Steroid Hormones Induce bcl-X Gene Expression through Direct Activation of Distal Promoter P4*

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Bel-X exists in at least five different isoforms with complex effects on programmed cell death. Glucocorticoids and progesterins control bcl-X expression and influence the ratio between bcl-Xs (antiapoptotic isoform) and bcl-Xa (proapoptotic isoform) in different tissues. The 5′-UTR region of the mouse bcl-X gene contains at least five different promoters, which exhibit a tissue-specific pattern of promoter usage. Several mRNAs with different 5′-leading exons can be generated upon promoter activation. Here we explore the potential of the various bcl-X gene promoters to be regulated by glucocorticoids or progesterins. We found that the region located immediately upstream of promoter 4 (P4) contains two hormone response element (HRE)-like sequences at positions −3040 (HRE I) and −3001 (HRE II) relative to the transcription initiation codon. These HRE-like sequences confer hormone responsiveness to a core promoter and bind glucocorticoid or progesterone receptors in vitro. Point mutations of both HREs that prevent steroid receptor binding also eliminate hormonal inducibility. In cells treated with glucocorticoids, the hormone receptor is recruited to the P4 region containing the HREs. Analysis of the products of the endogenous bcl-X in epithelial mammary cells showed that only transcripts originating from P4 increased upon hormone treatment. This observation correlates with the induction of the bcl-Xs mRNA, suggesting that P4 is one of the bcl-X promoters responsible for the generation of this antiapoptotic isoform.

bcl-X is one of the members of Bcl-2 family proteins and plays a critical role in the control of apoptosis. At least five different isoforms produced by alternative splicing of a unique gene have been described previously (1). Some of these isoforms exert opposite effects on programmed cell death (2–5), i.e. the ubiquitous large isoform Bcl-Xl and the tissue-specific isoform Bcl-Xs protect cells against apoptosis (2, 5), whereas the short isoform, Bcl-X, antagonizes cell death inhibition by interacting with Bcl-Xl and Bcl-2 (2). Thus, the control of apoptosis in some cell types could involve changes in the relative proportions of various Bcl-X isoforms, suggesting an accurate regulation not only of transcription but also of splicing of bcl-X transcripts.

Steroid hormones, in particular glucocorticoids and progesterins, control programmed cell death in several tissues. Although the molecular mechanism of hormone-dependent apoptosis is still not completely known, a link between glucocorticoids and genes from the Bcl-2 family has been demonstrated in several systems (6–9). bcl-X has been postulated as a key target gene on hormone-dependent apoptosis. In fact, the steroid hormones, glucocorticoids, and progesterone, have been shown to control bcl-X expression and to influence the ratio between bcl-Xs (antiapoptotic isoform) and bcl-Xa (proapoptotic isoform) in different cell types (6, 7, 9–11). The promoter region of the mouse bcl-X gene exhibits a complex structure. It contains at least five different promoters (P1–P5), which are used in a tissue-specific manner (Fig. 1) (1). Thus, several mRNAs differing in at least their 5′-leading exon can be generated upon alternative promoter usage. Although the physiological relevance of this mechanism is still not clear, it might be involved in the tissue-specific gene expression. Moreover, the activation of a particular promoter might generate specific splice isoforms. In this way, external signals like steroid hormones could influence the outcome of the splice process by regulating promoter choice by their activated hormone receptors.

Steroids exert their action by interacting with their intracellular receptors, which are ligand-dependent transcription factors (12). After hormone binding, the hormone receptor complex regulates the expression of target genes by binding to specific DNA sequences in chromatin called hormone response elements (HREs). In particular, progesterone and glucocorticoid receptors (hereafter denoted PR and GR, respectively) bound to the same palindromic consensus sequence TG-TCTXXXACGAR (12) located in the promoter or enhancer regions of target genes.

To explore the possibility of a direct effect of steroid hormones on the bcl-X gene expression, we performed transient transfection assays of expression vectors containing the luciferase gene under the control of different bcl-X promoter regions. In several cell lines, mainly P4 responds selectively to the synthetic glucocorticoid dexamethasone or to the progesterin agonist R5020. The screening of the region around this promoter revealed the presence of two HRE-like sequences located at positions −3040

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‡ The abbreviations used are: P, promoter; HRE, hormone response element; PR, progesterone receptor; GR, glucocorticoid receptor; cons, consensus; Dex, dexamethasone; EMSA, electrophoretic mobility shift assays; gapdh, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation.
FIG. 1. Mouse bcl-X promoter region structure. The numbers preceded by a negative sign refer to the distance upstream of the translation initiation codon. The dotted lines starting from exons E, D, C, and B and stopping with a cross-line correspond to putative 5'- and 3'-spliced sites according to the primary sequence. ORF, open reading frame; INR, initiator.

