Vol. 189, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1992 Pages 134-140

FUNCTIONAL STUDY OF THE GERMINAL ANGIOTENSIN I-CONVERTING ENZYME PROMOTER

Sophie Nadaud, Anne-Marie Houot, Christine Hubert, Pierre Corvol, and Florent Soubrier*

INSERM unit 36, Collège de France, 75005 PARIS, France

Received October 4, 1992

SUMMARY : Polymerase chain amplification experiments indicate that the germinal specific promoter of the angiotensin I-converting enzyme (ACE) is completely extinguished in somatic tissues. Despite this very strict specificity of expression, the germinal ACE promoter is active in transient transfection experiments in two somatic cell lines and one cell line of germinal origin. The analysis of the promoter shows the existence two regulatory elements within the first 350 bp : a proximal positive element and a distal negative element.

The structure and expression of the angiotensin I-converting enzyme (dipeptidyl carboxypeptidase, DCP-1, 3.4.15.1, ACE) gene results from a duplication of an ancestral gene (1). Two isoforms, somatic and germinal ACE, are expressed from the ACE gene. Somatic ACE is encoded by exons 1 to 26 (except exon 13) and has a molecular weight of 170 kD (2). This enzyme is widely distributed in endothelial, epithelial and neuroepithelial cells (3) and is composed of two highly homologous domains each containing an active site (1, 4).

The germinal isoform is encoded by exons 13 to 26 (2), has a smaller molecular weight of 90 kD and has been localized in developing haploid spermatids and spermatozoa (5). Germinal ACE contains one of the two homologous domains of somatic ACE and, therefore, a single active site (6). The germinal ACE mRNA is transcribed from an internal promoter located upstream from exon 13, as demonstrated by ribonuclease protection assays and by transgenic mouse experiments (2, 7, 8, 9). The germinal ACE promoter contains a TATA-like box (TATT), a cAMP-responsive element (CRE) and two binding sites for AP2 with, for each of these motifs, a single base different from the consensus sequence (2).

* To whom correspondence should be addressed.

Abbreviations : ACE : angiotensin I-converting enzyme, RE cell : rabbit endothelial cell RSV : Rous sarcoma virus, CAT: chloramphenicol acetyl transferase, CREM : cAMP responsive element modulator.

0006-291X/92 $4.00

The physiological role of ACE in endothelial cells is well established (2) and consists in the metabolism of two vasoactive peptides, via the conversion of angiotensin I into angiotensin II and the inactivation of bradykinin. However, the role of ACE at other locations, most notably in germinal cells, is not yet known.

In this study, we have used primers specific of the two isoforms mRNA in polymerase chain amplification (PCR) experiments on different tissues RNA. The results indicate that the activity of the germinal ACE promoter is completely extinguished in somatic cells. Using transient transfection experiments in different cell types, we demonstrate the existence of a proximal positive element and a distal negative regulatory element within the first 350 bp of intron 12.

MATERIALS AND METHODS

Materials

All DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA, USA). Oligonucleotides were synthesized on an Applied Biosystems Synthesizer. Purified luciferase from Photinus pyralis, synthetic D-luciferin and forskolin were obtained from Sigma Biochemical Co. (St Louis, MI, USA).

RNA extraction and PCR analysis

Total RNA was extracted from adult Swiss mice organs or cell lines using the guanidine thiocyanate method (10). 5 µg of total RNA was used to prepare cDNA using the MMLV reverse transcriptase (Boehringer, Mannheim) in the presence of 7.5 mM oligo dT. PCR was performed using 20 to 25 bases synthetic oligonucleotides as primers which had been purified on acrylamide gels. 1/10th of the cDNA reaction sample was incubated with 1 U/25 µl of *Taq* DNA polymerase (Boehringer) in the Boehringer buffer, in the presence of 500 nM of each primer. The PCR reaction was performed (Perkin Elmer apparatus) for 30 cycles : 94°C,1 min - 55°C, 1 min - 72°C, 2 min. We used two primers couples (7): Primer 1 (TTGGGCTGTCCGGTCATACT) and primer 2 (AGCACTGTGCAAGGAGGCA) specific to the mouse somatic form and primer 1 and primer 3 (ATGGGCCAAGGTTGGGCTACT) specific to the mouse germinal form.

