Second messenger up-regulation of androgen receptor gene transcription is absent in androgen insensitive human prostatic carcinoma cell lines, PC-3 and DU-145

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Abstract A theoretical pathway of transcriptional regulation of the androgen receptor (AR) gene is via a cAMP response element (CRE) present in its promoter region (−508 to −501). After 20 h of stimulation with 8-bromo-cAMP, AR mRNA was upregulated in LNCaP but not in either PC-3 or DU-145 cell lines. We have demonstrated that the level of CRE binding protein (CREB) was the same in all cell lines and that the putative AR-CRE forms specific and competable protein interactions with CREB. The ability to regulate AR gene transcription via the second messenger pathway is lost in the PC-3 and DU-145 cell lines. This may be an important primary mechanism of androgen insensitivity in prostate cancer.

Key words: Androgen receptor; Electromobility shift assay; Prostate cancer

1 Introduction

Prostate cancer is the most common malignancy and the second commonest cause of death from cancer in men [1]. The growth and differentiation of the normal prostate gland is dependent on androgen stimulation. It is observed that a though 70–80% of prostate cancers are initially responsive to androgen deprivation therapies, progression to an androgen-insensitive state occurs and the tumour becomes refractory to treatment [2]. The mechanism underlying this switch from androgen sensitivity (AS) to insensitivity (AI) is poorly understood. Although androgens are regulated via the AR most prostatic carcinomas even in the hormone insensitive stage contain a structurally intact AR with retained androgen binding capacity [3].

Potential mechanisms postulated for the change from AS to AI involve mutations in the AR coding region [4], amplification of th AR gene [5], and mutations in the AR gene promoter, although the latter has not been explored. A point mutation in the hormone binding domain (codon 877, exon 8) has been associated with metastatic prostate cancer [6]. However, such genetic alterations are rare and inconsistent and would not explain the observed frequency of the emergence of AI in human tumours.

The aim of our study was to stimulate AR gene transcription in androgen sensitive and insensitive prostatic carcinoma cells using a stable cAMP analogue, 8-bromo-cAMP and to determine whether the putative CRE located in the AR gene promoter (−508 to −501) functioned as a known CRE.

Our results show that the AR/CRE forms specific and competable protein interactions with cAMP response element binding protein (CREB) as compared with a known CRE sequence from adenovirus E4 promoter. In AR positive cells (LNCaP), AR mRNA is upregulated in the presence of 5 mM 8-bromo-cAMP but this induction is lost in the AR negative cell lines DU-145 and PC-3. The protein specific interactions of the AR/CRE sequence in the three prostatic cell lines were demonstrated by an electromobility shift assay (EMSA). Our results demonstrate that levels of CREB specific AR/CRE interactions in all three cell lines are similar. Furthermore, the lack of AR mRNA induction in DU-145 and PC-3 with 8-bromo-cAMP does not occur at the level of the CREB transcription factor. This may be an important primary mechanism of androgen insensitivity in prostate cancer.

2. Materials and methods

All chemicals were of the highest purity and purchased from Sigma chemicals unless otherwise stated.

2.1. Cell culture

LNCaP, PC-3 and DU-145 cell lines were routinely cultured in Dulbecco's modified Eagles medium without phenol red (DMEMwoPR) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL). Cells were plated at a concentration of 10^5 cells per 25 cm^2 tissue culture flask. After 24 h the culture medium was replaced with either fresh medium or with DMEMwoPR supplemented with 10% charcoal stripped FBS (CSFBS) and cultured for a further 48 h (to 80% confluence). The cells were then either stimulated with 8-bromo-cAMP (5 mM) or maintained under the same culture conditions for a further 20 h.

