

Blocking BRE expression in Leydig cells inhibits steroidogenesis by down-regulating 3 β -hydroxysteroid dehydrogenase

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Abstract

Conversion of cholesterol to biologically active steroids is a multi-step enzymatic process. Along with some important enzymes, like cholesterol side-chain cleavage enzyme (P450_{scc}) and 3 β -hydroxysteroid dehydrogenase/isomerase (3 β -HSD), several proteins play key role in steroidogenesis. The role of steroidogenic acute regulatory (StAR) protein is well established. A novel protein, BRE, found mainly in brain, adrenals and gonads, was highly expressed in hyperplastic rat adrenals with impaired steroidogenesis, suggesting its regulation by pituitary hormones. To further elucidate its role in steroidogenic tissues, mouse Leydig tumor cells (mLTC-1) were transfected with BRE antisense probes. Morphologically the BRE antisense cells exhibited large cytoplasmic lipid droplets and failed to shrink in response to human chorionic gonadotropin. Although cAMP production, along with

StAR and P450_{scc} mRNA expression, was unaffected in BRE antisense clones, progesterone and testosterone yields were significantly decreased, while pregnenolone was increased in response to human chorionic gonadotropin stimulation or in the presence of 22(R)OH-cholesterol. Furthermore, whereas exogenous progesterone was readily converted to testosterone, pregnenolone was not, suggesting impairment of pregnenolone-to-progesterone conversion, a step metabolized by 3 β -HSD. That steroidogenesis was compromised at the 3 β -HSD step was further confirmed by the reduced expression of 3 β -HSD type I (3 β -HSDI) mRNA in BRE antisense cells compared with controls. Our results suggest that BRE influences steroidogenesis through its effects on 3 β -HSD action, probably affecting its transcription.

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Introduction

Steroid hormones are synthesized from cholesterol in the adrenal cortex and the gonads in response to pituitary tropic hormones such as adrenocorticotrophic hormone, angiotensin II, follicle-stimulating hormone, luteinizing hormone or its placental counterpart human chorionic gonadotropin (hCG) via the classical first messenger/second messenger pathway. The adrenal corticoids play roles in carbohydrate metabolism, stress management and salt balance. The sex steroids induce and maintain secondary sexual characteristics and are essential for reproduction. An additional class of steroids, neurosteroids, is synthesized in the central nervous system, where they function as neurotransmitters (Mensah-Nyagan *et al.* 1999, Compagnone & Mellon 2000).

The synthesis of steroid hormones from cholesterol is a multi-enzymatic process, in which free cholesterol is released from cytoplasmic lipid droplets or cell membranes

by cholesterol ester hydrolase and transferred to the inner mitochondrial membrane. The latter is a rate-limiting process that requires *de novo* protein synthesis (Stevens *et al.* 1993, Stocco & Clark 1996). Inside a mitochondrion, cholesterol side-chain cleavage enzyme (P450_{scc}) converts cholesterol to pregnenolone. Pregnenolone migrates to smooth endoplasmic reticulum to undergo the next enzyme reaction. A modified steroid precursor shunts between various cell organelles, undergoing a further enzyme reaction at each stop, until finally exiting a cell as an organ-specific steroid hormone (Miller 1988).

Several proteins acutely regulate steroid hormone synthesis. Among these, the role of steroidogenic acute regulatory (StAR) protein in transporting free cholesterol to the inner mitochondrial membrane has been well established (Clark *et al.* 1994, Stocco 1999, Strauss *et al.* 1999, Bose *et al.* 2002). Another candidate, sterol carrier protein 2 (SCP2), also called the non-specific lipid-transfer protein (nsLTP), maintains sterol movement within the

cell, including the mitochondrion, by affecting the utilization of peroxisome-derived cholesterol (Chanderbhan *et al.* 1982, Vahouny *et al.* 1987, Stocco & Clark 1996). However, SCP2 levels did not change rapidly in response to acute stimulation or in the presence of cycloheximide (Stocco & Clark 1996). Moreover, SCP2 transfers phospholipids and glycosphingolipids between membranes (Bloj & Zilversmit 1981, Gadella & Wirtz 1991, Seedorf *et al.* 2000), which suggests that the protein is not a specific steroid regulatory protein.

We recently identified a new human gene that was down-regulated in cells that were treated with DNA-damaging agents such as ultraviolet light, 4-nitroquinoline-1-oxide and retinoic acid (Li *et al.* 1995). The gene, termed *BRE* after brain and reproductive organs expressed, transcribes a 1.9 kb mRNA that is supposedly translated into a 383-amino-acid protein. *BRE* is expressed in multiple isoforms in human and mouse tissues (Ching *et al.* 2001), but shows no significant homology with any known gene. Upon examining 50 different human tissues, *BRE* protein and mRNA were highly expressed in the adrenal gland, the ovary, the testis and the brain (Miao *et al.* 2001). The protein was up-regulated in the adrenal glands of rats (Miao *et al.* 2001) with impaired steroidogenesis and displaying large lipid droplets (Panesar & Chan 2000), akin to lipoidal congenital adrenal hyperplasia which may arise from P450_{scc} (White *et al.* 1987) or StAR protein (Stocco 1999) deficiency. The high expression of *BRE* and its mRNA in the aforementioned tissues suggest a role for the protein in steroidogenic process.

We have studied the consequences of blocking the expression of *BRE* on steroidogenesis in response to hCG and steroid precursors in mouse Leydig tumor cells (mLTC-1). In view of the results obtained we further examined the expression of StAR, 3 β -hydroxysteroid dehydrogenase/isomerase type I (3 β -HSDI) and P450_{scc} mRNAs.

