Cross-talk between glucocorticoid and retinoic acid signals involving glucocorticoid receptor interaction with the homoeodomain protein Pbx1

Nanthakumar SUBRAMANIAM[1], Javier CAMPIÓN, Ingalill RAFTER and Sam OKRET[2]

Department of Medical Nutrition, Karolinska Institutet, Huddinge University Hospital, Novum, SE-141 86 Huddinge, Sweden

Glucocorticoid (GC) signalling influences the response of the cell to a number of other signals via a mechanism referred to as 'cross-talk'. This cross-talk may act at several levels, including an interaction between the transcription factors involved in the signalling pathways. In the present paper, we demonstrate a novel functional interaction between GC and all-*trans*-retinoic acid (RA) signalling. We show that, in P19 embryonal carcinoma cells, GCs potentiate RA-induced expression of the murine *Hoxb*-1 gene through an autoregulatory element, b1-ARE, recognized by the Pbx1 and HOXB1 homoeodomain proteins. The synergistic effect of GC did not involve GC receptor (GR) binding to the b1-ARE, and the GC–GR complex alone was unable to activate transcription via the element. Furthermore, the ability of the GR to transactivate was not required, excluding expression of a GC-induced protein as the mechanism for the GC/RA synergy. Additional transfection experiments showed that the Pbx1/HOXB1 heterodimer was the target for the GC effect. Furthermore, functional dissection of the GR demonstrated that the DNA-binding domain (DBD) of the GR was required for the synergy. A physical interaction between the GR and Pbx1 proteins was demonstrated *in vivo* by co-immunoprecipitation experiments. These results are compatible with a model in which the GC/RA synergy is mediated by a direct interaction between the GR and Pbx1. On the basis of the ubiquitous expression of both GR and Pbx1, a number of genes regulated by Pbx are likely to be important targets for GC-mediated 'cross-talk'.

Key words: autoregulatory element, HOXB1, P19 embryonal carcinoma cells, protein–protein interaction.

INTRODUCTION

Glucocorticoids (GCs) play a key role in regulating diverse physiological processes, such as metabolism, salt and water balance, cell proliferation, differentiation, inflammation and immune responses [1]. The GC signal is transduced via the GC receptor (GR), which regulates the expression of a subset of genes in both a promoter- and a tissue-specific manner. The activated GR can either activate or repress transcription of specific genes. Activation of the target genes is preceded by interaction of the GR with GC-responsive elements (GREs), most often localized in the promoter region. Following the interaction with GREs, transcriptional activation is supported by the interaction of the GR with the basal transcription machinery, co-activators and other transcription factors [2]. Chromatin organization and chromatin-associated proteins also influence the transcriptional response of the GR. Co-operative stimulation of target gene expression very often involves the interaction of GR with other transcription factors, where each of the factors bind to juxtaposed DNA target sequences [3]. However, more recently it has also been demonstrated that the GR can stimulate the expression of target genes without directly contacting a GRE ([4–7]; see also the Discussion). Similarly, negative gene regulation by the GR occurs, in principle, in two ways. This involves either an interaction of the GR with negative GREs (nGREs), where the GR most likely causes a displacement of a positively acting transcription factor, or by an interaction between the GR and other transcription factors that is independent of GR binding to the DNA [1]. Examples of the latter situation include the well-described repression of AP-1-controlled target genes in cases where the AP-1 complex consists of a c-Jun/c-Fos heterodimer, and repression of transcriptional activation by the nuclear factor κB (NF-κB) complex. Although DNA binding by the GR, in itself, is not involved in these repressive mechanisms, the DNA-binding domain (DBD) of the GR is [8–11]. In addition to the DBD, the GR contains a C-terminal ligand-binding domain, which includes a ligand (agonist)-dependent activation domain (AF2) and an N-terminal domain accommodating a non-ligand-dependent transactivation domain (AF1 or τ_1) [2].

Pbx proteins are members of the three-amino-acid loop extension (TALE) class of homoeodomain proteins (for a review, see [12]). Genetic and biochemical analyses have shown that Pbx proteins regulate developmental pathways by serving as heterodimerization partners for other homoeodomain transcription factors, e.g. the HOX proteins. The Pbx proteins have been shown to enhance both HOX DNA-binding specificity and affinity [13,14]. Unlike the *Hox* genes, which are, for the most part, expressed temporally during development and differentiation, the Pbx proteins are expressed more ubiquitously [15]. This suggests that Pbx proteins also play a role in maintaining cellular differentiation in adult tissues. In fact, Pbx heterodimerization with other TALE proteins, such as Meis or Prep1,

Abbreviations used: b1-ARE, *Hoxb*-1 gene autoregulatory element; DBD, DNA-binding domain; Dex, dexamethasone; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; GC, glucocorticoid; GR, GC receptor; GRE, GC-responsive element; NF-κB, nuclear factor κB; NF-IL6, nuclear factor for interleukin 6; nGRE, negative GRE; Oct-1, octamer transcription factor-1; P19 EC cells, P19 embryonal carcinoma cells; R3, sequence motif repeat 3 (a subregion of b1-ARE); RA, all-*trans*-retinoic acid; RARE, RA-response element; TALE, three-amino-acid loop extension; TR, thyroid hormone receptor.

[1] Present address: Muscle Development Unit, Children's Medical Research Institute, Wentworthville, NSW 2145, Australia.

[2] To whom correspondence should be addressed (e-mail Sam.Okret@mednut.ki.se).

has been shown to mediate pancreas-specific transcription of the somatostatin [16] and glucagon [17] genes. Other genes known to depend on Pbx proteins for their proper regulation include the human a2(V) collagen gene [18], the elastase 1 gene [19], the malic enzyme gene [20], the *CYP17* gene [21], the bovine prolactin gene [22] and the mouse *Ren1*^{c} gene [23]. An important role for one of the Pbx proteins *in vivo*, Pbx1, was recently demonstrated in Pbx1-deficient mice, which were characterized by severe hypoplasia or aplasia of multiple organs and widespread patterning defects of the skeleton [24].

One of the Pbx-binding elements examined in most detail is the autoregulatory enhancer of the *Hoxb*-1 gene (b1-ARE). This element, located upstream of the *Hoxb*-1 transcriptional start site, contains three nearly identical sequence repeats with close similarity to the consensus binding site for Pbx proteins [25,26]. In the case of the b1-ARE, it has been demonstrated that Pbx1 recruits HOXB1 to form a heterodimer able to drive the transcription, thus forming an autoregulatory loop for *Hoxb*-1 gene transcription [25,27].

all-*trans*-Retinoic acid (RA) is a natural morphogen in vertebrate development, and is a regulator of the *HOX* gene cluster *in vivo*. This includes the *Hoxb*-1 gene [28]. Up-regulation of *Hoxb*-1 gene expression following RA administration can also be demonstrated in P19 embryonal carcinoma cells (P19 EC cells) [29,30]. Like GCs, RA modulates its actions via nuclear receptors, the RA receptors (RARs), that stimulate transcription through RA-response elements (RAREs) present in target genes. In the case of the *Hoxb*-1 gene, regulation by RA involves multiple regulatory regions containing functionally different RAREs that co-operate with the b1-ARE to maintain expression [28]. Although the b1-ARE does not contain RAREs in itself, it participates indirectly in mediating the RA-dependent activation via its autoregulatory ability. This involves an RA-stimulated increase in the cellular expression levels of Pbx and HOXB1 that will bind to the b1-ARE [29–31]. That this is the case has been demonstrated in, e.g. P19 EC cells by the ability of RA to stimulate expression of a reporter gene controlled by the b1-ARE alone [31].

