Regulation of the expression and activity by progestins of a member of the *SOX* gene family of transcriptional modulators

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ABSTRACT

The mammalian testis-determining gene *Sry* and the related *Sox* genes define a family of transcriptional regulators widely expressed during embryogenesis. Tightly controlled temporal profiles of expression are a feature of the *Sox* gene family and may be required for initiation of a cascade of gene expression, yet the molecular mechanisms that control *Sox* gene expression are unknown. We now show that human *SOX4* is expressed in the normal breast and in breast cancer cells. In these cells *SOX4* is a progesterone-regulated gene, the expression of which is increased by progestins, leading to a marked increase in SOX-mediated transcriptional activity. Treatment of T-47D breast cancer cells with the synthetic progestin ORG 2058 directly increased *SOX4* transcription, resulting in a 4-fold increase in *SOX4* mRNA levels within 4 h of treatment. No effect of ORG 2058 was noted on other *SOX* genes measured, nor were other hormone-regulated HMG box proteins detected in this system, suggesting that the observed ability of

progestin to increase *SOX* mRNA expression was confined to *SOX4*. The increase in *SOX4* transcription was reflected in increased *SOX4* protein expression, as progestin treatment of T-47D cells transfected with a *SOX*-responsive reporter resulted in a marked increase in reporter gene expression. Progesterone is essential for normal development and differentiation of the female reproductive system, plays an essential role in regulating growth and differentiation of the mammary gland and is required for opposing the proliferative effects of estrogen in specific cell types. The detection of *SOX4* expression in the normal and malignant breast and the demonstration that *SOX4* expression is under progesterone control suggests that changes in *SOX4* gene expression may play a role in commitment to the differentiated phenotype in the normal and malignant mammary gland.

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INTRODUCTION

The *SOX4* gene is a member of a family of transcription regulators which all contain an HMG box DNA-binding domain, and interact with DNA in a sequence-specific fashion (Pevny & Lovell-Badge 1997). Many *SRY*-related HMG box (*SOX*) genes have now been identified in the human, as well as other species, on the basis of their homology in this region. Where analysed, all have been shown to bind specifically to the double-stranded DNA motif, **A/TA/TCAAA/TG** (Denny

et al. 1992, van de Wetering & Clevers 1992, Laudet *et al.* 1993, van de Wetering *et al.* 1993, Connor *et al.* 1995, Hosking *et al.* 1995). Interaction of HMG box proteins with this motif results in bending of the DNA, suggesting that transactivation by these factors may occur by facilitating protein–protein or protein–DNA interactions, bringing normally distal components into proximity with each other (Ferrari *et al.* 1992). Although it is still not possible to ascribe functions to all members of the *Sox* gene family, there is increasing evidence that *Sox* proteins are likely to be involved in many

aspects of transcriptional regulation (Pevny & Lovell-Badge 1997). Their role as transcriptional regulators, combined with their limited tissue distribution and diverse patterns of distribution during embryogenesis, suggest that members of the *Sox* gene family play a pivotal role in cell fate determination in a wide range of developmental processes (Pevny & Lovell-Badge 1997). More recent evidence has implicated aberrant expression of *Sox* genes in malignancy: SOX21 expression has been associated with metastatic potential in melanoma cell lines (Tani *et al.* 1997), and aberrant expression of SOX4 in colon carcinoma has been implicated in regulation of the expression of p56^{lck}, a member of the *src* family of tyrosine kinases (McCracken *et al.* 1997).

