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Mechanisms of 17-β-Estradiol Regulation of the Proto-oncogene *Bcl-2* in MCF-7 Human Breast Cancer Cells

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ABSTRACT

Approximately 65% of human breast tumours express estrogen receptors and are dependent on estrogen for growth and tumourgenicity. However, it remains unclear as to the specific mechanisms by which estrogen promotes the survival of these estrogen-dependent breast cancer cells. In previous studies it has been demonstrated that Bcl-2 expression, an anti-apoptotic protein, is regulated by estrogen (17-β-estradiol or E2) in estrogen-responsive breast cancer cells. Culture of estrogen receptor-positive MCF-7 human breast cancer cells in E2-free medium resulted in decreased Bcl-2 expression at both the transcript and protein levels, whereas re-exposure to estrogen markedly induced Bcl-2 mRNA and protein. In the present studies, the mechanisms of estrogen regulation of Bcl-2 were investigated by analyzing the expression of different *Bcl-2* promoter-driven constructs stably transfected into MCF-7 cells. An approximately 1.7 kilobase (kb) sequence which directed estrogen-dependent regulation of *Bcl-2* expression in these stable MCF-7 clones was identified. Deletion mutants of this 1.7 kb region revealed a 500 base pair minimal element which retained E2-responsiveness and constitutive activity of the promoter.

Another agent which may play a role in the regulation of Bcl-2 expression is the tumour suppressor gene, *p53*. About 30 to 50% of human breast carcinomas carry a mutant p53, and in many of these cases there exists an inverse relationship between p53 and Bcl-2 expression. To further characterize this relationship we investigated the effects of various mutant p53 proteins and low levels of p53 on Bcl-2 expression in MCF-7 cells. Neither MCF-7/E6 cells expressing virtually undetectable levels of p53 nor MCF-7/173L cells expressing a DNA binding domain mutant p53, showed altered E2-mediated induction of Bcl-2 mRNA or protein levels. However,

MCF-7 cells expressing a truncated mutant p53 protein (Δ291) resulted in a dramatic decrease in Bcl-2 protein levels, but not mRNA levels, upon E2 treatment. These results suggest that a p53 protein lacking a carboxy-terminus may cooperate with E2 post-transcriptionally to negatively regulate Bcl-2 levels in MCF-7 human breast cancer cells.

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LIST OF ABBREVIATIONS

AF	activation function
AIF	apoptosis-inducing factor
Amp	Ampicillin
Bcl-2	B-cell lymphoma/leukemia-2
BH	Bcl-2 homology
bHLH	basic helix-loop-helix
bp	base pair
°C	degrees Celsius
$CaHPO_4$	calcium phosphate
CBP	cAMP-responsive element-binding protein (CREB)-binding protein
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CIAP	calf intestinal alkaline phosphatase
CMV	cytomegalovirus
CoA	co-activator
CoR	co-repressor
cpm	counts per minute
DBD	deoxyribonucleic acid binding domain
DCC	dextran-coated charcoal
dCTP	deoxycitidine triphosphate
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modification of Eagle's medium
DNA	deoxyribonucleic acid

dNTP	deoxynucleic triphosphate
DTT	dithiothreitol
E2	17-β-estradiol, estrogen
E6-AP	E6-associated protein
E6BP	E6 binding protein
E6TP1	E6-targeted protein 1
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetracetic acid
ERAP-160	estrogen receptor associated protein 160
ERE	estrogen response element
ER	estrogen receptor
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HAT	histone acetylase
HCl	hydrochloric acid
HDAC	histone deacetylase
hDLG	human discs large
HEPES	4-(2-hydroxylethyl)-1-piperazine ethanesulfonic acid
hER	human estrogen receptor
HPV	human papillomavirus
HRE	hormone responsive element
Hsp	heat shock protein
IL-3	Interleukin-3
kb	kilobase
KCl	potassium chloride

kDa	kilodalton
L	litre
LB	Luria-Bertani
LBD	ligand binding domain
LiCl	lithium chloride
M	molar
mAb	monoclonal antibody
Mcm-7	minichromosome maintenance 7
mg	milligram
mJ	millijoule
ml	millilitre
mM	millimolar
MOPS	3-[N-morpholino] propanesulfonic acid
mRNA	messenger ribonucleic acid
MUG	methylumbelliferyl galactosidase
NaCl	sodium chloride
NaOH	sodium hydroxide
N-CoR	nuclear co-repressor
neo	neomycin
PAGE	polyacrylamide gel electrophoresis
PBP	peroxisome proliferator-activated receptor (PPAR)-binding protein
PBS	phophate buffered saline
pCAF	p300/CBP-associated factor
pGL3B	pGL3-Basic

PMSF	phenylmethylsulfonyl fluoride
PNRE	p53-responsive negative regulatory element
pol II	RNA polymerase II
puro	puromycin
PVDF	polyvinylidenedifluoride
RIP-140	receptor interacting protein 140
RNA	ribonucleic acid
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
SERM	selective estrogen receptor modulator
SMRT	silencing mediator of retinoid and thyroid hormone receptors
SRC-1	steroid receptor coactivator-1
SSC	standard saline citrate
SSPE	standard saline phosphate EDTA
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline-Tween
TE	Tris-EDTA
TM	transmembrane
µg	microgram
µl	microlitre
UTR	untranslated region
UV	ultraviolet
Zn^{2+}	zinc ion

CHAPTER I

INTRODUCTION

ESTROGENS

The sex steroid hormone estrogen is important in both men and women for a variety of physiologic processes. Estrogen affects growth, differentiation, and function of tissues of the reproductive system, including the mammary glands, uterus, vagina and ovaries in females, and testes, epididymes, and prostate in males (Greenspan and Baxter, 1994). It also plays critical roles in maintaining bone density and protecting against osteoporosis, and it is thought to be cardioprotective, largely through its effects on blood lipids (Lufkin et al., 2001; Gray et al., 2001).

Estrogens are required for the normal maturation of the female. The key estrogens produced by women are estradiol (17-β-estradiol, E2), estrone (E1), and estriol (E3). They stimulate the development of the vagina, uterus, and fallopian tubes as well as the secondary sex characteristics. Estrogens also control cell proliferation in normal and transformed mammary epithelial cells, where they induce expression of hormone-responsive genes important for cell cycle progression (Altucci et al., 1996). However, factors which stimulate cellular proliferation have interplay with molecular mechanisms that control programmed cell death (PCD). PCD, also referred to as apoptosis, is a gene-directed, intrinsic cell suicide program that plays a crucial role in the normal development, maintenance of homeostasis, defence and aging of the individual. Estrogens inhibit apoptosis in the mammary gland, which cyclically undergoes a period of cell death at the end of the menstrual cycle (Ferguson and Anderson, 1981), and in human breast cancer MCF-7 cells expressing functional estrogen receptor (ER) (Teixeira et al., 1995; Wang and Phang, 1995).

ESTROGEN RECEPTOR

Estrogens exert nearly all their physiological effects by binding to nuclear proteins called estrogen receptors (ERs) which belong to the superfamily of nuclear hormone receptors. The members of this family, which include the steroid, vitamin D3 and thyroid hormone receptors, function as ligand-inducible transcription factors that can initiate or enhance the transcription of genes containing specific hormone responsive elements (HREs) (Beato et al., 1995).

Natural and synthetic compounds other than estrogen are also capable of binding to the ER. All ER ligands bind exclusively to the carboxy-terminal ligand-binding domain (LBD), which recognizes a variety of compounds diverse in their size, shape and chemical properties (Jordan and Morrow, 1999). Some of these ligands, including the endogenous estrogen 17-β-estradiol, function as pure agonists, whereas others, such as ICI 164,384, function as pure antagonists. Synthetic ligands such as tamoxifen and raloxifene belong to a growing class of molecules known as selective estrogen receptor modulators (SERMs), which function as antagonists or agonists depending on the specific tissue and promoter contexts (Osborne et al., 2000).

Tamoxifen and other similar drugs are effective in the treatment of breast cancer by inhibiting the proliferative effects of estrogen that are mediated through the ER. However, tamoxifen also has many estrogenic qualities including agonist effects on bone, blood lipids and the endometrium (Osborne, 1998). This tissue-specific behaviour of tamoxifen was demonstrated in a recent study. In a group of women at high risk for breast cancer who received tamoxifen treatment, there was

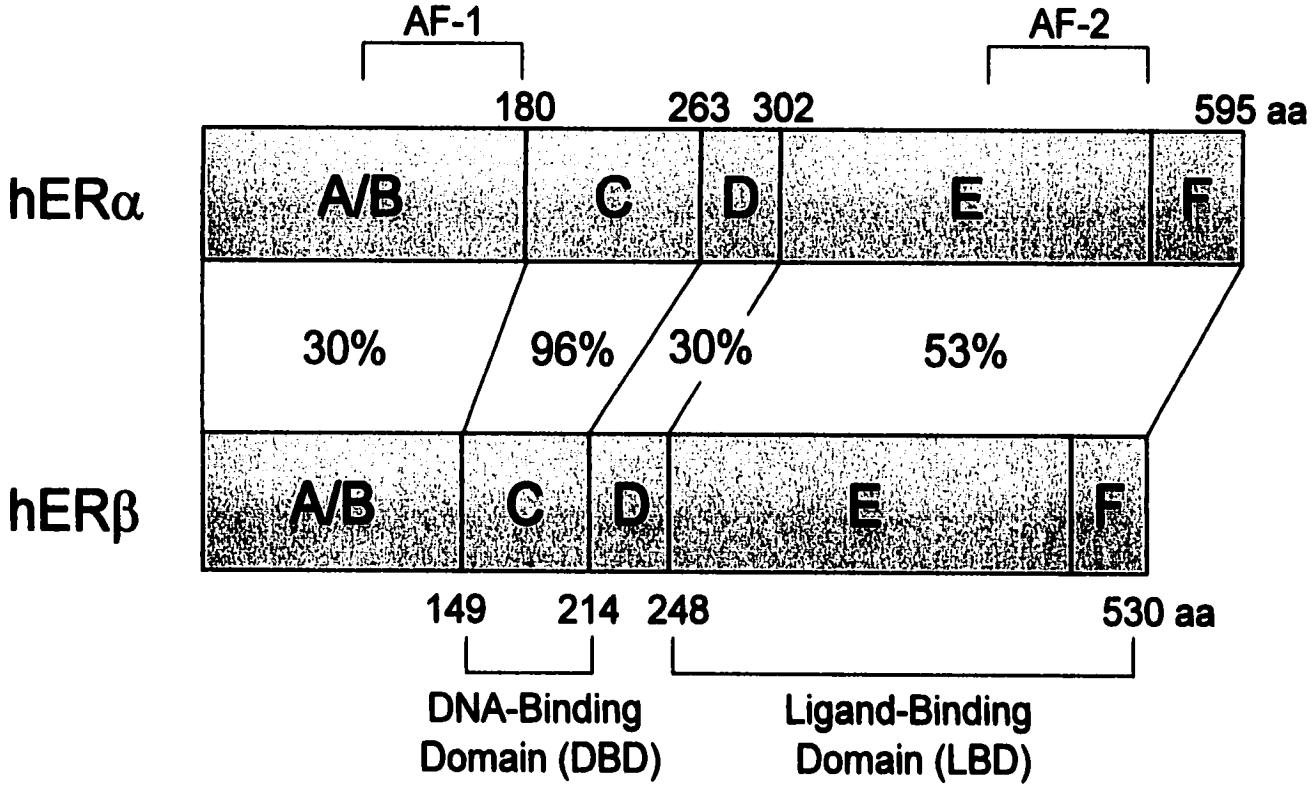
an increased incidence of endometrial cancer, but a reduced occurrence of certain bone fractures and a dramatic 45% reduction in breast cancer incidence (Smigel, 1998). To properly understand the effects of these molecules one must first understand the different ligand chemistries and structures upon ER transcriptional activity.