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was extracted by the RNAzol B method (Biogenesis Ltd, UK). Briefly, cells were washed twice with phosphate-buffered saline (PBS) pH 7.2–7.4 and lysed directly in the flask with 2 ml of RNAzol B. Chloroform was added to supernatant (1/10 of the volume) and the mixture vortexed for 15 s followed by centrifugation at 13 000 × *g* for 15 min at 4°C (MSE MicroCentur). The aqueous phase was removed and RNA precipitated with an equal volume of isopropanol and centrifuged for a further 15 min at the same temperature and speed. The resultant RNA pellet was washed with 75% ethanol, dried at 65°C in a heating block for 5 min and then resuspended in diethyl pyrocarbonate (DEPC)-treated water. Total RNA (2 μg) was denatured at 70°C for 10 min and chilled on ice for 2 min. cDNA was synthesised from 2 μg of denatured RNA in 50 mM Tris-Cl pH 8.3, 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$ (Gibco BRL), 40 U RNAsin (ribonuclease inhibitor, Promega), 500 μM dNTPs (Promega), 1 ml oligo$(dT)_{12-18}$ (Gibco BRL) and 50 U of StrataScriptase (Statagene)

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Table 1
Double-stranded DNA sequences used for electromobility shift assay

E4/CRE	5′-T T C C G T T A C G T C A T T T T T T A G -3′ 3′-G G C A A T G C A G T A A A A A A T C T T -5′
AR/CRE	5′-G A T C T C C C T A T G A C G G A A T C T A A G -3′ 3′-A G G G A T A C T G C C T T A G A T T C C T A G -5′

Underlined sequence represents CREB specific region.

in a final volume of 20 μl. This mixture was incubated at 37°C for 1 h and the resultant RNA/cDNA was stored at −20°C until use.

PCR for both AR and glyceraldehyde phosphate dehydrogenase (GAPDH) consisted of 100 ng cDNA, 10 mM Tris-Cl (pH 9), 50 mM KCl, 0.01% gelatin w/v, Triton X-100 (Promega), 5 μM of each primer either AR specific [7] (forward primer 5′-AGCTACTCCGGACCTTACG-3′ and reverse primer 5′-AGGTGCCATGGGAGGGTTAG-3′) or GAPDH specific [8] (forward primer 5′-GCCACATCGCTCAGACACCA-3′ and reverse primer 5′-GATGACCCTTTTGGCTCCCC-3′), 200 μM dNTPs (Promega), 1.5 mM $MgCl_2$ and 1.5 U Taq polymerase (Promega) in a final volume of 25 μl. Thermal cycling parameters for AR were 94°C for 1 min, 60°C for 1 min, 72°C for 3 min with a final extension of 72°C for 10 min, for, 20, 25, 30 or 35 cycles, and GAPDH were 94°C for 1 min, 65°C for 1 min and 72°C for 3 min for 15, 20, 25 or 30 cycles in a Techne-PHC3 thermal cycler. PCR products were separated on either a 1% or 1.5% TAE-agarose gel and visualised with ethidium bromide (0.5 mg/ml). Bands migrating at the predicted size of AR (1.4 kb) or GAPDH (483 bp) were gel purified using the Wizard PCR Preps (Promega) and sequenced with the fmol Sequencing System (Promega).

2.3. Nuclear extracts

Cells were harvested and washed twice in PBS then resuspended in 300 ml of lysis buffer (20 mM Tris-Cl pH 8, 20 mM NaCl, 0.5% Nonidet NP-40 (v/v), 20% sucrose (v/v), 2 mM DTT and 0.5 mM PMSF) and incubated on ice for 10 min then centrifuged at 13000×*g* for 1 min at 4°C. The resultant nuclear pellet was resuspended in 100 ml extraction buffer (20 mM Hepes pH 7.9, 0.45 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol (vol/vol), 2 mM DTT and 0.5 mM PMSF) and left on ice for 30 min followed by centrifugation at 13000×*g* for 1 min at 4°C. The final supernatant was aliquoted and stored at −70°C. The protein concentration was determined using the BioRad protein assay kit with bovine serum albumin as a standard.

2.4. Electromobility shift assay (EMSA)

Nuclear extracts (1–2 μg) were incubated for 2 h at 4°C in the presence or absence of an anti-CREB antibody followed by a further 20 min at room temperature with either 0.1 ng of ^{32}P-end-labelled adenovirus ATF-2 sequence [9] (Table 1) or 17.5 pM ^{32}P-end-labelled AR/CRE (Table 1) dsDNA, in a gel retardation assay buffer (25 mM Hepes pH 7.9, 0.15 M NaCl, 1 mM EDTA, 2 mM DTT, 10% glycerol (v/v), 1 μg of poly(dI·dC), 350 pM unrelated 30 bp dsDNA) with or without unlabelled specific probe (E4/CRE or AR/CRE). Products were separated on a 4% acrylamide gel (80:1 acrylamide:bisacrylamide, 0.5×TBE, 2.5% glyercol v/v) in 0.5×TBE (10×TBE: 890 mM Tris, 890 mM boric acid, 20 mM EDTA pH 8) transferred to Whatman 3MM paper, dried and exposed at −70°C overnight to Kodak X-OMAT film.