Materials and Methods

Construction of recombinant *BRE* antisense plasmids

Full-length human *BRE* cDNA (GenBank accession no. L38616) was amplified from a recombinant phage of λ gt11 as a template (Li *et al.* 1995) with the upstream primer, *BRE*-human-F1 (5'-GGA GAT CTG GGT ACT GTG GGG AAA AAC AC-3'), and the downstream primer, *BRE*-human-R2 (5'-TAG TCG ACG AAC AGC GAG GGG CAT TTA-3'), together with an extra *SalI* site introduced after the stop codon of *BRE*. The 1.5 kb PCR product of *BRE* was extracted and first inserted into a compatible plasmid pGEM-T (Promega, Madison, WI, USA). It was then subcloned in the mammalian expression vector pCDNA3 (Invitrogen, Carlsbad, CA, USA) at the *XhoI* site to produce the antisense p3 *BRE*(-) plasmid. The

complementary RNA for *BRE* was generated by cytomegalovirus promoter in the same vector.

The establishment of *BRE* antisense-transfected mLTC-1 clones

mLTC-1 cells were grown as monolayers in 100 mm culture dishes in RPMI 1640, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin. Cultures were maintained at 37 °C in 5% CO₂ atmosphere. To generate the stable *BRE* antisense-transfected cell lines, 5×10^5 mLTC-1 cells were seeded per 35 mm culture dish. After cells had reached 80% confluency, a mixture of 3 μ g p3 *BRE*(-) plasmid DNA and 10 μ l LipofectAMINE 2000 (Invitrogen) in 200 μ l Opti-MEM medium was added per culture dish. After 4 h incubation, the medium was replenished. After 2 days, cells were trypsinized, washed and transferred to a 100 mm culture dish and incubated for 24 h. Thereafter fresh medium containing 100 μ g/ml G418 (Invitrogen) was added and replaced every 4 days. 2 weeks after the transfection, G418-resistant colonies were harvested and cultured in a 96-well plate. Selected clones were amplified and further analyzed by reverse transcriptase (RT)-PCR and Western-blotting techniques. To negate the influence of G418 on results, neomycin-resistant mLTC-1 clone transfected with pCDNA3 vector alone (V-1) was similarly selected and used as a control.

BRE antibody and protein expression

The polyclonal rabbit antibody to *BRE* was commissioned from Research Genetics (Huntsville, AL, USA). The antibody was raised against a recombinant polypeptide comprising amino acid residues 40–224 of *BRE* expressed in *Escherichia coli* using the pRSET vector (Miao *et al.* 2001). The specificity of the antibody has been confirmed by immunohistochemistry and Western-analysis studies (Miao *et al.* 2001, Poon *et al.* 2004).

Generation of glutathione S-transferase (GST)-*BRE* fusion protein

GST-*BRE* fusion protein was produced by inserting full-length *BRE* into *E. coli* expression vector pGEX-2T fused to the *GST* gene. A 70 kDa GST-*BRE* protein was purified from the bacterial lysate using the MicroSpin GST Purification kit (Amersham Pharmacia, Piscataway, NJ, USA) according to the manufacturer's protocol.

The selection of *BRE* antisense-transfected mLTC-1 cells by RT-PCR and Western analysis

Transfected mLTC-1 cells (8×10^5 viable cells, >90% viability) were grown in 60 mm dishes. After 2 days, the

cells were disaggregated and total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). A 1 µg RNA sample was reverse-transcribed with oligo(dT)₁₅ primers (Promega) to obtain single-stranded cDNA. One-tenth of the RT product was used as the template for the PCR amplifications involving 30 cycles in a thermal cycler. Each cycle consisted of 1 min of denaturation at 94 °C, 1 min of annealing at 56 °C and 1 min of extension at 72 °C.

The primer sequences for antisense BRE confirmation were as follows: forward primer (T7 universal), 5'-TAA TAC GAC TCA CTA TAG GG-3'; reverse primer (BRE-human-F5), 5'-AGA TCT GAT GCC CAC ATA CCT TCT CAA G-3'. The PCR products were analyzed using 1.2% agarose gel electrophoresis and the DNA bands were viewed under ultraviolet trans-illumination. For Western blotting, 8×10^5 cells (>90% viability) grown on 60 mm culture dishes were washed three times with PBS and lysed in RIPA buffer containing protease inhibitors, 0.1 mg/ml PMSF, 30-fold dilution of aprotinin solution and 1 mM sodium orthovanadate. 25 µg of protein extracts were electrophoresed by SDS/PAGE (10% gel). The proteins were transferred from the gel to PVDF membrane (Millipore, Bedford, MA, USA) by electroblotting at 15 V for 20 min. The non-specific sites on the membrane were blocked with 5% non-fat milk overnight at 4 °C. The membrane was incubated with 1:1000 dilution of rabbit anti-BRE antibody or tubulin (internal control) for 1 h, followed by a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antibody (Promega) for another hour. The membrane was washed with Tris-buffered saline containing 0.1% Tween-20. The proteins were detected by means of the ECL reagent kit (Amersham Biosciences, Little Chalfont, Bucks, UK) with X-ray film exposures between 30 s and 5 min.

cAMP, pregnenolone, progesterone and testosterone production in response to hCG in wild-type (wt), V-1 and BRE antisense-transfected mLTC-1 cells

At 40–50% confluency, wt, V-1 and BRE antisense-transfected mLTC-1 cells were disaggregated with trypsin/EDTA (0.05% and 0.53 mM, respectively) dissolved in calcium- and magnesium-free Hank's balanced salt solution (HBSS; Sigma Chemical Co., St Louis, MO, USA). After washing once with the culture medium, approximately 66 000 cells in 1 ml growth medium were seeded per well of a 24-well plate and cultured. After 2 days, the cells were washed once with 1 ml HBSS and incubated with 200 µl Hepes/phosphate-buffered Ringer (HPBR) containing 135 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 0.83 mM Na₂HPO₄, 3.33 mM NaH₂PO₄, 5 mM Hepes, 10 mM glucose and 0.5% BSA (pH 7.4) with various concentrations of hCG for 1 h at 37 °C. Supernatants were collected and stored at –20 °C for the analysis of pregnenolone, progesterone and

testosterone. 1 ml 1.7% HCl in ethanol was added per well to extract cAMP from cells. cAMP and testosterone were assayed by the highly sensitive in-house RIA techniques described previously (Panesar *et al.* 2003). The intra- and inter-assay coefficients of variation for these assays were less than 8%. Pregnenolone was assayed by means of enzyme immunoassay according to the manufacturer's instructions (ALPCO Diagnostics, Windham, NH, USA). Progesterone was determined by means of the electrochemiluminescence immunoassay (ECLIA) on the Roche Elecsys Modular Analytics E170 immunoassay analyzer (Indianapolis, IN, USA). All experiments were performed in triplicate or quadruplicate and on two or three separate occasions.

