgrowth at puberty have also been observed [7]. The spatially selective expression of E- and P-cadherins during branching morphogenesis is thought to be involved in tissue integrity and normal growth rate of the epithelium [8]. Knock-out mice deficient in the expression of either the progesterone receptor or cyclin-D1 exhibit impaired branching morphogenesis of the mammary epithelium at puberty [9–11].

In an attempt to molecularly characterize genes involved in the pubertal growth phase of the mammary epithelium, we have applied the differential display cloning method [12] to identify genes specifically expressed in the mouse mammary gland during this particular developmental window. This approach has led to the identification of a novel gene, *pmg*-1, whose expression is restricted to the early pubertal growth phase of the mammary epithelium thereby implicating *pmg*-1 in the hormonally induced initiation and/or promotion of mammary epithelial cell growth.

2. Materials and methods

2.1. Animals and organoid preparation

Mammary glands at various stages of development were isolated from Swiss Moro female mice (BRL, Füllinsdorf, CH). Immature mammary glands were taken at the age of 2–3 weeks, pubertal mammary glands were collected over the period of 3.5–6 weeks of age and mature mammary glands represented the entire estrus cycle [13]. The preparation of multicellular organoids comprising myoepithelial and epithelial cells was performed essentially as described ([14].

2.2. RNA preparation and differential display

Total RNA was prepared and enriched for poly(A) RNA according to Andres et al. [13]. The differential display analysis was performed using the GenHunter mRNA Differential Display Kit according to the protocol of the manufacturer (Bio/Gene, Bolnhurst, UK). Briefly, 0.2 μg DNA-free mRNA from immature, pubertal and mature mammary gland organoids was reverse transcribed using the 3′-primer 5′-AAGCTTTTTTTTTTTTTC-3′. A tenth of the reaction volume was amplified by PCR using the random 5′-primers. The amplificates were separated on standard sequencing gels, differentially displayed fragments excised from the gel and re-amplified using the same primer set. PCR products were cloned into pBluescript and sequenced using the dideoxynucleotide chain termination method (Sequenase Version 2.0 kit, Amersham Switzerland, Zürich, CH).

2.3. cDNA library construction and screening

Double-stranded cDNA was made from mRNA of intact pubertal mammary glands and cloned into HybriZapII vector using the HybriZapII cloning kit according to the manufacturer protocol (Stratagene, Heidelberg, Germany). Phage plaques were screened with the PCR cloned fragment and positive colonies plaque purified. Phage DNA was prepared according to standard protocols [15], the insert excised

*Corresponding author. Fax: (41) 31-308-8014.
E-mail: Andres@akef.unibe.ch

PII S0014-5793(97)00836-3

and re-cloned into pBluescript for further analysis. The full-length sequence was determined using the Exonuclease III Erase-a-base system (Promega, Madison, WI) and Sequenase Version 2.0 (Amersham, Switzerland). Sequence comparisons were made using the BLAST program [16].

2.4. Southern blot analysis

Genomic DNA was prepared as described [15]. 10 μg of *Bam*HI- or *Hin*dIII-digested DNA was electrophoresed in a 1% agarose gel and blotted onto cellulose nitrate (Schleicher and Schuell, Dassel, Germany). Filters were prehybridized and hybridized with the full-length *pmg*-1 cDNA labeled with [^{32}P]dCTP by random priming (Boehringer-Mannheim, Rotkreuz, CH) at 25°C in 50% formamide, 4×SSC, 5×Denhardt's, 0.2% SDS 0.1% tetra-Na-diphosphate and 100 μg/ml denatured salmon sperm DNA. Filters were washed to a final stringency of 2×SSC, 0.5% SDS at 30°C and exposed to X-ray film (Kodak, Lausanne, CH).

2.5. Northern blot analysis and in situ hybridization

Poly(A)-enriched (5 μg) or 10 μg of total RNA were separated on 1% agarose gels as described [17] and blotted onto cellulose nitrate. The filters were hybridized to the ^{32}P-labeled full-length *pmg*-1 cDNA (hybridization solution as above) at 42°C, washed to a final stringency of 0.25×SSC, 0.2% SDS at 65°C and exposed to X-ray film. In situ hybridization of 5-μm-thick paraformaldehyde-fixed paraffin sections of pubertal mammary glands was performed using the RNA Colour Kit according to the manufacturers instructions (Amersham International). The anti-sense probe and the sense control was synthesized by in vitro transcription of the *pmg*-1 cDNA using T3 or T7 RNA polymerase.