Characterization of the common structural features that nuclear receptors share have identified several functional domains, designated A to F, which are important in the biological activity of the receptors (Kumar et al., 1986, 1987). The classical human ER (ERα) protein consists of 595 amino acids with a molecular weight of 66 kDa and possesses a central DNA-binding domain (DBD) found in region C, along with a ligand-binding domain (LBD) located in the E domain (Green et al., 1986) (Fig.1).

ER-mediated gene transcription is stimulated through at least two distinct transcriptional activation function (AF) domains located in the highly variable amino-terminal A/B region (called AF-1) and the carboxy-terminal E region (called AF-2) of the receptor. The AF-1 domain is hormone-independent, whereas the AF-2 is hormone-dependent (Webster et al., 1989). Both AF-1 and AF-2 are required for maximal ER transcriptional activity, however, with certain promoters, AF-1 and AF-2 can function independently (Katzenellenbogen et al., 1995). The activity of AF-1 has been found to be regulated by growth factors acting through the MAP kinase pathway (Kato et al., 1995). MAP kinase was shown to phosphorylate a specific serine residue (Ser 118) in the AF-1 region of the human ERα which functionally activated AF-1 thus establishing a mechanism for cross-talk between membrane-

Figure 1. Structural comparison of human ERα and ERβ.

A to F regions represent classical nomenclature for the structurally similar amino acid sequences. Transcriptional activation function domains are named AF-1 and AF-2. Percent amino acid homology in the functional domains between the two receptors is also shown.


AF-1
AF-2
180
263
302
595 aa
hERα
A/B
C
D
E
F
30%
96%
30%
53%
hERβ
A/B
C
D
E
F
149
214
248
530 aa
DNA-Binding
Domain (DBD)
Ligand-Binding
Domain (LBD)

type growth factors and nuclear receptors. Unlike AF-1, which has been shown to be constitutively active, AF-2 is induced upon hormone binding (Tora et al., 1989). The binding of agonists triggers AF-2 activity, whereas the binding of antagonists does not (Berry et al., 1990).

Recent structural studies suggest that ligands regulate AF-2 activity by directly affecting the structure of the LBD. Comparison of the structure of unliganded human retinoid X receptor α LBD (Bourguet et al., 1995) with the structures of the agonist-bound LBDs of human retinoic acid receptor γ (Renaud et al., 1995) and other nuclear receptors suggests that an agonist-induced conformational change involving the repositioning of helix 12, the most C-terminal helix of the LBD, is essential for AF-2 activity (Moras and Gronemeyer, 1998). Since certain point mutations in helices 3, 5, and 12 abolish AF-2 activity but have no effect on ligand or DNA binding, these regions of the LBD have been predicted to form part of a recognition surface, created in the presence of agonist, for molecules that link the receptor to the general transcriptional machinery (Wrenn and Katzenellenbogen, 1993; Henttu et al., 1997; Feng et al., 1998).

Similarly, recent crystal structures of the ER bound to various ligands has revealed that the ER also undergoes extensive conformational changes after ligand binding (Brzozowski et al., 1997; Shiau et al., 1998). In the estrogen-liganded complex, helix 12 is repositioned over the ligand-binding cavity to form a secure lid over the ligand. This ER conformation exposes several amino acids that are critical for binding to specific regulatory proteins called coactivators. In contrast, the repositioning of helix 12 over the ligand-binding cavity is prevented by SERMs such

as tamoxifen or raloxifene, therefore gene transcription does not occur because coactivators cannot bind.

Thus it appears that the ER does not act alone to influence gene expression. It has been shown that nuclear receptors interact with coregulatory factors which modulate the activity of the receptors (Horwitz et al., 1996). Current models of ER action suggest that it modulates the rate of transcription initiation through interactions with the basal transcription machinery and through alterations in the state of chromatin organization at the promoter of target genes via the recruitment of coactivators. The p160/SRC-1 type coactivators include such proteins as steroid receptor coactivator 1 (SRC-1), estrogen receptor associated protein 160 (ERAP-160) and receptor interacting protein 140 (RIP-140) (Onate et al., 1995; Halachmi et al., 1994; Cavailles et al., 1995). These proteins possess basic helix-loop-helix (bHLH) domains characterized by the presence of multiple LXXLL motifs also called the NR box, which directly interact with the LBD of nuclear receptors (Heery et al., 1997), and these associations are increased by the binding of ligand. According to structural studies, the orientation of the helix 12 in the LBD cleft, when the receptor is bound to E2, permits the binding of p160/SRC-1 coactivator via the NR box. Conversely, the binding of ER by tamoxifen or raloxifene precludes this interaction. Other coactivators such as CBP, p300, and the p300/CBP-associated factor, pCAF have been shown to bind a wide variety of nuclear receptors including the ER and activate transcription (Kamei et al., 1996; Chakravarti et al., 1996; Blanco et al., 1998). Importantly, interactions with CBP and provide physical links to basal transcription since, for instance, the transactivation domain of CBP can directly

interact with TFIIB machinery (Hanstein et al., 1996; Kamei et al., 1996). Most interestingly, both p160/SRC-1 family and CBP/p300 have histone acetyltransferase (HAT) activity (Chen et al., 1997; Spencer et al., 1997). These acetyl transferases add acetyl groups to specific lysine residues found in core nucleosomal histones (Schiltz et al., 1999), thereby loosening their interaction with DNA, which may expose important residues to the basal transcriptional machinery.

Traditionally it was thought that ER bound to its response element in target genes and remained bound as long as the stimulus (E2) was present. Recently, it has been postulated that in the presence of agonist, ER transcription complexes are stimulated to assemble and disassemble on target promoters in a cyclic fashion (Shang et al., 2000). Using chromatin immunoprecipation (ChIP) analysis Shang et al. (2000) found that endogenous p160 coactivators CBP, p300, pCAF, and PBP are recruited in specific order to the ER transcription complex after E2 stimulation in MCF-7 breast cancer cells. More specifically, p300 followed by CBP and then pCAF are sequentially recruited to the ERα complex in the first cycle of transcription initiation. PBP, although recruited around the same time as p300, CBP and pCAF, is thought to act at a later stage in gene activation, perhaps after histone acetylation by the p160 complex has made the chromatin more accessible. Based on these findings, Shang et al. (2000) have proposed a cyclic model for the assembly of ER transcriptional complexes. Briefly, the binding of activated ER to response elements in target genes is almost immediately followed by recruitment of both a HAT-containing p160-p300 and a PBP complex. The HAT complex modifies local chromatin structure through histone acetylation to facilitate RNA polymerase II (pol

II) recruitment. Upon the onset of transcription the pol II C-terminal domain is phosphorylated and p300 is replaced in the complex by CBP and its associated factor pCAF. Subsequently, acetylation of p160 by CBP causes the release of p160 along with ER. Finally, CBP and pCAF disassemble and the cycle is repeated.

In contrast, nuclear receptors have also been shown to be regulated by corepressor proteins. At least two well characterized corepressors called nuclear receptor co-repressor (N-CoR) and silencing mediator of retinoid and thyroid hormone receptors (SMRT) exist (Horlein et al., 1995; Chen et al., 1995). They usually bind the receptors near the N-terminal end of region E involving helices 3, 4, and 5. This interaction occurs in the absence of ligands and suppresses the transcriptional activation of target genes by the receptor (Shiau et al., 1998). Both N-CoR and SMRT have a NR box and interact with the previously mentioned region of the nuclear receptors (Hu et al., 1999). Generally speaking, the corepressors possess histone deacetylase (HDAC) activity, and this activity silences transcription by allowing the DNA to wrap more tightly around core histone proteins. Ligand binding then releases the corepressors from the transcription complex, enabling the receptor to recruit coactivators with their associated histone acetylase activity.