The *Sox4* gene, first isolated from a mouse embryo cDNA library as the Sry-related gene a4 (Gubbay *et al.* 1990), is specifically expressed in the ovary, testis and thymus of adult mice and in mouse T and pre-B lymphocytic cell lines (van de Wetering *et al.* 1993). *Sox4* has transactivating capacity when cotransfected into Sox4-negative T cells with a reporter plasmid containing seven copies of the AACAAAG motif. It was found that binding of at least two *Sox4* transactivation domains was required to see this effect in the absence of other enhancer elements, suggesting that *Sox4* may cooperate with other transcription factors to exert tissue-specific control of gene expression (van de Wetering *et al.* 1993). Evidence is now emerging that cooperative function between Sox proteins and other transcription factors may be a general feature: Sox2 cooperates with one of the POU domain factors, Oct-4, in the regulation of osteopontin expression in preimplantation development (Botquin *et al.* 1998). Sox2 is also involved in transcriptional regulation of the *FGF-4* gene, in cooperation with Oct-3 and Sp1/Sp3 (Lamb & Rizzino 1998). Sox11 and Sox4 cooperate with another POU protein, Brn-1, in glial cells (Kuhlbrodt *et al.* 1998). Sox4 cooperates also with the transcription factor Ets-1 in regulating the expression of p56^{lck} (McCracken *et al.* 1997).

During embryogenesis *Sox4* is essential for normal cardiac development. Transgenic mice harbouring a homozygous mutation in *Sox4* die during embryogenesis because of a failure in endocardial ridge development which prevents proper formation of semilunar valves, resulting in circulatory failure (Schilham *et al.* 1996). Furthermore, introduction of bone marrow cells from these animals into an irradiated host results in a block in B-cell lineage progression at the stage of pro-B-lymphocyte expansion (Schilham *et al.* 1996). This indicates that *Sox4* is involved in developmental

processes occurring both in embryogenesis and in the mature animal.

Although murine *Sox4* is expressed in the ovary and testis, which are steroid hormone-responsive tissues, there is no information on steroidal regulation of *SOX4* gene expression. *SOX4* was identified in searching for genes that were early targets of progestin action: human *SOX4* was detected in the progesterone receptor (PR)-positive T-47D breast cancer cell line as a progestin-regulated gene. Progestin regulation of *SOX4* may represent an intermediate step in downstream progestin effects in the normal and malignant breast.

MATERIALS AND METHODS

Materials

The general chemicals used were of molecular biological or analytical reagent grade, as required by specific methodology, and were from the same sources as previously listed (Clarke *et al.* 1990). Radiochemicals, ORG 2058, Moloney murine leukaemia virus reverse transcriptase, *Taq* DNA polymerase and Megaprime DNA labelling system were obtained from Amersham Australia (North Ryde, Sydney, Australia). The pSG5-hPR1 plasmid was a gift from Dr P Chambon (Strasbourg, France) and has been described previously (Kastner *et al.* 1990). Oestradiol-17 β was a Sigma (St Louis, MO, USA) product. ICI 182 780 was a gift from Dr Alan Wakeling, Zeneca (Macclesfield, UK) and RU 38486 was a gift from Dr J P Raynaud, Roussel-Uclaf (Romainville, France). The pBL2CAT-[SOX4]₄ plasmid containing four SOX4-binding motifs (AACAAAG) upstream of the thymidine kinase promoter and chloramphenicol acetyltransferase (CAT) sequences was a gift from Dr G E O Muscat, University of Queensland (Brisbane, Australia). The following oligonucleotide sequences were identified from the GenBank DNA sequence database and were obtained from Bresatec (Adelaide, SA, Australia) or from Pacific Oligos (Lismore, NSW, Australia) and used as probes or PCR primers as described in the text: SOX4F1, 5'-AGGAGTTGAGGGGCCAGTTC-3'; SOX4R1, 3'-CACTGCCACCGACCTTGTCTC-5'; SOX2R, 3'-CACATGTGTGAGAGGGGCGAGTGTG-5' (nucleotides 942–965); SOX3R, 3'-CAGTCTCCAGAAGGCTGTACATTG-5' (nucleotides 665–688); SOX11R, 3'-GTTGCTCTCCGCTTCAAGCTCTC-5' (nucleotides 91–114); mSox21F1, 5'-CTCAGGCTTGGCAGTGGA-3' (nucleotides 2363–2380); mSox21R1, 3'-AGGCTGGGTGGA TTGCCT-5' (nucleotides 2982–2999); SOX22F1,

5'-TG TAGAGCGATCCTGGGA-3' (nucleotides 1894-1911); SOX22R1, 3'-CAGTCTCACTGGC CAAGT-5' (nucleotides 2486-2503). The plasmid pSox9-5a (Wright *et al.* 1995) containing a 500 bp fragment of Sox9 cDNA was a gift from Dr P Koopman, University of Queensland. A partial rat glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA was a gift from Mr G Brown (CSIRO, North Ryde, Australia) and was used as a probe for human GAPDH.