3. Results and discussion

The differential display method comparing RNA isolated from immature, pubertal and mature mammary organoids led to the identification of a cDNA fragment present in the pubertal RNA only. This fragment was cloned and used as a probe to screen a cDNA library derived from pubertal mammary glands. A full-length cDNA clone with a 858 bp insert was isolated for further characterization. Sequence determination and comparison with the GenBank database revealed

```
1    GCACGAGATTCTCCTTATCCCATCCTCAAACTCCTAGCTCCTCTCAACATGTCCTGCAAC  60
                                                    M  S  C  N
61   AGCTGCTCTGGAACTTTCTCCCAGTCCTCTGGGGGCCAACTGCAGTATCCGATCTCTTCA 120
     S  C  S  G  T  F  S  Q  S  F  G  G  Q  L  Q  Y  P  I  S  S
121  TGCGGTTCCTCCTACCCCAACAACGTCTTCTACAGCACTGACCTCCAAACTCCCATCACC 180
     C  G  S  S  Y  P  N  N  V  F  Y  S  T  D  L  Q  T  P  I  T
181  CACCAGCTGGGCTCTTCTCTTCACAGTGGGTGCCAGGAAACCTTCTGTGAGCCCACCAAC 240
     H  Q  L  G  S  S  L  H  S  G  C  Q  E  T  F  C  E  P  T  N
241  TGCCAGACAGCCTATGTGGTCTCCAGACCCTGCCAGAGGCTTTCTACAGTCAGAGGATTC 300
     C  Q  T  A  Y  V  V  S  R  P  C  Q  R  L  S  T  V  R  G  F
301  GAGGGCCCTGCAGGCTGCCAGTCAACTTTCTCGGGATCCCTGGGATTTGGTTCCAGGGGT 360
     E  G  P  A  G  C  Q  S  T  F  S  G
361  TTCCAGTCTTTTGGCTGTGGCTACCCATCCCAGGGCTTTGGATCCCATGGTTTCCAGTCA 420
421  GTAGGATGTGGTACCCCTACTTTCTCATCCCTAAATTGTGGATCCAGCTTTTACCGCCCA 480
                                T  F  S  S  L  N  C  G  S  S  F  Y  R  P
481  ACCTGCTTCTCTACCAAAAGCTGCCAGTCTGTTTCTTATCAGCCAACCTGTGGGACTGGC 540
     T  C  F  S  T  K  S  C  Q  S  V  S  Y  Q  P  T  C  G  T  G
541  TTCTTCTGATCTCTATTGGGGAAAATTAGAAATGTAAAAGGTGCCTACTACCTACTGTGT 600
     F  F  *
601  TAAGAGCCGTGCTGTCTTCAGAGTTTCTATATTAGTCTAGAGATTTGACTTCTACTCATG 660
661  AGTTCCTGTAACTGGCAAAACGTATTTGATTAGAGAAAGATGCTAAATTGTTTCTTGTA 720
721  TATTATAGGTATGCAAAAATCAAGCATAATTCTTTCTTCGCATATCTCTTAGTAATTACT 780
781  CAGACTTCCGGTCTTGATTTTTGCAAAGAGGAAGGTTGTAAGTTTAAATAAAGATATGCA 840
841  ACTGGAAAAAAAAAAAAAA
```

Fig. 1. Nucleotide sequence and deduced amino acid sequence of *pmg-1*. The amino acid repeat is shaded and the putative polyadenylation signal is given in bold and italic. The murine *pmg-1* cDNA sequence has been deposited with GenBank under the accession number AF003691.

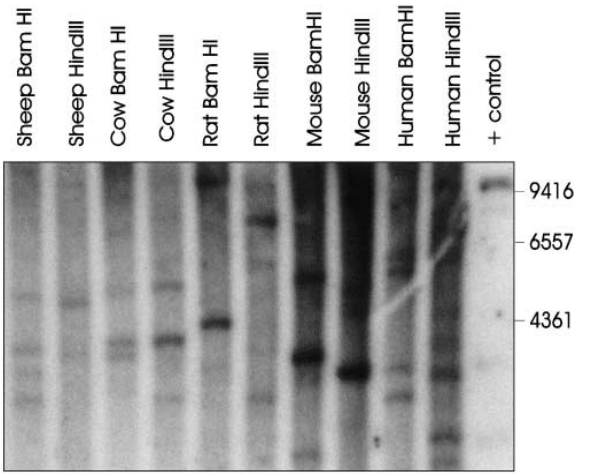

Fig. 2. The *pmg-1* gene is conserved in mammals. Genomic Southern blot of DNA from the different mammals indicated digested with either *Bam*HI or *Hin*dIII. The positive control consists of the linearised full-length *pmg-1* clone. The probe used for hybridization corresponded to full-length *pmg-1*. Fragment sizes of the *Hin*dIII digested lambda phage DNA are indicated.