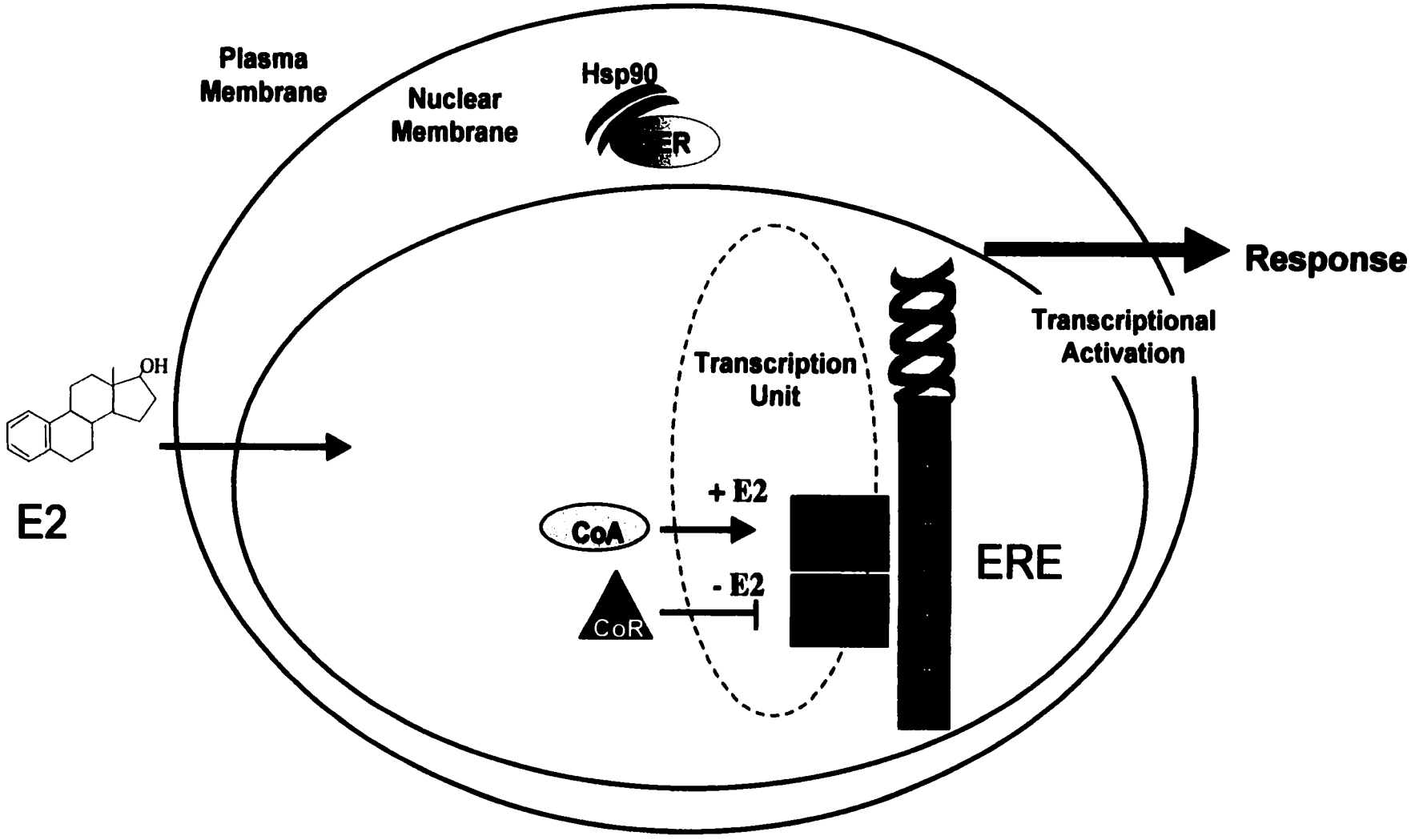
The C region of the receptors is where the very highly conserved DNA binding domain and a dimerization domain are located. The DBD contains two zinc fingers that consist of several tandemly repeated cysteine residues coordinated by zinc atoms. ER deletion mutants lacking the DBD cannot bind DNA *in vitro* or *in vivo* demonstrating the necessity of these zinc (Zn^{2+}) finger motifs (Kumar and Chambon, 1988). It is believed that specificity of a certain receptor is afforded by the first of the

two zinc fingers. Mutagenesis in the region of the first zinc finger revealed that the zinc finger modules are joined primarily through interaction between the amphipathic helices formed by amino acids at the base of the two fingers (Umesono and Evans, 1989). The amino acids in the stem of the first Zn^{2+} finger form an α-helix and make base contacts with the major groove of the DNA. This α-helix coincides with the P-box which has been implicated in the determination of specificity of binding site recognition. The second Zn^{2+} finger is responsible for stabilizing this interaction through ionic bonds with the phosphate groups in the DNA backbone.

E2 diffuses through the plasma membrane of cells and binds to the ER, which in the absence of estrogen is found predominantly in the nucleus (King and Greene, 1984; Welshons et al., 1984). The ER exists as part of a multiprotein complex consisting of a dimer of heat shock protein 90 (Hsp90), a p23 monomer, and one of several immunophilins (Knoblauch et al., 1999). It has been proposed that this Hsp90-based chaperone complex inactivates the ER's transcriptional regulatory capabilities and maintains the ER in a conformation competent for steroid binding. Once E2 binds to the ER, the Hsp90 complex dissociates and a change in conformation and homodimerization occurs. Nuclear-liganded, dimerized receptor then binds its cognate E2 responsive elements (EREs) in the genome, stimulating the transcription of associated promoters. The classical ERE consists of an inverted repeat of two half sites of the motif GGTCA separated by any three nucleotides (Fig. 2).

Besides the established mechanism of direct DNA binding to classical ERE motifs, the ER can also activate other pathways. AP-1 response elements, for

Figure 2. A subcellular model of 17-β-estradiol (E2) action in a target tissue. Estradiol diffuses into all cells but binds to the estrogen receptor (ER) specifically located in estrogen target tissues. The steroid receptor complex undergoes a conformational change and dimerizes before binding to an estrogen response element (ERE) usually found in the promoter region of an estrogen-responsive gene. A transcription unit is formed by interaction with coactivator (CoA) molecules to initiate RNA synthesis and ultimately the estrogen-stimulated cellular response. Corepressor (CoR) modules are believed to prevent transcription by interacting with unliganded ER complexes.


Plasma Membrane
Nuclear Membrane
Hsp90
ER
Response
Transcriptional Activation
Transcription Unit
OH
E2
+ E2
CoA
- E2
CoR
ERE

instance, are regulated indirectly through interactions between ER and AP-1 transcription factors c-fos and c-jun (Webb et al., 1995). Estrogen receptors have also been shown to bind imperfect EREs, or half-sites, and form functional complexes with Sp1 transcription factors to mediate gene regulation. E2-dependent transactivation via ERα/Sp1 interactions have been characterized in promoters of several E2-responsive genes, including *creatine kinase B*, *c-myc*, *cathespin D*, *heat shock protein 27*, *c-fos*, *retinoic acid receptor α1*, *E2F1*, *adenosine deaminase*, *insulin-like growth factor binding protein 4*, *transforming growth factor α*, and *Bcl-2* (Dubik and Shiu 1992; Krishnan et al.,1994; Rishi et al., 1995, Porter et al., 1996, 1997; Duan et al., 1998; Sun et al., 1998; Wang et al., 1998, 1999; Dong et al., 1999; Qin et al., 1999; Xie et al., 1999; Vyhlidal et al., 2000). Both ERα and Sp1 proteins physically interact, however, transactivation is mediated by at least two pathways, namely: (I) interaction of ERα/Sp1 proteins with GC-rich genomic sites in which only Sp1 protein bind DNA; and (II) interaction of ERα/Sp1 with Sp1$(N)_x$ERE half-site motifs in which both proteins bind DNA.

Until recently it was believed that a single ER (ERα) existed however, in 1996 another isoform was cloned from a rat prostate cDNA library and named ERβ (Kuiper et al., 1996). The human ERβ is somewhat shorter than ERα, containing only 530 amino acids with a molecular weight of 59.2 kDa (Mosselman et al., 1996) and is expressed in several tissues such as prostate, ovary, uterus and bladder. The region of highest homology between ERβ and ERα is in the DBD (96%), however, there is only 53% homology in the LBD and much less conservation in all other regions (Ogawa et al., 1998) (Fig. 1). Correspondingly, the ERβ binds and

transactivates from consensus EREs but binds with slightly lower affinity to E2 than does ERα. Like ERα, ERβ activity can be upregulated by phosphorylation of serine residues in the AF-1 domain by MAP kinase which results in SRC-1 recruitment to this region both in presence and absence of E2 (Tremblay et al., 1997).

BREAST CANCER

Breast cancer is a leading and feared cause of cancer deaths, affecting about one-in-nine women in North America alone (Harrell 1999). In general, cancer is believed to be the result of multiple genetic events which causes the loss of growth control and/or inhibition of appropriate cell death. The most common risk factors associated with breast cancer are familial association (Plu-Bureau and Thalabard, 1998) and the levels of exogenous and endogenous hormones (i.e. estrogen and progesterone) (Soderqvist, 1998). Other factors that have also been implicated are diet and environment (Snyderwine, 1998; Mannisto et al., 1999).

Primary breast carcinomas arise from the epithelial cells of the lobular epithelium. They can present as either non-invasive lesions, either intraductal or intralobular carcinomas, or as invasive cancers. Tumours that are invasive can spread rapidly by infiltrating through tissue spaces and can invade both lymphatic and blood vessels. They can produce a high incidence of distant metastases, mostly in the lungs, soft tissues, liver, bones and adrenals (Lippman et al., 1989).

The first evidence for a connection between estrogen and breast cancer growth was presented in 1896 when Beatson, a British physician, discovered that by removing the ovaries of premenopausal women, he could cause a regression of

advanced breast tumours. Since then, substantial evidence has accumulated demonstrating that many human breast cancers are dependent on estrogen for growth and tumourgenicity. Between 60 and 70% of human breast tumours contain ERs and about half of these are estrogen-dependent (Lippman, 1985; Lippman and Dickson, 1989; McGuire et al., 1975). Accordingly, about one third of metastatic breast cancers regress in response to estrogen ablation. In fact, estrogen withdrawal is one of the principal treatment strategies for breast cancer (reviewed in Santen et al., 1990; Jordan and Murphy, 1990). Many breast tumours appear to follow this type of predictable clinical pattern, being apparently confined to local structures, responsive to endocrine manipulation, and/or sensitive to cytotoxic chemotherapy. However, it has been suggested that over time many breast tumours evolve into an estrogen-independent phenotype that is often associated with a more malignant and aggressive stage of the disease. It is the development of a metastatic, hormone-independent and drug-resistant phenotype that is responsible for the high percentage of treatment failures among breast cancer patients (Clarke et al., 1994).