The conversion of exogenous 22(R)OH-cholesterol, pregnenolone and progesterone to testosterone by unstimulated wt, V-1 and BRE antisense-transfected mLTC-1 cells

Approximately 66 000 cells were seeded per well in 24-well plates and cultured. After 2 days, the cells were washed once with 1 ml HBSS and incubated with 200 µl HPBR containing 10^{-4} M 22(R)OH-cholesterol (Steraloids, Newport, RI, USA), 10^{-4} M progesterone or 10^{-5} M pregnenolone (Sigma) for 1 h at 37 °C in triplicate or quadruplicate in a 5% CO₂ incubator. After the incubation, the supernatant from each well was removed and stored at –20 °C until assayed for testosterone.

The expression of 3-βHSDI, P450_{scc} and StAR mRNA in wt, V-1 and BRE antisense-transfected mLTC-1 cells

For the extraction of total RNA, 8×10^5 cells (>90% viability) were seeded on 60 mm dishes and cultured for 2 days in 37 °C CO₂ incubator. Total RNA was isolated from different mLTC-1 cell lines using the RNeasy Mini kit according to the manufacturer's instructions. RT-PCRs were performed using the Access one-step RT-PCR system (Promega). The reaction mixture contained 0.2 µg total RNA, 0.2 mM dNTP, 1 mM MgSO₄, 2 U avian myeloblastosis virus (AMV) RT, 2 U *Tfi* DNA polymerase, 1 µM forward and 1 µM reverse primers in 20 µl AMV/*Tfi* buffer. The primer sequences and their expected product sizes in bp were: mouse 3β-HSDI-forward, 5'-CTC AGT TCT TAG GCT TCA GCA ATT AC-3', and -reverse, 5'-CCA AAG GCA GGA TAT GAT TTA GGA-3' (98 bp; O'Shaughnessy *et al.* 2002); mouse L19 (internal control) forward, 5'-GAA ATC GCC AAT GCC AAC TC-3', and reverse, 5'-TCT TAG ACC TGC GAG CCT CA-3' (395 bp; Manna *et al.* 1999); mouse StAR-forward, 5'-GAC CCT GAA AGG CTC AGG AAG AAC-3', and -reverse, 5'-TAG CTG AAG ATG GAC AGA CTT GC-3' (980 bp; Manna *et al.* 1999); and mouse P450_{scc} forward,

5'-AGG ACT TTC CCT GCG CT-3', and reverse, 5'-GCA TCT CGG TAA TGT TGG-3' (950 bp; Greco & Payne 1994).

The RT-PCR thermocycler sequence for 3 β -HSDI, L19 and P450_{scc} mRNAs was 48 °C for 45 min, 94 °C for 2 min and 22 cycles of 94 °C for 30 s, 56 °C for 1 min and 68 °C for 2 min. For StAR mRNA the sequence was 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min for 30 cycles. Under these conditions, the amplifications occurred in linear exponential phase. Negative controls were performed without the RNA template. 15 μ l RT-PCR products were loaded onto a 2% agarose gel with 0.5 μ g/ml ethidium bromide and electrophoresed. The DNA bands were visualized under ultraviolet transillumination, and the fluorescent signals were determined using the Quantity One Software (Bio-Rad, Hercules, CA, USA). The amounts of RT-PCR products of the three genes were determined as ratios of optical densities relative to the concurrently run L19 gene (internal control). All RT-PCRs were performed on two or more occasions.

Statistical analyses

The interactions between different hCG concentrations and mLTC-1 clones were tested by ANOVA with Scheffe's *post hoc* test using Statistica software (Statsoft, Tulsa, OK, USA). For steroid hormone production from exogenous steroid precursors or stimulation with a single concentration of hCG in different clones, the data were compared by Student's *t* test. In the latter case, and in view of the transfection procedure, the data were compared with the control V-1 clone. *P* values of less than 0.05 were regarded as significant.

Results

The BRE antisense-transfected mLTC-1 clones and the Western analyses of BRE in cell lysates

Figure 1A shows the reaction of BRE antiserum with cell lysates from wt mLTC-1, which yielded a major 52 kDa band and two minor 43 and 32 kDa bands (Fig. 1A, lane 1). The staining of 52 kDa band was markedly decreased following the reaction with anti-BRE antiserum that had been pre-treated with a purified recombinant GST-BRE protein (Fig. 1A, lane 2).

32 p3 BRE(-) and eight pCDNA3-transfected mLTC-1 clones were selected and amplified for screening. Figure 1B shows the positive RT-PCR bands amplified from the RNA extracted from three p3 BRE(-)-transfected clones – AS-A6, AS-E4 and AS-F2 – for the forward T7 primer (within the plasmid of pCDNA3) and the reverse BRE-human-F5 primer (within the cDNA sequence of BRE). These results indicated that