We have shown previously that the GR can repress transcription from an nGRE present in the bovine prolactin promoter [32]. This nGRE (PRL3) is recognized by two homoeodomain-containing proteins, namely Pbx and Pit-1/GHF-1 in pituitary cells, or Pbx and octamer transcription factor-1 (Oct-1) in non-pituitary cells ([22,33]; but see [33a]). Both proteins contribute to the enhanced expression from the PRL3 element, and Pbx is required for GC repression to occur ([22,33]; but see [33a]). The involvement of the Pbx in GC-mediated repression led us to speculate whether Pbx also is a target for the GR in other promoter contexts. To investigate this, we analysed the effect of the GR on the activity from the Pbx1/HOXB1-binding b1-ARE. We demonstrate here that, in contrast with the repressive effect of GR on the Pbx-containing PRL3 nGRE, the GR enhances the Pbx1/HOXB1-mediated activity from the b1-ARE, most likely by acting as a co-activator or modulating cofactor interaction via a protein–protein contact with Pbx1.

EXPERIMENTAL

Plasmids

The luciferase reporter construct pAdMLARE containing the complete b1-ARE and the pAdMLR3 containing three copies of repeat 3 from the b1-ARE are described elsewhere [27]. The pAdML reporter lacking the b1-ARE was created by removing the autoregulatory element from the pAdMLARE by cutting with restriction endonucleases *Hin*dIII and *Xho*I, followed by religation. The GR expression vectors pSVGR1, GR_{nx}, GTG, R488Q, K490E and $GR\Delta\tau_1$ have been described previously [11]. HOXB1 (pSG-HOXB1) and Pbx1a (pBK-RSVPbx1a) expression vectors used in our co-transfection experiments were from Di Rocco et al. [27] and Kagawa et al. [34] respectively.

Cell culture and transient transfections

Mouse P19 EC cells were grown as monolayers in Dulbecco's modified Eagle medium ('DMEM') supplemented with 5% (v/v) fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin (all from Gibco BRL) at 37 °C in 10% CO_2. For transfection, P19 EC cells were plated at 30% confluence in 24-well plates overnight, and the next day cells were transfected with 200 ng of reporter plasmid by Lipofectin® following the protocol of the manufacturer (Gibco BRL). As the P19 EC cells contain very low amounts of endogenous GR, they were co-transfected with 50 ng of wild-type or mutant GR expression plasmid. In some cases, 25 ng of HOXB1 and/or Pbx1a expression vectors were co-transfected. After 6 h incubation at 37 °C in 10% CO_2, the medium containing the DNA/Lipofectin® mix was removed, and fresh medium supplemented with 5% fetal calf serum containing the indicated concentrations of dexamethasone (Dex; Sigma) and/or RA (Sigma) was added. To cells not treated with hormone, the same amount of solvent was added [final concentration 0.01% (v/v) ethanol or DMSO]. After incubation for 16–18 h, the cells were lysed and luciferase activity was determined as described previously [11]. Each experimental condition was measured in triplicates or quadruples, and the values shown indicate the means $\pm$ S.D. from two or three different experiments. Since RA affected the expression of several tested internal transfection controls in the P19 EC cells, including rous sarcoma virus ('RSV')-galactosidase, cytomegalovirus ('CMV')-galactosidase and simian virus 40 ('SV40')-alkaline phosphatase, these were excluded. Instead, to guarantee equal transfection efficiency, transfections were performed in 10 cm plates, after which the cells were trypsin-treated, mixed and divided into 24-well plates, followed by hormone treatment. In addition, cell extracts were analysed for equal protein concentration.

Establishing P19 EC GR stable cell lines

For establishing P19 EC cells stably expressing GR, cells were transfected as described above with 100 ng of pSVGR1 expression plasmid and 10 ng of the neomycin (Neo) resistance expression plasmid pSV-Neo. After 6 h incubation at 37 °C in 10% CO_2, the medium containing the DNA/Lipofectin® mix was removed, and fresh medium was added. After 24 h, the cells were trypsin-treated and diluted 10 times into complete growth medium containing 250 μg/ml G418. Clones were isolated and screened for GR expression by Western blotting using the GR antibody 250 [11]. The stable cell clones used contained approximately 50000 GR molecules per cell as determined by ligand binding assay.

Nuclear extract preparation, Western blot analysis and electrophoretic mobility-shift assays (EMSA)

GR-transfected P19 EC cells treated with the indicated hormones for 18 h were washed with PBS and harvested by scraping into 1 ml of PBS using a rubber policeman. Nuclear extracts from the cell pellets were prepared as described by Gough [35]. Nuclear extract (25 μg) was mixed with an equal volume of 2 $\times$ SDS buffer containing Bromophenol Blue and boiled for 5 min, before

SDS/PAGE (10 % polyacrylamide gels). Gels were blotted on to Hybond-C Extra nitrocellulose membrane (Amersham Biosciences). To check for equal protein loading/transfer, the membranes were stained with Ponceau S solution (Sigma). The membranes were blocked with 10 % dried milk powder in PBS, and probed with a 1:1000 dilution of Pbx (C-20; Santa Cruz Biotechnology) or HOXB1 (PRB-231P, CRP Inc.) antiserum. Membranes were subsequently incubated with goat anti-(rabbit-horseradish peroxidase) secondary antibody (Amersham Biosciences) and developed using the ECL® system (Amersham Biosciences) for 30 s to 15 min, depending on signal intensity. Non-specifically appearing bands were used as controls, in addition to the Ponceau S staining, to guarantee equal protein loading and transfer.

For EMSA, oligonucleotides encompassing the sequence motif repeat 3 element (R3), a subregion of b1-ARE known to bind Pbx and HoxB1 [27,34], or 2 × GREII from the tyrosine aminotransferase gene [10,11] were end-labelled by [γ-^{32}P]ATP (3000 Ci/mmol; Amersham Biosciences) using T4 polynucleotide kinase. DNA binding reactions of 20 μl were allowed to proceed in buffer containing 20 mM Tris/HCl, pH 8.0, 10 % (w/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 μg of poly(dI-dC) (Amersham Biosciences), 25 mM KCl, 3 % (w/v) BSA, 0.1–0.3 ng of radiolabelled GRE or R3 and 5 μg of nuclear extract. Binding reactions were carried out at room temperature for 20 min. Where indicated, a 50-molar excess of unlabelled GRE or R3 element was included in the binding reaction. For supershift experiments, 1 μl of polyclonal antibody was added during the incubation period (for the antibody source, see above). A polyclonal anti-Sp1 antibody [X-7(X); Santa Cruz Biotechnology] was used as control. Free and bound DNA were separated on a 4 % polyacrylamide gel, which was run at a constant voltage of 200 V in 22 mM Tris/borate/0.5 mM EDTA, pH 8.5.

Immunoprecipitation experiments

Nuclear extracts from Dex and RA-treated P19 EC cells and RA- and/or Dex-treated P19GR stable cells were prepared by lysing the cells in 200 μl of Buffer A [10 mM Hepes (pH 7.5)/1.5 mM $MgCl_2$/10 mM KCl/0.5 mM DTT/0.5 % Nonidet P40]. After microcentrifugation for 10 s, nuclear pellets were extracted with 200 μl of Buffer B [20 mM Hepes (pH 7.5)/0.2 M NaCl/0.5 mM DTT/25 % (v/v) glycerol/0.5 mM PMSF/0.2 mM EDTA]. Samples were diluted to the same protein concentration with Buffer B, and then diluted further 4-fold with ETG buffer [20 mM Tris/HCl (pH 7.4)/2 mM EDTA/10 % glycerol/2 mM 2-mercaptoethanol] to approx. 800 μl. This extract was incubated with 50 μl of the anti-GR antibody 250 coupled with cyanogen-bromide-activated Sepharose (Amersham Biosciences) pretreated with 10 % dried milk powder and washed once in ETG buffer. The nuclear extract 250/Sepharose mixture was kept for 18 h at 4 °C under continuous rotation. The 250/Sepharose mixture was then washed three times in ice-cold ETG buffer containing 50 mM NaCl. Proteins bound to the 250/Sepharose were eluted with 40 μl of ETG/1 M NaCl (incubated on ice for 1–2 h with occasional stirring), and the supernatant (eluate), after spinning down the 250/Sepharose, was mixed with 2 × SDS buffer and analysed by SDS/PAGE (10 % gels), before blotting and analysis using the anti-GR and anti-Pbx antibodies, as described above. An aliquot of the nuclear extracts prior to the incubation with the 250/Sepharose mixture was also analysed by Western immunoblotting to check for the input of GR and Pbx respectively. The bands were detected using the ECL® chemiluminescent detection system (Amersham Biosciences). In the case of analysing Pbx-associated proteins, immunoprecipitation was performed as above, except that 20 μl of the Pbx antibody was bound to Protein A–Sepharose (Amersham Biosciences).