Many laboratory models have been developed for the study of breast cancer. Among these are ER-negative, hormone-independent cell lines that exhibit a down-regulation of hormone receptors (e.g. MDA-MB-231). In contrast, the MCF-7, T-47D and ZR-75-1 cell lines represent ER-positive, hormone-dependent human breast carcinomas (Brunner et al., 1990). These cell lines are invaluable tools to study the mechanisms controlling proliferation of both hormone-dependent and -independent breast cancer cells. They provide a readily available source of propagating cells

with infinite growth potential, and in many cases, these lines retain *in vivo* properties allowing for physiological studies to be performed.

The MCF-7 cells that were used in the following studies were initially derived from pleural effusions of a patient with metastatic mammary adenocarcinoma (Soule et al., 1973). They are an immortalized cell line from which the human ER was cloned and sequenced (Green et al., 1986; Greene et al., 1986). The MCF-7 cell culture system was also used in the characterization of potential ER modulators such as tamoxifen (Coradini et al., 1994), which is frequently used in the treatment of breast cancer. This SERM has the advantage of low acute toxicity and high rate of compliance, however, the success of treatment is often limited due to the development of an anti-estrogen resistant phenotype. In addition to expressing significant levels of functional ER, MCF-7 cells also express Bcl-2 and wild-type p53 (O'Connor et al., 1997). Therefore, the MCF-7 cell line represents an excellent model system to investigate the mechanism of estrogen regulation of these factors (Bcl-2 and p53) in the context of hormone-dependent breast carcinomas.

BCL-2

The *Bcl-2* (B-cell lymphoma/leukemia-2) gene, as its name implies, was initially discovered at the breakpoints of the t(14:18) chromosomal translocations found in the majority of non-Hodgkin's B-cell lymphomas (Tsujimoto et al., 1985; Bakhshi et al., 1985, Cleary and Sklar, 1985), and has since been found to be highly expressed in other malignancies including breast carcinoma (Bhargava et al., 1994; Joensuu et al., 1994). These t(14;18):(q32;q21) translocations constitute the most common type

in human lymphoid malignancies, being present in 85% of follicular and 20% of diffuse B-cell lymphomas. In these t(14;18) translocations, the *Bcl-2* gene is moved from its normal location on chromosome 18q21 into juxtaposition with powerful transcriptional enhancer elements associated with the immunoglobulin heavy-chain locus at 14q32. This places *Bcl-2* in the same transcriptional orientation as the immunoglobulin heavy chain locus, giving rise to chimeric RNAs. However, translocation does not interrupt the protein-encoding region so the wild-type and translocated alleles produce the same-sized 25-kDa protein. As a result, the t(14;18)-bearing B cells have deregulated overexpression of the translocated *Bcl-2* gene and inappropriately elevated levels of Bcl-2 protein production in the germinal center B cells from whence follicular lymphomas arise (Cleary et al., 1986; Seto et al., 1988).

The consequence of deregulated Bcl-2 overexpression was shown both *in vivo* and *in vitro*, to be prolonged cell survival. A minigene representing the *Bcl-2*-Ig fusion gene found at the t(14;18) chromosomal breakpoint was generated and placed into the germ line of mice to assess the effects of this translocation during development. (McDonnell et al., 1989; 1990). These transgenic mice that overexpressed *Bcl-2* in the B cell lineage demonstrated extended cell survival and progress to high grade lymphomas. Cell cycle analysis confirmed that close to 97% of the expanded B cells resided in G_0/G_1 and these recirculating B cells accumulated because of extended survival rather than increased proliferation. In contrast, gene knock-out experiments have identified critical lineages that require the anti-apoptotic function of Bcl-2. Newborn *Bcl-2*$^{-/-}$ knockout mice are viable, but the majority die at a

few weeks of age (Veis et al., 1993). They develop polycystic kidneys that contain very few nephrons and greatly increased apoptosis in metanephric structures, resulting in renal failure. In normal fetal kidney Bcl-2 maintains cell survival during inductive interactions between epithelium and mesechyme. The *Bcl-2*$^{-/-}$ mice turn gray at 5-6 weeks of age, this hypopigmentation reflecting decreased melanocyte survival. The lymphoid organs, thymus and spleen, are initially normal; however, at 4-8 weeks of age they undergo massive cell death. All these characteristics result from increased cell death due to the absence of Bcl-2.

Bcl-2 overexpression studies were also performed on immature pre-B cells that are dependent on lymphokine Interleukin-3 (IL-3) for their growth and survival in culture. Vaux et al. (1988) noticed that stable transfer of *Bcl-2* expression vectors permitted prolonged cell survival in the absence of IL-3, but without concomitant cell proliferation. Hockenbery et al. (1990) later demonstrated that overexpression of *Bcl-2* in an IL-3-deprived pro-lymphocyte cell line conferred survival by blocking apoptosis. Indeed, overexpression of *Bcl-2* has been shown to prevent or markedly reduce cell death induced by a wide variety of stimuli including neurotrophic factor withdrawl (Garcia et al., 1992; Allsopp et al., 1993), chemotherapeutic drugs as well as UV- and γ-irradiation (Miyashita and Reed, 1992, 1993; Lotem and Sachs, 1993), c-Myc (Bissonnette et al., 1992), glucocorticoids and calcium (Sentman et al., 1991; Strasser et al., 1991; Siegel et al., 1992; Miyashita and Reed, 1992, 1993). These data are more intriguing when taken with the fact that Bcl-2 protein overproduction was found to prevent apoptosis without affecting cellular proliferation rates, placing *Bcl-2* in a new category of oncogenes.

Bcl-2 is a member of a growing family of cell death regulatory proteins which include death antagonists (Bcl-2, Bcl-X_L, Mcl-1, Bcl-w, Bfl-1, Brag-1, A1) as well as proapoptotic molecules (Bax, Bak, Bcl-Xs, Bad, Bid, Bik, Bim and Hrk) (Kroemer, 1997 and references therein). Protein-protein interactions between Bcl-2 family members to form homo- and heterodimer pairs appear to determine the susceptibility of a cell to a given apoptotic stimulus depending on the ratio of death inducers to death protectors (White, 1996). The family shares homology in four conserved domains designated (Bcl-2 homology) BH1, BH2, BH3 and BH4 (Fig. 3). The multi-dimensional NMR and X-ray crystallographic structure of a Bcl-X_L monomer indicated that the BH1-4 domains corresponded to seven α-helices. The helices of BH1, 2 and 3 domains are in close proximity and create a hydrophobic pocket presumably involved in interactions with other Bcl-2 family members (Muchmore et al., 1996). The second amphipathic helix of dimerized Bcl-2 family proteins inserts into this groove, like a peptide ligand binding to a receptor (Sattler et al., 1997). As the BH3 domain corresponds to this inserting α-helix (ligand) but also forms part of the surface pocket (receptor), the implication is that Bcl-2 family members assume at least two conformations, with one dimerizing partner playing the role of the receptor and the other the ligand. Located near the C-terminus of the Bcl-2 protein is a stretch of hydrophobic amino acids (219-237) that account for the posttranslational insertion of Bcl-2 into intracellular membranes.

In humans, the Bcl-2 protein is 239 amino acids in length with a molecular weight of about 26 kDa. For the most part, the Bcl-2 protein is found in association with outer mitochondrial membranes where it is thought to function as a pore or

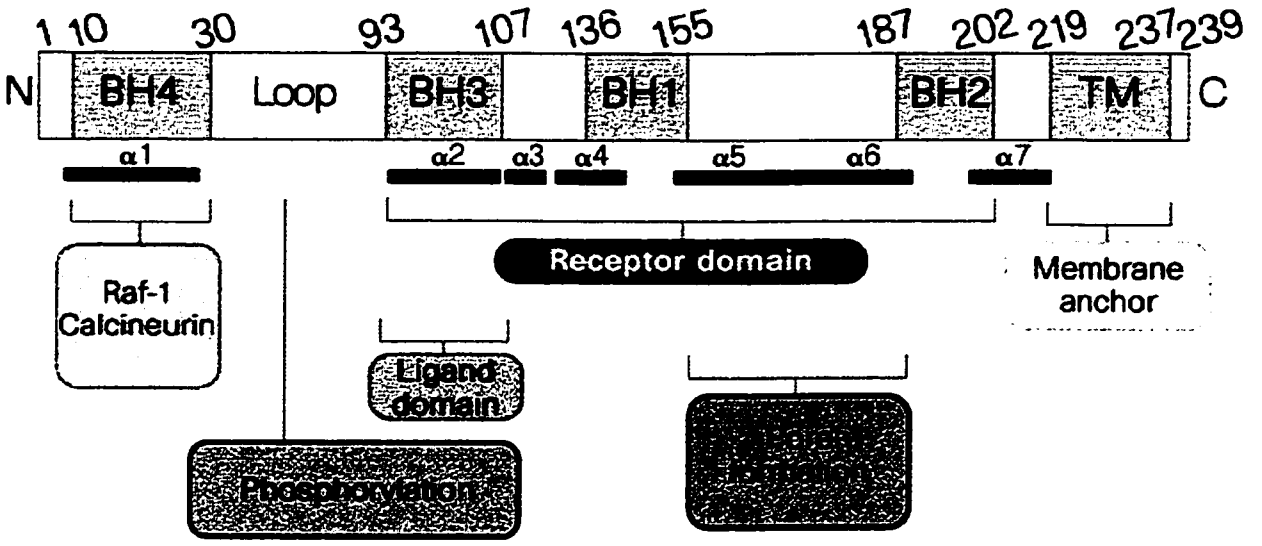
channel. The three-dimensional structure of Bcl-X_L also revealed a striking similarity to the pore-forming domains of certain bacterial toxins that act as channels for either ions or proteins. Interestingly, direct evidence of *in vitro* ion-channel activity has been obtained for certain family members (Minn et al., 1997; Schendel et al., 1997; Antonsson et al., 1997) suggests that the may participate in some of the cellular phenomena recently associated with apoptosis, particularly mitochondrial permeability transition (megapore opening) and release of apoptogenic protease activators (cytochrome c and apoptosis-inducing factor (AIF)) from mitochondria.

