cAMP-response element modulator τ is a positive regulator of testis angiotensin converting enzyme transcription

(cAMP-response element modulator/spermatogenesis)

YUDONG ZHOU*, ZUOMING SUN†, ANTHONY R. MEANS†, PAOLO SASSONE-CORSI‡, AND KENNETH E. BERNSTEIN*

*Department of Pathology and Laboratory Medicine, Emory University, Atlanta, GA 30322; †Department of Pharmacology, Duke University Medical Center, Durham, NC 27710; and ‡Institut de Génétique et de Biologie Moléculaire et Cellulaire, BP 163, 67404 Illkirch, C.U. de Strasbourg, France

Communicated by Douglas C. Wallace, Emory University School of Medicine, Atlanta, GA, August 13, 1996 (received for review January 23, 1996)

ABSTRACT Testis angiotensin-converting enzyme (ACE) is a unique form of ACE, only produced by male germ cells, and results from a testis-specific promoter found within the ACE gene. We have investigated the role of cAMP-response element modulator (CREM) τ in testis ACE transcription. In gel shift experiments, testes nuclear proteins retard an oligonucleotide containing the cAMP-response element (CRE) found at position –55 in the testis ACE promoter. Anti-CREM antibody supershifts this complex. Competitive gel shift shows that recombinant CREM τ protein and testes nuclear proteins have a similar specificity of binding to the testis ACE CRE. Functional analysis using *in vitro* transcription and transfection studies also demonstrate that CREM τ protein is a transcriptional activator of the testis ACE promoter. Western blot analysis identifies CREM τ protein in the protein–DNA complex formed between nuclear proteins and the testis ACE CRE motif. This analysis also identified other CREM isoforms in the gel-shifted complex, which are thought to be CREM τ 1/2, CREM α / β , and S-CREM. These data indicate that CREM τ isoforms play an important role as a positive regulator in the tissue-specific expression of testis ACE.

Spermatogenesis is a complex developmental process that is associated with unique patterns of gene expression (1). One example is the production of a testis isozyme of angiotensin-converting enzyme (ACE). There are two isozymes of ACE in mammals (2). Somatic ACE is produced by endothelium and other somatic tissues and is responsible for the conversion of angiotensin I into the potent vasoconstrictor angiotensin II (3). A second isozyme, testis ACE, is a tissue-specific gene product made only by round and elongating spermatids (4). Somatic ACE is composed of two homologous catalytic domains; testis ACE contains a single catalytic domain identical to the carboxyl half of somatic ACE (5–7). Both ACE isozymes are encoded by the same gene (8). The study of ACE knockout mice has demonstrated that testis ACE is important for fertility (9).

Previous studies have identified a germ cell-specific testis ACE promoter located within the 12th intron of the somatic ACE gene; as few as 91 bp of this promoter can target the expression of a reporter gene to round and elongating spermatids in transgenic mice (10, 11). *In vitro* analyses of the testis ACE promoter have identified two important transcriptional motifs within the 91-bp promoter (12). One element, TCT-TAT, is at position –32 relative to the start of transcription and appears to be a nonconsensus TATA box. The other motif, TGAGGTCA, is located at position –55 and is highly homologous to the consensus cAMP-response element (CRE), TGACGTCA. The consensus CRE, or variants of it, have been found in the promoter regions of cAMP-responsive genes (13, 14). Upon hormonal stimulation, a signal transduction cascade

leads to the phosphorylation of a number of CRE-binding proteins, which then exert positive or negative effects on the transcription of cAMP-responsive genes. A unique member of this group of transcription factors is CREM (cAMP-response element modulator) (15). The CREM gene encodes both transcriptional repressors and activators. By alternative splicing, three repressor isoforms, CREM α , CREM β , and CREM γ are produced. In contrast, CREM τ is an alternative splice form of the CREM gene containing two glutamine-rich regions and functions as a transcriptional activator. CREM τ is abundant within male germ cells. Other forms of CREM τ , termed τ 1 and τ 2, contain a single glutamine-rich region and are also transcriptional activators. S-CREM is a truncated form of CREM τ produced by alternative translational initiation and functions as a repressor of transcription.