Statistical analysis

P values for comparison of samples were assessed using the two-tailed *t*-test for two samples with equal variance using the Microsoft Excel program.

RESULTS

GCs enhance RA-induced expression of HOXB1 in P19 EC cells

We have identified previously a capacity of GR to interfere with the transcriptional activity of the homoeodomain-containing protein Pbx binding to an nGRE in the bovine prolactin promoter ([22,33]; but see [33a]). This raised the possibility that GCs will also interfere with the transcriptional control of other promoter elements regulated by Pbx. To test this hypothesis, we analysed the effect of GCs on the *Hoxb*-1 gene, which, in addition to RAREs, contains in its promoter the autoregulatory element b1-ARE recognized by the Pbx1 and HOXB1 homoeodomain proteins [25,27]. Western blot experiments demonstrated that RA treatment of P19GR cells resulted in increased expression of HOXB1 protein (Figure 1A). Dex treatment in itself did not result in an increased HOXB1 expression (Figure 1A, compare lanes 1 and 2). However, co-treatment with RA and Dex synergistically activated HOXB1 expression (compare lanes 3 and 4, or 5 and 6). Although RA also induced Pbx expression, co-treatment with Dex did not result in further expression (Figure 1B). These experiments demonstrated that GCs potentiate RA-induced HOXB1 expression in P19 cells.

GCs potentiate RA-induced transcription through the b1-ARE

In order to investigate whether the synergistic effect by Dex on the RA-induced HOXB1 expression was mediated through the b1-ARE in the *Hoxb*-1 promoter, luciferase-based reporter genes containing (pAdMLARE) or lacking (pAdML) the b1-ARE

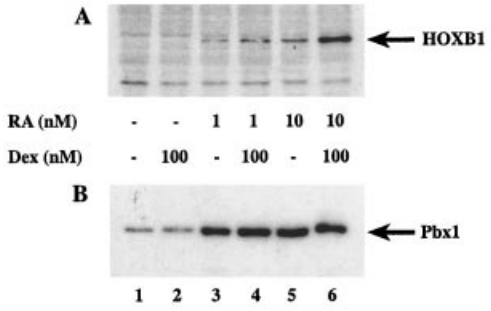

Figure 1 Activation of endogenous HOXB1 expression synergistically by Dex and RA

P19GR cells were treated with the indicated concentrations of hormones for 20 h and analysed by Western immunoblotting for endogenous HOXB1 (**A**) and Pbx1 (**B**) expression, as described in the Experimental section.

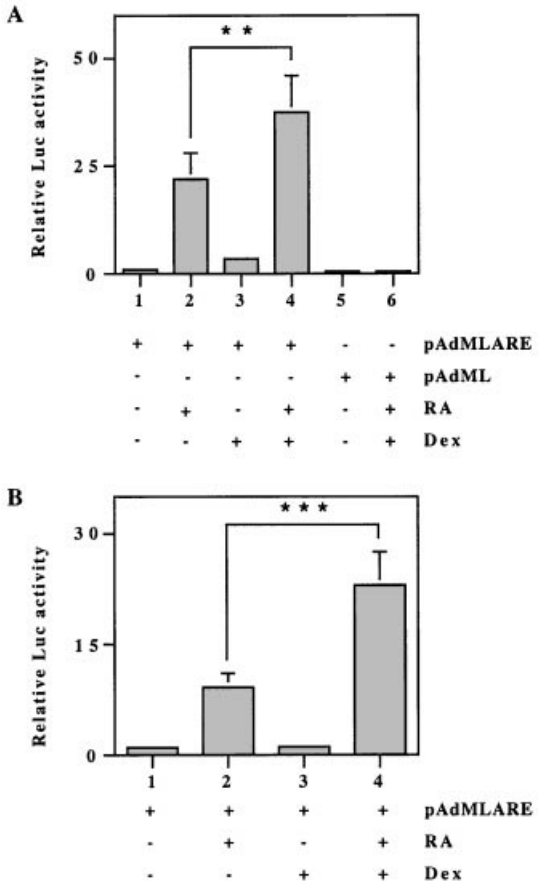

Figure 2 Enhancement by GCs of RA-induced transcription through the b1-ARE

(**A**) The b1-ARE-containing reporter plasmid pAdMLARE (200 ng; lanes 1–4) or pAdML lacking the b1-ARE (200 ng; lanes 5 and 6) were transiently transfected into P19 EC cells, together with an expression vector for wild-type GR (pSVGR1; 50 ng; lanes 1–6) into parent P19 EC cells, as described in the Experimental section. Following transfection, cells were stimulated with or without 1 μM Dex and/or 1 μM RA, as shown in the Figure. After 16 h of incubation, cells were lysed and analysed for luciferase (Luc) activity. Luc activities were related to the activity for pAdMLARE in the absence of hormone. Each bar represents the mean $\pm$ S.D. (**$P < 0.01$ comparing lane 2 with lane 4; $n = 4$). (**B**) As in (**A**), but in this case P19 EC cells stably transfected with the pSVGR1 expression vector were used (***$P < 0.001$ comparing lane 2 with lane 4; $n = 3$). The experiment was repeated three times (**A**) or twice (**B**), yielding similar results.

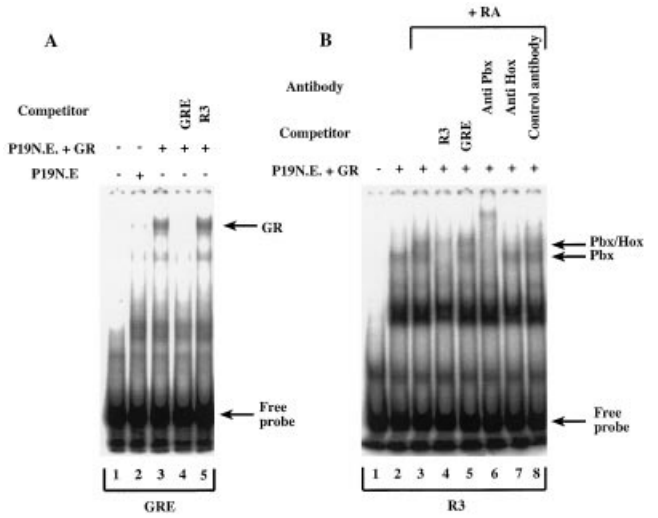

Figure 3 The GR does not bind to the R3 element

Radiolabelled double-stranded oligonucleotides [GRE (**A**) or R3 (**B**)] were incubated with 5 μg of nuclear extract (N.E.) from non-transfected or GR-transfected P19 EC cells treated with 1 μM Dex and, when indicated, with 1 μM RA for 16 h, and analysed by EMSA. Competition experiments were performed with a 50-fold excess of unlabelled R3 or GRE, as indicated in the Figure. When indicated, 1 μl of polyclonal anti-Pbx, anti-HOXB1 or control antibody (anti-Sp1) was added during the incubation.