that this cDNA has no homology to any known sequence (Fig. 1). We have designated the name *pmg*-1 (*p*ubertal *m*ammary *g*land specific) for this novel gene reflecting its particular expression pattern (see below). *pmg*-1 has an open reading frame encoding a protein with a predicted length of 166 amino acids (calculated molecular weight 19.9 kDa). The start methionine is preceded by a 46 bp 5′-untranslated sequence and is flanked by a hexanucleotide conforming to the Kozak consensus sequence [18]. The predicted amino acid sequence does not contain any known structural motifs which would allow a prediction about its function or localization. In particular, the lack of a hydrophobic signal peptide and of possible N-glycosylation sites suggests that *pmg*-1 does not encode a secreted or membrane-bound protein. An interesting feature of the amino acid sequence, however, is an almost perfect internal repeat of 17 amino acids at amino acid position 97–130. The 3′-untranslated region contains 314 bp and terminates with a classical polyadenylation signal.

Analysis of genomic DNA prepared from a variety of species showed that *pmg*-1 is conserved in mammals including humans (Fig. 2). The appearance of multiple bands may reflect that *pmg*-1 constitutes a novel gene family consisting of more than one member. Indeed, comparison of the *pmg*-1 sequence to the dbEST database [19] identified a cDNA sequence from a human fetal heart cDNA library exhibiting 67% homology in the coding sequence. Furthermore, a mouse skin cDNA exhibited 61% homology over 100 bp corresponding to the second 17 amino acid of the observed repeat in *pmg*-1.

The expression of *pmg*-1 during mammary gland development was analyzed by Northern blotting of RNA prepared from all the different developmental stages of the mammary gland (Fig. 3). *pmg*-1 detected a single transcript of about 900 bp corresponding well to the size of the cloned full-length cDNA sequence. In the mouse, sexual maturation begins at 3.5 weeks and between 4 and 6 weeks multi-layered mammary epithelial end buds are formed which penetrate into the adipose stroma [1]. *pmg*-1 expression was barely detected in the immature gland at 3 weeks but was evident at the onset of

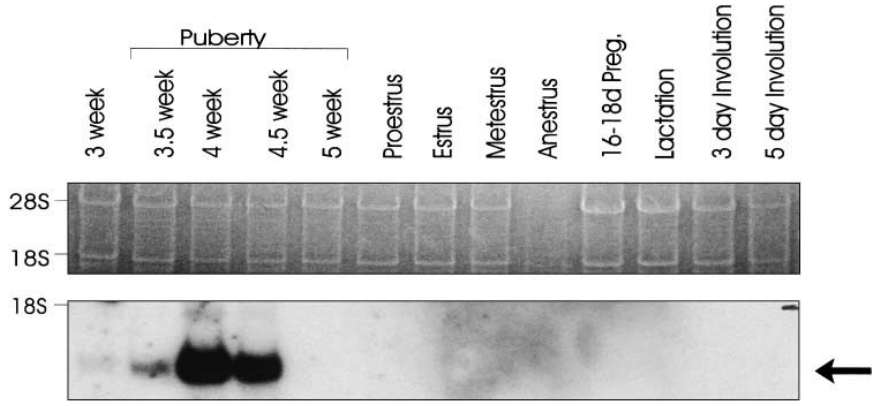

Fig. 3. *pmg-1* expression during mouse mammary gland development. Poly(A)-enriched RNA prepared from the mammary gland at the developmental stages indicated was analyzed by Northern blotting. Equal loading was verified by acridine orange staining of the gel (upper panel). The probe used for hybridization corresponded to full-length *pmg-1*. The position of the 18S and 28S rRNA are indicated. The arrow indicates the position of *pmg-1* RNA.

puberty at 3.5 weeks. Expression was highly induced at 4 weeks of age, declined at 4.5 weeks and was undetectable at 5 weeks. No expression was detected in mature mammary glands during the estrus cycle, pregnancy, lactation and involution. Beside the pubertal mammary gland, no expression of *pmg*-1 was detected in any other organs analyzed (Fig. 4A) including the ovaries of pubertal mice (Fig. 4B). These results demonstrate that *pmg*-1 expression is restricted to the mammary gland at the onset of pubertal maturation, the time point at which end bud formation is induced [20] and high cell proliferation is seen in the myoepithelial and epithelial cells lining the ducts and in the cells constituting the terminal end buds [2].