Cell culture

T-47D human breast cancer cells (Keydar *et al.* 1979) were obtained from E G and G Mason Research Institute, Worcester, MA, USA and stocks were maintained in virtually continuous exponential growth in phenol red-free RPMI 1640 medium containing 10% fetal calf serum (FCS) as described previously (Clarke *et al.* 1990).

Differential display reverse transcription (RT) PCR and cDNA cloning

T-47D cells, growing exponentially in phenol red-free RPMI 1640 medium containing 10% FCS, were treated in duplicate for 6 h with 10 nM ORG 2058 or vehicle. The cells were harvested and total RNA was prepared separately from duplicate control and progesterone-treated pairs. The differential display method used was derived from the protocols of Liang and colleagues (Liang *et al.* 1992, 1993). Total RNA (5 µg) was annealed to a degenerate primer mix (2.5 µM each oligonucleotide) with general sequence T₁₁YC (where Y=A, C or G). RT was performed using Moloney murine leukaemia virus reverse transcriptase with 50 mM Tris, pH 8.2, 50 mM KCl, 6 mM MgCl₂, 20 µM dNTP mix and 20 U RNasin RNase inhibitor (Promega Corp., Annandale, Australia), by incubation at 35 °C for 1.5 h. PCR mixtures containing 3 µl RT product, 2.5 µM 3'-primer mix (as for reverse transcription), 0.5 µM each 5'-CTGACCAGCC and 5'-GGGAG ACATC oligonucleotide primers, 2 µM dNTP mix, 12 µCi [³⁵S]dATP (1500 Ci/mmol), 1.5 mM MgCl₂, 1 U *Taq* DNA polymerase and reaction buffer (supplied), were denatured at 90 °C for 1 min, followed by 40 PCR cycles. PCR conditions were: denaturation for 30 s at 90 °C, annealing at 40 °C for 1 min, and extension at 72 °C for 30 s, followed by a final incubation at 72 °C, 5 min. Amplified products were analysed by electrophoresis through denaturing 8% acrylamide gels at 50 °C. Gels were dried on to Whatman 3MM paper and exposed to film overnight.

A differentially displayed band of 99 bp was excised and reamplified using the same PCR conditions as above except that the dNTP concentration in the reaction was increased 10-fold to 20 µM. The reamplified PCR products were inserted into the pGEM-T vector (Promega Corp.) and sequenced using an ABI 373A automated DNA sequencing system (Applied Biosystems, Warrington, Cheshire, UK). Sequences were compared with the GenBank and EMBL databases using the National Center for Biotechnology Information BLAST local alignment network service (Altschul *et al.* 1990).

cDNA clones were obtained by screening a human breast carcinoma λgt11 cDNA library derived from the ZR-75-1 cell line (Clontech, Palo Alto, CA, USA). Positive phage clones were propagated and purified essentially as described by Sambrook *et al.* (1989). cDNA inserts were excised using EcoRI and subcloned into the EcoRI site of pGEM-7Zf(+). The identity of positive clones to SOX4 was confirmed by DNA sequencing and alignment to the GenBank and EMBL database using the BLAST network service (Altschul *et al.* 1990). An 800 bp SOX4 fragment corresponding to sequences [+1612,+2412] of the gene was isolated using this approach.