enhancer element were transfected into P19 EC cells transiently (Figure 2A) or stably (Figure 2B) expressing GR. Following transfection, the cells were treated with RA or Dex, or with a combination of the two hormones. RA treatment of the cells induced pAdMLARE activity approx. 20-fold in the P19 EC cells transiently expressing GR (Figure 2A, lane 2) and 9-fold in the P19GR cells stably expressing GR (Figure 2B, lane 2). The induction was dependent on b1-ARE, since no RA induction of pAdML was observed (Figure 2A, lane 6). Dex in itself had no, or only a very minor, effect on pAdMLARE (Figures 2A and 2B, lanes 3). However, co-treatment with RA and Dex (Figures 2A and 2B, lanes 4) activated pAdMLARE almost 40-fold in the transiently transfected cells and 23-fold in the stably transfected P19 EC cells, demonstrating a synergistic activity. The synergy required the b1-ARE because no effect was seen on the pAdML reporter gene (Figure 2A, lane 6). Furthermore, in the absence of the GR, no synergy was observed (results not shown). Similar results were produced when one of three related Pbx1/HOXB1 binding sequence motifs (R3) present in the b1-ARE was used to drive the reporter gene (pAdMLR3 [27]; also see Figure 5B). Taken together, these results suggest that GCs potentiate RA-induced HOXB1 expression through the Pbx1/HOXB1 binding-sequence motifs in b1-ARE.

The GR does not bind to the R3 element

The sequence motif repeat 3 (R3), a subregion of the b1-ARE known to bind Pbx and HOXB1 [25,27], was sufficient to confer the RA/Dex synergy (see above, and Figure 5B). To investigate whether GC potentiation of the RA effect was mediated via binding of the GR to the R3 motif, nuclear extract from P19 EC cells transfected with the GR expression vector and treated with Dex was incubated with labelled GRE oligonucleotide and analysed by EMSA. As shown in Figure 3(A), incubation with nuclear extract from GR-transfected cells resulted in a GR–GRE complex (lane 3), which was absent in P19 EC cells not transfected with the GR expression vector (lane 2). The specificity of the complex was demonstrated further by adding an excess of unlabelled GRE to the incubation mixture (lane 4). In contrast, an equal molar excess of the R3 element had no effect on the GR–GRE complex (lane 5), demonstrating that the GR has no affinity for binding to R3.

To confirm the lack of GR binding to the R3 element, GR-containing nuclear extracts from cells treated with Dex were also incubated with labelled R3. Two specific R3 complexes could be detected that were competed with an excess of unlabelled R3, but not with GRE (Figure 3B, compare lanes 4 and 5 with lane 3). The inability of an excess of unlabelled GRE oligonucleotide to compete away the two specific R3 complexes demonstrated that the Dex–GR complex had no affinity for binding to R3. It is noteworthy that the slower migrating complex of the two was formed in the presence of RA (compare lanes 3 and 2). Supershift

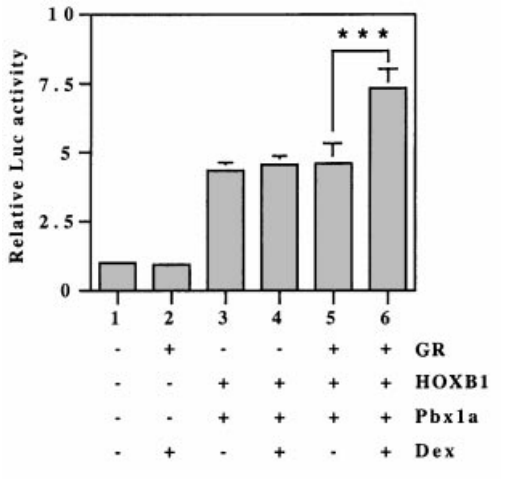

Figure 4 Enhancement of the transcriptional activity of HOXB1/Pbx1 by GR

The reporter gene pAdMLARE (200 ng) was co-transfected with expression vectors for wild-type GR (50 ng), Pbx1a (25 ng) and HOXB1 (25 ng) into P19 EC cells, as indicated in the Figure. Following transfection, cells were, when indicated, stimulated with 1 μM Dex for 16 h, after which luciferase (Luc) activity was measured. Luc activities were related to the activity for pAdMLARE in the presence of GR, but in the absence of hormone (lane 1). Each bar shows the mean $\pm$ S.D. (***$P < 0.001$ comparing lane 5 with lane 6; $n = 3$). Similar results were obtained in two independent experiments.

experiments using anti-Pbx1 antibody (lane 6) or anti-HOXB1 antibody (lane 7) revealed that the slower migrating complex contained Pbx and HOXB1, whereas the faster non-RA-inducible complex contained Pbx, but not HOXB1. This correlates with the former observation that HOXB1 expression is seen only after RA treatment, whereas a basal Pbx1 expression is seen also prior to exposing the cells to RA (see Figure 1B). In summary, these experiments showed that the GC potentiation of the RA effect is not mediated through a direct interaction of GR with the R3 element.

Pbx1a/HOXB1-stimulated transcription from the b1-ARE is potentiated by GCs

Several studies have demonstrated that transcriptional regulation via the b1-ARE involves binding and co-operative interaction between the Pbx1 and HOXB1 proteins [25,27]. In order to investigate whether indeed the b1-ARE and the Pbx1–HOXB1 complex, and not other factors induced by RA, were the targets for the GR-mediated synergy, we transiently transfected P19 EC cells with expression vectors for Pbx1a, HOXB1 and GR, together with the pAdMLARE reporter gene. As can be seen in Figure 4, co-transfection with Pbx1a and HOXB1 resulted in an approx. 4-fold stimulation of pAdMLARE reporter gene activity. Transfection with either Pbx1a or HOXB1 alone, or with one of them alone together with the GR in the presence of Dex, did not result in enhanced or synergistic expression (results not shown; see also [27]). However, the synergistic effect was reconstituted following Dex treatment of cells transfected with Pbx1a–HOXB1 and GR (Figure 4, lane 6), suggesting that the Pbx1a–HOXB1 heterodimer is the target for the GC effect. No effect of the proteins on the pAdML reporter lacking the b1-ARE was seen (results not shown).

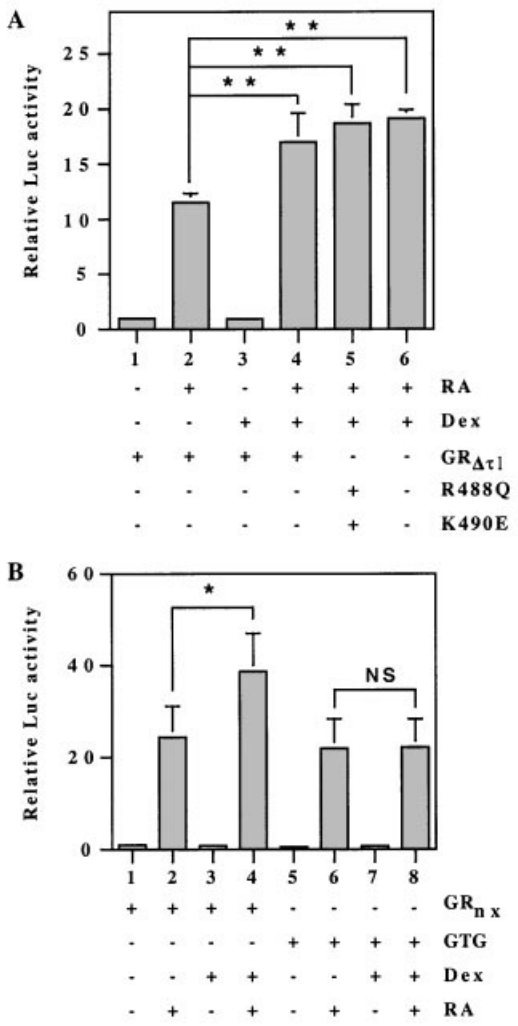

Figure 5 Requirement of the DBD, but not the transcriptional activity, of the GR for the synergistic effect