3. Results

The presence of AR transcription in LNCaP was demonstrated by an appropriate 1.4 kb PCR product and verification of specificity was achieved by sequence analysis by the fmol Sequencing System (Promega, UK). Standardisation of the semiquantitative PCR determination of AR and GAPDH mRNA expression was determined by repeating PCR thermal cycling for 20, 25, 30 and 35 cycles (for AR) or 15, 20, 25 and 30 cycles (for GAPDH). Linearity for AR PCR was achieved at 25 cycles (Fig. 1) and for GAPDH after 15 cycles (data not shown). Fig. 1 represents the results of AR PCR amplification of samples 1–4 (with FBS), 5–8 (with CSFBS) and 9–12 (CSFBS and 5 mM 8-bromo-cAMP) for 20 (1,5,9), 25 (2,6,10), 30 (3,7,11) and 35 (4,8,12) cycles. It is clear that the expression of AR mRNA is reduced in the presence of FBS (1–4) as compared to those cultured with CSFBS (5–8) or stimulated with 8-bromo-cAMP (9–12). GAPDH expression was the same under all experimental conditions.

To examine the ability of the putative AR/CRE to form specific DNA-protein complexes, an electromobility shift assay was performed using nuclear extracts from Hela cells, CREB protein produced from rabbit reticulolysate and a known CRE from adenovirus (Fig. 2). In the absence of CREB specific antibody (lanes 2,6) a single major band was observed with both the E4 and AR/CRE sequence (labelled B) both of which were abolished by a 20-fold excess of unlabelled E4 or AR/CRE (lanes 3 and 7, respectively). In the presence of a CREB specific antibody (Fig. 2, lanes 1,4,8) two specific bands were observed (A,C). Band A was competed out with either a 20-fold excess of unlabelled E4 (lane 5) or AR/CRE (lane 9) whereas band C remained unchanged during the same competition. Using CREB protein produced in a rabbit reticulolysate, it is clear that band A represents CREB specific-DNA interactions (Fig. 2, lane 1). This experiment demon-

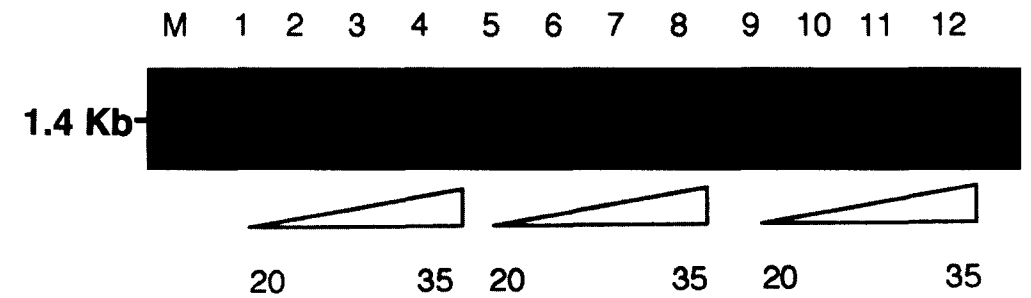

Fig. 1. Expression of AR mRNA in LNCaP cells. PCR was performed on total RNA as described in the text and the products were resolved on a 1% TAE agarose gel. Levels of AR mRNA in the presence of: (lanes 1–4) FBS, (lanes 5–8) SCFBS, (lanes 9–12) SCFBS+5 mM 8-bromo-cAMP, and (lane M) 100 bp ladder. Numbers 20–35 refer to PCR cycle number.

strates that the AR/CRE forms specific competable DNA interactions with CREB which are comparable to a known CRE [9].