Premeiotic male germ cells express only the repressor isoforms of CREM. However, as these cells mature into pachytene spermatocytes, large amounts of CREM τ mRNA are expressed. This developmental switch of CREM expression is induced by follicle-stimulating hormone (16). CREM τ protein has been implicated in the transcriptional regulation of several testis-specific genes such as caldesmon, TP1, and RT7 (17–19).

We sought to define the role of CREM τ in the expression of rodent testis ACE. In part, this stems from the previous observation that testis ACE mRNA is first expressed in the germ cells of 22-day-old mice (20). This is associated with the appearance of early spermatids and is a stage at which large amounts of CREM τ protein is first detected (21). Here we present data showing that CREM τ is important in the function of the testis ACE promoter. These studies support the role of CREM τ as a positive regulator in the unique tissue-specific expression of testis ACE.

MATERIALS AND METHODS

Gel Retardation Assays. Whole testis nuclear extracts were prepared from adult Sprague Dawley rats as described (11). The oligonucleotides used to generate a double-stranded fragment containing the testis ACE-55 element were: ACE.327 5'-ACATTGCTCTATGAGGTCACTGCAG-GCTTG-3' and ACE.328 5'-CAAGCCTGCAGTGTGAC-CTCATAGAGCAAT-3'. The double-stranded oligonucleotides were blunted with T₇ DNA polymerase, [α -³²P]dGTP and [α -³²P]dTTP. The gel retardation assays were performed as follows: 10 μ g of rat testis nuclear extract was preincubated in a 15- μ l reaction volume containing 20 mM Hepes (pH 7.6), 32 mM KCl, 80 mM EDTA, 8% glycerol, 0.8 mM DTT, and 1 μ g double-stranded poly(dI-dC) at room temperature for 5 min. Probe (3.0 $\times$ 10⁴ cpm) was then added and incubated at room

temperature for another 15 min. The reaction mix was loaded onto a 5% native polyacrylamide gel (12). The gel was run in $0.5 \times$ TBE buffer (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) at 4°C , dried, and exposed to film.

For gel supershift analysis, 3–6 μg of anti-CREM antibody (Santa Cruz Biotechnology) was added to the gel shift reaction and incubated at room temperature for 30 min. The reaction mix was electrophoresed as above. This antibody was prepared against whole human CREM-1 protein.

For preparative gel retardation followed by Western blot analysis, a large-scale binding reaction was performed using 150 μg rat testis nuclear extract, 10 ng unlabeled double-stranded oligonucleotide containing the testis ACE CRE, 15 μg double-stranded poly(dI-dC), and 10^5 cpm labeled probe. After incubating at room temperature for 20 min, the reaction mix was loaded onto a 5% preparative native polyacrylamide gel containing 2.5% glycerol. The gel was run in $0.5 \times$ TBE at 4°C , and exposed to x-ray film without drying. The gel slices containing the retarded bands were excised and dialyzed against $1 \times$ SDS/PAGE running buffer at room temperature overnight. Proteins were then electroeluted into $1 \times$ SDS/PAGE running buffer at 100 V for 2 hr, dialyzed against $0.5 \times$ SDS/PAGE running buffer at room temperature for 2 hr, and dried under vacuum. Proteins were suspended in water and passed through a Bio-Spin 6 column (Bio-Rad). The sample was then mixed with an equal volume of $2 \times$ SDS/PAGE loading dye, denatured, and loaded onto a 10% SDS/PAGE mini-gel. The gel was run at room temperature and transferred to a polyvinylidene difluoride membrane (22). The membrane was blocked in 5% nonfat milk in TTBS (20 mM Tris-HCl, pH 6.8/150 mM NaCl/0.05% Tween 20) solution and incubated with primary antibodies according to the protocol from the vendor, Santa Cruz Biotechnology. A donkey anti-rabbit Ig horseradish peroxidase-conjugated antibody (Amersham) was used as the secondary antibody. The blot was developed using enhanced chemiluminescence reagents (Amersham).