(**A**) The reporter plasmid pAdMLARE (200 ng) was co-transfected with expression vectors coding for various GR mutants, GR$\Delta\tau$1 (50 ng), R488Q (50 ng) and K490E (50 ng) into P19 EC cells, as shown in the Figure. Following transfection, cells were stimulated with or without 1 μM Dex and/or 1 μM RA for 16 h. Each bar represents the mean $\pm$ S.D. (**$P < 0.01$ for lane 2 compared with lanes 4, 5 or 6; $n = 3$). Similar results were obtained in two independent experiments. (**B**) Experiments were performed as in (**A**), except that the reporter plasmid pAdMLR3 (200 ng) was co-transfected with expression vectors for wild-type GR (GR$_{nx}$; 50 ng) or a GR in which the GR DBD has been exchanged with the DBD of TRβ (GTG; 50 ng). Luciferase (Luc) activities were related to the activity of the reporter plasmid in the absence of hormones. (*$P < 0.05$ for lane 2 compared with lane 4; $n = 3$). 'NS' denotes no significant difference between lanes 6 and 8. Similar results were obtained in two independent experiments.

The DBD, but not the transactivation function of the GR, is required for the RA/GC synergy on the b1-ARE

A second possibility for the GC/RA synergy through the b1-ARE is that GCs induce the expression of a protein, e.g. a co-activator that will enhance the transcriptional activity of Pbx1 and HOXB1. In order to test this, we used a GR devoid of its major transactivation domain τ_1 (GR$\Delta\tau_1$) localized in the N-terminal domain and two GR point mutants that have lost their ability to transactivate target genes via GREs. The two trans-activation-deficient mutants used contain point mutations of a

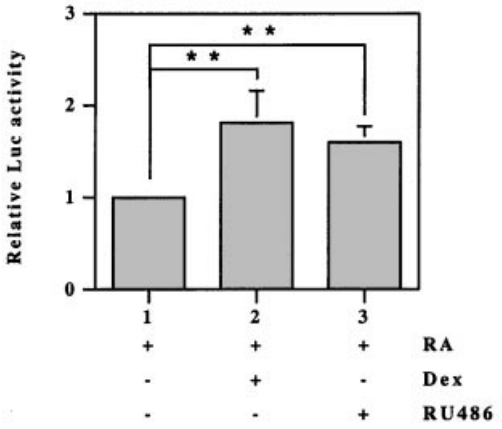

Figure 6 Enhancement of RA-induced transcriptional activity of the b1-ARE by the GC antagonist RU486

The reporter plasmid pAdMLARE was co-transfected with wild-type pSVGR1 into P19 EC cells, as described in the Experimental section. Following transfection, cells were stimulated with or without 1 μM RA and/or 1 μM Dex or 100 nM RU486 for 16 h, as indicated in the Figure. All luciferase (Luc) activities were related to the activity for pAdMLARE in the presence of RA, but in the absence of Dex/RU486. Each bar represents the mean $\pm$ S.D. ($^{**}P < 0.01$ for bar 1 compared with bars 2 or 3; $n = 3$). Similar results were obtained in two independent experiments.

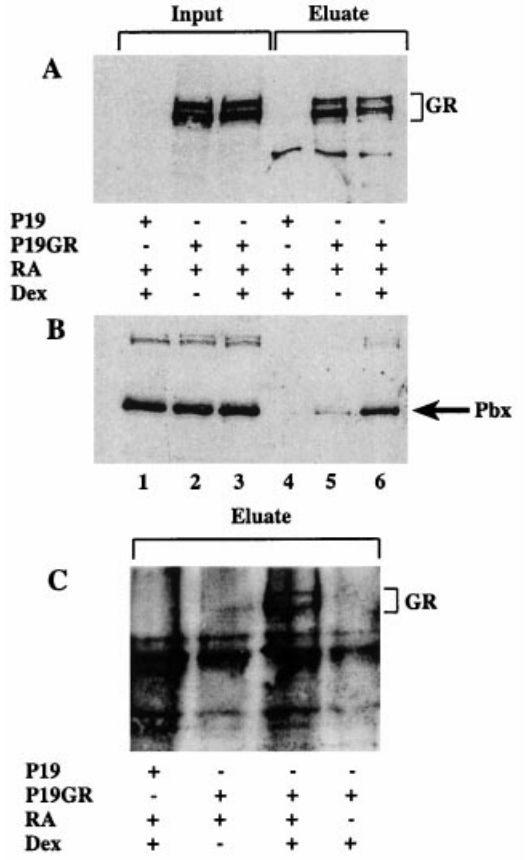

Figure 7 Physical interaction of GR with Pbx *in vivo*

Nuclear extracts from parent P19 or P19 cells stably transfected with a GR expression vector (P19GR), treated with or without Dex and/or RA, were analysed by immunoprecipitation with an anti-GR antibody, followed by Western immunoblotting using an anti-GR (**A**) or anti-Pbx (**B**) antibody, as described in the Experimental section. Lanes 1–3 shows the amount of GR and Pbx present in the nuclear extracts prior to immunoprecipitation, whereas lanes 4–6 show the amount of GR and Pbx bound to the anti-GR antibody following immunoprecipitation. (**C**) Western blot analysis of GR in the eluates of immunoprecipitates using a Pbx antibody. Lane 1, P19 cells treated with RA and Dex; lane 2, P19GR cells treated with RA alone; lane 3, P19GR cells treated with RA and Dex; and lane 4, P19GR cells treated with Dex alone.

conserved arginine or lysine in the second zinc finger of the GR DBD ($Arg^{488} \rightarrow Gln$ and $Lys^{490} \rightarrow Glu$) [11]. Interestingly, when P19 EC cells were transfected with either GR$\Delta\tau_1$, Arg^{488}Gln or Lys^{490}Gln, the RA/Dex synergy of pAdMLARE activation remained intact (Figure 5A), demonstrating that the RA/Dex synergy does not involve enhanced expression of a second protein involved in the regulation of pAdMLARE activation. Furthermore, the major transactivation domain τ_1 of GR is not required for the synergy to occur.

In an attempt to elucidate further which domain of the GR was involved in the synergistic effect, we analysed the ability of a GR in which the DBD of the GR had been exchanged for the DBD of the thyroid hormone receptor (TR) β to enhance transcriptional activity of HOXB1 and Pbx1. This construct, termed GTG, is unable to transactivate from a GRE, but transactivates from a palindromic thyroid response element [36]. Interestingly, the RA/Dex synergy was not observed when the wild-type GR was replaced by GTG, illustrating that the DBD of the GR is necessary for the synergistic effect (Figure 5B).

The GR antagonist RU486 also mediates synergy with RA signalling via the b1-ARE

In order to investigate the effect of a GR antagonist on the RA signalling from the b1-ARE, P19 EC cells transfected with the expression vector for the wild-type GR and the reporter gene pAdMLARE were treated with RA either in the absence of GR ligands or in the presence of Dex or the GR antagonist RU486 respectively. Interestingly, as shown in Figure 6, the GR antagonist RU486 was, like the agonist Dex, able to mediate the synergy with RA signalling from the b1-ARE. This suggests that the C-terminal activation domain (AF2) is not necessary for the synergy between the GR and the homoeodomain proteins, because this activation domain is only functional in the presence of an agonist [37].