It is apparent that Bcl-2 is an important regulator of proper cellular homeostasis, yet little is known about the molecular mechanisms controlling its expression. Characterization of its genomic organization revealed that *Bcl-2* consists of three exons, with the second and third exon separated by an extremely large, 225-kb intron (Seto et al., 1988). Two promoters are responsible for the initiation of *Bcl-2* transcription. The principal promoter, P1, is a GC-rich, TATA-less promoter that displays multiple start sites and includes seven consensus binding sites for the Sp1 transcription factor. It has been mapped to an area about 1.7 kb upstream of the open reading frame and is where close to 95% of the transcripts initiate. The second promoter, P2, is located approximately 1.3 kb downstream of the first. It includes both a CCAAT box and a TATA element and displays two discrete initiation sites, but fewer than 5% of transcripts initiate from this region. Analysis of the chromatin structure of the P1 promoter region by DNase I hypersensitivity assays supports the possibility that this promoter may be constitutively active, and implies that the expression of the human *Bcl-2* gene could

Figure 3. Topology of the human Bcl-2 protein

The locations of the Bcl-2 homology (BH) domains, the predicted α-helical segments, and the transmembrane (TM) anchor of the human Bcl-2 protein are depicted. The BH3 domain (second α-helix) plays the role of 'ligand' during dimerization of the Bcl-2 family proteins, whereas the combination of the BH1, BH2 and BH3 domains appears to be required for forming the hydrophobic groove into which the BH3 domain inserts. The fifth and sixth α-helices are predicted to participate in channel formation, presumably by penetrating lipid bilayers.

Human Bcl-2 protein



Reed, 1997. *Nature* **387**: 775

be regulated by negative or positive regulatory elements located elsewhere in the gene (Young and Korsmeyer, 1993).

p53

It is becoming increasingly apparent that a central cause of cancer is genetic instability, and p53, by regulating normal responses to DNA damage and other forms of genotoxic stress, is a key element in maintaining genomic stability. The *p53* gene has become the center of intensive study ever since it was recognized that slightly more than 50% of human cancers contain mutations in this gene (Hollstein et al., 1994). The nature of these genetic changes in cancer cells is most commonly a missense mutation in one allele, producing a faulty protein that is then observed at high concentrations in these cells, followed by a reduction to homozygosity. As well, *p53*$^{-/-}$ mice are highly prone to spontaneous tumour formation and predominantly develop lymphomas. The p53 protein resides primarily in the nucleus where it functions at least in part as a transcriptional regulator. The wild-type p53 protein has been shown to act as a tumour suppressor by causing growth arrest and apoptotic cell death when transfected into transformed cell lines (Yonish-Rouach et al., 1991). The growth arrest and apoptosis are separable events, although both require the presence of p53 protein in the cell nucleus during the G1 phase of the cell cycle (Ryan et al., 1993).

The human p53 protein contains 393 amino acids and has been divided structurally and functionally into four domains (reviewed in Ko and Prives, 1996). The first 42 amino acids at the N-terminus constitute a transcriptional activation

domain that interacts with the basal transcriptional machinery in positive regulating gene expression. The sequence-specific DNA binding domain of p53 is localized between amino acid 102 and 292. It is a protease-resistant and independently folded domain containing a zinc ion that is required for its DNA binding activity. The p53 protein binds DNA as a tetramer to its consensus sequence containing two copies of the 10-mer (5'RRRCA/TT/AGYYY-3'). The residues required for oligomerization of the protein lie within the carboxyl terminus (324-355) and are linked to the DBD by a flexible linker of 37 residues (287-323).

As p53 is such a potent inhibitor of cell growth, its function must be tightly controlled to allow normal growth development. This is achieved through several mechanisms that include regulation of p53 transcription and translation, protein stability, subcellular localization and activity (Woods and Vousden, 2001). One of the key regulators of p53 is the MDM2 protein, which can both inhibit p53's transcriptional activity and target p53 for degradation (Chen et al., 1994; Nakamura et al., 2000). MDM2 can function as an E3 ligase, the final component of the enzyme cascade that results in the conjugation of ubiquitin to proteins, targeting them for degradation by the proteasome. MDM2 ubiquitinates p53 and itself, contributing to the rapid turnover of both proteins (Woods and Vousden, 2001). As MDM2 is a transcriptional target of p53, an autoregulatory feedback loop is generated in which increased p53 activity leads to increased expression of its own negative regulator. The importance of this regulatory loop is demonstrated in mice, where loss of MDM2 expression leads to a very early embryonal lethality caused by aberrant p53-driven apoptosis (de Rozieres et al., 2000).

In most cases, induction of p53 in response to stress involves the inhibition of MDM2 function, which is achieved through several different and independent pathways, depending on the stress signal. Mechanisms that regulate the MDM2-induced degradation of p53 include direct repression of MDM2 expression, post-translational modification of p53 and MDM2, expression of proteins that inhibit MDM2 function and regulation of the subcellular localization of p53 or MDM2 (reviewed in Woods and Vousden, 2001). Overall, there appear to be many pathways that allow stress-induced stabilization of p53 and defects in these pathways have been identified in cancers that retain wild-type p53.

The critical role played by p53 in human breast cancer is well established. About 30 to 50% of the cases carry a mutant *p53* gene, and about an additional 30% carry "non-functional" wild-type p53 because the protein is sequestered in the cytoplasm of tumour cells and is stabilized (Moll et al., 1992). The exclusion of the p53 from the cell nucleus eliminates the ability of this protein to inhibit cell proliferation and/or apoptosis. Interestingly, in many breast carcinomas there exists an inverse relationship between p53 and Bcl-2 expression, which is exacerbated in tumours with mutant p53 (Haldar et al., 1994; Baba et al., 1996). p53-induced apoptosis can be blocked by overexpression of Bcl-2 (Wang et al., 1993), suggesting that p53 and Bcl-2 may participate in a common pathway for regulation of cell life and death. One interpretation of this finding is that Bcl-2 contributes to the survival of breast cancer cells in the presence of wild-type p53 protein.

There is evidence that p53 can down-regulate *Bcl-2* gene expression both *in vivo* and *in vitro* (Miyashita et al., 1994a). Specifically, expression of a temperature-

sensitive p53 mutant in *p53*-deficient murine myeloblastic leukemia cell line M1 was shown to result in reductions in Bcl-2 mRNA and protein levels upon shift to the permissive temperature. In addition, immunohistochemical and immunoblot analysis of Bcl-2 protein levels in *p53*-deficient transgenic mice revealed elevated levels of Bcl-2 protein in several tissues including spleen, thymus, lymph nodes, and prostate. The mechanism by which this repression occurs remains to be elucidated, however it appears to involve, at least in part, a p53-responsive negative regulatory element (PNRE) located in the 5'-untranslated region of the *Bcl-2* promoter (Miyashita et al., 1994b).

OBJECTIVES

Although Bcl-2 is one of the most recognized regulators of apoptosis, little is known about the molecular mechanisms controlling its expression. I have examined this aspect regarding Bcl-2 by addressing the following objectives:

(I) Determining the functional relationship of estrogen and its receptor with the Bcl-2 promoter;

(II) Investigating the potential interaction between p53 and estrogen in the regulation of Bcl-2.

CHAPTER II

MATERIALS AND METHODS

Cell Culture

MCF-7 ER-positive breast cancer cells (a gift of Dr. Leigh Murphy, Winnipeg) were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco BRL) containing phenol red and supplemented with 5% v/v heat-inactivated fetal bovine serum (FBS) (Gibco BRL), 1% non-essential amino acids, 4.5g/L glucose, 110mg/L sodium pyruvate, and 2 mg/ml gentamicin sulphate. Cells were incubated at 37°C in 5% CO_2. For experiments requiring E2 depletion, cells were pre-cultured for 7-10 days, with three changes of medium, in phenol red free DMEM (Gibco BRL) containing 5% fetal bovine serum stripped of steroids by absorption to dextran-coated charcoal (see below). E2 (Sigma) was added for the indicated times at a final concentration of 10^{-8}M from a 1mM stock solution in ethanol. The pure anti-estrogen ICI 164,384 (the gift of A. E. Wakeling, Zeneca Pharmaceuticals) was used for the indicated times at a final concentration of 10^{-8}M from a 1mM stock in ethanol.

Preparation of Dextran-Coated Charcoal (DCC) Stripped Serum

Fetal bovine serum was stripped of endogenous steroid hormones by treatment with DCC (0.25% Norit A, 0.0025% dextran in 0.01M Tris-HCl pH 8.0). The serum was incubated with the DCC for 30 minutes at 45°C, and the charcoal removed by centrifugation (3000 rpm) for 10 minutes at 4°C.

Plasmids

The *Bcl-2* promoter constructs were the gift of Stanley J. Korsmeyer (Harvard Medical School). The complementary DNA (cDNA) for the human papillomavirus

(HPV) 16-E6 oncoprotein was provided by Peter Howley (Harvard Medical School). The p53 mutant constructs (173L, Δ291) were a generous gift from Karen Vousden (Ludwig Institute for Cancer Research). The glyceraldehyde phosphate dehydrogenase (GAPDH) plasmid was acquired from Balwant Tuana (University of Ottawa).

Construction of Luciferase Reporters

Luciferase plasmid vectors for Bcl-2 promoter expression were generated by standard recombinant DNA techniques (Sambrook *et al.*, 1989). A promoterless luciferase reporter gene expression vector, pGL3-Basic (pGL3B, Promega), was digested with *KpnI* and *HindIII* simultaneously, and the phosphate groups were removed by treatment with calf intestinal alkaline phosphatase (CIAP, Gibco). The reaction was extracted twice with phenol/chloroform and the vector was separated by electrophoresis. The band of interest was exicised from the gel and purified using the Qiagen gel extraction kit. The 1.7 kb estrogen responsive sequence identified in the Long P1 construct was released from the backbone pBluescript vector (Young and Korsmeyer, 1993) by digestion with *KpnI* and *HindIII*. The 1.7 kb band was separated on a 1% agarose gel, excised and purified as described above. The 1.7 kb fragment was ligated into the *KpnI-HindIII* digested pGL3B vector using T4 ligase and buffer (Gibco BRL) at 16°C for 4 to 16 hours to generate 1.7 kb-Bcl-2Pluc. Similarly, a *NarI-Hind*III 0.5 kb fragment of Long P1 was blunted using a dNTP mix and T4 polymerase (Gibco), and ligated into the *Sma*I site of pGL3-Basic to generate 0.5-Bcl-2Pluc. Ligation reactions were used to transform *E. coli* DH5α

and positive clones were identified by restriction enzyme mapping of miniprep plasmid DNA. Proper orientation of the Bcl-2 promoter inserts in these luciferase plasmids was analyzed by restriction enzyme digest and subsequently verified by DNA sequencing (ABI Automated Sequencer).