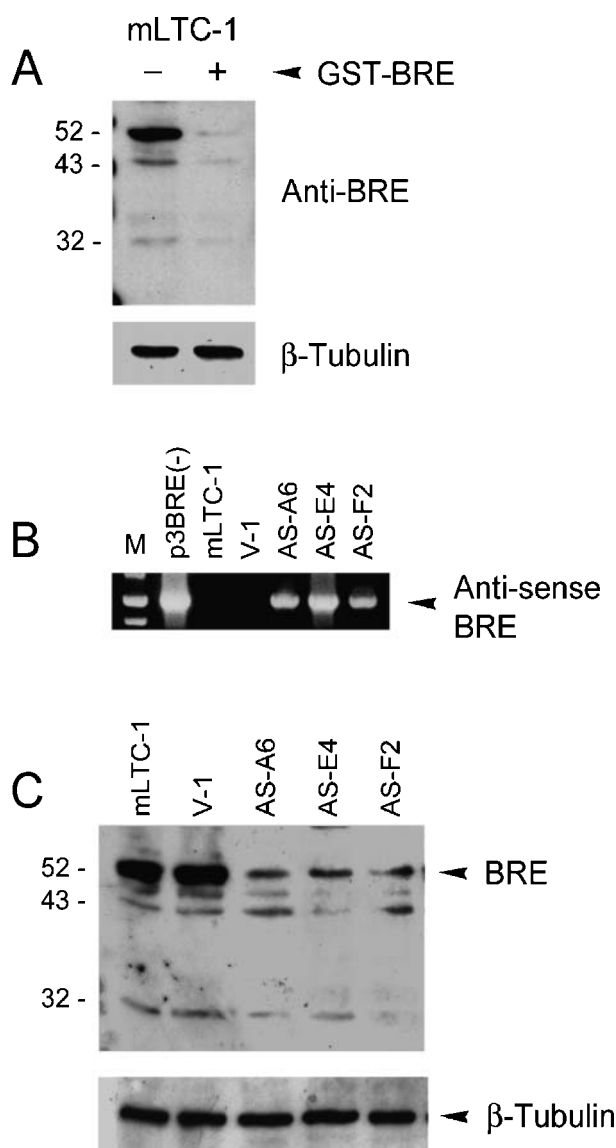


Figure 1 (A) Western blot of extracts from mLTC-1 before (–) and after (+) treatment with purified GST-BRE recombinant protein (primary antibody-BRE). (B) RT-PCR of RNA extracted from wt mLTC-1 (mLTC-1), mLTC-1 transfected with pCDNA3 (clone V-1) and mLTC-1 transfected with p3 BRE(-) (clones AS-A6, AS-E4 and AS-F2) using primers T7 and BRE-human-F5. Positive amplification bands from AS-A6, AS-E4 and AS-F2 indicate the generation of antisense BRE transcripts. Plasmid p3 BRE(-) was used as a positive control. M, molecular mass markers. (C) Western blot for BRE protein in cells extracts of wt mLTC-1, clone V-1, and the AS-A6, AS-E4 and AS-F2 clones. Note the marked reduction of BRE proteins in the antisense clones. β -Tubulin served as a control for sample loading.

clones AS-A6, AS-E4 and AS-F2 expressed anti-sense BRE mRNA. No amplification band was observed for RNA extracted from pCDNA3-transfected V-1 clone or wt mLTC-1 (Fig. 1B). BRE protein level in p3

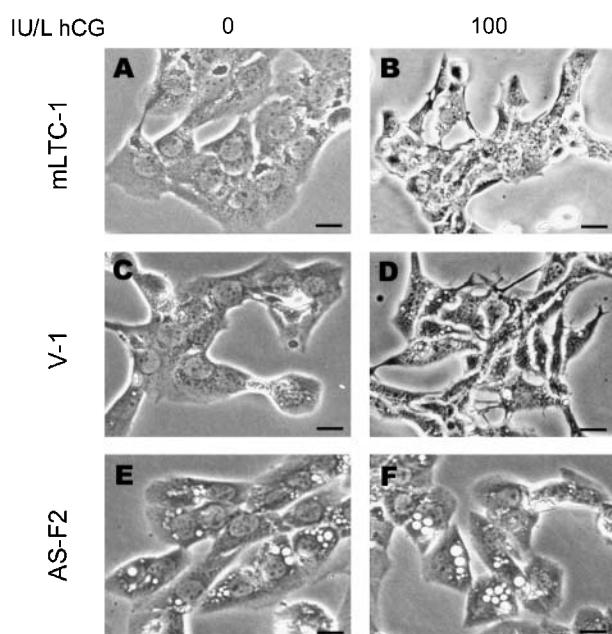


Figure 2 Cell morphology of wt mLTC-1 (upper row), pCDNA3-transfected V-1 (middle row) and the BRE antisense AS-F2 clone (lower row) before (A, C and E) and after (B, D and F) hCG stimulation. Pictures were taken under phase-contrast microscopy. The experiments were repeated three times, each with similar results. Scale bars, 15 μ m.

BRE(-)-transfected clones was approximately one-eighth of the wt and V-1 mLTC-1 cells, determined densitometrically by Quantity One software (Fig. 1C).

Cell morphology of wt, V-1 and BRE antisense-transfected mLTC-1 cells before and after hCG stimulation

Compared with the wt (Fig. 2A) or V-1 (Fig. 2C) cells, almost 90% of BRE-antisense mLTC-1 cells had large lipid droplets in the cytoplasm (Fig. 2E; only AS-F2 cells shown), while 5% of the control cells showed these lipid droplets. The number of the lipid droplets also increased with culture time (results not shown). After stimulation with 100 IU/l hCG for 1 h, all the wt and V-1 cells appeared shrunken (Fig. 2B and D, respectively), whereas none of AS-F2 cells showed either a change in morphology or lipid droplets (Fig. 2F). Similar results were also obtained with AS-E4 and AS-A6 clones (results not shown).

The production of cAMP, testosterone, pregnenolone and progesterone by wt, V-1 and BRE antisense-transfected mLTC-1 cells in response to hCG

Figure 3A and B show the cAMP and testosterone productions in response to increasing concentrations of hCG in the five mLTC-1 clones. cAMP production

within and between the five clones was not significantly different under basal and 1 IU/l hCG stimulation. The differences became significant ($P < 0.0000$ or better; ANOVA with Scheffe's *post hoc* test) between 1 and 10 IU/l hCG for all five clones, and thereafter (at 10 and 100 IU/l hCG) were again insignificant, whether within or between the five clones. Testosterone production under basal conditions was not significantly different between the five clones. However, after stimulation with hCG, both wt and V-1 cells showed a significant increase in testosterone at 1, 10 and 100 IU/l hCG when compared individually with the three antisense clones. Testosterone production between different antisense clones was not significantly different. Table 1 shows ANOVA with Scheffe's *post hoc* probabilities for cAMP and testosterone production between the five cell lines relative to all concentrations of hCG. Over the whole range of hCG, cAMP production was slightly decreased in all three BRE antisense clones, but, with the exception of AS-E4, the decrease was not significantly different from wt and V-1 cells (Fig. 3A and Table 1, upper diagonal matrix). On the other hand, the production of testosterone in the three BRE antisense clones was significantly reduced compared with wt and V-1 clones (Fig. 3B and Table 1, lower diagonal matrix).