The GR physically interacts with Pbx *in vivo*

The transfection experiments and the lack of GR binding to the R3 element suggested that the effect may be mediated by an interaction between the GR and the b1-ARE-binding proteins. A possible intracellular association of endogenous Pbx with GR *in vivo* was examined by co-immunoprecipitation. Nuclear extracts from both parental P19 EC cells and P19 cells stably expressing the GR (P19GR) were immunoprecipitated with anti-GR antibodies and the precipitates were analysed for GR (Figure 7A) and Pbx (Figure 7B) by Western immunoblotting. As shown in Figure 7(A), GR was detected only in P19GR nuclear extract ('Input', lanes 2 and 3) and precipitates from these cells ('Eluate', lanes 5 and 6), whereas no precipitated GR was found in the parental P19 cells (lanes 1 and 4). Only a minor amount of Pbx co-precipitated with GR in the absence of Dex, but the amount increased dramatically when the cells had been treated with Dex (Figure 7B, compare lanes 5 and 6). No Pbx was precipitated in cells lacking GR (Figure 7B, lane 4). Notably, the amount of Pbx present in the parental and stably GR expressing P19 cells

were identical (Figure 7B, 'Input', lanes 1–3). Conversely, co-immunoprecipitation using a Pbx antibody demonstrated that Pbx was able to 'pull down' the GR, but only in the presence of RA and Dex (Figure 7C). These results demonstrate that the GR can interact with Pbx *in vivo*, thus explaining the functional synergy on the b1-ARE.

DISCUSSION

In the present paper we have demonstrated a cross-talk between RA and GC signalling on the *Hoxb*-1 gene that is mediated through an interaction between the GR and a Pbx1–HOXB1 complex binding to the b1-ARE. This results in a potentiation by GCs of the ability of RA to induce HOXB1 expression. GCs by themselves showed no stimulatory effect on HOXB1 expression. The co-operativity could be confirmed on reporter genes under the control of isolated b1-ARE or the repeat 3 (R3) motif, the latter a subregion of the b1-ARE known to bind Pbx1–HOXB1 [25,27]. We also demonstrated that the co-operativity was not mediated via an interaction of the GR with the R3 element. This was not unexpected, since no sequence similarity with a GRE can be found in R3 (or in b1-ARE). Because the b1-ARE and R3 elements interact with the RA-induced Pbx1 and HOXB1 proteins [25,27], we hypothesized that the GC effect instead is mediated via these proteins. We obtained several experimental results that support such a conclusion. GR-mediated synergy could be reconstituted by expressing directly the Pbx1 and HOXB1 proteins. This excludes the possibility that an unknown RA-activated or -induced factor is the target for the GR. This also argues against an interaction between the GR and the RA receptor as the mechanism for the observed synergy. In addition, our results exclude GC-induced expression of Pbx1–HOXB1 (Figure 1) or an unknown factor as the cause for the GC potentiation of the RA effect. The latter possibility can be excluded because co-operativity was still seen using either GR mutants devoid of transactivation capacity or the GR antagonist RU486. Instead, we suggest a mechanism for the co-operativity in which GR acts as a co-activator itself for the Pbx1–HOXB1 proteins by directly or indirectly interacting with these proteins. Alternatively, if the GR, in itself, does not act as a cofactor for the Pbx1–HOXB1 complex, the GR-mediated synergy may be mediated by a GR-dependent recruitment of additional cofactors or stabilization of their interaction. Like nuclear hormone receptors, the Pbx1–HOXB1 complex has recently been shown to achieve transcriptional activation or repression through differential recruitment of co-activators and co-repressors [31,38]. One such cofactor may be Prep1, which has been shown to form a ternary complex with Pbx1–HOXB1 without Prep1 interacting with the DNA. This will result in enhancement of Pbx1–HOXB1 transcriptional activity in a co-operative manner [39]. A third possibility is that GR represses or displaces transcriptional repressors, e.g. histone deacetylase 2 ('HDAC2') present in the Pbx–HOXB1 complex [38].

The immunoprecipitation experiments support an interaction between the GR and the Pbx1 protein. However, this interaction can be indirect, occurring via HOXB1 or other proteins present in a complex with Pbx–HOXB1, such as Meis/Prep or repressing histone deacetylase/N-Cor factors. To elucidate this will require further experiments. Moreover, the ability to detect a GR–Pbx complex in nuclear extracts only in the presence of Dex does not necessarily indicate a ligand-dependent interaction. Instead, it may reflect a Dex-induced nuclear translocation of GR and hence co-localization of GR and Pbx in the nuclear compartment. Furthermore, we were unable by EMSA to detect a supershift of the Pbx1–HOXB1 complex on the R3 element in the presence of GR (results not shown). This may due to instability of such a complex during EMSA conditions. This is not unlikely, since other well-established GR–protein interactions, e.g. with NF-κB or AP-1, also fail to demonstrate a supershift in EMSA.

It has been suggested previously that the GR can stimulate transactivation by other transcription factors without having to bind to the DNA. This is, for example, the case for AP-1-controlled target genes where the AP-1 complex is composed of a c-Jun homodimer [4]. In addition, GCs enhance prolactin-stimulated transcription of the β-casein gene via the interaction of the GR with STAT5 (signal transducer of transcription 5) [6]. Furthermore, the GR appears to potentiate transcriptional activation by STAT3 from a nuclear factor for interleukin 6 (NF-IL6) recognition sequence [5], or NF-IL6 from a CCAAT/enhancer-binding protein ('C/EBP')-binding site [7].

In the case of the PRL3 nGRE, the GR represses the activity of Pbx and Pit-1 by displacing them from the DNA ([33]; but see [33a]). This is in contrast with the enhancing activity that the GR confers on Pbx1–HOXB1 on the b1-ARE. The reason for this difference in response, despite the fact that the GR in both cases interacts with homoeodomain-containing proteins, is unclear, but could relate to the ability of GR to interact with the PRL3 nGRE, the specific nature of the response element or the particular combination of homoeodomain proteins.

Our experiments demonstrated that the GR DBD is necessary for the GR–Pbx1–HOXB1 synergy to occur, since exchanging the GR DBD with the DBD from the TRβ abolished the co-operativity. This does not necessarily mean that the GR DBD is sufficient for the effect. A function of the DBD could be to bring in an activation domain (present in the GR or in a second protein) or a co-activator that enhances the transcriptional activity. Indeed, the GR DBD has been suggested not only to be involved in DNA binding and homodimer formation, but also in interactions with different proteins, such as AP-1, NF-κB, Oct-1/2, NF-IL6 and Nur77 [7,9,10,40–43].

Interestingly, the GR antagonist RU486, like the agonist dexamethasone, was also able to induce the synergy. As it has been reported that the RU486–GR complex is able to translocate to the nucleus and bind DNA, but not to transactivate from a GRE, these effects may be sufficient for allowing the synergistic effect with the Pbx1–HOXB1 proteins. That these RU486-induced changes in the GR can be enough to confer some of the GR effects is supported by the ability of the RU486–GR to partially repress AP-1 and NF-κB activity in transfection experiments [10,11]. In addition, RU486 has been shown to cause activation of the non-ligand-dependent AF1 activation domain, but not the ligand (agonist)-dependent AF2 activation domain [44].

The ability of steroid/thyroid receptors to interact with a subgroup of homoeodomain-containing proteins, namely POU-homoeodomain proteins, and to modulate transcription of their target genes, has been described previously. For example, interaction of the TR and oestrogen receptor ('ER') with the POU-homoeodomain protein Pit-1/GHF-1 resulted in the synergistic activation of growth hormone and prolactin gene transcription respectively [45,46]. With regard to the GR, interaction with the POU-homoeodomain-containing proteins Oct-1, HoxC4 and Pit-1/GHF-1 has been described [43,47–49], as well as with non-mammalian homoeodomain proteins, such as Dlx2, Hoxd4 and MsxB (from zebrafish) and Ftz and Prd (from *Drosophila*) [49]. In all cases, the highly conserved homoeodomain of these proteins seems to be involved in the interaction with the GR [43,47,49]. The outcome of GR interaction with homoeodomain proteins (in situations where the GR does not bind to DNA

itself) can be repressive [47,48], whereas under other conditions, as one can see for the GR–Pbx1–HOXB1 interaction, it can result in co-operativity [43,49]. The mechanisms explaining the different responses are still unclear.