In Vitro Transcription Assays. The template DNA for *in vitro* transcription was a linear fragment containing the mouse testis ACE promoter from –91 to –9 followed by a 380-bp G-free cassette. Each reaction contained 50 μg rat testis nuclear extract, 300 ng testis ACE template, 200 ng pADML(190) as an internal control, and 300 ng double-stranded poly(dI-dC) in a total volume of 20 μl (11, 12). In the indicated experiments, 1–5 μg of anti-CREM antibody (Santa Cruz Biotechnology) or rabbit IgG (Sigma) was preincubated with the rat testis nuclear extract for 1 hr on ice before the addition of templates. The reaction was performed at 30°C for 45 min. Quantitation of the transcripts was performed using a PhosphorImager (Molecular Dynamics). The quantitation ratio of the 372 band to the 190 band was used as the measurement of each transcription reaction.

Transfection and Chloramphenicol Acetyltransferase (CAT) Assays. The ptACE-CAT reporters contained the testis ACE promoter from –688 to +17 or from –91 to +17 cloned in front of the CAT gene in pBLCAT₃ (11, 23). The pSom-CAT contains the CRE from the somatostatin promoter in pBLCAT₂ (24). Two micrograms of the reporter constructs were cotransfected with 200 ng pC_αEV, a vector encoding the catalytic subunit of protein kinase A (PKA), 0.5 μg pSVCREM τ , 0.5 μg pSVCREM β , or 0.5 μg pSVCREB as indicated in Fig. 4 (18, 25). The calcium phosphate precipitation method was used for transfection, and CAT activity was analyzed by standard methods.

RESULTS

We have used a gel-mobility shift assay to identify proteins that interact with the testis ACE CRE-like motif. A 32-bp oligonucleotide encoding positions –66 to –35 of the testis ACE promoter was mixed with nuclear proteins prepared from rat

testes. This oligonucleotide contains the CRE motif TGAGGTCA at positions –55 to –48 (12). Gel retardation analysis showed a major retarded band (Fig. 1, band I) that on shorter exposure resolved into a closely spaced series of bands. A minor gel retardation species migrated faster in the gel (Fig. 1, band II). We asked if a rabbit polyclonal anti-CREM antibody active against all CREM isoforms would supershift the gel retarded bands. Increasing quantities of this antibody resulted in a supershift of the major gel shift band but not the minor species (Fig. 1, lanes 3 and 4). Recombinant CREM τ and CREM β proteins also bind to this oligonucleotide (Fig. 1, lanes 5 and 6). These recombinant proteins retarded a single major band that migrated slightly faster than the endogenous (nonrecombinant) proteins in a testis nuclear extract.

To investigate the specificity of the gel shift reaction, we created oligonucleotides that differ from the testis ACE CRE-like region by a point mutation at either position –48 (A–G) or position –51 (G–A) (Fig. 2A). We also obtained a cAMP-response element binding protein (CREB) consensus oligonucleotide encoding the CRE sequence from the rat somatostatin gene and an oligonucleotide, called t2, containing sequence from positions –97 to –66 of the testis ACE promoter. Within the t2 oligonucleotide is the sequence TGGGGTCA, which varies from the putative testis ACE CRE motif TGAGGTCA by one nucleotide. First we studied the ability of each unlabeled oligonucleotide to compete with the gel shift of the testis ACE CRE oligonucleotide. When gel shift was performed using a rat testis nuclear extract, the testis ACE CRE oligonucleotide competed efficiently with itself (Fig. 2B). Among the other oligonucleotides, the point mutation at –51 and the CREB oligonucleotide were more effective competitors than the –48 point mutation. The t2 oligonucleotide was the least effective competitor. We also performed a similar competition experiment substituting recombinant CREM τ protein in place of the rat testis nuclear extract (Fig. 2C). The pattern of competition using CREM τ was similar to that using the testis nuclear extract. The testis ACE CRE oligonucleotide competed well against itself. The –51 point mutation and the CREB oligonucleotide were somewhat less effective in competition. Neither the point mutation at –48 nor the t2 oligonucleotide were very effective competitors, even at a 250-fold excess. These data are evidence that the proteins in a testis nuclear extract responsible for gel shift have a specificity of binding similar to that observed with recombinant CREM τ .

FIG. 1. Gel shift and supershift of the testis ACE CRE element. Lane 1 is the migration of a 32-bp oligonucleotide encoding the testis ACE CRE motif. Lanes 2–4 show the gel shift pattern after the addition of 10 μg of rat testis nuclear proteins. In lanes 3 and 4, 3 and 6 μg of anti-CREM antibody were added to the testis nuclear proteins. This antibody supershifts the major complex of bands (I) but not a rapidly migrating minor band (II). Anti-CREM antibody in the absence of nuclear proteins had no effect on free probe (data not shown). Purified recombinant CREM τ (lane 5) and CREM β (lane 6) protein also bind to and retard the 32-bp oligonucleotide.