Pregnenolone production in response to 100 IU/l hCG was significantly lower in the three antisense clones compared with V-1 (Fig. 3C; $P = 0.0000$, 0.0000 and 0.0003 respectively for AS-A6, AS-E4 and AS-F2; Student's *t*-test). The yields compared with V-1 cells were decreased by 80, 70 and 40% in AS-A6, AS-E4 and AS-F2, respectively. On the other hand, pregnenolone production in response to 100 IU/l hCG was significantly increased in all antisense clones compared with V-1 cells, despite pregnenolone output in AS-E4 and AS-F2 not being as high as in AS-A6 (Fig. 3D; $P = 0.0002$, 0.0000 and 0.0000 respectively for AS-A6, AS-E4 and AS-F2; Student's *t*-test).

Testosterone production from exogenous 22(R)OH-cholesterol, pregnenolone and progesterone in wt, V-1 and BRE antisense-transfected mLTC-1 cells

Figure 4A and B respectively show the production of testosterone and progesterone from 22(R)OH-cholesterol in various mLTC-1 clones. In all cases the amount of testosterone or progesterone produced in BRE antisense clones were significantly lower than the V-1 cells (Fig. 4A; $P = 0.0016$, 0.0019 and 0.0022 respectively for AS-A6, AS-E4 and AS-F2; Fig. 4B, $P = 0.0000$, 0.0004 and 0.002 respectively for AS-A6, AS-E4 and AS-F2; all Student's *t*-test).

Incubations with exogenous pregnenolone and progesterone induced an increase in testosterone production in both wt and V-1 mLTC-1 cells (Fig. 4C and 4D). However, testosterone yields from exogenous pregnenolone were significantly reduced in the BRE