PCR amplification of SOX sequences

Partial cDNA fragments were amplified by PCR from human genomic DNA using SOX4- or SOX22-specific primers and from mouse genomic DNA using Sox21-specific primers. PCR reactions containing 50 ng DNA, 0.3 µM each oligonucleotide primer SOX4F1 and SOX4R1, 2 µM dNTPs, 1.5 mM MgCl₂, 1.25 U *Taq* DNA polymerase and 1 × reaction buffer (supplied), were denatured at 95 °C for 5 min followed by 30 PCR cycles (95 °C, 30 s; 60 °C, 30 s; 72 °C, 30 s) and a final extension step at 72 °C for 10 min. The PCR product, SOX22F1R1, was used as the HMG-box specific probe. Similar reactions were set up for Sox21 and SOX22 using msox21f1 and msox21r1 or SOX22F1 and SOX22R1 except that an annealing temperature of 52 °C was used. Amplification of a single band of expected size was confirmed by analysis of an aliquot of the PCR reaction on a 1.2% agarose gel. The PCR products were purified from the remaining reactions using the JETquick kit (Genomed Inc, Research Triangle Park, NC, USA) and labelled using the Amersham Megaprime DNA labelling system using [α -³²P]ATP for use as HMG-box, SOX21- and SOX22-specific probes respectively. Oligonucleotides SOX2R, SOX3R and SOX11R were

end-labelled using [γ - 32 P]ATP and T4 polynucleotide kinase and used as probes for *SOX2*, *SOX3* and *SOX11* respectively.

RNA isolation and Northern blot analysis

T-47D cells growing exponentially in phenol red-free RPMI 1640 medium containing 5% FCS were changed 3 or 4 days after plating into phenol red-free RPMI 1640+1% charcoal-stripped FCS and incubated for a further 24 h, then treated as described in the figure legends and text. After harvesting the cells, total RNA was isolated by the guanidinium isothiocyanate/cesium chloride method and poly(A)⁺ RNA extracted using the PolyATtract mRNA isolation kit (Promega, Madison, WI, USA). Northern blot analysis was performed as described previously (Clarke *et al.* 1990). The *SOX4* and hPR1 cDNA probes were labelled using the Amersham Megaprime DNA labelling system. Total RNA was also isolated from normal breast tissue derived from reduction mammoplasty and analysed in the same way. The 18S oligonucleotide probe was end-labelled using [γ - 32 P]ATP and T4 polynucleotide kinase. Results were analysed using a Molecular Dynamics (Sunnyvale, CA, USA) PhosphorImager and Imagequant software.

Nuclear run-on

SOX4 and PR gene transcription rates were estimated using the method of Greenberg & Ziff (1984) with modifications, as described previously (Clarke *et al.* 1991). Labelled run-on transcripts were hybridized to nitrocellulose filters, on which 5 μ g each of linearized *SOX4*[+1612,+2412]-pGEM7, pSG5-hPR1, pGEM-7Zf(+) and α 1 (α -tubulin) (Cowan *et al.* 1983) plasmids had been slot blotted and fixed by UV irradiation. Filters were washed (Clarke *et al.* 1991), dried and exposed to a phosphor screen and later to film. The results were scanned and analysed using a Molecular Dynamics PhosphorImager and Imagequant software. To control for differences in hybridization, *SOX4* and PR signals were normalized using the α -tubulin signal on the same filter.

Gene transfection

T-47D cells were plated into 25 cm² flasks (5 $\times$ 10⁵ cells/flask) in RPMI 1640 phenol red-free medium containing 5% FCS 2 days before transfection, and changed to Dulbecco's modified Eagle's medium supplemented with 5% FCS on the day of transfection. Cells were transfected by the calcium

Human *SOX4*

FIGURE 1. Identification of *SOX4* fragments by differential display. The figure shows the schematic map of the *SOX4* cDNA indicating the locations of the 99 bp fragment identified by differential display RT-PCR and the 800 bp *SOX4* fragment isolated from the cDNA library. The coding region of *SOX4* is boxed and the HMG box is indicated as the hatched region.

phosphate precipitation method (Gorman 1985) as described previously (Clarke *et al.* 1991), with pBL2CAT-[*SOX4*]₄ (5 μ g/flask) and 1 μ g pCH110 β -galactosidase control plasmid. The cells were subjected to osmotic shock 4 h after addition of precipitates, after which the precipitates were returned to the flasks and drug treatments were added. Cells were given fresh medium (containing the appropriate drug concentrations) 16 h after osmotic shock, and harvested 8 h later. Enzyme activities and protein concentrations were measured in cell lysates as described previously (Clarke *et al.* 1991).