With regard to the Pbx-family homoeodomain proteins, the present study is the first demonstration of an interaction with a steroid-activated nuclear receptor. Recently, it was demonstrated that the TRα can interact with Pbx1 in thyroid hormone regulation of the malic enzyme gene [20]. Thus co-operative interaction between nuclear receptors and the TALE class of homoeodomain proteins may serve as an important mechanism to differentially control tissue-specific gene expression. It is noteworthy that the GC–RA cross-talk demonstrated in the present study does not involve an interaction with the respective nuclear receptor, but an interaction of the GR with RA-induced gene products. The GR–Pbx1 cross-talk may be an important mechanism contributing to tissue-specific RA responses, and to fine-tune a complex network of hormone responses using a limited number of transcription factors. Finally, as a result of the ubiquitous expression of GR and Pbx proteins [1,15] and the highly conserved homoeodomain regions, one might expect a GC effect on additional genes controlled by Pbx or other homoeodomain proteins. In fact, GR interaction with homoeodomain proteins has been shown to interfere with developmental processes in *Xenopus laevis* and in zebrafish [49,50].

This work was supported by a grant from the Swedish Cancer Society to S.O. This research has also been supported by a Marie Curie fellowship to J.C. of the European Community program 'Quality of Life and Management of Living Resources' under contract number QLK4-CT-2000-51274. We thank Dr M. R. Capecchi (Howard Hughes Medical Institute, University of Utah, Salt Lake City, UT, U.S.A.), Dr M. S. Featherstone (McGill Cancer Centre, McGill University, Montreal, Quebec, Canada) and Dr F. Mavilio (TIGET, Istituto Scientifico H. S. Raffaele, Milan, Italy) for providing plasmids. Dr. A.-C. Wikström and Dr M. Rönnholm (of this department) are acknowledged for providing 250GR-Sepharose. We also thank Dr M. Kamps (University of California School of Medicine, La Jolla, CA, U.S.A.) and Dr F. Mavilio for some valuable comments on our results, and Dr G. Faulds and Dr J. Webster (of this department) for valuable comments on the manuscript. Dr T. Murata is acknowledged for the help with formatting the manuscript.

REFERENCES

1 Newton, R. (2000) Molecular mechanisms of glucocorticoid action: what is important? Thorax **55**, 603–613
2 Aranda, A. and Pascual, A. (2001) Nuclear hormone receptors and gene expression. Physiol. Rev. **81**, 1269–1304
3 Schüle, R., Muller, M., Kaltschmidt, C. and Renkawitz, R. (1988) Many transcription factors interact synergistically with steroid receptors. Science **242**, 1418–1420
4 Teurich, S. and Angel, P. (1995) The glucocorticoid receptor synergizes with Jun homodimers to activate AP-1-regulated promoters lacking GR binding sites. Chem. Senses **20**, 251–255
5 Zhang, Z., Jones, S., Hagood, J. S., Fuentes, N. L. and Fuller, G. M. (1997) STAT3 acts as a co-activator of glucocorticoid receptor signalling. J. Biol. Chem. **272**, 30607–30610
6 Stocklin, E., Wissler, M., Gouilleux, F. and Groner, B. (1996) Functional interactions between Stat5 and the glucocorticoid receptor. Nature (London) **383**, 726–728
7 Boruk, M., Savory, J. G. A. and Hache, R. J. G. (1998) AF-2-dependent potentiation of CCAAT-enhancer-binding protein β-mediated transcriptional activation by glucocorticoid receptor. Mol. Endocrinol. **12**, 1749–1763
8 Schüle, R., Rangarajan, P., Kliewer, S., Ransone, L. J., Bolado, J., Yang, N., Verma, I. M. and Evans, R. M. (1990) Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. Cell **62**, 1217–1226
9 Yang-Yen, H.-F., Chambard, J.-C., Sun, Y.-L., Smeal, T., Schmidt, T. J., Drouin, J. and Karin, M. (1990) Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein–protein interaction. Cell **62**, 1205–1215
10 Heck, S., Kullmann, M., Gast, A., Ponta, H., Rahmsdorf, H. J., Herrlich, P. and Cato, A. C. (1994) A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. EMBO J. **13**, 4087–4095
11 Liden, J., Delaunay, F., Rafter, I., Gustafsson, J. and Okret, S. (1997) A new function for the C-terminal zinc finger of the glucocorticoid receptor. Repression of RelA transactivation. J. Biol. Chem. **272**, 21467–21472
12 Burglin, T. R. (1997) Analysis of TALE superclass homeobox genes (MEIS, PBC, KNOX, Iroquois, TGIF) reveals a novel domain conserved between plants and animals. Nucleic Acids Res. **25**, 4173–4180
13 Knoepfler, P. S. and Kamps, M. P. (1995) The pentapeptide motif of Hox proteins is required for cooperative DNA binding with Pbx1, physically contacts Pbx1, and enhances DNA binding by Pbx1. Mol. Cell. Biol. **15**, 5811–5819
14 Knoepfler, P. S. and Kamps, M. P. (1997) The highest affinity DNA element bound by Pbx complexes in t(1;19) leukemic cells fails to mediate cooperative DNA-binding or cooperative transactivation by E2a-Pbx1 and class I Hox proteins – evidence for selective targetting of E2a-Pbx1 to a subset of Pbx-recognition elements. Oncogene **14**, 2521–2531
15 Monica, K., Galili, N., Nourse, J., Saltman, D. and Cleary, M. L. (1991) PBX2 and PBX3, new homeobox genes with extensive homology to the human proto-oncogene PBX1. Mol. Cell. Biol. **11**, 6149–6157
16 Goudet, G., Delhalle, S., Biemar, F., Martial, J. A. and Peers, B. (1999) Functional and cooperative interactions between the homeodomain PDX1, Pbx, and Prep1 factors on the somatostatin promoter. J. Biol. Chem. **274**, 4067–4073
17 Herzig, S., Fuzesi, L. and Knepel, W. (2000) Heterodimeric Pbx-Prep1 homeodomain protein binding to the glucagon gene restricting transcription in a cell type-dependent manner. J. Biol. Chem. **275**, 27989–27999
18 Penkov, D., Tanaka, S., Di Rocco, G., Berthelsen, J., Blasi, F. and Ramirez, F. (2000) Cooperative interactions between PBX, PREP, and HOX proteins modulate the activity of the α2(V) collagen (COL5A2) promoter. J. Biol. Chem. **275**, 16681–16689
19 Swift, G. H., Liu, Y., Rose, S. D., Bischof, L. J., Steelman, S., Buchberg, A. M., Wright, C. V. E. and MacDonald, R. J. (1998) An endocrine–exocrine switch in the activity of the pancreatic homeodomain protein Pdx1 through formation of a trimeric complex with Pbx1b and Mrg1 (Meis2). Mol. Cell. Biol. **18**, 5109–5120
20 Wang, Y., Yin, L. and Hillgartner, F. B. (2001) The homeodomain proteins PBX and MEIS1 are accessory factors that enhance thyroid hormone regulation of the malic enzyme gene in hepatocytes. J. Biol. Chem. **276**, 23838–23848
21 Bischof, L. J., Kagawa, N. and Waterman, M. R. (1998) The bovine CYP17 promoter contains a transcriptional regulatory element cooperatively bound by tale homeodomain proteins. Endocr. Res. **24**, 489–495
22 Subramaniam, N., Cairns, W. and Okret, S. (1998) Glucocorticoids repress transcription from a negative glucocorticoid response element recognised by two homeodomain proteins, Pbx-1 and Oct-1. J. Biol. Chem. **273**, 23567–23574
23 Pan, L., Xie, Y., Black, T. A., Jones, C. A., Priutt, S. C. and Gross, K. W. (2001) A Abd-B class HOX-PBX recognition sequence is required for expression from the mouse *Ren-1^c* gene. J. Biol. Chem. **276**, 32849–32494
24 Selleri, L., Depew, M. J., Jacobs, Y., Chanda, S. K., Tsang, K. Y., Cheah, K. S., Rubenstein, J. L., O'Gorman, S. and Cleary, M. L. (2001) Requirement for Pbx1 in skeletal patterning and programming chondrocyte proliferation and differentiation. Development (Cambridge) **128**, 3543–3557
25 Popperl, H., Bienz, M., Studer, M., Chan, S. K., Aparicio, S., Brenner, S., Mann, R. S. and Krumlauf, R. (1995) Segmental expression of Hoxb-1 is controlled by a highly conserved autoregulatory loop dependent upon exd/pbx. Cell **81**, 1031–1042
26 Chang, C. P., Shen, W. F., Rozenfeld, S., Lawrence, H. J., Largman, C. and Cleary, M. L. (1995) Pbx proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins. Genes Dev. **9**, 663–674
27 Di Rocco, G., Mavilio, F. and Zappavigna, V. (1997) Functional dissection of a transcriptionally active, target-specific Hox–Pbx complex. EMBO J. **16**, 3644–3654
28 Ogura, T. and Evans, R. M. (1995) A retinoic acid-triggered cascade of HOXB1 gene activation. Proc. Natl. Acad. Sci. U.S.A. **92**, 387–391
29 Knoepfler, P. S. and Kamps, M. P. (1997) The Pbx family of proteins is strongly upregulated by a post-transcriptional mechanism during retinoic acid-induced differentiation of P19 embryonal carcinoma cells. Mech. Dev. **63**, 5–14
30 Oulad-Abdelghani, M., Chazaud, C., Bouillet, P., Sapin, V., Chambon, P. and Dolle, P. (1997) Meis2, a novel mouse Pbx-related homeobox gene induced by retinoic acid during differentiation of P19 embryonal carcinoma cells. Dev. Dynam. **210**, 173–183
31 Saleh, M., Rambaldi, I., Yang, X. J. and Featherstone, M. S. (2000) Cell signaling switches HOX–PBX complexes from repressors to activators of transcription mediated by histone deacetylases and histone acetyltransferases. Mol. Cell. Biol. **20**, 8623–8633
32 Cairns, C., Cairns, W. and Okret, S. (1993) Inhibition of gene expression by steroid hormone receptors via a negative glucocorticoid response element: evidence for the involvement of DNA-binding and agonistic effects of the antiglucocorticoid/antiprogestin RU486. DNA Cell Biol. **12**, 695–702
33 Subramaniam, N., Cairns, W. and Okret, S. (1997) Studies on the mechanism of glucocorticoid-mediated repression from a negative glucocorticoid response element from the bovine prolactin gene. DNA Cell Biol. **16**, 153–163