Plasmid Isolation

Plasmids were routinely purified from overnight bacterial cultures grown in Luria-Bertani (LB) broth (1% Bacto-tryptone, 0.5% yeast extract, 1% sodium chloride (NaCl)) containing 50μg/ml ampicillin (Amp) (Sambrook *et al.*, 1989). Plasmid DNA was isolated by the alkaline lysis method (Birnboim and Doly, 1979) in the presence of RNase A (500μg/ml) prior to purification on a mega prep column (Qiagen) according to the manufacturer's protocol.

Generation of Stable Clones in MCF-7 Cells

To generate stable cell lines, MCF-7 cells were trypsinized and plated in 100mm dishes at a confluence of 30 to 50%. The following day cells were co-transfected with 7.5μg of the desired expression plasmid and 7.5μg of cytomegalovirus-puromycin (CMV-puro), a selectable expression construct, using the calcium phosphate co-precipitation method (Chen and Okayama, 1987). Cells were incubated with the precipitate for 8 hours, then washed with 1X phosphate buffered saline (137mM NaCl, 27mM potassium chloride, 10mM di-sodium hydrogen orthophosphate, 1.76mM potassium dihydrogen orthophosphate) (PBS) and subjected to hypotonic shock for 3 minutes using DMEM containing 20% glycerol

and 2% FBS. Cells were selected for puromycin resistance in medium containing 2μg/ml of puromycin over a 2-3 week period with several changes of medium. When no further cell death was evident, individual colonies were isolated using a sterile pipette tip and transferred to a multiwell plate, expanded, and assayed for the presence or expression of the transfected gene.

Stable Expression of Bcl-2 Promoter Constructs in MCF-7 Cells

To generate stable cell lines expressing the different Bcl-2 promoter constructs, MCF-7 cells were co-transfected with expression plasmid (Long P1+P2, Short P1+P2, Long P1, Short P1, Long P2 and Short P2) or pTZ18 (negative control) plasmid and CMV-puro. The plasmids are described in detail in the Results section. The clones were assayed for the presence of specific promoter constructs by Southern blot analysis.

Generation of p53-deficient MCF-7 Cells

To generate p53 deficient clones, MCF-7 cells were co-transfected with an expression plasmid encoding the HPV16-E6 gene, and CMV-puro. Individual colonies were screened for expression of the E6 gene by Northern blot analysis. E6-expressing clones were further analyzed by Western blot to confirm deficits in p53 expression.

Stable Expression of Mutant p53 Proteins in MCF-7 Cells

MCF-7 cells were co-transfected with an expression plasmid encoding one of the mutant p53 proteins (173L, Δ291) (Marston *et al.*, 1994) and CMV-puro. The expression of mutant p53 was detected by Northern and Western blot analysis.

Transient Transfections

MCF-7 cells were transfected in 60mm dishes using the calcium phosphate co-precipitation method. To correct for differences in transfection efficiencies between dishes, 2.5μg of pCMV-βgalactosidase (pCMV-βgal) plasmid were co-transfected with 2.5μg of luciferase reporter constructs and either 2.5μg of a plasmid encoding the human estrogen receptor (hER) or pcDNA3 (control vector). Cells were incubated with $CaHPO_4$ for 6-8 hours in phenol red free DMEM containing charcoal-treated FBS as well as other appropriate supplements (see Cell Culture). Following transfection, the cells were incubated for 48-60 hours in fresh medium before assaying for luciferase activity. Cells were treated prior to harvest with either vehicle (0.1% ethanol) or E2.

DNA Isolation and Analysis

Total genomic DNA from MCF-7 cells stably transfected with the different Bcl-2 promoter constructs was isolated as described previously (Sambrook *et al.*, 1989). In brief, cells were washed with PBS and harvested in 2ml of extraction buffer (10mM Tris-HCl pH 8.0, 10mM ethylenediaminetetracetic acid (EDTA) pH 8.0, 10mM NaCl, 0.5% sodium dodecyl sulfate (SDS)). The cells were scraped into 15ml

polypropylene tubes (Falcon) and incubated with 50μg/ml proteinase K (Sigma) overnight at 37°C. An equal volume of phenol (pH 8.0) was added to the tubes and gently mixed by inversion. The aqueous and organic phases were separated by centrifugation at 5000 rpm for 10 minutes. The aqueous layer was transferred to a fresh tube where an equal volume of chloroform/isoamyl alcohol (24:1) was added and mixed by inversion. The two phases were again separated by centrifugation at 5000 rpm for 5 minutes. The aqueous layer was transferred to a new tube and the phenol-chloroform extraction step was repeated 2 times. After the last extraction, the aqueous layer was incubated with 50μg/ml RNase A (Sigma) at 37°C for 30 minutes, followed by a final phenol-chloroform extraction. To the aqueous layer, 0.1 volumes of 3M sodium acetate (pH 5.2) and 2 volumes of ice-cold ethanol were added to precipitate the genomic DNA. The DNA was spooled out on a glass rod, resuspended in 500μl TE (10mM Tris-HCl pH 8.0, 1mM EDTA) and quantified using a Beckman DU 640 spectrophotometer. DNA samples (10μg) were digested overnight with *EcoR*I and electrophoresed on a 1% agarose gel. Following size fractionation, the DNA was denatured in an alkaline denaturation solution (1.5M NaCl, 0.5M NaOH) for 20 minutes and then neutralized (1.5M NaCl, 0.5M Tris-HCl pH 7.0) for the same amount of time. Next, the DNA was transferred by capillary electrophoresis to a positively charged MSI nylon membrane (Fisher), using 20X SSC. The membranes were briefly rinsed with 2X SSC before the DNA was immobilized by UV cross-linking (1200 mJ for 30 seconds). The membrane blots were pre-hybridized for a minimum of 4 hours at 42°C in hybridization cocktail (50% deionized formamide, 5X SSPE (0.75M NaCl, 50mM sodium orthophosphate, 5mM

EDTA, pH 7.0), 5X Denhardts (0.5% polyvinylpyrrolidone, 0.5% bovine serum albumin, 0.5% Ficoll 400), 0.1% SDS and 250μg/ml denatured, sonicated herring sperm DNA) prior to addition of radiolabeled probes. Blots were incubated overnight at 42°C with a 900 bp cDNA fragment corresponding to a region of the neomycin resistance gene that was labeled with [α-32P] dCTP (DuPONT NEN) to a specific activity of a least 1 x 10^9 cpm/μg of DNA using the Rediprime labeling kit (Amersham). Following hybridization, blots were washed twice at low stringency (2X SSC, 0.1% SDS; room temperature, 20 minutes) and once at high stringency (0.1X SSC, 0.1% SDS; 65°C, 5-30 minutes). Membranes were exposed to Kodak Biomax MR X-ray film for several days at -70°C.

RNA Isolation and Northern Analysis

Total cellular RNA was isolated using the lithium chloride-urea (LiCl-urea) method (Auffray and Rougeon, 1980). Briefly, cells were washed with PBS and harvested in 2ml of 8M urea/3M LiCl. The cells were scraped into 15ml polypropylene tubes (Falcon), homogenized for 2 minutes using a polytron (Fisher), and incubated overnight at 4°C. Samples were then centrifuged (Beckmen J2-MC) for 15 minutes at 7000 rpm, and the pellets were washed with 3M LiCl using a 22-gauge syringe. The samples were transferred to 1.5ml microfuge tubes and centrifuged (Eppendorf 5417R) for 10 minutes at 4°C. The resulting RNA pellet was resuspended in 300μl of diethylpyrocarbonate (DEPC)-treated water. RNA samples (20μg) were size fractionated in a 1% agarose gel containing 2% formaldehyde and 1X MOPS buffer (20mM 3-[N-morpholino]propanesulfonic acid (MOPS), 5mM sodium acetate, 1mM

EDTA, pH 7.0). The RNA was subsequently transferred to a positively charged, nylon MSI membrane using 10X SSC (1.5M sodium chloride, 0.15M sodium citrate, pH 7.0). The membranes were briefly rinsed with DEPC-treated water before RNA immobilization by UV cross-linking. The blots were pre-hybridized as previously described for Southern analysis. Blots were incubated overnight at 42°C with various random-primed cDNA probes labeled with [α-32P] dCTP to a specific activity of at least 1 x 10^9 cpm/μg of DNA using the Rediprime labeling kit. Following hybridization, blots were washed twice at low stringency and once at high stringency (as described for Southern analysis). Membranes were exposed to Kodak Biomax MR X-ray film for anywhere from 2 hours to several days at 70°C, depending on the intensity of the signal detected by a Geiger counter. Accuracy of RNA loading was estimated using a 600 bp probe from the *GAPDH* gene whose mRNA levels do not change during estrogen treatment of MCF-7 cells. All experiments were repeated at least three times and compared to *GAPDH* loading controls.

Western Blot Analysis

Cultured cells were washed twice with PBS and then incubated in 400 μl/10^7 cells of lysis buffer (50mM Tris (pH 8.0), 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 10 μg/ml phenylmethylsulphonyl fluoride, 1 μg/ml aprotinin, and 0.02% sodium azide) for 30 min on ice. Insoluble material was removed by centrifugation at 12 000 x *g* for 15 min at 4°C. Protein concentrations were determined using the BioRad DC protein assay kit. Proteins (25μg) were denatured in sample buffer (63mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5% β-

mercaptoethanol and 0.25% bromophenol blue), resolved on 11% SDS-polyacrylamide gels by electrophoresis (SDS-PAGE) (Laemmli, 1970), and transferred to polyvinylidenedifluoride (PVDF) polyscreen membranes (DuPONT NEN). The blots were blocked with 5% Carnation skim milk powder dissolved in Tris buffered saline (20mM Tris-HCl pH 7.6, 137mM NaCl) containing 0.1% Tween-20 (TBS-T). The filters were incubated for 1 hour at room temperature with the appropriate primary antibodies diluted to a final concentration of 1:1000 in blocking solution. The blots were rinsed two times 5 minutes with TBS-T prior to incubation with horseradish peroxidase conjugated goat anti-mouse or anti-rabbit IgG diluted 1:5000 in TBS-T for 30 minutes at room temperature, rinsed four times (5 minute incubations) with TBS-T and detected using the enhanced chemiluminescence (ECL) system (DuPONT NEN) according to manufacturer's protocol. All experiments were repeated at least three times and compared to α-actin loading controls.