FIG. 2. Competition analysis. (A) The coding sequence of oligonucleotides used to compete the gel shift of a 32-bp oligonucleotide encoding the testis ACE CRE element. (B) Gel shift pattern using 10 μ g of a rat testis nuclear extract. Lanes: 1, free probe; 2, no competitor; 3 and 4, a 10- and 250-fold excess of unlabeled probe, respectively; 5–12, a 10- and 250-fold excess of the indicated competitor oligonucleotides. (C) Gel shift pattern using 0.5 μ g of recombinant CREM7 protein. Lanes: 1, no competitor; 2 and 3, a 50- and 250-fold excess of unlabeled probe, respectively; 4–11, a 50- and 250-fold excess of the indicated competitor oligonucleotides. The patterns of competition observed in B and C are similar.

In vitro transcription can be used to study the transcriptional regulation of the testis ACE promoter (11). In this assay a nuclear protein extract prepared from adult rat testes is used to transcribe a construct consisting of the testis ACE promoter from position –91 to –9 and, as a reporter, a 380-bp G-free cassette. Newly synthesized RNA is 372 nucleotides (Fig. 3) and is dependent on RNA polymerase II. As an internal control, we concurrently transcribe a second construct composed of the adenovirus major late promoter and a G-free cassette of 190 bp. Previously we showed that DNA constructs lacking the testis ACE CRE element transcribed at about 30% the level of the native testis ACE promoter (12).

To establish the functional involvement of CREM in testis ACE transcription, we measured the *in vitro* activity of the testis ACE promoter in the presence of increasing amounts of a polyclonal anti-CREM antibody. Addition of this antibody resulted in a dose-dependent decrease of testis ACE promoter activity (Fig. 3); in the presence of 5 μ g of anti-CREM antibody (lane 4) transcription was reduced to about 23% of that in the absence of antibody ($n = 4$). No reduced transcription from the control adenovirus promoter was observed, suggesting that the reduction of testis ACE transcription is not

Rabbit anti-CREM

FIG. 3. *In vitro* transcription. For each *in vitro* transcription reaction a rat testis nuclear extract was used to transcribe 300 ng of a linear fragment containing the testis ACE promoter from –91 to –9 followed by a 380-bp G-free cassette. This produced a 372 base RNA transcript (solid arrow). In each reaction we also included 200 ng of the adenovirus major late promoter followed by a 190-bp G-free cassette (open arrow). Lanes 2–4 show transcription in the presence of 1, 3, and 5 μ g of anti-CREM antibody, respectively. This antibody inhibits transcription from the testis ACE promoter but not from the adenovirus major late promoter control. These data are representative of four separate experiments.

the result of RNase contamination or nonspecific degradation of the nuclear protein extract. In contrast to the results with the anti-CREM antibody, neither pooled rabbit IgG nor an irrelevant rabbit polyclonal antibody had an effect on testis ACE promoter transcription (data not shown). These experiments support a functional role for CREM in testis ACE transcription.

The functional importance of CREM7 in testis ACE transcription has been measured by cotransfection experiments using JEG-3 cells (Fig. 4). These are human choriocarcinoma cells with an efficient cAMP-dependent signal transduction pathway and were chosen because no cultured haploid male germ cell lines are available. The reporter constructs contained the testis ACE promoter in the plasmid pBLCAT3, and promoter strength was reflected in CAT activity. One construct contained the testis ACE promoter from –688 to +17, whereas a second construct contained this promoter from –91 to +17. Cells were cotransfected with expression constructs encoding the catalytic subunit of PKA, CREM7, CREM β , or CREB. As a positive control, we also used a construct in which CAT gene expression is under the control of the CRE from the somatostatin gene, a known cAMP-dependent promoter region, cloned into pBLCAT2 (24). These data show a marked stimulation of the testis ACE promoter activity by PKA. Furthermore, cotransfection of PKA and CREM7 augmented transcription as compared with transfection of PKA or CREM7 alone. CREM7 was a better activator of the testis ACE promoter than CREB. In contrast, CREM β was an efficient suppressor of transcription. These effects were observed with both the testis ACE promoter constructs.