Southern blot analysis

PCR reaction samples were electrophoresed on agarose gels (2 %), capillary blotted onto a nylon membrane (Hybond N+, Amersham) and hybridized with synthetic end-labeled oligonucleotides. The oligonucleotide M 949, obtained from mouse intron 13 sequence (7), overlaps on exon 13 and exon 14 and only hybridizes with germinal ACE cDNA.

Plasmids construction

The cloning and isolation of the human angiotensin converting enzyme gene has already been described (2). The promoter-less plasmid pCAT (11) which comprises the coding sequence for the bacterial gene chloramphenicol acetyltransferase (CAT) was used for cloning germinal ACE promoter fragments. The following fragments were subcloned into pCAT : Apa I (-80)-PstI (+3), BstX I (-224)-Pst I (+3), AvaI (-349)-PstI (+3) and ApaI (-900)-PstI (+3), generating the plasmids p80+, p224+, p349+ and p900+ respectively. The 349 bp insert was also subcloned in the reverse orientation generating p349-. All plasmids contain the transcription start site up to nucleotide +3. A vector pRSVCAT with the Rous Sarcoma Virus (RSV) enhancer/promoter fused to the CAT coding region (12) was used as a positive control. A pRSVluci vector with the RSV enhancer/promoter fused to the coding sequence for the firefly gene luciferase (13), was used to correct the results for transfection efficiencies.

Cell culture and transfection

Human testicular Tera-1 cells derived from an embryonic carcinoma were obtained from ATCC (HTB 105). Rabbit endothelial (RE) cells were immortalized with the SV40 T antigen under the control of the vimentine promoter (14, 15). Hep G2 cells are derived from human hepatoma cells. All cells were transfected in Dulbecco's Modified Eagle's Medium (Boehringer) with FCS (10%), penicillin (10 mg/ml), streptomycin (10 mg/ml) and glutamine (0.5 mM). Transient transfections were performed using the calcium phosphate precipitation method with supercoiled DNA purified on $CsCl_2$ gradient

essentially as described (16). Each plate received 18 µg of DNA consisting of 3 µg of pRSVluci (internal control) and 15 µg of CAT reporter gene or 5 µg of pRSVCAT and 10 µg carrier pBluescript. After 48 hours, whole-cell extracts were prepared by three freeze-thaw cycles. After removal of cell debris by centrifugation, cell extracts were analyzed for luciferase and CAT activities.

Reporter assays

Luciferase activity was assayed as described (17) using a ß scintillation counter integrating luminescence for 1 minute. CAT assays were performed as described (18). After chromatography, the radioactive spots of acetylated and non-acetylated forms of ^{14}C chloramphenicol were cut and quantitated in a ß scintillation counter.

RESULTS AND DISCUSSION

Tissue-specificity of germinal ACE expression

We took advantage of the splicing of exon 13 from the endothelial ACE mRNA and of its presence in the germinal ACE mRNA to specifically amplify germinal ACE mRNA (Fig. 1). Germinal ACE primers are located on exon 13 and 14, separated by one intron, thus enabling the differentiation of cDNA amplification (258 bp) from amplification of genomic DNA (390 bp). The sensitivity of detection of amplified cDNA was further increased by Southern hybridization with a germinal ACE cDNA specific oligonucleotide probe (M 949). The PCR process was monitored by the amplification of the ACE genomic band. The quality of RNA was checked by parallel amplification with endothelial ACE specific primers, resulting in a 350 bp product present in all samples (data not shown).

The 258 bp fragment amplified from germinal ACE cDNA was only detected in testis and in epididymis, even after Southern-blotting which enhances the degree of sensitivity (Fig. 2). An additional 340 bp fragment was detected from the germinal cDNA amplification, corresponding to a hybrid between amplified cDNA and genomic DNA. The detection of a low amount of this mRNA in the epididymis suggests that a few mature spermatids or pre-spermatozoa may be present. No germinal ACE mRNA amplification was observed in any other tissue, even as trace.

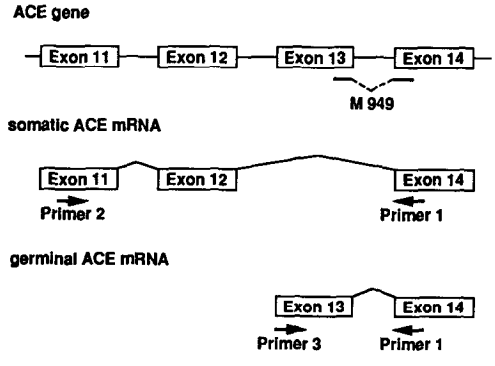

Fig. 1 : Schematic representation of the exon 11 to 14 of the ACE gene and of the two ACE mRNA. The location of the PCR primers (1, 2 and 3) is indicated by arrows underneath the corresponding exon. The probe M 949 is represented by a solid line interrupted by a broken line which indicates that the sequence of the oligonucleotide overlaps exon 13 and exon 14.