FIG. 2. Hormonal control of bcl-X promoter activity. A, bcl-X promoter fragments were subcloned upstream of the luciferase gene as described under “Experimental Procedures.” B–C, COS-1 cells were cotransfected with either 1 μg of GR (B) or 1 μg of PR (C) and 5 pmols of each bcl-X promoter vector. D–E, T47D cells (D) or HC11 cells (E) were transfected with 5 pmols of each bcl-X promoter vector. 3 μg of cytomegalovirus-LacZ vector were also introduced. Cells were incubated for 36 h with ethanol (Control), 10 nM R5020 alone (R5020), or with 0.1 μM RU38486 (R5020+RU), 10 nM Dex alone, or with 0.1 μM RU38486 (Dex+RU), and luciferase activity was measured. After correcting for β-galactosidase activity, the values are expressed as fold induction relative to the controls. The means ± S.D. from three independent experiments are shown. nt, nucleotide; Luc, luciferase.
(HRE I) and ~3001 (HRE II) relative to the translation initiation site. Both HREs differ from the consensus HRE (cons-HRE) by three mismatches. Here we show that recombinant GR and PR bind specifically to a bcl-X oligonucleotide containing HRE I and II sequences. An analysis of the endogenous gene expression showed that only those transcripts generated by the activation of P4 increased their levels upon steroid treatment in mouse epithelial mammary cells. This observation was confirmed by chromatin immunoprecipitation assays, which demonstrated the loading of GR and increased occupancy by the RNA polymerase II at the P4 region after the addition of dexamethasone. P4 activation correlates with the induction of the bcl-X mRNA, suggesting that P4 may be one of the bcl-X promoters responsible for the generation of this antiapoptotic isoform. These results contribute to the understanding of the molecular basis of hormone-dependent apoptosis.

**EXPERIMENTAL PROCEDURES**

**Steroids and Reagents**—R5020 was purchased from PerkinElmer Life Sciences. Dexamethasone (Dex) and RU 38486 (RU) from Sigma were used for all of the hormonal treatments. Hormones were dissolved in absolute ethanol. For in vitro assays, 1000× solutions were prepared. Dulbecco’s modified Eagle medium and fetal calf serum were purchased from Invitrogen. RPMI 1640 medium was purchased from Sigma. Fetal calf serum was previously charcoal-stripped to deplete it of steroid hormones (13).

**Expression Vectors**—The vector pGAW (kindly provided by Dr. Guntram Suske, IMT, Philips Universitaet, Marburg, Germany) is a derivative plasmid from pGL3-basic (Promega). This vector was used to subclone the bcl-X promoter fragments upstream of the luciferase gene. The expression vector P1 contained a genomic sequence of mouse bcl-X gene from nucleotides ~594 to ~95 relative to the translation initiation codon. It was generated by subcloning a HindIII–BamHI fragment from pNM1–9 SalI plasmid (11) into pGAW. Vector P5–P2 contained a genomic sequence from nucleotides ~3420 to ~537. This fragment includes from P5 to P2 a promoter region. This vector was generated by digesting a 2884-bp fragment of pNM1–9EagI (1) with SacI enzyme and cloning it into SacI site of pGAW. The vector P2 contained the P2 promoter region from nucleotides ~1000 to ~537, and it was generated by digestion of P5–P2 with EcoRI enzyme followed by religation. P3 vector was generated by amplifying from pNM1–9 SalI plasmid, a fragment of 497 bp with the oligonucleotides 5’-CATGGAATTCGGTACAGTATGCTA-CATAG-3’ and 5’-CGGCTCGAGTTCCTTTCCATCCACTG-3’ as forward and reverse primers, respectively. The PCR product was cloned into the EcoRI and XhoI sites of pGAW. Vector P4 “core" was generated by digesting P4-P3 vector with XhoI enzyme followed by Klenow treatment and then by digestion with SspI enzyme followed by religation. Vector P4-P3 was generated by subcloning an EcoRI-XhoI fragment from P5–P2 into pGAW. Vector P4 “extended" was generated by digestion of P5–P2 with BamHI enzyme followed by religation. The obtained vector was then digested with XhoI enzyme followed by Klenow treatment. This vector then was cut with SspI enzyme and religated. Vector P4-extended AHRE was generated by cutting from P4 extended, a fragment of 95 bp between the nucleotides ~3045 and ~2950 with XmnI and MscI enzymes followed by Klenow treatment and religation.