In order to define which cell type in the mammary gland is responsible for *pmg*-1 expression, we performed in situ hybridization on mammary glands isolated from 4-week-old females using the full-length *pmg*-1 cDNA as a probe (Fig. 5). Hybridization of the anti-sense probe was clearly localized to the epithelial cells of the ducts (Fig. 5B) and more prominently to the epithelial cells of the multi-layered alveolar end buds

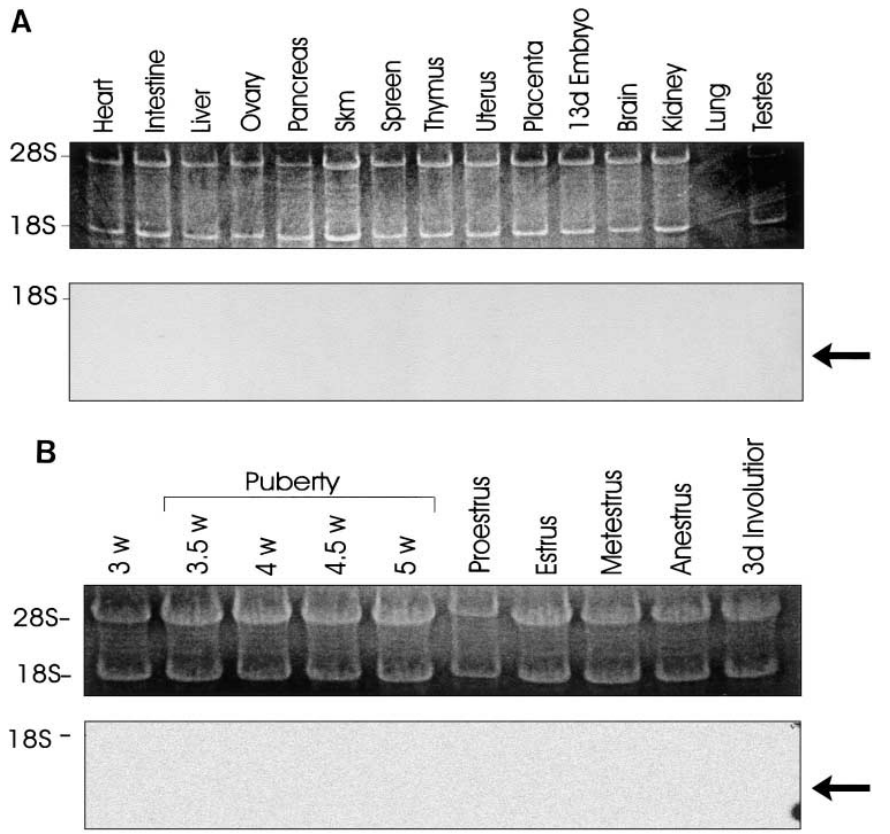

Fig. 4. *pmg-1* expression in (A) mouse organs (B) during ovarian development. Poly(A)-enriched RNA prepared from the organs indicated and total RNA prepared from ovaries at the indicated developmental stages was analyzed by Northern blotting using full-length *pmg*-1 as the probe. The position of the 18S and 28S rRNA are indicated. The arrow indicates the expected position of *pmg-1* RNA.