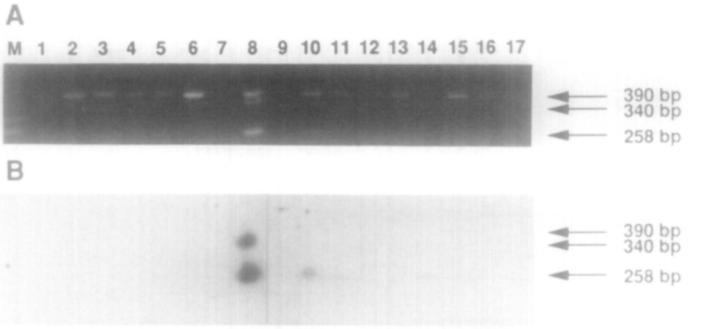

Fig. 2 : PCR ANALYSIS OF GERMINAL ACE EXPRESSION
PCR experiments were performed on cDNA obtained from various mouse organs: 1-kidney, 2-lung, 3-spleen, 4-large intestine, 5-heart, 6-small intestine, 7-liver, 8-testis, 9-pancreas, 10-epididymis, 11-hypothalamus, 12-adrenal gland, 13-brain, 14-ovaries, 15-seminal vesicules, 16-aorta. The size marker (ϕ X 174 DNA cut by Hae III) was run in lane M.
Panel A : amplification with primers 1 and 3, specific for germinal ACE.
Panel B : Southern hybridization of the products obtained from amplification with the germinal ACE specific primers using probe M 949.

Using transgenic animal technology, Langford et al showed that the transcription of a reporter gene drived by a 650 bp fragment upstream from exon 13 was restricted to spermatids (9). However, a low extra-testicular transcription could not be excluded due to the lower sensitivity of the method of detection as compared with PCR amplification. A previous PCR study (19) demonstrated the existence of an "illegitimate transcription" of a gene occurring in tissues where the encoded protein was not detected. In contrast with this gene, the total absence of germinal ACE mRNA expression in tissues other than testis and epididymis indicates that the activity of the germinal promoter is completely extinguished in somatic tissues. This might result either from the absence of a germinal-specific transcription factor or from the existence of a specific gene extinguisher.

Analysis of the germinal ACE promoter

To study the function of the germinal ACE promoter, different constructs were transfected in three cell lines. As no haploid cell lines can be developed, we used the Tera-1 cells, derived from primary diploid germ cells, which express endothelial ACE and a very low amount of germinal ACE (detected by PCR and hybridization). We also used the endothelial RE cells which only express the endothelial ACE, and the HepG2 cells which do not express either forms.

The p349+ plasmid, containing the 12th intron of the ACE gene, was transcriptionally active in all cell types as compared with the pRSVCAT activity (Fig. 3). Control experiments with the p349- construct or with no promoter (pCAT) indicate that this activity can be ascribed to the insert, which contains a functional promoter. The *in vivo* cell specificity is not reproduced *in vitro* and might result from titration of transcription inhibiting-factors or from a non genomic chromatin organization and methylation pattern of the plasmid DNA.

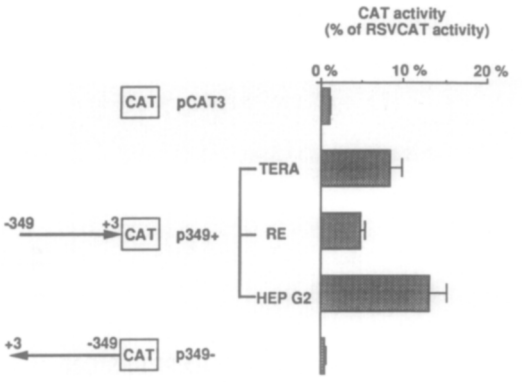

Fig. 3 : ACTIVITY OF THE GERMINAL ACE PROMOTER IN THREE CELL LINES
The Ava I-Pst I fragment of the germinal ACE promoter (-349 to +3) was inserted in both orientations in the pCAT vector resulting in the p349+ and p349- plasmids. Each cell line : Tera-1, RE or Hep G2 was transfected with pCAT, p349+, p349- (15 µg) or pRSVCAT (5 µg). Results are expressed as a percentage of pRSVCAT activity, normalized to luciferase activity for each plasmid transfected. Data are the means ±S.E.M. of triplicate CAT activity measurements from 3 to 8 separate transfection experiments.