**Cell Cultures and Transfection Assay**—Cells were cultured at 37 °C under humidified atmosphere with 5% CO₂ in p100 plates. COS-1 cells were grown in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum containing penicillin (100 IU/ml), streptomycin (100 μg/ml), and glutamine (2 mM). Mammary epithelial T47D and HC11 (the latter one was kindly provided by Dr. Nancy Hynes, Basel, Switzerland) were grown in RPMI 1640 medium plus 10% fetal calf serum and 1% penicillin/streptomycin. 5 μg/ml insulin were added to HC11 cell cultures. For transient transfections, 5 × 10⁵ cells plated in 60-mm plates were transfected with Lipofectin 2000 (Invitrogen) following the instructions of the manufacturer. 5 pmol of each expression vector expressing luciferase under the control of bcl-X promoters were transfected into HC11, T47D, or COS-1 cells. 1 μg of PR (expressing human progesterone isoforn B receptor) (14) or GR (expressing human glucocorticoid receptor) (15) was cotransfected into COS-1 cells. 3 μg of pCMV-LacZ were also introduced as control of transfection. The plasmids were diluted in 100-μl medium and added dropwise to an equal volume of medium containing 4 μl of Lipofectin 2000. After 20 min, the transfection mixture was added dropwise to the cells. 6 h later, the medium was replaced by medium containing 10% charcoal-stripped fetal calf serum and the antibiotics described above and incubated overnight at 37 °C in 5% CO₂ atmosphere. The cells were then incubated with the corresponding steroids for 36 h. After incubations, cells were harvested in lysis buffer (Promega, catalog number E3871) and luciferase activity was measured with luciferase kit according to manufacturer protocol (Promega, catalog number E1501). β-Galactosidase activity was measured as described previously (16).

**In Silico Analysis**—Screening for the potential HREs was performed using MatInspector software (17).

**Electrophoretic Mobility Shift Assays (EMSA)**—EMSA were performed with the synthetic oligonucleotides: cons-HRE (5’-TCCAGTTG- CCTAGAAGAAACACTGTGCTCAAC-3’) (18); bcl-X HRE (5’-GA- GTTGAAAACAACTTTCTGTTGCTCCATCCACATGGGCTCAGCTC-TGCCGAGCACACAAATTTCAAGTCAGGAGGG-3’); and mutated bcl-X HRE(mut) (5’-GAGTTTGGAAAATCTAGTATGCTATTTCCACAT-
EXT, extended.

A DNA complex is plotted as the percentage of the values in the absence of competitor. The amount of PR/H18528 were incubated with the labeled DNA and the PR/H18528 DNA complex was supershifted by a monoclonal GR antibody (lanes 6) but not by a nonspecific oligonucleotide (lanes 8). The GR-DNA complex was supershifted by a monoclonal GR antibody (lane 9). The arrows indicate the free DNA, the GR-DNA, and the GR-DNA antibody complexes. C. Increasing amounts (0-, 10-, 20-, 100-, 200-, and 500-fold molar excess) of unlabeled bcl-X HREs competed with the 32P-labeled bcl-X HRE for binding to PR (lanes 2–8). The arrows indicate the free DNA and the PR-DNA complex. D. 32P-labeled cons-HRE was incubated with increasing amounts of either cons-HRE or bcl-X HREs to measure their relative affinities to PR. The amount of PR-DNA complex is plotted as the percentage of the values in the absence of competitor. AB, antibody; EXT, extended.

GGGCTCACTTCCACATACACCGATTTTCAAGGGG-3') as described previously (19). The complementary strands were annealed in equimolar amounts (100 pmol each) in annealing buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 30 mM KCl) by denaturation at 95 °C for 5 min and cooling down to room temperature. Double-stranded oligonucleotides were radiolabeled with T4 polynucleotide kinase and [γ-32P]ATP. Recombinant PR and GR were expressed in baculovirus and purified as described previously (19). Nuclear extracts containing PR were prepared from T47D cells treated for 2 h with 10 nM R5020 as described previously (20). Binding reactions were carried out in 30-μl reaction buffer containing 10 mM Tris-HCl, pH 8, 0.5 mM EDTA, 5% glycerol, 0.5 mM 2-mercaptoethanol, 90 mM NaCl, 1 μg of poly(dI-dC), 50 ng of radiolabeled probe, 100 ng of calf thymus DNA, and 3 μg/μl bovine serum albumin. 1–8 μl aliquots of the nuclear extract or 15–120 ng of recombinant PR/GR were added to the binding reaction and incubated for 30 min at room temperature. Specific competition assays were performed by adding 10–500-fold molar excess of unlabeled bcl-X HRE. For the detection of the complexes, the reaction mixture was subjected to electrophoresis for 3 h on 3.5% acrylamide, 20% glycerol, 0.5% agarose, 0.3× Tris borate-EDTA gel. Results were visualized by autoradiography of the dried gel and analyzed using a PhosphorImager (Fuji FLA 3000G) and quantification software (Image Gauge, version 3.1).