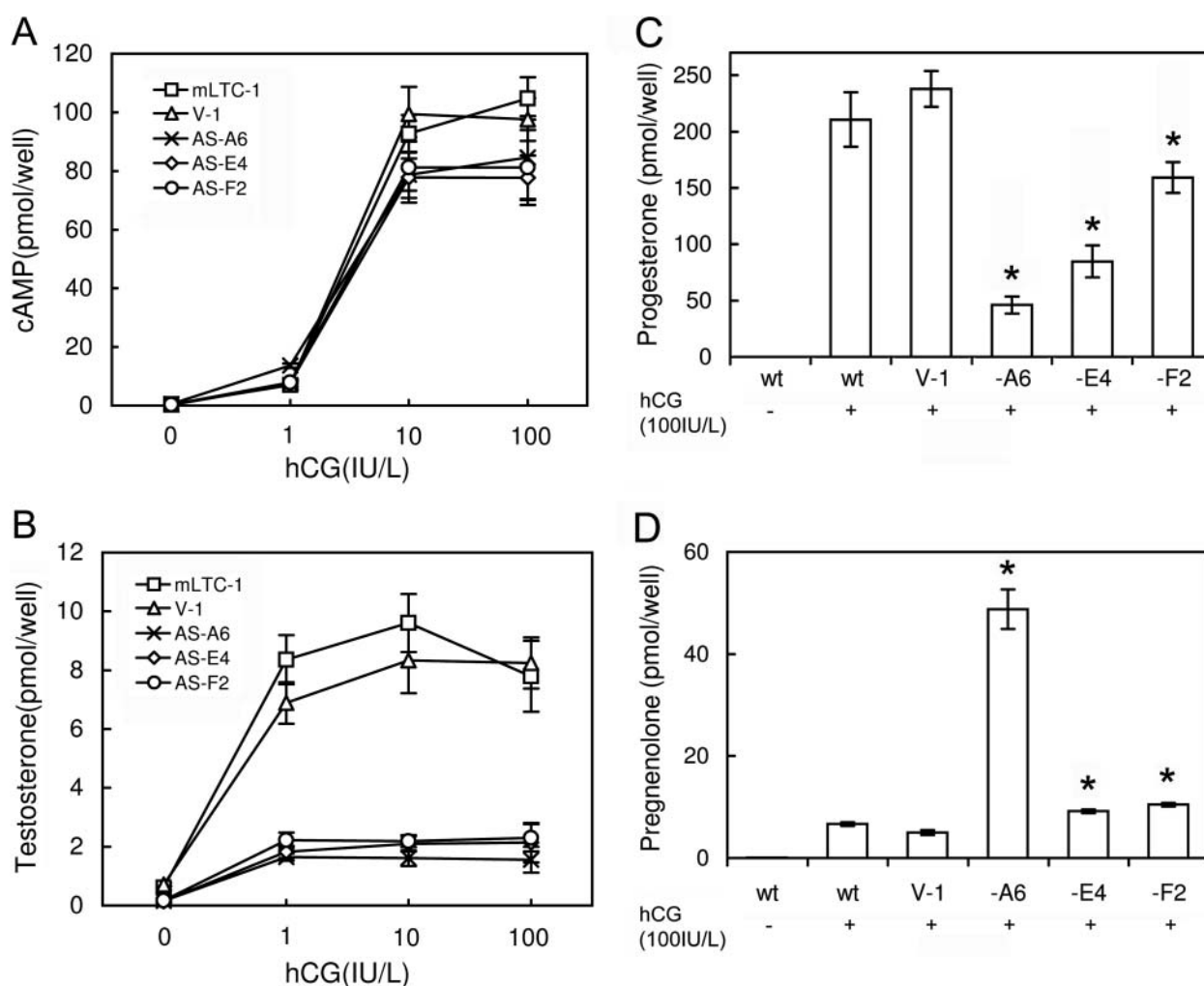


Figure 3 (A, B) cAMP and testosterone production in wt mLTC-1 cells (\square), vector-transfected mLTC-1 (V-1; \triangle), and BRE antisense clones AS-A6 (\times), AS-E4 (\diamond) and AS-F2 (\circ) in response to different concentrations of hCG (plotted logarithmically). Values are means \pm s.d. from four independent experiments. The statistical analysis with ANOVA and Scheffé's *post hoc* probabilities as function of hCG are presented in Table 1. (C, D) Progesterone and pregnenolone productions in wt, V-1 and BRE antisense clones in response to 100 IU/l hCG stimulation for 1 h. Values are means \pm s.d. of three or four independent experiments. Asterisks in show significant differences between each antisense clone versus V-1 (C, $P=0.0000$, 0.0000 and 0.0003 ; D, $P=0.0002$, 0.0000 and 0.0000 , respectively for AS-A6, AS-E4 and AS-F2; Student's *t*-test).

antisense clones compared with V-1 cells (Fig. 4C; $P=0.0000$, 0.0000 and 0.0003 respectively for AS-A6, AS-E4 and AS-F2; Student's *t*-test). Testosterone yields from exogenous progesterone were similar to V-1 cells (Fig. 4D; $P=0.3898$, 0.4427 and 0.3216 respectively for AS-A6, AS-E4 and AS-F2; Student's *t*-test).

The expression of 3 β -HSDI, P450_{scc} and StAR mRNA in the wt, V-1 and BRE antisense mLTC-1 clones without or with hCG stimulation

Figure 5 shows the electrophoretograms and the densitometric expression ratios of RT-PCR fragments of

mouse StAR (Fig. 5A), 3 β -HSDI (Fig. 5B) and P450_{scc} (Fig. 5C) relative to L19 in different mLTC-1 clones. The expression of StAR mRNA was unaffected by BRE down-regulation and increased 3.2-fold in response to hCG stimulation in all clones. The expression of 3 β -HSDI showed a variable degree of down-regulation in the three BRE antisense clones, with AS-A6 showing the highest repression. After 100 IU/l hCG stimulation, the expression of 3 β -HSDI mRNA in AS-A6, AS-E4 and AS-F2 was 38, 80 and 87%, respectively, compared with the wt mLTC-1 cells. The expression of P450_{scc} mRNA showed no variation in any of the clones without or with hCG stimulation.

Table 1 ANOVA with Scheffe's *post hoc* probabilities for cAMP (upper diagonal matrix) and testosterone (lower diagonal matrix) production between wt mLTC-1 cells, V-1 and three BRE-transfected antisense mLTC-1 clones over the whole range of hCG concentrations (0–100 IU/l)

Cell type	<i>P</i> value				
	mLTC-1	V-1	AS-A6	AS-E4	AS-F2
mLTC-1	—	0.999999	0.189148	0.012014*	0.055868
V-1	0.285063	—	0.206036	0.013636*	0.062269
AS-A6	0.000000*	0.000000*	—	0.792542	0.981581
AS-E4	0.000000*	0.000000*	0.796511	—	0.978384
AS-F2	0.000000*	0.000000*	0.417570	0.971681	—

*Significant *P* value.

Discussion

Although BRE shows no significant homology with any known gene, there is 89% conservation in the coding region between mouse (GenBank accession no. AF440752) and human (L38616) BRE cDNAs, and 99% similarity in mouse (AAL40809.1) and human (AAA64231.1) protein structures based on an NCBI Blast search. These similarities permit the use of human BRE cDNA for blocking mouse BRE expression, and the antiserum raised against human protein for detecting rodent BRE. Our previous results support this point of view, where *in situ* hybridization and immunohistochemical staining were successfully applied in the examination of BRE mRNA and protein in rat tissues (Miao *et al.* 2001). A 1.9 kb BRE mRNA should decode a 383-amino-acid protein with a predicted molecular mass of 43 kDa. However, the Western analysis of mLTC-1 cells showed a weak reaction with the 43 kDa protein. The main band detected corresponded to a 52 kDa protein, which was significantly decreased in all three BRE antisense clones established. The increased molecular mass may thus be due to post-translational modifications of the protein, such as glycosylation, which needs to be verified.

Because of its strong presence in steroidogenic tissues, the three BRE antisense-transfected mLTC-1 clones, AS-A6, AS-E4 and AS-F2, were established to determine if BRE had a direct role in steroidogenesis. Down-regulation of BRE yielded interesting results with regard to steroid hormone production. All three BRE antisense mLTC-1 clones showed a significant 80% decrease in testosterone production, but an insignificant 20% or lower decrease in cAMP production in response to hCG. This suggests that the receptor-coupled activation of G_s heterotrimer complex and adenylate cyclase (Farndale *et al.* 1992) were relatively unaffected by the BRE down-regulation. Although wt mLTC-1 cells produce relatively more progesterone than testosterone (Panesar *et al.* 2003), the BRE antisense clones displayed a variable decrease in progesterone production in response to hCG, amounting

to 80, 70 and 40% in AS-A6, AS-E4 and AS-F2, respectively, compared with the controls. Conversely, pregnenolone production showed a reciprocal increase in the BRE antisense clones, with the AS-A6 clone showing the biggest increase. These results suggest that the inhibition of steroidogenesis in the BRE antisense clones occurred upstream of progesterone synthesis but downstream of pregnenolone production. The incubation of BRE antisense clones with 22(R)OH-cholesterol, which freely diffuses into mitochondria without StAR and is acted upon by P450_{scc} and 3 β -hydroxysteroid dehydrogenase/isomerase (3 β -HSD), or with pregnenolone, a substrate for 3 β -HSD only, resulted in significantly lower progesterone (and testosterone) yields compared with control, whereas progesterone was readily metabolized to testosterone in all mLTC-1 clones. These data further indicate that the blocking of steroidogenesis involved the conversion of pregnenolone to progesterone, a step metabolized by 3 β -HSD. Thus, the reduced steroid hormone synthesis in BRE antisense cells is suggested to be associated with the action of 3 β -HSD.