We have demonstrated that a testis nuclear extract gel shifts a 32-bp oligonucleotide encoding the testis ACE promoter CRE motif. Given the results of *in vitro* transcription and transfection experiments supporting a role of CREM7 in testis ACE transcription, we asked whether the gel-shifted bands

participation in the transcriptional regulation of the testis ACE gene. High level CREM τ expression is found in round spermatids, the precise cells that actively transcribe testis ACE. Whereas most CREM isoforms act as inhibitors of transcription, CREM τ , CREM τ 1, and CREM τ 2 have been shown to be activators of the transcription of other male germ cell restricted gene products (15). In this paper we provide evidence by gel shift and Western blot analysis that CREM τ and other CREM isoforms can bind to the testis ACE promoter CRE element. The removal of CREM isoforms from a testis nuclear extract using anti-CREM antibody, markedly reduced the ability of this extract to transcribe from the testis ACE promoter. Finally, transfection studies showed that the co-transfection of CREM τ and the catalytic region of PKA led to high level transcription from the testis ACE promoter.

An interesting observation of the gel shift studies is that CREM isoforms other than τ isoforms can bind to and gel shift the testis ACE CRE motif. In fact, Western blot analysis of proteins bound to gel-shifted bands identified proteins consistent in size with CREM α , CREM β , and S-CREM. In analyzing these data, one must consider that the testis nuclear extract used for the gel shift studies was prepared from whole rat testis. Within this preparation are diploid spermatocytes that produce only the repressor forms of CREM. As these cells mature into pachytene spermatocytes, follicle-stimulating hormone triggers a change in the pattern of CREM gene expression leading to an abundance of CREM τ (16). These cells and round spermatids produce large quantities of testis ACE mRNA (20). Second, it is clear that the designation of CREM α as a strictly inhibitory form of CREM is inaccurate. Loriaux and colleagues (28) have shown that CREB-CREM α heterodimers can significantly contribute to gene transcription as long as both proteins are appropriately phosphorylated. Goraya *et al.* (29) recently published transfection studies showing that CREM α stimulated the expression of a testis ACE promoter CAT construct in HepG2 cells. They concluded that the effects of CREM α depended on cell type and the exact sequence in which the CRE site is found. Thus, it is possible that within germ cells the CREM α / β isoforms may contribute to the high levels of testis ACE transcription.

We have also detected small amounts of CREB protein in the gel-shifted band of the testis ACE CRE element. It appears from transfection experiments that CREB is a weak stimulator of testis ACE promoter transcription. It is difficult to know the precise role of CREB in the testis expression of testis ACE because previous studies have demonstrated that the majority of CREB transcripts produced by male germ cells encode proteins that lack a nuclear translocation signal (30, 31). Thus, we postulate that CREB plays only a minor role in the tissue-specific expression of testis ACE.

In summary, the testis ACE promoter contains a CRE region recognized by CREM proteins. CREM τ isoforms appear to play an important role in the tissue-specific expression of testis ACE. We predict that animals lacking CREM τ isoforms would not produce testis ACE.

We wish to thank Drs. M. Brown, G. Benian, D. Hayzer, B. Schieffer, M. Marrero, and C. Esther for helpful discussions and Qing Chai for technical assistance. This work was supported by National Institutes of Health Grants DK39777, DK44280, and DK45216.