The monoclonal GR antibody (BuGR, Affinity Bioreagents, Golden, CO) was included in the incubation mixture for supershift experiments.

Dimethyl Sulfate Methylation Assay—Methylation was carried out by the addition of 1 μl of 10% dimethyl sulfate to 20 μl of binding buffer (see above) containing 50 ng of end-labeled DNA, 250 ng of poly(dI-dC), 3 μg/μl bovine serum albumin, and 15–75 ng of recombinant PR. After 1 min at room temperature, the reaction was stopped by the addition of 2 μl of 250 mM dithiothreitol. After extraction with phenol-chloroform and ethanol precipitation, the samples were treated with 1 μl piperidine during 30 min at 90 °C, dried under vacuum, and analyzed in 6.5% sequencing gels.

RNA Analysis—Cells were resuspended in denaturing solution (4 μM guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.1 μM β-mercaptoethanol, and 0.5% sarcosyl), and total RNA was extracted by the single step method (21). For reverse transcription, 4 μg of total RNA were used. The first cDNA strand was synthesized with Superscript reverse transcriptase (Invitrogen) and 25 ng/μl oligo(dT) (Invitrogen) as reverse complementary primer. For PCR amplification of the P4 5′-leading exon, the oligonucleotide 5′-exonD (5′-CCAGGATGAGTCC-CACTTTGAAAGGAGAGAGTCAAAAC-3′) corresponding to the nucleotides 2694 to 2655 and the oligonucleotide 3′-exonD (5′-AAATGAGC-TATAACGTATTTTACA-3′) corresponding to the nucleotides 2551 to 2516.
to −2573 were used as forward and reverse primers, respectively. The reaction yielded a 143-bp length cDNA fragment. For the amplification of the cDNAs generated by the activation of P1 the oligonucleotides, bcl-X P1 (5′-CTCTAGGAAGCTTTCTGTTCTCTTCA-3′) corresponding to the nucleotides −137 to −114 and exonStop (5′-CCCGTAGAGATCCAGCTCTGGCTCTC-3′), which hybridized with the 5′-region of the second coding exon of bcl-Xb or bcl-Xc, were used as forward and reverse primers, respectively. The reaction yielded a 730-bp length cDNA fragment. PCR amplification of the cDNAs containing exon B were performed with the oligonucleotides bcl-X P2 (5′-GACTAGTCCAGGTGACGGGGGCGAGGCATGGCGGACT-3′), which specifically hybridize with gapdh mRNA, were used. The reaction yielded a 357-bp length cDNA fragment. To achieve semiquantitative conditions, reverse transcriptase-PCRs were terminated and the products were quantified when all of the samples were in the linear range of amplification. The cDNA pool (2 μl), 1.25 units Thermus aquaticus Taq polymerase (Invitrogen), and amplification primers (20 pmol each) in 50 μl of PCR mixture (1× polymerase buffer, 2 mM MgCl₂, 200 μM each dNTP) denatured 3 min at 98 °C followed by 8, 15, 25, and 30 cycles of amplification by using a step program (96 °C for 40 s; 65 °C (for gapdh), 60 °C (for exonD), 58 °C (for bcl-X P1 and bcl-X P2) for 30 s; and 72 °C for 1 min) and a final extension at 72 °C for 10 min. 10 μl of PCR products were analyzed by electrophoresis in 1.5% agarose gels and visualized under UV light. The negative was scanned, and the density was quantified with ImageQuant software (Molecular Dynamics, Amersham Biosciences).

RNome protection analysis was performed as described previously (22). For preparing the bcl-X probe, plasmid pGDL3 was digested with HinfI and transcribed by T3 RNA polymerase. The complete sequence is shown on the right, and the nucleotides nt −1 are found below.

Fig. 5. Identification of PR/bcl-X HREs contacts with dimethyl sulfate. Increasing amounts of recombinant PR were incubated with a 32P end-labeled DNA fragment containing the bcl-X-HREs (lanes 3–6), and the samples were subjected to dimethyl sulfate (DMS) treatment and DNA digestion. The products were analyzed by electrophoresis on a 6.5% sequencing gel. The arrows denote protected guanine residues. Lanes 1 and 2, A+G chemical sequencing reactions of bcl-X HRE region. The complete sequence is shown on the right, and the locations of HRE I and HRE II are indicated. nt, nucleotide.