Antibodies

A rabbit polyclonal antiserum specific for human Bcl-2 (generous gift from John Reed, Burnham Institute) and a mouse mAb (Pab 240) specific for both mutant and wild-type p53 (Santa-Cruz Biotechnology) were used for Western blot analysis. Proteins were visualized after incubation with peroxidase-conjugated secondary antibody (Sigma) and chemiluminescent substrate (DuPONT NEN). A rabbit antibody against α-actin (Sigma) was used as an internal control for protein loading.

Luciferase Activity and β-galactosidase Assays

Cells were washed once with PBS buffer and harvested in 400µl 1X Reporter Lysis Buffer (Promega). After one freeze-thaw cycle, the insoluble material was removed by centrifugation at 12 000 x g for 2 minutes at 4°C. Room temperature extract (20µl) was mixed with 100µl of room temperature Luciferase Assay Reagent (Promega) and immediately placed in a BioOrbit 1250 luminometer where the luciferase activity (light produced by the reaction for a period of 10 seconds) was measured. β-galactosidase activity was determined in the same samples using a MUG (methylumbelliferyl galactosidase) assay (Ausubel et al., 1989). Equal amounts of extract and MUG substrate (0.3mM MUG (Sigma), 15mM Tris-HCl pH 8.8) were incubated at 37°C for 30 minutes. The reaction was terminated with MUG stop solution (300mM glycine, 15mM EDTA pH 11.2), mixed with 2ml of Z-buffer (60mM Na_2HPO_4, 40mM $NaHPO_4$, 10mM KCl, 1mM $MgSO_4$) and measured with a Beckman fluorometer. Luciferase activity was divided by β-galactosidase activity to normalize for transfection efficiency. These values were then normalized to the activity of the vector (pGL3-Basic). All experiments were repeated at least three times and compared to negative (pGL3-Basic, Promega) controls.

Densitometry

Quantification of data was done using photo densitometry (MCID Imaging Research Incorporated) of the autoradiogram.

Statistical Analysis

Data were analyzed using one-way analysis of variance (ANOVA) with additional Tukey post-hoc tests where appropriate.

CHAPTER III

RESULTS

Transcriptional activation of *Bcl-2* promoter-reporter constructs by E2

Previous data have demonstrated that estrogen treatment can induce *Bcl-2* mRNA and protein levels in MCF-7 cells, and that this upregulation of *Bcl-2* expression conferred resistance to drug-induced apoptosis in these human breast cancer cells (Teixeira et al., 1995; Kandouz et al., 1996; Lapointe et al., 1999). To further investigate this E2-dependent regulation of *Bcl-2* expression, MCF-7 cells were stably co-transfected with each of the promoter-reporter constructs generated from the 7.8 kb fragment of the *Bcl-2* 5'-UTR linked to the *neo* gene illustrated in Figure 4 (Young and Korsmeyer, 1993) and a puromycin resistance marker. These plasmids contained *Bcl-2* gene promoter inserts subcloned into pBluescript KS(-) (Stratagene), using primer-generated restriction enzyme sites. Stable cell lines were isolated by selection in puromycin and stable integration of the *Bcl-2* promoter constructs was determined by Southern blot analysis using a probe for the *neomycin* reporter gene (Fig. 5). Our previous results showed that MCF-7 cells cultured in the presence of estrogenic compounds (e.g. phenol red) express the *Bcl-2* transcript while depletion of E2 from the medium results in decreased expression of the mRNA to very low levels. Reexposure to estrogen stimulated a robust induction of *Bcl-2* transcript levels within 24 hours (Teixeira et al., 1995). Therefore, stable clones were pre-cultured in phenol red-free medium (i.e. E2-free) for 7 to 10 days before estrogen treatment. Northern blot analysis of *neo* reporter gene expression in MCF-7 cells reflected the activity of the various *Bcl-2* promoter plasmids in cells in the absence or presence of E2 (Figs. 6 and 7). The longest promoter construct (Long P1+P2), which contains both gene promoters, exhibited E2-responsiveness with

Figure 4. Bcl-2 promoter constructs

Map of a 7.8-kb HindIII subclone of the *Bcl-2* 5' UTR from which the Bcl-2Neo promoter constructs were generated by joining *Bcl-2* fragments to the *neomycin* (*neo*) reporter gene. The P1 and P2 promoters are indicated by arrows representing transcriptional initiation sites. The hatched region represents intronic sequences. The constructs that were used to stably transfect MCF-7 cells are shown below.

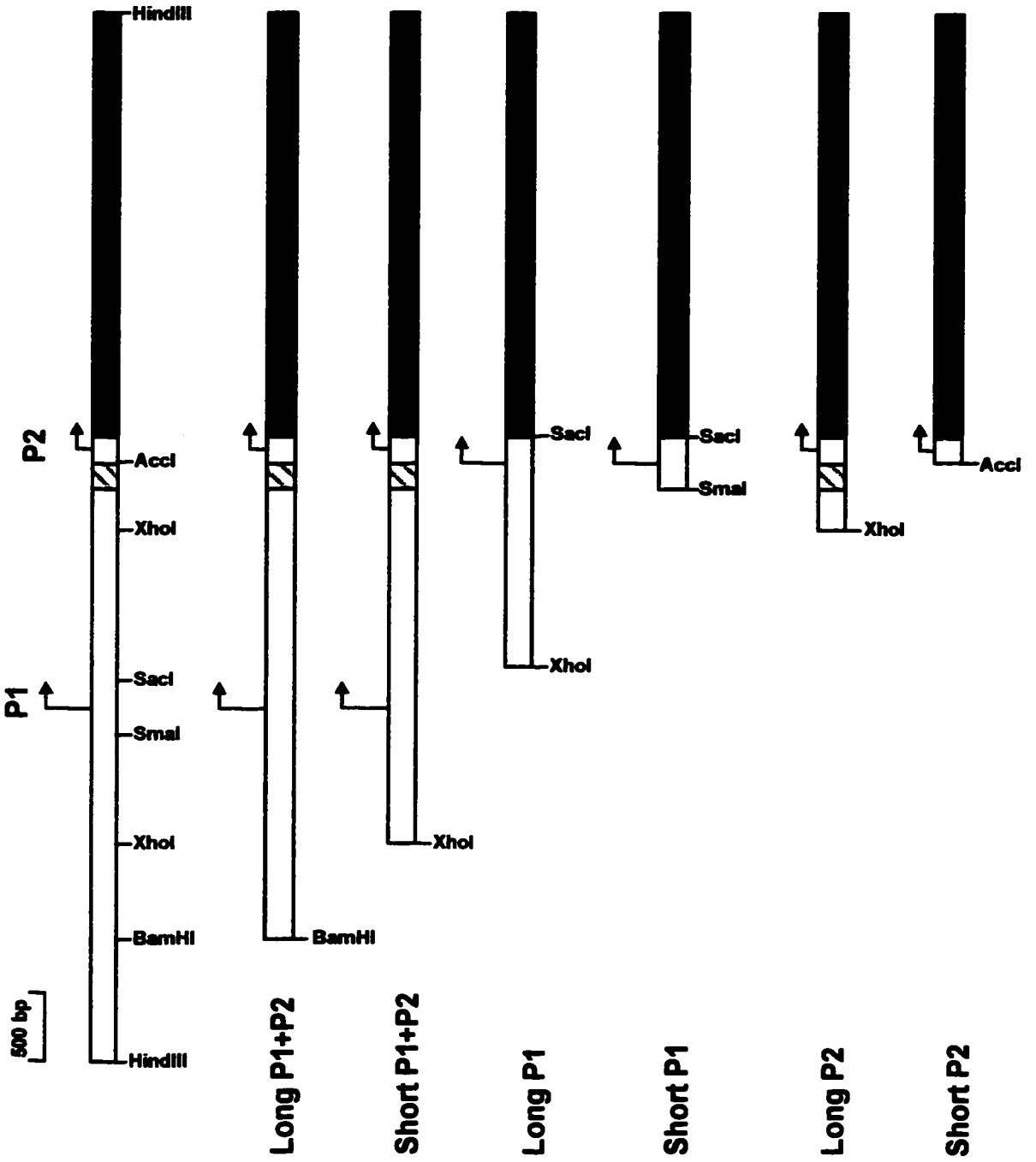

HindIII
P2
AccI
XhoI
P1
SacI
SmaI
XhoI
BamHI
HindIII
500 bp
BamHI
XhoI
SacI
XhoI
SacI
SmaI
XhoI
AccI
Long P1+P2
Short P1+P2
Long P1
Short P1
Long P2
Short P2

Figure 5. Identification of MCF-7 cells stably transfected with Bcl-2 promoter constructs

Detection of neomycin sequences in genomic DNA extracted from MCF-7 clones stably transfected with different Bcl-2Neo promoter constructs (see Figure 4). Cell lines were selected by co-transfection with a plasmid expressing the puromycin resistance gene driven by the cytomegalovirus (CMV) promoter. EcoRI-digested genomic DNA (10 μg) was analyzed by Southern blot with a 900 bp MluI-BglII neo fragment derived from the RSV-neomycin plasmid. Numbers (1,2,3) indicate individual stable clones used for further analysis.