33a Erratum. (1997) DNA Cell Biol. **16**, 670
34 Kagawa, N., Ogo, A., Takahashi, Y., Iwamatsu, A. and Waterman, M. R. (1994) A cAMP-regulatory sequence (CRS1) of CYP17 is a cellular target for the homeodomain protein Pbx1. J. Biol. Chem. **269**, 18716–18719
35 Gough, N. (1988) Cytoplasmic RNA extraction protocol. Anal. Biochem. **173**, 93–95
36 Thompson, C. C. and Evans, R. M. (1989) Trans-activation by thyroid hormone receptors: functional parallels with steroid hormone receptors. Proc. Natl. Acad. Sci. U.S.A. **86**, 3494–3498
37 Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A. and Carlquist, M. (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. Nature (London) **389**, 753–758
38 Asahara, H., Dutta, S., Kao, H. Y., Evans, R. M. and Montminy, M. (1999) Pbx–Hox heterodimers recruit coactivator–corepressor complexes in an isoform-specific manner. Mol. Cell. Biol. **19**, 8219–8225
39 Berthelsen, J., Zappavigna, V., Ferretti, E., Mavilio, F. and Blasi, F. (1998) The novel homeoprotein Prep1 modulates Pbx–Hox protein cooperativity. EMBO J. **17**, 1434–1445
40 Nishio, Y., Isshiki, H., Kishimoto, T. and Akira, S. (1993) A nuclear factor for interleukin-6 expression (NF-IL6) and the glucocorticoid receptor synergistically activate transcription of the rat α1-acid glycoprotein gene via direct protein–protein interaction. Mol. Cell. Biol. **13**, 1854–1862
41 Caldenhoven, E., Liden, J., Wissink, S., Van de Stolpe, A., Raaijmakers, J., Koenderman, L., Okret, S., Gustafsson, J. A. and Van der Saag, P. T. (1995) Negative cross-talk between RelA and the glucocorticoid receptor: a possible mechanism for the anti-inflammatory action of glucocorticoids. Mol. Endocrinol. **9**, 401–412
42 Drouin, J., Maira, M. and Philips, A. (1998) Novel mechanism of action for Nur77 and antagonism by glucocorticoids: a convergent mechanism for CRH activation and glucocorticoid repression of POMC gene transcription. J. Steroid Biochem. Mol. Biol. **65**, 59–63
43 Prefontaine, G. G., Lemieux, M. E., Giffin, W., Schild-Poulter, C., Pope, L., LaCasse, E., Walker, P. and Hache, R. J. (1998) Recruitment of octamer transcription factors to DNA by glucocorticoid receptor. Mol. Cell. Biol. **18**, 3416–3430
44 Wagner, B. L., Norris, J. D., Knotts, T. A., Weigel, N. L. and McDonnell, D. P. (1998) The nuclear corepressors NCoR and SMRT are key regulators of both ligand- and 8-bromo-cyclic AMP-dependent transcriptional activity of the human progesterone receptor. Mol. Cell. Biol. **18**, 1369–1378
45 Schaufele, F., West, B. L. and Baxter, J. D. (1992) Synergistic activation of the rat growth hormone promoter by Pit-1 and the thyroid hormone receptor. Mol. Endocrinol. **6**, 656–665
46 Day, R. N., Koike, S., Sakai, M., Muramatsu, M. and Maurer, R. A. (1990) Both Pit-1 and the estrogen receptor are required for estrogen responsiveness of the rat prolactin gene. Mol. Endocrinol. **4**, 1964–1971
47 Kutoh, E., Stromstedt, P. E. and Poellinger, L. (1992) Functional interference between the ubiquitous and constitutive octamer transcription factor 1 (OTF-1) and the glucocorticoid receptor by direct protein–protein interaction involving the homeo subdomain of OTF-1. Mol. Cell. Biol. **12**, 4960–4969
48 Adler, S., Waterman, M. L., He, X. and Rosenfeld, M. G. (1988) Steroid receptor-mediated inhibition of rat prolactin gene expression does not require the receptor DNA-binding domain. Cell **52**, 685–695
49 Wang, J. M., Préfontaine, G. G., Lemieux, M. E., Pope, L., Akimenko, M.-A. and Hache, R. J. G. (1999) Developmental effects of ectopic expression of the glucocorticoid receptor DNA binding domain are alleviated by an amino acid substitution that interferes with homeodomain binding. Mol. Cell. Biol. **19**, 7106–7122
50 Gao, X., Stegeman, B. I., Lanser, P., Koster, J. G. and Destree, O. H. (1994) GR transcripts are localized during early *Xenopus laevis* embryogenesis and overexpression of GR inhibits differentiation after dexamethasone treatment. Biochem. Biophys. Res. Commun. **199**, 734–731

Received 27 March 2002/28 October 2002; accepted 17 December 2002
Published as BJ Immediate Publication 17 December 2002, DOI 10.1042/BJ20020471