RESULTS

bcl-X P4 Promoter Region Contains HRE-like Sequences—To check the hormone responsiveness of the bcl-X promoters, expression vectors containing the luciferase reporter gene under the control of different promoter regions of the mouse bcl-X gene (Fig. 2A) were cotransfected with GR (Fig. 2B) or PR (Fig. 2C) in COS-1 cells. With a fragment containing all of the five promoters, we observed around a 2-fold induction by the synthetic glucocorticoid, dexamethasone (Fig. 2B, lane 2), or by the progesterin agonist R5020 (Fig. 2C, lane 2). Of the five promoter fragments tested, only the one containing an extended P4 region from −3288 to −2652 relative to the translation initiation codon exhibited a robust response to dexamethasone (Fig. 2B, lane 3) and to R5020 (Fig. 2C, lane 3). In all of the cases, the coincidence with the antagonist RU38486 completely abolished the hormone effects (Fig. 2, B and C, lanes 3 and 6). Vectors with P1, P2, or core P4 did not respond either to glucocorticoid or to progesterin treatment (Fig. 2, B and C, lanes 8, 14, and 17, respectively). However, whereas treatment with dexamethasone had no effect (Fig. 2B, lane 11) in cells transfected with the P3 vector, treatment with R5020 increased luciferase expression <2-fold (Fig. 2C, lane 11). This construct contains one of the three HRE-like sequences reported recently by Gascoyne et al. (11). With another vector containing these three HRE-like sequences, we also observed a steroid hormone-dependent expression; however, it was lower than that obtained with extended P4 vector (data not shown).

An increase in luciferase expression was also observed when extended P4 vector was transfected in the human mammary cell line T47D and treated with R5020 (Fig. 2D, lane 2). T47D cells contain high amounts of endogenously expressed PR (25), which probably mediated the response to R5020, because it was blocked by RU38486 (Fig. 2D, lane 3). No induction was observed when cells were transfected with core P4 vector (Fig. 2D, lane 5). As the expression of Bcl-Xb is induced by dexamethasone in the mouse epithelial mammary cell line HC11 (9), we assayed the effect of dexamethasone on P4 activation in these cells (Fig. 2B). Luciferase expression was enhanced after glucocorticoid treatment in HC11 cells transfected with extended P4 vector (Fig. 2E, lane 2) but not with core P4 vector (Fig. 2E,
All of the constructs showed similar basal activities in the various cell lines tested (data not shown). Similar results were obtained in the RENTROP cell line, which is derived from rat epithelial endometrial cells and responds to steroid hormones with inhibition of programmed cell death through the induction of bcl-X expression (data not shown) (6).

Taken together, these results suggest that the region located between the nucleotides $-3288$ and $-2766$ relative to the translation initiation codon contains sequences responsible for the hormone-dependent P4 activation. A sequence analysis of the promoter region revealed the presence of several putative HRE sites located upstream of core P4. Two of these HRE-like sequences located between positions $-3040$ and $-3027$ (HRE I = TGTTGgtcTGGTTCC) and between $-3014$ and $-3001$ (HRE II = AGCCTCtCAGCACA) are included in the extended P4 construct (Fig. 3). Both these HREs are relatively well conserved when compared with the palindromic cons-HRE identified in the mouse mammary tumor virus gene (TGTYCTTnnnACARGA) (26). HRE I and HRE II are imperfect palindromes separated by 3 and 2 bp, respectively, and differ from the cons-HRE by three mismatches.

To test whether these two putative HREs bind GR or PR, we performed EMSA with increasing amounts of nuclear extracts from the T47D cell line treated with R5020. We observed a retarded band (Fig. 4A, lanes 2–5), which was abolished by a 500-fold molar excess of unlabeled cons-HRE (Fig. 4A, lane 6). Increasing amounts of recombinant GR generated a similar retarded complex (Fig. 4B, lanes 2–6), which was also abolished by a 500-fold molar excess of unlabeled cons-HRE oligonucleotide (Fig. 4B, lane 7), but not of nonspecific oligonucleotide (Fig. 4B, lane 8). The identity of the complex was confirmed by a supershift assay with a monoclonal GR antibody (Fig. 4B, lane 9). Similar results were obtained with recombinant PR (data not shown).

Since the bcl-X HREs are degenerated, we tested whether they were able to compete for the binding of PR to cons-HRE (Fig. 4C). The binding of PR to a labeled cons-HRE was competed by the unlabeled bcl-X HREs in a concentration-dependent manner (Fig. 4C, lanes 3–8). A 50% reduction in PR-DNA complex formation was observed with 10-fold molar excess of the unlabeled cons-HRE. In contrast, approximately a 20-fold molar excess of the bcl-X HRE oligonucleotide was required to reduce by 50% binding of PR to the labeled cons-HRE (Fig. 4D). These results indicate that PR binds to the bcl-X HREs with approximately half of the affinity compared with the cons-HRE.