- Russell, L. D., Ettlin, R. A., Sinha Hikim, A. P. & Clegg, E. D., eds. (1990) *Histological and Histopathological Evaluation of the Testis* (Cache River, Clearwater, FL), pp. 1–36.
- Bernstein, K. E. (1992) *Semin. Nephrol.* **12**, 524–530.
- Soffer, R. L. (1981) in *Biochemical Regulation of Blood Pressure*, ed. Soffer, R. L. (Wiley, New York), pp. 123–164.
- Strittmatter, S. M. & Snyder, S. H. (1984) *Endocrinology* **115**, 2332–2341.
- Kumar, R. S., Kusari, J., Roy, S. N., Soffer, R. L. & Sen, G. C. (1989) *J. Biol. Chem.* **264**, 16754–16758.
- Ehlers, M. R., Fox, E. A., Strydom, D. J. & Riordan, J. F. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7741–7745.
- Lattion, A.-L., Soubrier, F., Allegrini, J., Hubert, C., Corvol, P. & Alhenc-Gelas, F. (1989) *FEBS Lett.* **252**, 99–104.
- Howard, T., Shai, S., Langford, K., Martin, B. & Bernstein, K. E. (1990) *Mol. Cell. Biol.* **10**, 4294–4302.
- Krege, J. H., John, S. W. M., Langenbach, L. L., Hodgins, J. B., Hagaman, J. R., Bachman, E. S., Jennette, J. C., O'Brien, D. A. & Smithies, O. (1995) *Nature (London)* **375**, 146–148.
- Langford, K., Shai, S., Howard, T., Kovac, M., Overbeek, P. & Bernstein, K. (1991) *J. Biol. Chem.* **266**, 15559–15562.
- Howard, T., Balogh, R., Overbeek, P. A. & Bernstein, K. E. (1993) *Mol. Cell. Biol.* **13**, 18–27.
- Zhou, Y., Delafontaine, P., Martin, B. M. & Bernstein, K. E. (1995) *Dev. Genet.* **16**, 201–209.
- Montminy, M. R., Gonzalez, G. A. & Yamamoto, K. K. (1990) *Trends Genet.* **13**, 84–88.
- Meyer, T. E. & Habener, J. F. (1993) *Endocr. Rev.* **14**, 269–290.
- Lee, J. S., Lalli, E., Masquillier, D., Schlotter, F., Molina, C. A., Foulkes, N. S. & Sassone-Corsi, P. (1995) in *Inducible Gene Expression*, ed. Baeuerle, P. A. (Birkhauser, Boston), Vol. 2, pp. 1–38.
- Foulkes, N. S., Schlotter, F., Pevet, P. & Sassone-Corsi, P. (1993) *Nature (London)* **362**, 264–267.
- Sun, Z., Sassone-Corsi, P. & Means, A. R. (1995) *Mol. Cell. Biol.* **15**, 561–571.
- Delmas, V., van der Hoorn, F., Mellstrom, B., Jegou, B. & Sassone-Corsi, P. (1993) *Mol. Endocrinol.* **7**, 1502–1514.
- Kistler, M. K., Sassone-Corsi, P. & Kistler, W. S. (1994) *Biol. Reprod.* **51**, 1322–1329.
- Langford, K. G., Zhou, Y., Russell, L. D., Wilcox, J. N. & Bernstein, K. E. (1993) *Biol. Reprod.* **48**, 1210–1218.
- Foulkes, N. S., Mellstrom, B., Benusiglio, E. & Sassone-Corsi, P. (1992) *Nature (London)* **355**, 80–84.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1993) *Current Protocols in Molecular Biology* (Wiley, New York), pp. 10.0.1–10.19.12.
- Luckow, B. & Schutz, G. (1987) *Nucleic Acids Res.* **13**, 5490.
- Foulkes, N. S., Borrelli, E. & Sassone-Corsi, P. (1991) *Cell* **64**, 739–749.
- Mellon, P. L., Clegg, C. H., Correll, L. A. & McKnight, S. G. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 4887–4891.
- Delmas, V., Laoide, B. M., Masquillier, D., de Groot, R. P., Foulkes, N. S. & Sassone-Corsi, P. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 4226–4230.
- Cushman, D. W. & Cheung, H. S. (1971) *Biochim. Biophys. Acta* **259**, 261–265.
- Loriaux, M. M., Brennan, R. G. & Goodman, R. H. (1994) *J. Biol. Chem.* **269**, 28839–28843.
- Goraya, T. Y., Kessler, S. P., Stanton, P., Hanson, R. W. & Sen, G. C. (1995) *J. Biol. Chem.* **270**, 19078–19085.
- Waerber, G., Meyer, T. E., LeSieur, M., Hermann, H. L., Gerard, N. & Habener, J. F. (1991) *Mol. Endocrinol.* **5**, 1418–1430.
- Ruppert, S., Cole, T. J., Boshart, M., Schmid, E. & Schütz, G. (1992) *EMBO J.* **11**, 1503–1512.