EMSA was used to identify the protein specific interaction of this AR/CRE sequence in these prostatic cell lines. Lack of second messenger stimulation in PC-3 and DU-145 may be due to the inability of the cells to form AR/CRE-CREB complexes. In Fig. 3 CREB specific interactions (band A) with Hela cell extracts (lane 1) are compared with those from the LNCaP cell line in the absence of CREB specific antibody (lanes 2) and in the presence of CREB specific antibody (lane 3–5). CREB-AR/CRE was competed out with 20-(lane 4) and 100-fold (lane 5) excess of unlabelled AR/CRE. The presence of this protein-AR/CRE interaction was identified in LNCaP (Fig. 4), DU-145 and PC-3. The levels of these protein-DNA interactions in LNCaP cells are similar under different experimental culture conditions of FBS (lanes 2,3), SCFBS (lanes 4,5) and SCFBS with 5 mM 8-bromo-cAMP (lanes 6,7). The levels of CREB specific interactions in both DU-145 and PC-3 (data not shown) were similar in all experimental conditions studied.

4. Discussion

To understand the mechanism by which androgen insensitivity occurs in prostate cancer, we have analysed the regulation of the human AR transcription in both androgen sensitive (LNCaP) and insensitive (DU-145 and PC-3) cell lines.

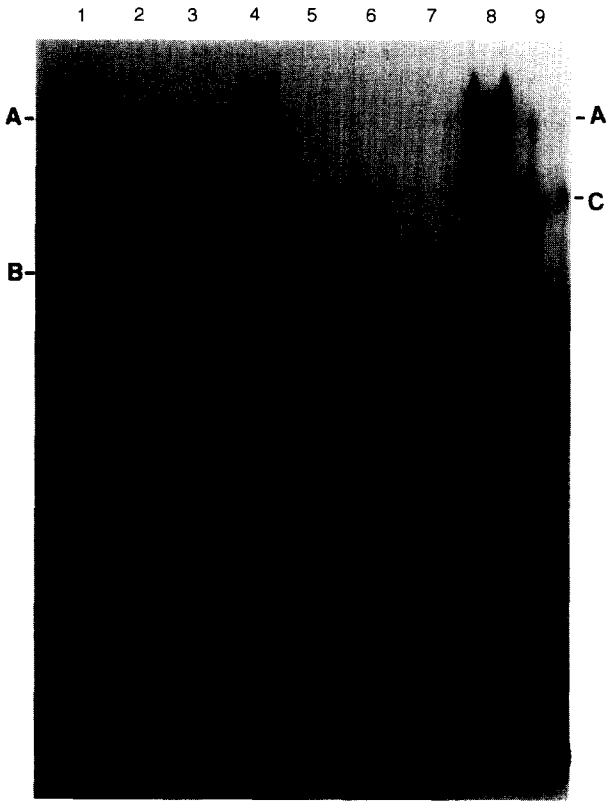

Fig. 2. EMSA of nuclear extracts from Hela cells and CREB protein produced from rabbit reticulolysate (RL). (Lane 1) CREB (RL), (lanes 2–9) Hela nuclear extract, (lanes 1,4,5,8,9) CREB specific antibody, (lanes 1–5) E4/CRE, (lanes 6–9) AR/CRE. Band B represents specific binding to both E4/CRE and AR/CRE in the absence of CREB specific antibody and band A, CREB specific interactions with both E4/CRE and AR/CRE.