To identify the guanines contacted by PR, we next performed dimethyl sulfate methylation experiments. Increasing amounts of recombinant PR protected specific guanine residues located within both HRE I and HRE II sites (Fig. 5, lanes 3–6). These protected guanines correspond to those previously reported on murine mammary tumor virus HRE1 (18).

To test the relevance of the identified receptor contacts, we mutated all of the contacted guanines of the HREs to adenines (Fig. 6A) and tested the effect of these mutations on receptor binding. In binding competitions experiments, an unlabeled bcl-X mutHRE oligonucleotide was a very weak competitor for the binding of PR to the labeled cons-HRE in comparison with wild type bcl-X HREs (Fig. 6B, lanes 2–11). >100-fold molar excess of the mutated form was required to reduce by 50% binding of PR to the labeled cons-HRE (Fig. 6C). These experiments suggest that PR binds to the bcl-X mutHREs with approximately five times less affinity compared with the bcl-X HREs.

bcl-X HREs Are Necessary to Confer Steroid Responsiveness to P4—To test the functional relevance of HRE I and II, we performed transient transfection assays with the extended P4ΔHRE expression vector, which contains a deletion of 95 nucleotides including both putative HREs (Fig. 7A). This construct did not respond to either R5020 (Fig. 7B, compare lane 5 with lane 2) in T47D cells or dexamethasone in HC11 cells (Fig. 7C, compare lane 5 with lane 2). These results suggest that HRE I and HRE II are bona fide functional HREs, which confer hormone responsiveness to P4.

P4 Is Activated by Steroid Hormones in Vivo—Transcri-
tional activation of P4 by hormones in vivo was confirmed by RNase protection assay and reverse transcriptase-PCR in HC11 cells. As previously shown (6), hormonal treatment lead to accumulation of transcripts for both bcl-X<sub>L</sub> and bcl-X<sub>S</sub> isoforms but the proportion of bcl-X<sub>L</sub> mRNA increased more markedly (Fig. 8A, lane 2). No hormonal effect was observed when we analyzed the PCR products obtained from transcripts generated by the proximal promoters P1 or P2 (Fig. 8B, lanes 2 and 5, respectively). However, using specific primers for the amplification of the P4 5'-leading exon, an increase of the product was observed in samples obtained from cells treated with dexamethasone (Fig. 8C, upper panel, lane 2). This hormone effect was abolished by the coincubation with RU38486 (Fig. 8C, upper panel, lane 3). As a control, no change was detected in gapdh RNA levels after hormone treatment (Fig. 8C, lower panel).

ChIPs experiments demonstrated that, 30 min after treatment of HC11 cells with dexamethasone, GR was recruited to the P4 promoter region of bcl-X containing the putative HREs (Fig. 9A, upper panel, lane 2). No differences in the binding of GR were observed in the bcl-X proximal promoter region encompassing P1 or P2 (Fig. 9A, middle panel, lane 2).

We next tested whether the bcl-X P4 promoter region was transcriptionally active after the hormone treatment. ChIP experiments using a monoclonal RNA-polymerase II (RNApol II) antibody demonstrated a specific recruitment of the enzyme to this region after incubating HC11 cells with dexamethasone (Fig. 9B, upper panel, lane 4). Hormonal treatment did not influence the binding of RNApol II to the bcl-X proximal promoter region (Fig. 9B, middle panel, lane 4). In all of the cases, ChIPs of the gapdh gene were used as control (Fig. 9A and B, lower panel). Thus, the specific binding of GR to the bcl-X P4 promoter region correlates with an increase in the binding of the RNApol II to this region, suggesting that GR would mediate in vivo activation of P4. The results demonstrate that glucocorticoid treatment induces the transcription of endogenous bcl-X gene, mainly through the activation of P4 in mammary epithelial cells.

**DISCUSSION**

Among other genes, bcl-X has been postulated to be crucial in hormone-dependent apoptosis. In many cases, the patterns of Bcl-X<sub>L</sub> or Bcl-X<sub>S</sub> expression are different from or opposite to those reported for Bcl-2, suggesting that Bcl-X<sub>L</sub> and Bcl-2 regulate cell survival and death at different stages of cell differentiation through tissue-specific control of their expression (27). In mammary gland, Bcl-X<sub>L</sub> is the most abundant cell survival member from the Bcl-2 family. However, it has been demonstrated that this protein is not essential during mammosogenesis but is critical for controlled apoptosis during the first phase of involution (28). Although the ratio of bcl-X<sub>L</sub>/bcl-X<sub>S</sub> remained stable in the virgin, pregnant, and lactating gland, it increased 6-fold during the first 2 days of involution (29). To ensure a correct control of apoptosis, the ratio between antiapoptotic and proapoptotic proteins (i.e. Bcl-2/Bax or Bcl-X<sub>L</sub>/Bcl-X<sub>S</sub>) must be precisely regulated. Thus, the regulation of cell death requires not only an accurate control of transcription but also control of splicing of bcl-X gene. The complex structure of mouse bcl-X gene with at least four different exons located upstream of the unique open reading frame suggests that promoter choice and alternative splicing may provide tissue-specific response to different stimuli.