Different constructs were used to map regulatory elements. The results obtained are similar in the three cell lines (Fig. 4) and show a significant ($p<0.05$) increase of transcription with addition of the sequence between -80 and -224 and a significant ($p<0.05$) decrease between -224 and -349. This suggests the presence of two regulatory regions within the first 350 bp : a proximal positive regulatory region (-80 to -224) and a distal regulatory element (-224 to -349). These elements seem to respond to ubiquitous factors as also shown for the rat testis specific histone H1t promoter transfected in mouse fibroblasts (20). In the appropriate chromatin environment, *in vivo*, this negative element located between -224 and -349 may have a role in inhibiting transcription in somatic cells and in early germ cells. The location of these elements is consistant with a previous study which demonstrated that the first 650 bp of the 5' region are sufficient to confer a germinal specific pattern of expression in transgenic mice (9). A negative element has also been implicated in the testis specific regulation of the sea urchin sperm specific H2B gene (21).

Effect of cAMP

The analysis of the 12th intron sequence (300 bp) does not show many known regulatory *cis* elements with the exception of 2 sequences close to AP2 binding sites and 1 sequence close to a cAMP responsive element (2).

To test the activity of this CRE we performed transfection experiments in the presence of forskolin 10^{-6}M without serum in Tera-1 cells. The activity of the plasmids pRSVCAT and pCAT, used as controls, were not modified by the addition of forskolin to the culture medium. In our system, we were not able to detect any effect of forskolin on the germinal ACE promoter (results not shown). However, the pattern of expression of