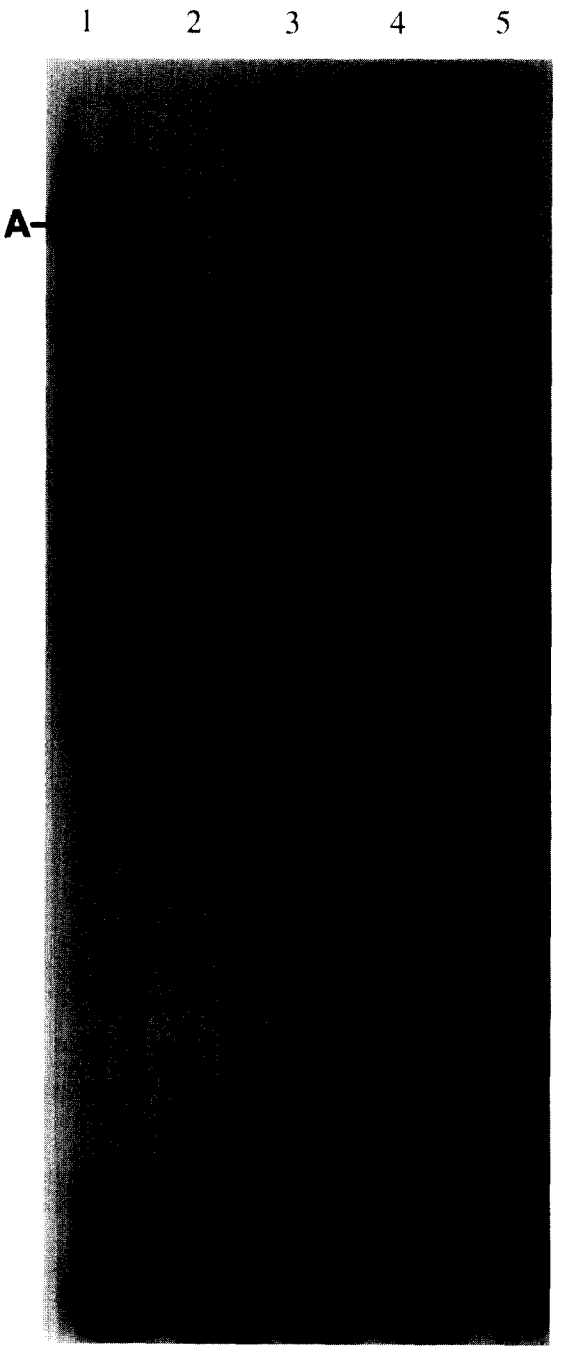

Fig. 3. EMSA of LNCaP nuclear extract with AR/CRE. (Lane 1) Control (Hela), (lanes 2–5) LNCaP, (lanes 1,3–5) with anti-CREB antibody, (lanes 4,5) with 20× and 100× AR/CRE. Band A represents CREB specific interaction.

Based on sequence homology the AR promoter region contains a putative cAMP response element (located at −508 to −501).

We were able to demonstrate upregulation of AR gene transcription in the presence of charcoal-stripped fetal bovine serum and 8-bromo-cAMP (5 mM). Charcoal stripping results in the removal of factors such as steroids which inhibit AR mRNA levels and thus the results represent a true measure of the effect of cAMP and cAMP analogues on AR gene transcription. Although previous reports have demonstrated a 2-fold induction of AR mRNA with 5 mM $(Bu)_2$cAMP [10] we were unable to detect AR mRNA expression in DU-145 and

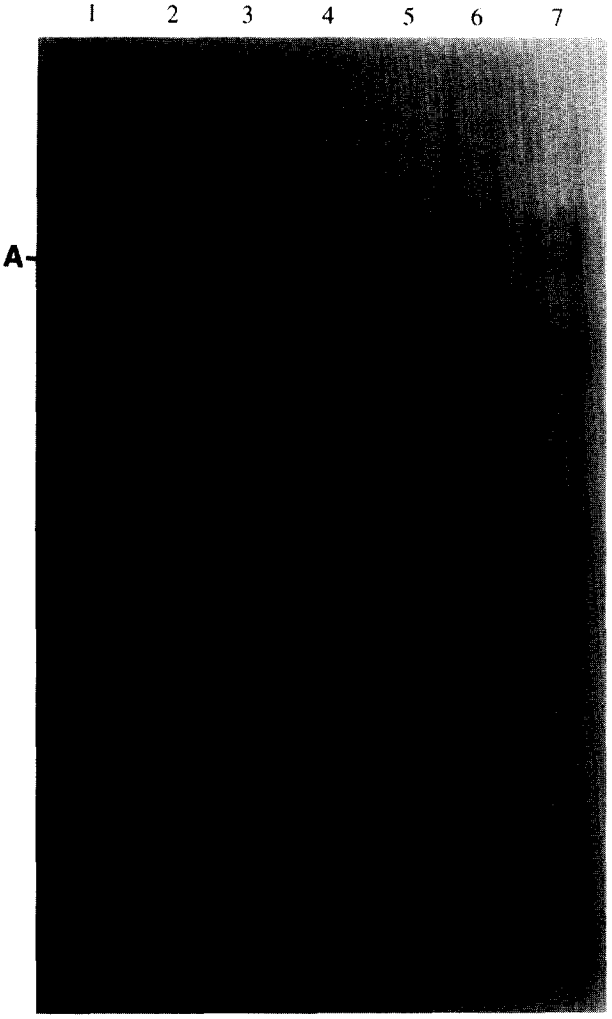

Fig. 4. EMSA of CREB specific LNCaP nuclear extract with AR/CRE. (Lane 1) Control (Hela), (lanes 2–7) LNCaP, (lanes 2,3) FBS, (lanes 4,5) SCFBS, (lanes 6,7) SCFBS+5 mM 8-bromo-cAMP. Band A represents CREB specific interaction.