The experiments summarized in this paper support the notion that steroid hormones modulate bcl-X gene expression through the activation of a distal promoter. We identified two novel HREs immediately upstream of the bcl-X promoter P4 that specifically bind GR and PR in vitro with an affinity only 2-fold lower than a canonical HRE. Deletion of both bcl-X HREs contacted by the hormone receptors in vitro eliminates hormone induction of the P4 reporter gene in transfection assays, supporting a role of the HREs in the in vivo response of the bcl-X gene. Following hormone treatment of HC11 cells, in vivo binding of GR to the bcl-X P4 promoter region was detected. This recruitment of the hormone receptor correlates with an increased binding of RNApol II with accumulation of mRNA transcripts generated from the P4 promoter and with an increase in the ratio of bcl-X<sub>L</sub> isoform to bcl-X<sub>S</sub> isoform. These
results support the hypothesis that GR mediates in vivo activation of P4 in mouse mammary epithelial cells. Because P4 is the only known bcl-X promoter responding to steroid hormones in this cell line, our results are congruent with the notion that P4 activation by hormones generates mainly the antiapoptotic bcl-XL isoform, although the mechanism of this differential activation is still unknown.

After completion of the experimental part of this work, Gascoyne et al. (11) describe the existence of other HRE-like sequences on the mouse bcl-X gene and show an in vitro interaction of these elements with nuclear extract of dexamethasone-treated fibrosarcoma cells (11). However, these HREs are different from those we found and their studies do not address selective promoter usage controlled by glucocorticoids. The ChIP assays performed in our study did not discriminate whether GR binds in vivo to the HREs described here or to those described by Gascoyne et al. (11). However, according to our transient transfection assays, only HRE I and HRE II, as described here, confer steroid responsiveness to P4 in mammary epithelial cells. In fact, the vector P4ΔHRE, which contains two of the HRE-like sequences described by Gascoyne et al. (11), did not respond to dexamethasone or to the progestagen R5020 in HC11 and T47D cells, respectively. It is possible that the cell context determines GR or PR binding to different HREs in the proximity of P4. As suggested in a previous study (30), the cooperativity among several HRE sequences may contribute to the inducibility of the promoter. Thus, the degree of occupancy of the different HREs may mediate recruitment of different tissue-specific or ubiquitous factors, which consequently determine hormone-dependent bcl-X expression. In this sense, the presence of several HREs within a short DNA region may provide the basis for specifying context-dependent hormonal regulation of programmed cell death.

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REFERENCES
1. Pecchi, A., Viegas, L. R., Baranan, J. L., and Beato, M. (2001) J. Biol. Chem. 276, 21062–21069
2. Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turkson, L. A., Mao, X., Nunez, G., and Thompson, C. B. (1993) Cell 74, 597–608
3. Pang, W., Rivard, J. J., Mueller, D. L., and Behrens, T. W. (1994) J. Immunol. 152, 1438–1439
4. Shiraiwa, N., Inohara, N., Okada, S., Yuzaki, M., Ushijima, S., and Ohita, S. (1996) J. Biol. Chem. 271, 13258–13265
5. Yang, X. F., Weber, G. F., and Cantor, H. (1997) Immunity 7, 629–639
6. Pecchi, A., Scholz, A., Pelster, D., and Beato, M. (1997) J. Biol. Chem. 272, 11791–11798
7. Vosent, G. P., Pecchi, A., Ghani, A., Pivien-Pilsip, G., and Galgianina, M. D. (2002) Exp. Cell Res. 276, 142–154
8. Yamamoto, M., Fukuoka, K., Miuara, N., Suzuki, R., Kido, T., and Komatsu, Y. (1998) Hepatology 27, 969–966
9. Schorr, K., and Furtth, P. A. (2000) Cancer Res. 60, 5950–5953
10. Chang, T. C., Hung, M. W., Jiang, S. Y., Chu, J. T., Chu, L. L., and Tsai, L. C. (1997) FEBS Lett. 415, 11–15
11. Gascoyne, D. M., Kryla, R. M., and Vivanco, M. M. (2001) J. Biol. Chem. 278, 18022–18029
12. Beato, M., Herrlich, P., and Schutz, G. (1995) Cell 83, 851–857