RESULTS

Detection of *SOX4* as a progesterin-regulated gene

Progesterin induction of the *SOX4* gene was demonstrated by differential display RT-PCR in the PR-positive T-47D breast cancer cell line. Sequence analysis of the RT-PCR product showed it was identical to a 3' region of the human *SOX4* gene (Fig. 1). This region is unique to *SOX4* and has low identity with other *SOX* gene family members. The *SOX4* transcript was detected on Northern blots as a single band migrating ahead of the 28S ribosomal subunit in total RNA (Fig. 2A), consistent with the previous characterization of the *SOX4* mRNA as a major transcript of 5.2 kb (Farr *et al.* 1993).

Characterization of *SOX4* mRNA regulation

SOX4 mRNA levels were induced by treatment of T-47D cells with the synthetic progesterin ORG 2058 (Fig. 2A). The increase was detectable within 1 h of treatment and reached a maximum, approximately 4-fold the time-matched control, by 4 h after treatment (Fig. 2B). The induction was transient:

FIGURE 2. Induction of SOX4 mRNA by ORG 2058. T-47D cells growing in phenol red-free RPMI 1640 medium supplemented with 5% FCS were changed to medium containing 5% charcoal-treated FCS 24 h before treatment. (A) Cells were treated with 10 nM ORG 2058 or vehicle and harvested at the times shown. Total RNA was prepared and SOX4 mRNA was detected by Northern blot analysis. The locations of the 18S and 28S rRNA subunits are indicated. The same blot was probed using an oligonucleotide hybridizing to the 18S rRNA to check loading, shown in the lower panel. (B) SOX4 mRNA levels were quantified densitometrically and are expressed as a percentage of the time-matched vehicle-treated control. The data represent the combined results of three separate experiments. Inset: T-47D cells growing in phenol red-free RPMI 1640 medium supplemented with 5% FCS were changed to medium containing 5% charcoal-treated FCS 24 h before treatment. Cells were treated with the range of ORG 2058 concentrations shown or vehicle and harvested 4 h later. SOX4 mRNA levels are shown as a percentage of the vehicle-treated control (%C).

by 8 h after treatment the SOX4 mRNA level had decreased to less than 2-fold the time-matched control and by 10 h had returned to control levels (Fig. 2B). At 24 and 48 h after treatment SOX4 mRNA remained at control levels. The progestin effect on SOX4 mRNA was concentration-dependent (Fig. 2B inset). When

FIGURE 3. Effect of anti-progestins, oestrogen and anti-oestrogen on SOX4 mRNA levels. T-47D cells growing in phenol red-free RPMI 1640 medium supplemented with 5% FCS were changed to medium containing 5% charcoal-treated FCS 24 h before treatment. Cells were treated with vehicle, 10 nM ORG 2058, 100 nM RU 38486, 1 nM oestrogen (E2) or 100 nM ICI 182 780, in the combinations indicated, and harvested at 4 and 24 h for preparation of total RNA. The same blot was probed using an oligonucleotide hybridizing to the 18S rRNA to check loading, shown in the lower panel. SOX4 mRNA was quantified densitometrically and expressed as a percentage of the time-matched vehicle-treated control.

SOX4 mRNA levels were examined after 4 h treatment with 0.01 to 100 nM ORG 2058, maximal induction of SOX4 mRNA was observed at 1 nM. To determine whether progestin regulation of SOX4 was mediated by PR, the effects of a progestin antagonist were examined after 4 or 24 h of treatment (Fig. 3). The anti-progestin, RU 38486, had no effect on SOX4 mRNA levels alone at either time, but completely abrogated the ORG 2058 effect at 4 h, showing that the progestin effect was PR-mediated. Treatment with 17 β -oestradiol

decreased *SOX4* mRNA levels and partially abrogated the progesterin-mediated effect (Fig. 3). Treatment with the anti-estrogen ICI 182 780 abrogated the effects of oestradiol (Fig. 3).