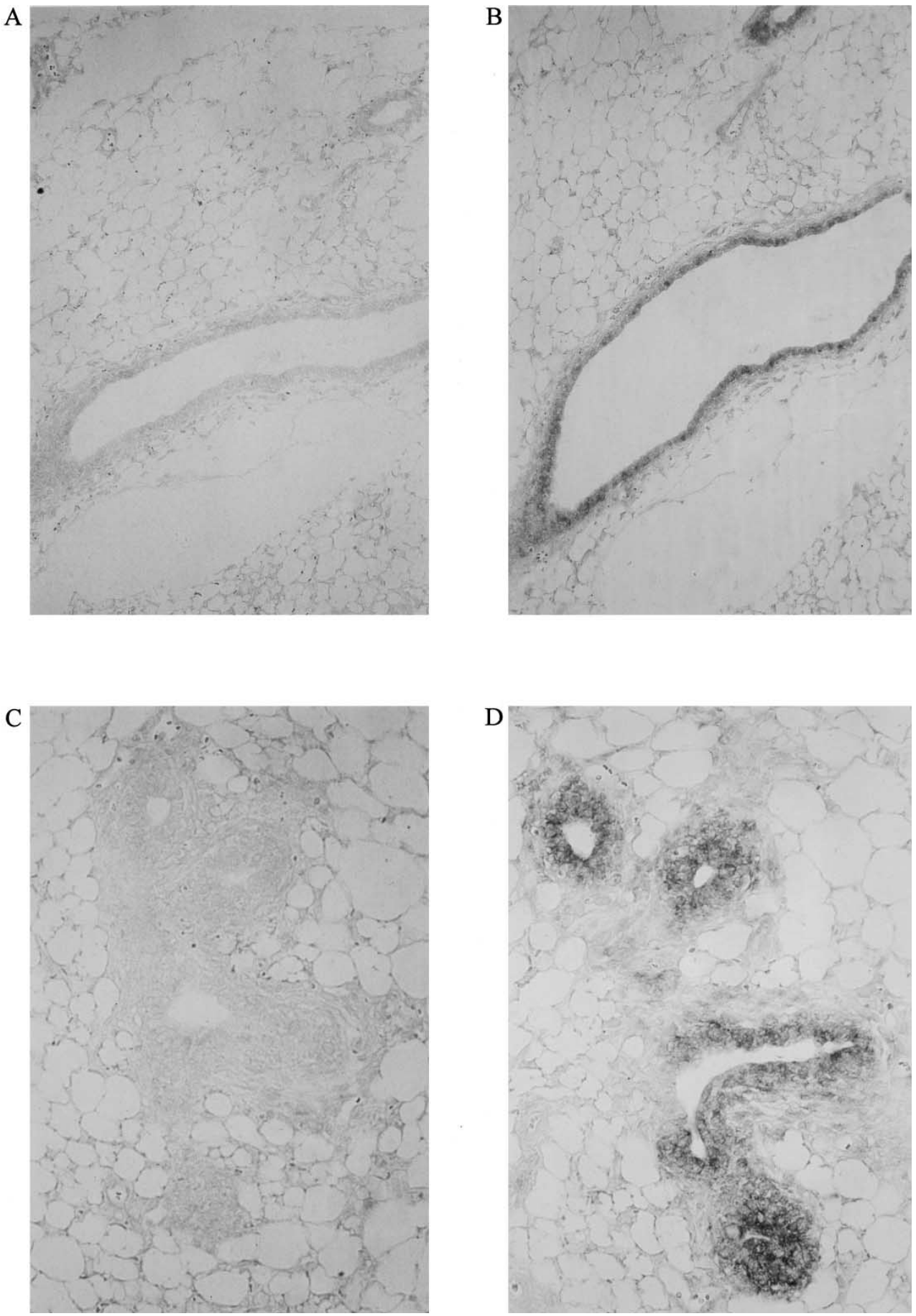
A
B
C
D

Fig. 5. In situ hybridization of *pmg-1* in the pubertal mammary gland. A,C: Sense probe; B,D: antisense probe. Panels A and B illustrate a mammary gland duct and panels C and D represent a cross-section through multi-layered mammary epithelium of a mammary gland from a pubertal 4.5-week-old female. Magnification: ×250 (A,B); ×300 (C,D).
←

(Fig. 5D). No signal was seen in the fibroblasts surrounding the epithelial structures, in the adipose stroma or after hybridization with the control sense probe (Fig. 5A,C). Interestingly, not all epithelial cells hybridized to the same extent and occasionally *pmg*-1 positive cells were seen adjacent to non-expressing cells. This may be due to asynchronous expression of *pmg*-1 in the mammary epithelial cells. Alternatively, the differential expression of *pmg*-1 could also reflect the different epithelial cell populations present in the mammary ductal tree. The pubertal terminal end buds are thought to originate from pluripotent stem cells which are interspersed in the determined ductal epithelial cells [20]. It remains to be investigated if these pluripotent stem cells are indeed those expressing *pmg*-1. The number of pubertal end buds, consisting of undifferentiated mitotically active cells, has been positively correlated with the development of mammary cancer [21] and thus a potential role of *pmg*-1 in mammary carcinogenesis has to be considered.