PC-3 either before or after stimulation with 8-bromo-cAMP even after 40 cycles of PCR amplification. This lack of induction is not due to gene deletion (data not shown).

To examine the molecular basis for the loss of cAMP induced AR transcription we have characterised the putative AR/CRE (−508 to −501) with a known CRE (E4/CRE), the ATF-2 site at −50 from the adenovirus E4 promoter [9]. Specific competable protein-DNA interactions were observed between Hela cell nuclear extracts (HCNE) and the E4/CRE as compared with the AR/CRE (Fig. 2). A further control of CREB synthesised from a rabbit reticulolysate was used to verify the specificity of these protein-DNA interactions (Fig. 2). In addition CREB specific interactions with AR/CRE were observed with nuclear extracts isolated from LNCaP (LCNE) cells which were comparable to those of the control HCNE. Mizokami and colleagues have suggested that protein-AR/CRE interactions with HCNE were different from those from LCNE [10], their interpretation being subjective as no antibodies were used in the EMSA. The presence of CREB specific proteins was also observed in both PC-3 and DU-145 nuclear extracts and their levels were comparable to those found in LNCaP cells. Thus disruption of the cAMP signalling pathway in androgen insensitive cell lines (DU-145 and PC-3) is not due to non-functioning CREB. A possible explanation for our observation is that cAMP response is down regulated by heterodimer formation of CREB with activating transcription factor-1 [11]. Thus, only cells which contain a significant proportion of homodimeric CREB will be responsive. The loss of AR gene transcription via a second messenger pathway may be an important primary mechanism of androgen insensitivity in prostate cancer.

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References

[1] Carter, H.B. and Coffey, D.S. (1990) Prostate 16, 39–48.
[2] Stearn, M.E. and McGarvey, T. (1992) Lab. Invest. 67, 540–552.
[3] Taplin, M.-E., Bubley, G.J., G.L., Shuster, T.D., Frantz, M. E., Spooner, A.E., Ogata, G.K., Keer, H.N. and Balk, S.P. (1995) N. Engl. J. Med. 332, 1393–1398.
[4] Ruizeveld-de-Winter, J.A., Janssen, P.J., Sleddens, H.M., Verleun-Mooijman, M.C., Trapman, J., Brinkmann, A.O., Santerse, A.B., Schroder, F.H. and Van der Kwast, T.H. (1994) Am. J. Pathol. 144, 735–746.
[5] Visakorpi, T., Hyytinen, E., Koivisto, P., Tanner, M., Keinanen, R., Palmberg, C., Palotie, A., Tammela, T., Isola, J. and Kallioniemi, O.-P. (1995) Nat. Gen. 9, 401–406.
[6] Gaddipati, J.P., McLeod, D.G. and Heidenberg, H.B. (1994) Cancer Res. 54, 2861–2864.
[7] Culig, Z., Klocker, H., Eberle, J., Kaspar, F., Hobisch, A., Cronauer, M.V. and Bartsch, G. (1993) Prostate 22, 11–22.
[8] Ercolani, L., Florence, B., Denaro, B. and Alexander, M. (1988) J. Biol. Chem. 263, 15335–15341.
[9] Hurst, H., Masson, N., Jones, N.C. and Lee, K.A.W. (1990) Mol. Cell. Biol. 10, 6192–6203.
[10] Mizokami, A., Yeh, S.Y. and Chawnshang, C. (1994) Mol. Endocrinol. 8, 77–88.
[11] Ellis, M.J.C., Lindon, C.A., Flint, K.J., Jones, C.N. and Goodbourn, S. (1995) Mol. Endocrinol. 9, 255–265.