That 3 β -HSDI activity was compromised was further confirmed by the markedly decreased expression of 3 β -HSDI mRNA in the BRE antisense clones, with the AS-A6 clone showing the greatest decrease. The 3 β -HSDI enzyme complex plays a crucial role in the conversion of Δ^5 -3 β -hydroxysteroids to Δ^4 -3-oxosteroids, which is an essential step in the production of all active steroid hormones (Payne & Hales 2004). Unlike the StAR mRNA which increased in response to hCG in all clones, the expression of 3 β -HSDI mRNA could not be up-regulated by the gonadotropin in the BRE antisense clones. Therefore it seems that BRE is involved in the expression of 3 β -HSDI enzyme. Low levels of gonadotropins up-regulate 3 β -HSDI, while high levels of gonadotropins down-regulate it through de-sensitization of the luteinizing hormone/hCG receptor (Tang *et al.* 1998). Steroids such as testosterone can suppress the expression of the enzyme at nuclear level. A transcription factor termed steroidogenic factor-1 (SF-1) is required for the expression of various P450 steroidogenic enzymes, including 3 β -HSDI; there are three potential SF-1 consensus

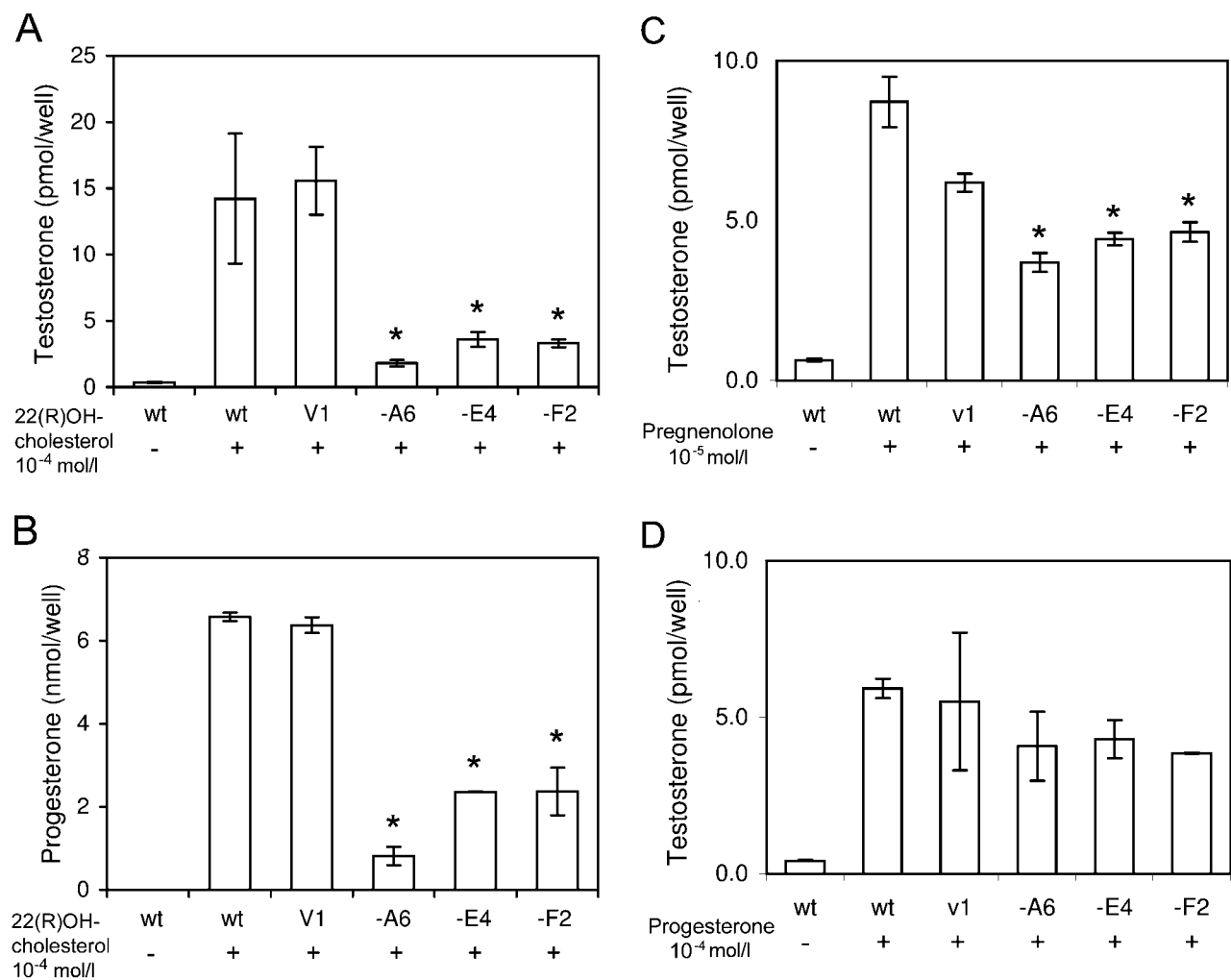


Figure 4 (A, B) Testosterone and progesterone productions in wt, V-1 and BRE antisense-transfected clones (AS-A6, AS-E4 and AS-F2) after 1 h incubation with 10^{-4} M 22(R)OH-cholesterol. Asterisks show significant differences between each antisense clone versus V-1 (A, $P=0.0016$, 0.0019 and 0.0022 ; B, $P=0.0000$, 0.0004 and 0.002 , respectively for AS-A6, AS-E4 and AS-F2; Student's *t*-test). (C, D) Testosterone production in wt, V-1 and BRE antisense clones after 1 h incubation with 10^{-5} M pregnenolone (C) or 10^{-4} M progesterone (D). Asterisks in (C) show significant differences between each antisense clone versus V-1 ($P=0.0000$, 0.0000 and 0.0003 respectively for AS-A6, AS-E4 and AS-F2; Student's *t*-test). The testosterone production between each antisense clone and V-1 showed no difference (D, $P=0.3898$, 0.4427 and 0.3216 respectively). Values are means \pm s.d. from four independent experiments.

binding sites in the proximal promoter of the 3β -HSDI gene (Payne & Hales 2004). Without referring to SF-1, Tang *et al.* (1998) mentioned an unidentified transcription factor that may be involved in the expression of 3β -HSDI, which may be BRE. There is no homology in DNA or protein structures of SF-1 and BRE. The fact that pregnenolone production was unaffected in BRE antisense clones suggests that P450_{scc} was unaffected by the protein's down-regulation. This was further confirmed by the intactness of RT-PCR results for P450_{scc} mRNA in all clones.