Progesterin regulation of *SOX4* mRNA was transcriptionally mediated

To determine whether the progesterin induction of *SOX4* expression was due to increased transcription, *SOX4* gene transcription was measured in nuclei isolated from T-47D cells which had been treated for 2 h with 10 nM ORG 2058 or vehicle (Fig. 4). Progesterins markedly increased *SOX4* transcription compared with the matched control, both in the presence and absence of the protein synthesis inhibitor, cycloheximide, showing that the progesterin effect was transcriptionally mediated and suggesting that it was direct.

Effect of progesterins on other *SOX* genes

Northern blots were hybridized with probes homologous to *SOX2*, *SOX3*, *Sox9*, *SOX11*, *Sox21* and *SOX22*. *SOX2*, *SOX3* and *SOX11* mRNA were undetectable in T-47D cells, and hormone treatment did not increase their levels to within the detectable range. *Sox9* was detected at a low level and its expression was not modulated by hormone treatment (data not shown). A single transcript migrating between the 28S and 18S rRNA transcripts was identified upon hybridization of Northern blots at low stringency with a mouse *Sox21* probe; as the human *SOX21* has yet to be described, this transcript was assumed to represent human *SOX21* mRNA: no regulation of this transcript by hormonal treatment was noted (not shown). *SOX22* was detected by Northern blot analysis of T-47D cells and no consistent effect of hormone treatment was noted (data not shown). In order to determine whether the expression of *SOX* genes, other than those tested above, was influenced by ORG 2058 treatment, poly(A)⁺ RNA from vehicle- or progesterin-treated cells was probed at low stringency with a cDNA flanking and including the *SOX4* HMG box (*SOXF1R1*; Fig. 5). This detected a progesterin-regulated transcript of a size consistent with *SOX4*, in keeping with the data shown above, but no other transcripts of significant abundance, and there was no evidence of progesterin regulation of transcripts other than *SOX4*.

Induction of *SOX4* transcriptional activity by progesterins

The induction of *SOX4* gene expression by progesterins was reflected in an induction of *SOX4*

FIGURE 4. Regulation of *SOX4* transcription rate. T-47D cells growing in phenol red-free RPMI 1640 medium supplemented with 5% FCS were changed to medium containing 5% charcoal-treated FCS 24 h before treatment for 2 h with 10 nM ORG 2058 or vehicle, in the presence or absence of 20 µg/ml cycloheximide (CHX). Cells were harvested and nuclei were prepared by lysis at 4 °C in a Nonidet P40 buffer. (A) *SOX4* and α -tubulin transcription rates were estimated using the nuclear run-on technique. The pGEM-7Zf(+) plasmid was used as a negative control. The results were scanned and analysed using a Molecular Dynamics PhosphorImager and Imagequant software. To control for differences in hybridization the *SOX4* signal was normalized using the α -tubulin signal on the same filter. (B) *SOX4* transcription rate was estimated by densitometry and is expressed as a percentage of the vehicle-treated control, after normalization for differences in hybridization using α -tubulin.

transcriptional activity. When the pBL2CAT-*[SOX4]₄* construct, which contains four *SOX* consensus motifs upstream of the thymidine kinase promoter and CAT reporter sequences, was transiently transfected into T-47D cells, ORG 2058 treatment resulted in a greater than 50-fold increase in CAT activity (Fig. 6). No effect of ORG 2058 treatment was seen if the same pBL2CAT vector

FIGURE 5. Effect of ORG2058 on expression of *SOX* genes other than *SOX4* in T-47D cells. T-47D cells growing in phenol red-free RPMI 1640 medium supplemented with 5% FCS were treated with vehicle (lane 1) or 10 nM ORG 2058 (lane 2) and harvested at 4 h. Poly(A)⁺ RNA was prepared, blotted and probed with the SOXF1R1 cDNA, which flanks and includes the SOX4 HMG box. The same blot was probed using a GAPDH probe to check loading, shown in the lower panel. Only one major transcript (arrowed), consistent in size and progestin regulation with *SOX4* mRNA, was detected. A second, minor, transcript was detected after prolonged exposure but no regulation of this transcript was noted (not shown).