FIG. 8. Hormonal-dependent expression of bcl-X. HC11 cells were incubated with ethanol (Control) with 10 nm Dex or with 10 nm dexamethasone and 0.1 µM RU38486 (Dex+RU) for 5 h. Total RNA was extracted, and RNase protection assay (A) or reverse transcription-PCR (B and C) was performed. A, a comparison of bcl-X transcripts from cells treated with ethanol (lane 1) or with Dex (lane 2). The protected bands corresponding to both bcl-X isoforms are indicated by arrows. Lane 3 shows tRNA as negative control. All of the samples were hybridized with the gapdh riboprobe as control of recovery. B and C, upper panels, linear PCRs were performed by using as 5′-primer bcl-X P1- or bcl-X P2-specific oligonucleotides and a 3′-primer that hybridizes with the stop codon of bcl-XL (B) and specific oligonucleotides for the amplification of bcl-X P4 leading exon (exonD) (C). 10 µl of PCR products were electrophoresed in 1.6% agarose gel. Amplification of gapdh cDNA was used as control (B and C, lower panel).

FIG. 9. Chromatin immunoprecipitation assay. HC11 cells were untreated or incubated for 30 min with 10 nm Dex. A, binding of GR to the bcl-X P4 promoter region (upper panel) and to the bcl-X proximal region (middle panel) was determined in vivo with the ChIP assay (lanes 1 and 2). A control without added antibody is shown in lane 3. Input chromatin is shown in lanes 4–6. B, binding of RNAPol II to the bcl-X P4 promoter region (upper panel) and to the bcl-X proximal region (middle panel) was determined in vivo with the ChIP assay (lanes 3 and 4). Input chromatin is shown in lanes 1 and 2. Amplification of gapdh gene was used as control (A and B, lower panel). Ab, antibody; Ip, immunoprecipitate.
Direct Steroid Induction of P4 bcl-X Promoter

13. Bottenstein, J., Hayashi, I., Hutchings, S., Massi, H., Mather, J., McClure, D. B., Ohana, S., Rizzino, A., Sato, G., Serrero, G., Wolfe, R., and Wu, R. (1979) Methods Enzymol. 58, 94–109
14. Kastner, P., Boequel, M. T., Turcotte, B., Garnier, J. M., Harwitz, K. B., Chambron, P., and Gronemeyer, H. (1990) J. Biol. Chem. 265, 12163–12167
15. Godowski, P. J., Rusconi, S., Miesfeld, R., and Yamamoto, K. R. (1987) Nature 325, 365–368
16. Truss, M., Bartsch, J., Schelbert, A., Hache, R. J., and Beato, M. (1995) EMBO J. 14, 1737–1751
17. Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T. (1995) Nucleic Acids Res. 23, 4878–4884
18. Chalepakis, G., Schauer, M., Cao, X. A., and Beato, M. (1990) DNA Cell Biol. 9, 355–368
19. Di Croce, L., Koop, R., Venditti, P., Westphal, H. M., Nightingale, K. P., Corona, D. F., Becker, P. B., and Beato, M. (1999) Mol. Cell 4, 45–54
20. Andrews, N. C., and Faller, D. V. (1991) Nucleic Acids Res. 19, 2499
21. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
22. Zinn, K., DiMaio, D., and Maniatis, T. (1983) Cell 34, 865–879
23. Strutt, H., and Paro, R. (1999) Methods Mol. Biol. 119, 455–467
24. Eberhardy, S. R., D'Cunha, C. A., and Farnham, P. J. (2000) J. Biol. Chem. 275, 33798–33805
25. Keysar, I., Chen, L., Karby, S., Weiss, F. R., Delarea, J., Ruda, M., Chaitrik, S., and Brenner, H. J. (1979) Eur. J. Cancer 15, 659–670
26. Scheidereit, C., Geisse, S., Westphal, H. M., and Beato, M. (1983) Nature 304, 749–752
27. Krajewski, S., Bodrug, S., Gaseyne, R., Berean, K., Krajewska, M., and Reed, J. C. (1994) Am. J. Pathol. 145, 515–525
28. Walton, K. D., Wagner, K. U., Rucker, E. B., III, Shillingford, J. M., Miyoshi, K., and Hennighausen, L. (2001) Mech. Dev. 109, 281–293
29. Heermeier, K., Benedict, M., Li, M., Furth, P., Nunez, G., and Hennighausen, L. (1996) Mech. Dev. 56, 197–207
30. Nordeen, S. K., Suh, B. J., Kuhnel, B., and Hutchison, C. D. (1990) Mol. Endocrinol. 4, 1866–1873
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