Computer analysis of the BRE amino acid sequence indicates that the polypeptide, although lacking the phos-

phorylation sites for protein kinase A, has six putative sites for protein kinase C and three for CKII (J Miao, unpublished observations). Protein kinase C regulates basal steroidogenesis primarily by determining the steady-state levels of steroid hydroxylase gene(s) expression (Reyland 1993, Reyland *et al.* 1998). BRE may thus be involved in protein kinase C signaling pathway, which in turn regulates the expression of 3β -HSD (Simard *et al.* 2005). Interestingly, StAR has an N-terminal mitochondrial pre-sequence (Clark *et al.* 1994), which helps to target the protein to mitochondrial membranes (Kiebler *et al.* 1993, Pfanner 2000). A similar structure also occurs in the N-terminus of BRE and has been confirmed by using

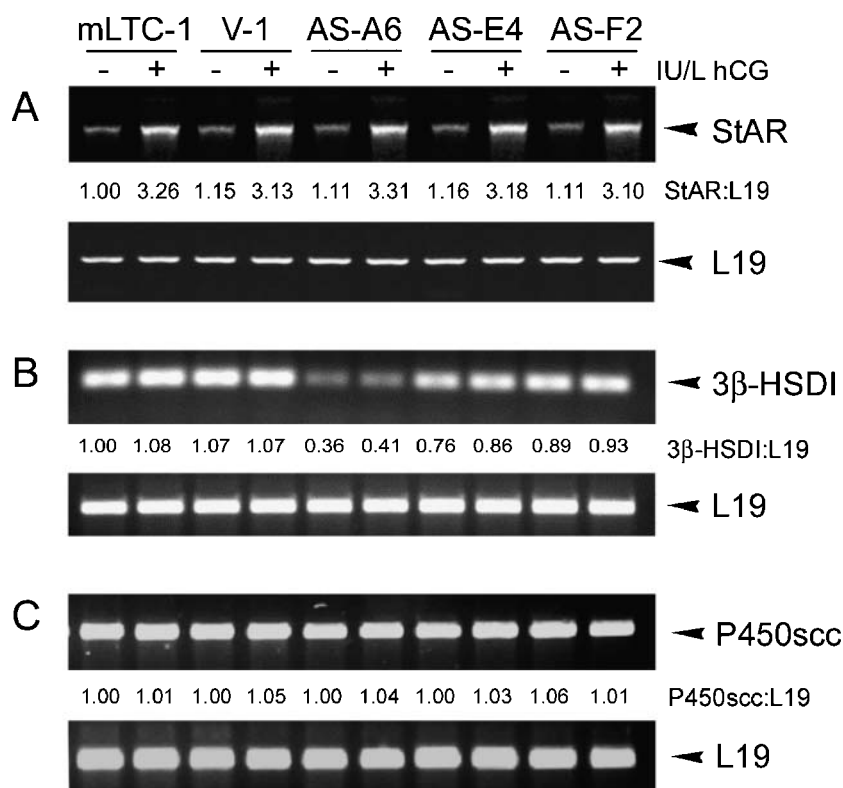


Figure 5 Concurrent electrophoretogram pairs for StAR (A), 3β-HSDI (B), P450scc (C) and L19 (internal control) mRNA RT-PCR products in wt, V-1 and BRE antisense clones (AS-A6, AS-E4 and AS-F2) without or with 1 h stimulation with 100 IU/l hCG. The data between each pair of electrophoretograms represent typical ratios of optical densities for StAR, 3β-HSDI or P450scc PCR bands relative to L19 in wt mLTC-1 cells.

green fluorescent protein as an expression marker (J Miao, unpublished observations). BRE has been suggested to be transported to endoplasmic reticulum (Ching *et al.* 2003). As 3β-HSD is primarily expressed in mitochondria and endoplasmic reticulum (Ishimura & Fujita 1997, Pelletier *et al.* 2001), it is possible that a direct interaction between BRE and 3β-HSD may occur in these sites. Whether the regulation of the enzyme by BRE is at nuclear or endoplasmic reticulum level needs further work.

The unstimulated BRE antisense mLTC-1 cells harbored large lipid droplets. Lipid droplets are normally due to the accumulation of cholesterol or its esters (Szabo *et al.* 1996, de Almeida *et al.* 1998), often seen in lipoidal congenital adrenal hyperplasia due to either StAR protein (Miller 1997) or P450scc (White *et al.* 1987) deficiency. However, all antisense clones displayed a similar expression of P450scc and StAR mRNA, without or with hCG stimulation. Although the primary action of BRE down-regulation was the impaired expression of 3β-HSDI and accumulation of pregnenolone, it is not inconceivable that this action tails back to a point where cholesterol and its esters start to accumulate and give rise to lipid droplets.

The BRE antisense clones also failed to show the morphological shrinking as seen in wt and V-1 mLTC-1 cells in response to hCG. The cytoskeleton in Leydig (and adrenocortical) cells changes in response to stimulation with hCG or cAMP and is linked to mobilization of cellular cholesterol, including its transport to a mitochondrion (Bilinska *et al.* 1999, Whitehouse *et al.* 2002). Therefore it seems BRE may also have an impact on the mobilization of intracellular cholesterol.

In conclusion, BRE is obviously involved in steroidogenesis. The down-regulation of BRE in mLTC-1 cells has shown that the protein is somehow involved in the action of 3β-HSDI, which may be at transcription level.

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