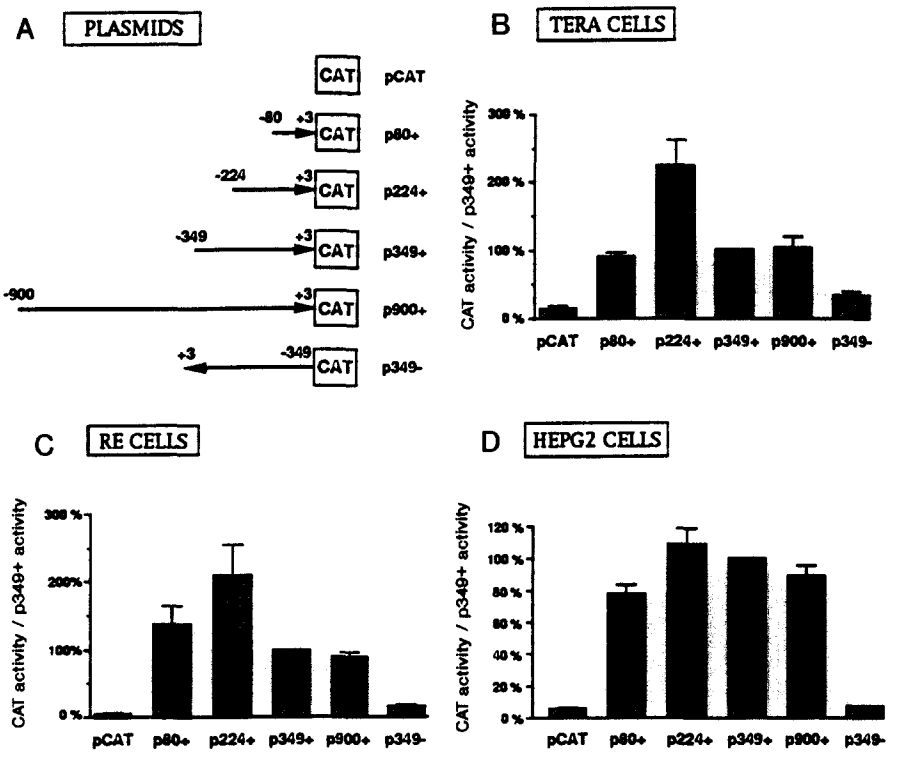

Fig. 4: GERMINAL PROMOTER ANALYSIS IN THREE CELL LINES
Panel A : different fragments of the germinal ACE promoter were inserted in pCAT generating the different plasmids used for transfection experiments. p80+: Apa I-Pst I (-80 to +3), p224+ : BstX I-Pst I (-224 to +3), p349+ : Ava I-Pst I (-349 to +3) and p900+ : Apa I-Pst I (-900 to +3).
These plasmids were transfected in the Tera-1 cells (panel B), in the RE cells (panel C) and in the HepG2 cells (panel D). Results are expressed as a percentage of the p349+ activity, normalized to luciferase activity to account for transfection efficiency. Data are the means ± S.E.M. of triplicate CAT activity measurements from 3 to 8 separate transfection experiments.

germinal ACE is compatible with an effect of CREM on transcription, since CREM is a transcription inhibitor except in spermatocytes and spermatids, due to an alternative splicing of the gene (22) Further experiments will be required to test its effect on the germinal ACE promoter cAMP responsive element.

ACKNOWLEDGMENTS

This work was supported by Institut National de la Santé et de la Recherche Médicale and Centre National de la Recherche Scientifique. We thank Drs.D. Paulin and P. Vicart for kindly providing the rabbit endothelial (RE) cells.

REFERENCES

1. Soubrier F., Alhenc-Gelas F., Hubert C., Allegrini J., John M., Tregear G. and Corvol P. (1988) Proc. Natl. Acad. Sci. USA. 85, 9386-9390.
2. Hubert C., Houot A. M., Corvol P. and Soubrier F. (1991) J. Biol. Chem. 266, 15377-15383.

3. Erdös E. G. and Skidgel R. A. (1987) Laboratory investigation. 56, 345-348.
4. Bernstein K. E., Martin B. M., Edwards A. S. and Bernstein E. A. (1989) J. Biol. Chem. 264, 11945-11951.
5. Brentjens J. R., Matsuo S., Andres G. A., Caldwell P. R. B. and Zamboni L. (1986) Experientia. 42, 399-402.
6. Lattion A. L., Soubrier F., Allegrini J., Hubert C., Corvol P. and Alhenc-Gelas F. (1989) FEBS Lett. 252, 99-104.
7. Howard T. E., Shai S. Y., Langford K. G., Martin B. M. and Bernstein K. E. (1990) Mol. Cell. Biol. 10, 4294-4302.
8. Kumar R. S., Thekumkara T. J. and Sen G. (1991) J. Biol. Chem. 266, 3854-3862.
9. Langford K. G., Shai S.Y., Howard T. E., Kovac M. J., Overbeek P. A. and Bernstein K. E. (1991) J. Biol. Chem. 266, 15559-.
10. Chominczynski P. and Sacchi N. (1987) Anal. Biochem. 28, 5311-5316.
11. Luckow B. and Schütz G. (1987) Nucleic Acid Res. 15, 5490.
12. Gorman C.M., Glenn T.M., Willingham M.C., Pastan I. and Howerd B. (1982) Proc. Natl. Acad. Sci. USA. 79, 6777-6781.
13. De Wet J.R., Wood K.V., Deluca M., Helinski D.R. and Subramani S. (1987) Mol. Cell. Biol. 7, 725-737.
14. Schwartz B., Vicart P., Delouis C. and Paulin D. (1991) Biol. Cell.73, 7.
15. Vicart P., Testut P., Schwartz B., Llorens-Cortes C., Delouis C. and Paulin D. Submitted.
16. Maniatis T., Fritsch E. F. and Sambrook J. (1982) Molecular cloning (a laboratory manual), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
17. N'Guyen V. T., Morange M. and Bensaude O. (1988) Anal. Biochem.171, 404-408.
18. Crabb D. W. and Dixon J. E. (1987) Anal. Biochem. 163, 88-92.
19. Chelly J., Concordet J. P., Kaplan J. C. and Kahn A. (1989) Proc. Natl. Acad. Sci. USA. 86, 2617-2621.
20. Kremer E. J. and Kistler W. S. (1992) Gene. 110, 167-173.
21. Lim K. and Chae C. B. (1992) J. Biol. Chem. 267, 15271-15273.
22. Foulkes N. S., Mellström B., Benusiglio E. and Sassone-Corsi P. (1992) Nature. 355, 80-84.