with an oestrogen-responsive sequence replacing the SOX consensus motifs was transfected (not shown), demonstrating that the effect was mediated through the SOX consensus region. RU 38486 had little effect alone but completely abolished the progestin effect, showing that the induction was PR-mediated. Transactivation of pBL2CAT-[SOX4]₄ could be contributed by other SOX proteins, as the motif represents a consensus binding element for SRY-related proteins. How-

FIGURE 6. Transfection of a *SOX4* reporter construct into T-47D cells. (A) T-47D cells were transfected with pBL2CAT-[SOX4]₄ and the pCH110 β -galactosidase control plasmid. At 4 h after addition of precipitates, cells were treated with 10 nM ORG 2058 and 100 nM RU 38486 separately or combined as indicated. The cells were harvested 28 h after transfection, and CAT and β -galactosidase activities were determined. The results are expressed as fold induction of CAT relative to control, after correction using β -galactosidase activity. Each data point represents the results of triplicate transfections and is representative of two experiments where the same results were seen. Error bars are not visible because the magnitude of the error fell below the resolution of the bar graph. No effect of ORG 2058 was noted when the plasmid lacked the SOX motif, shown when transfections were repeated with an oestrogen-responsive sequence in place of the SOX-responsive sequences (data not shown).

ever, the basal activation of the reporter was very low, suggesting that endogenous SOX protein expression is low in these cells. Furthermore, there was no evidence, from the experiments described above, of progestin regulation of *SOX* genes apart from *SOX4* in T-47D cells.

Expression of *SOX4* mRNA in normal breast and breast cancer cell lines

SOX4 mRNA was detected in the normal breast, albeit at levels lower than those seen in progestin-treated T-47D cells, and was detectable in total RNA (Fig. 7). The higher level of *SOX4* mRNA in T-47D breast cancer cells when compared with normal breast was noted more generally in a range of breast cancer cell lines: *SOX4* mRNA was detected in all cell lines tested, and the majority of cell lines had *SOX4* levels similar to those found in

FIGURE 7. Expression of *SOX4* mRNA in normal breast. Northern blots of total RNA from T-47D cells treated with 10 nm ORG 2058 for 4 h (lane 1) and from normal breast tissue (lane 2) were hybridized with a *SOX4* cDNA (top panel). The same blot was probed for 18S rRNA to check loading, shown in the lower panel.

T-47D cells. Interestingly, two breast cancer cell lines, MDA-MB-361 and the MDA-MB-134, expressed markedly higher *SOX4* levels (Fig. 8).

DISCUSSION

This is the first report of regulation by steroid hormones of a member of the *SOX* gene family. The *SOX4* gene was transcribed in PR-positive T-47D breast cancer cells, and *SOX4* mRNA was rapidly induced by progestins. The effect was concentration-dependent, with maximal induction being seen with 1nM ORG 2058 treatment. *SOX4* was the most abundant member of the *SOX* gene family detected in these cells; moreover, progestin regulation of *SOX* gene expression was restricted to *SOX4* and was not noted for a range of other *SOX* genes tested.

The induction of *SOX4* mRNA by progestins in breast cancer cells was reflected in an increase in *SOX* transcriptional activity, as progestins caused a marked induction of the pBL2CAT-[*SOX4*]₄ reporter containing four *SOX4* motifs inserted upstream of TK-CAT. This activity could be contributed by other *SOX* proteins, as the motif represents a consensus binding element for SRY-related proteins. However, two lines of evidence argue against this: first, there was no evidence of progestin modulation of *SOX* genes apart from *SOX4* in these cells. Secondly, the basal activation of the reporter gene was very low compared with the strong activity upon progestin induction, suggesting that endogenous *SOX* expression is low in these cells and that the observed effects are progestin specific, and likely to be mediated through *SOX4*.

FIGURE 8. Expression of *SOX4* mRNA in breast cancer cells. Total RNA isolated from nine breast cancer cell lines (ER+ cells: T-47D, MCF-7M, MCF-7, MCF-7Z, MDA-MB-361, MDA-MB-134 and ER-cells: MDA-MB-231, BT-20, MDA-MB-453), HBL-100-transformed breast epithelial cells and the LNCaP prostate carcinoma cell line was analysed by Northern blotting (20 µg/sample) as described in Materials and Methods (A). (B) *SOX4* mRNA levels were quantified by densitometry. The same blot was probed for 18S rRNA, *SOX4* levels were normalized for differences in loading and the data depicted as arbitrary units (AU).