In summary, our results document the first isolation of a gene whose expression is exquisitely specific for the onset of the branching morphogenesis of the mammary epithelium at puberty. This phase is governed mainly by estrogen, growth hormone and insulin-like growth factor-1, TGF-α and heregulin-α [22,23]. The responsiveness to progesterone is only acquired later around 7 weeks [24]. It will be important to elucidate, which hormones required for the induction of mammary gland maturation are inducing the expression of *pmg*-1.

Acknowledgements: The authors wish to thank Zariana Nikolova for assistance with the in situ hybridization, Peter Küng for providing the cDNA library and Gisela Zürcher for technical assistance. We thank Andrew Ziemiecki for helpful discussions and critical reading of the manuscript and Bob Friis for continued interest and support. This work was supported by the Roche Research foundation, the Bernese Cancer League and by the Swiss National Science Foundation (31-45599.95).

References

[1] Williams, J.M. and Daniel, C.W. (1983) Dev. Biol. 97, 274–290.
[2] Djonov, V., Altermatt, H.J., Arnold, A.-M., Gerber, H. and Andres, A.-C. (1995) Int. J. Oncol. 7, 311–318.
[3] Nandi, S., Guzman, R.C. and Yang, J. (1995) Proc. Natl. Acad. Sci. USA 92, 3650–3657.
[4] Harris, J.R., Lippman, M.E., Veronesi, U. and Willett, W. (1992) N. Engl. J. Med. 327, 319–328.
[5] Robinson, S.D., Silberstein, G.B., Roberts, A., Flanders, K.C. and Daniel, C.W. (1991) Development 113, 867–878.
[6] Pierce, D.F., Gorska, A.E., Chytil, A., Meise, K.S., Page, D.L., Coffey, R.J. and Moses, H. (1995) Proc. Natl. Acad. Sci. USA 92, 4254–4258.
[7] Snedeker, S.M., Brown, C. and diAugustine, R.P. (1991) Proc Natl Acad Sci USA 88, 276–280.
[8] Daniel, C.W., Strickland, P. and Firedmann, Y. (1995) Dev. Biol. 169, 511–519.
[9] Lydon, J.P., DeMayo, F.J., Funk, C.R., Mani, S.K., Highes, A.R., Montgomery, C.A., Shymala, G., Conneely, O.M. and O'Malley, B.W. (1995) Genes Dev. 9, 2266–2278.
[10] Sicinski, P., Donaher, J.L., Parker, S.B., Li, T., Fazeli, A., Gardnaer, H., Haslam, S.Z., Bronson, R.T., Elledge, S.J. and Weinberg, R.A. (1995) Cell 82, 621–630.
[11] Fantl, V., Stamp, G., Andrews, A., Rosewell, I. and Dickson, C. (1995) Genes Dev. 9, 2364–2372.
[12] Liang, P. and Pardee, A.B. (1992) Science 257, 967–971.
[13] Andres, A.-C., Zürcher, G., Djonov, V., Flück, M. and Ziemiecki, A. (1995) Int. J. Cancer 63, 288–296.
[14] Schönenberger, C.A., Zuk, A., Groner, B., Jones, W. and Andres, A.-C. (1990) Dev. Biol. 139, 327–337.
[15] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[16] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) J. Mol. Biol. 215, 403–410.
[17] Thomas, P. (1980) Proc. Natl. Acad. Sci. USA 77, 5201–5205.
[18] Kozak, M. (1991) J. Cell. Biol. 115, 887–903.
[19] Baguski, M.S., Lowe, T.M. and Tolstoshew, C.M. (1993) Nat. Genet. 4, 332–333.
[20] Daniel, C.W. and Silberstein, G.B. (1987) in: Neville, M.C. and Daniel, C.W. (Eds.), The Mammary Gland: Development, Regulation and Function, Plenum Press, New York, pp. 1-36.
[21] Russo, J. and Russo, I.H. (1994) Cancer Epidemiol. Biomarkers Prevent. 3, 353–364.
[22] Kleinberg, D.L. (1997) J. Mam. Gland Biol. Neoplasia 2, 49–57.
[23] Jones, F.E., Jerry, D.J., Guarino, B.C., Andrews, G.C. and Stern, D.F. (1996) Cell Growth Diff. 7, 1031–1038.
[24] Haslam, S.Z. (1989) Endocrinology 125, 2766–2772.