It is unlikely that transcription factors other than *SOX* proteins, for instance PR, bind and activate the pBL2CAT-[*SOX4*]₄ reporter. The *SOX4* motif has little resemblance to a progestin-responsive element (Gronemeyer 1991, Tsai & O'Malley 1994). Similarly, the AP1 transcription complex, which mediates the effects of hormones, growth factors and cytokines on a number of target genes (Angel & Karin 1991), is not likely to mediate the effects of

progestins on the pBL2CAT-[SOX4]₄ reporter. The AP1-recognition sequence differs markedly from the SOX motif and, furthermore, progestins have been shown to negatively regulate an AP1 reporter, rather than to induce its activity (Shemshedini *et al.* 1991, Alkhalaf & Murphy 1992).

Progestin regulation of SOX4 mRNA was clearly PR-mediated as the induction was completely abrogated by the progestin antagonist RU 38486. Transcriptional run-on analysis revealed that progestin induction of SOX4 mRNA occurred through an increase in the transcription rate of the *SOX4* gene. Cotreatment with the protein synthesis inhibitor, cycloheximide, was unable to abolish this effect, suggesting that it is directly mediated through PR binding to the *SOX4* gene. This was consistent with the rapidity of SOX4 mRNA induction, which resulted in almost 2-fold induction within 1 h of progestin exposure. The superinduction of SOX4 transcription by progestins in the presence of cycloheximide may reflect stabilization of SOX4 transcripts as the result of downregulation by the protein synthesis inhibitor of nuclear RNases normally responsible for turnover of nascent transcripts. It can be concluded that progestins increase the SOX4 transcription rate in both the presence and absence of cycloheximide, indicating that PR exerts direct transcriptional control on the *SOX4* gene.

Although SOX4 expression and regulation have been described in most detail here in malignant breast cancer cells, SOX4 mRNA was also detected in the normal human breast, highlighting the possibility that the *SOX4* gene plays a developmental role in normal progestin-responsive tissues in the adult. Such a role would be comparable, in some respects, to the part played by many of the *SOX* genes in the development of neural tissues, central and peripheral nervous systems and skeletal tissue during embryogenesis (Denny *et al.* 1992, Stevanovic *et al.* 1993, Connor *et al.* 1994, 1995, Foster *et al.* 1994, Wagner *et al.* 1994, Uwanogho *et al.* 1995), and may provide a mechanism for the complex cell specificity of progestin action in target tissues.

The mammary gland is remarkable in being present only in rudimentary form in the neonate, with the major stages of its development taking place consequent to exposure to ovarian hormones in the adolescent and during pregnancy. Progesterone plays an essential role in regulating the growth and differentiation of the mammary gland (Lydon *et al.* 1995) and is essential for opposing the proliferative effects of oestrogen in specific cell types (Clarke & Sutherland *et al.* 1990). The

mechanisms mediating the effects of progesterone in the reproductive system are not understood and the demonstration that expression of the *SOX4* gene is increased by progestin implies that SOX4 may be a candidate mediator of the effects of progesterone in target tissues. In addition, SOX4 may cooperate with ovarian hormones in controlling transcription; transactivation studies of *Sox* genes now indicate that this protein cooperates with other transcriptional regulators to affect gene expression (van de Wetering *et al.* 1993, McCracken *et al.* 1997, Botquin *et al.* 1998, Kuhlbrodt *et al.* 1998, Lamb & Rizzino 1998).

The demonstration of the effects of progestin on *SOX4* gene expression in breast cancer cells and the identification of *SOX4* gene expression in breast tumours (unpublished observations) suggest that hormonal alterations in *SOX4* gene expression may play a role in malignant breast tissue. More broadly, ovarian hormone control of *SOX4* expression may indicate a role for this gene family in postnatal development of the mammary gland.

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