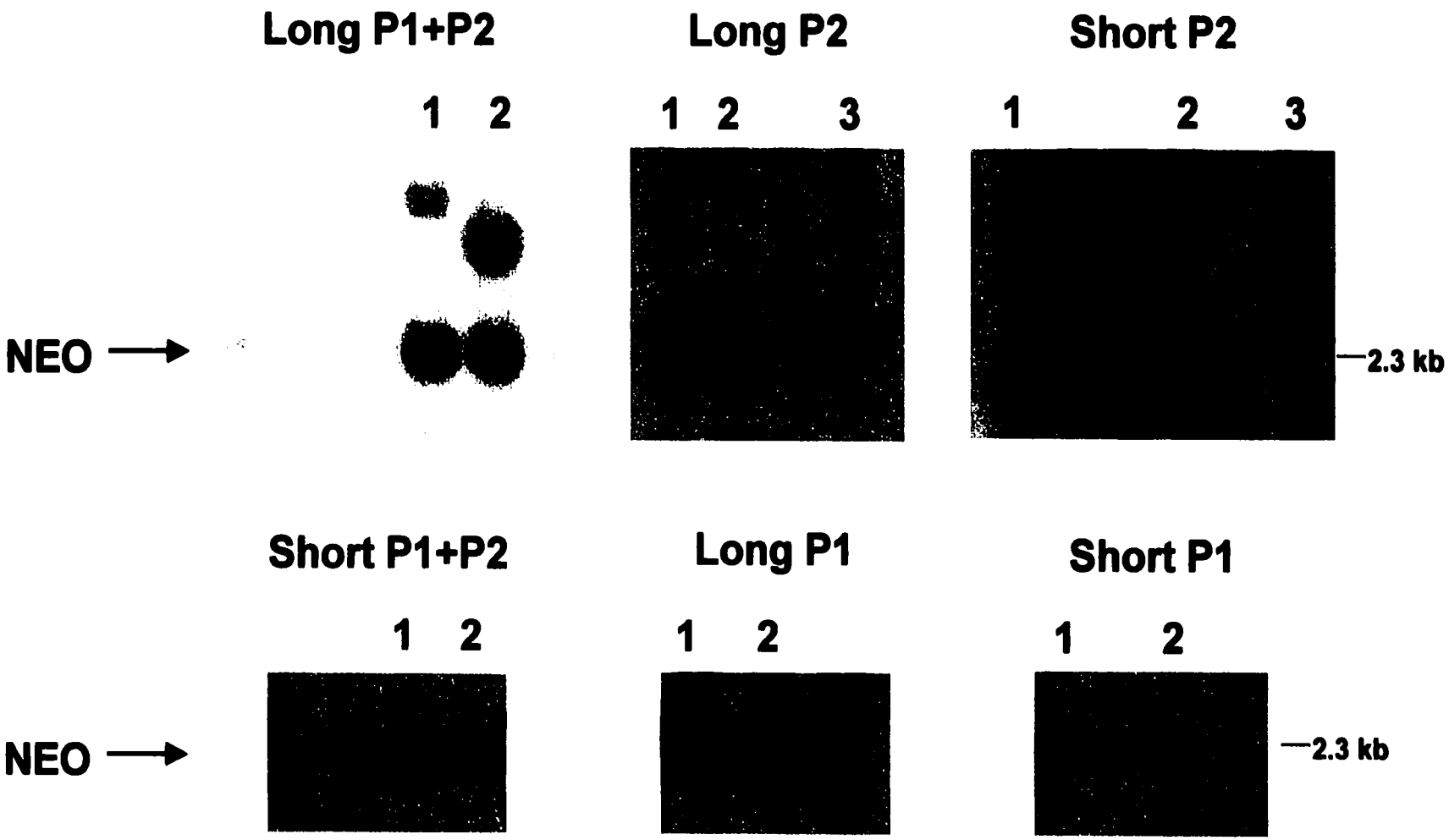
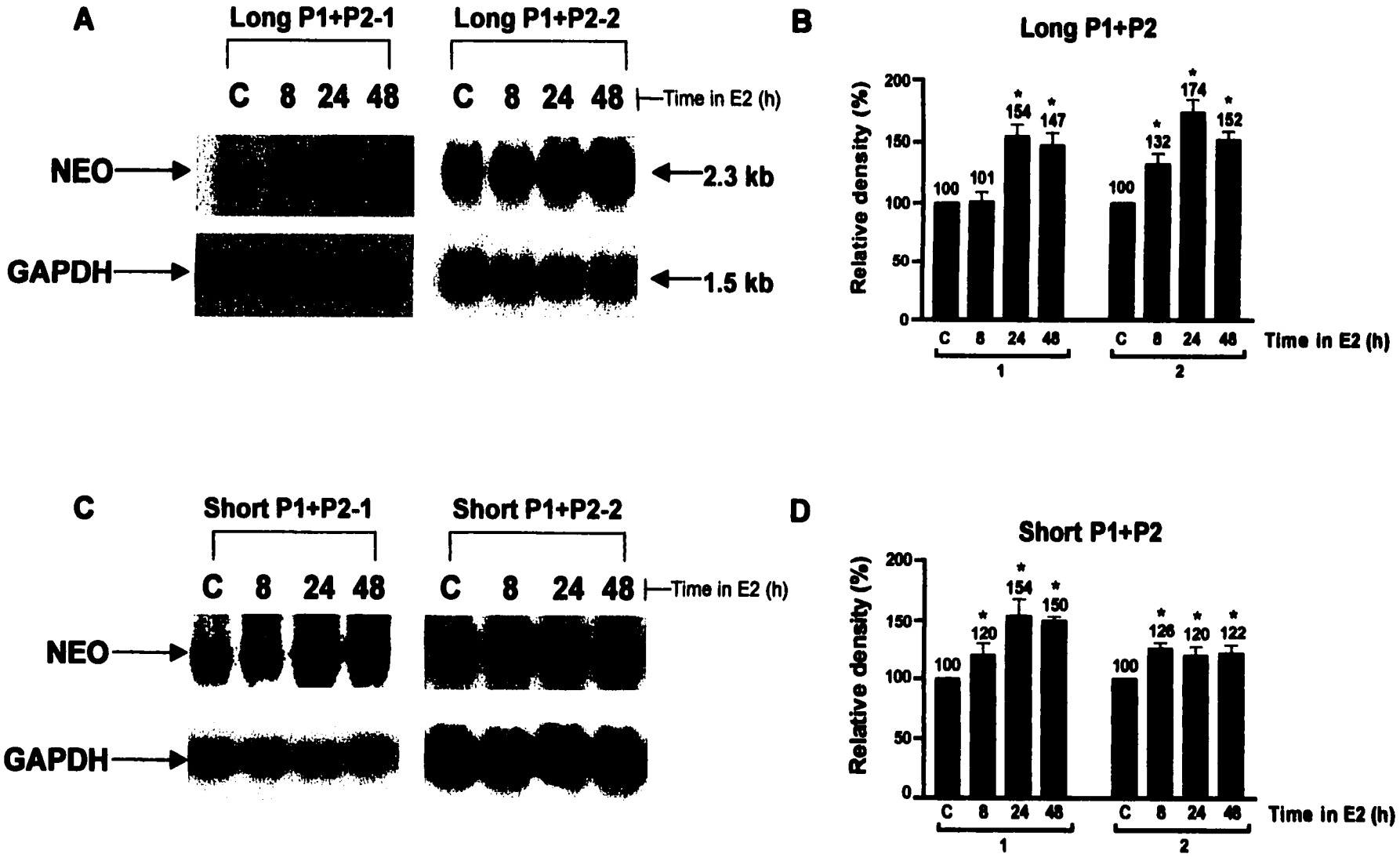

Long P1+P2
Long P2
Short P2
1 2
1 2 3
1 2 3
NEO
2.3 kb
Short P1+P2
Long P1
Short P1
1 2
1 2
1 2
NEO
2.3 kb

Figure 6. Effect of estrogen treatment on transcriptional activity of Bcl-2 promoter-transfected MCF-7 cells

The effect of E2 on *Bcl-2* promoter activity was examined in MCF-7 cells stably transfected with the indicated Bcl-2Neo constructs (see Figure 4 for illustration). **(A)** Northern blot analysis of 20 μg of total RNA isolated from MCF-7 stable clones transfected with the Long P1+P2 *Bcl-2* promoter construct, using a 900 bp MluI-BglII neo probe derived from the RSV-neomycin plasmid. The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) probe was used as an internal control for RNA loading. MCF-7 clones were E2-depleted by culture for 10 days in phenol red free DMEM before treatment for the indicated times (8, 24,and 48 hours) with either vehicle (C) for 48 hours or 10^{-8}M E2. RNA was harvested and analyzed from two separate clones, Long P1+P2-1 and Long P1+P2-2. **(B)** Densitometric analysis was performed separately on the two Long P1+P2 MCF-7 clones and used to determine the percent induction of *Bcl-2* activity relative to *GAPDH* in control and E2 treated cells. **(C)** Similarly, Northern blot analysis of 20 μg of total RNA isolated from MCF-7 stable clones transfected with the Short P1+P2 *Bcl-2* promoter construct and E2-depleted by culture before treatment for the indicated times with either vehicle (C) or 10^{-8}M E2. RNA was harvested and analyzed from two separate clones, Short P1+P2-1 and Short P1+P2-2. **(D)** The Short P1+P2-1 and Short P1+P2-2 Northern blots were further analyzed separately by densitometry to determine the percent induction of *Bcl-2* activity relative to *GAPDH* in control and E2 treated cells. **(E)** Northern analysis of the MCF-7 stable clones, Long P1-1 and Long P1-2, as previously described in section **A**, and **(F)** densitometric analysis of those blots as mentioned in section **B**. **(G)** Northern blot analysis of MCF-7 stable clones transfected with the Short P1 *Bcl-2* promoter construct (Short P1-1 and Short P1-2) was performed as explained in section **A**. **(H)** Analysis of the Short P1-1 and Short P1-2 blots by densitometry as described in section **B**.

Values represent the mean ± standard error of at least three independent experiments. ANOVA results: $^{*}p < 0.05$.


A
Long P1+P2-1
Long P1+P2-2
C 8 24 48
C 8 24 48
Time in E2 (h)
NEO
2.3 kb
GAPDH
1.5 kb
B
Long P1+P2
Relative density (%)
200
150
100
50
0
100
101
154
147
100
132
174
152
C 8 24 48
C 8 24 48
Time in E2 (h)
1
2
C
Short P1+P2-1
Short P1+P2-2
C 8 24 48
C 8 24 48
Time in E2 (h)
NEO
GAPDH
D
Short P1+P2
Relative density (%)
200
150
100
50
0
100
120
154
150
100
126
120
122
C 8 24 48
C 8 24 48
Time in E2 (h)
1
2

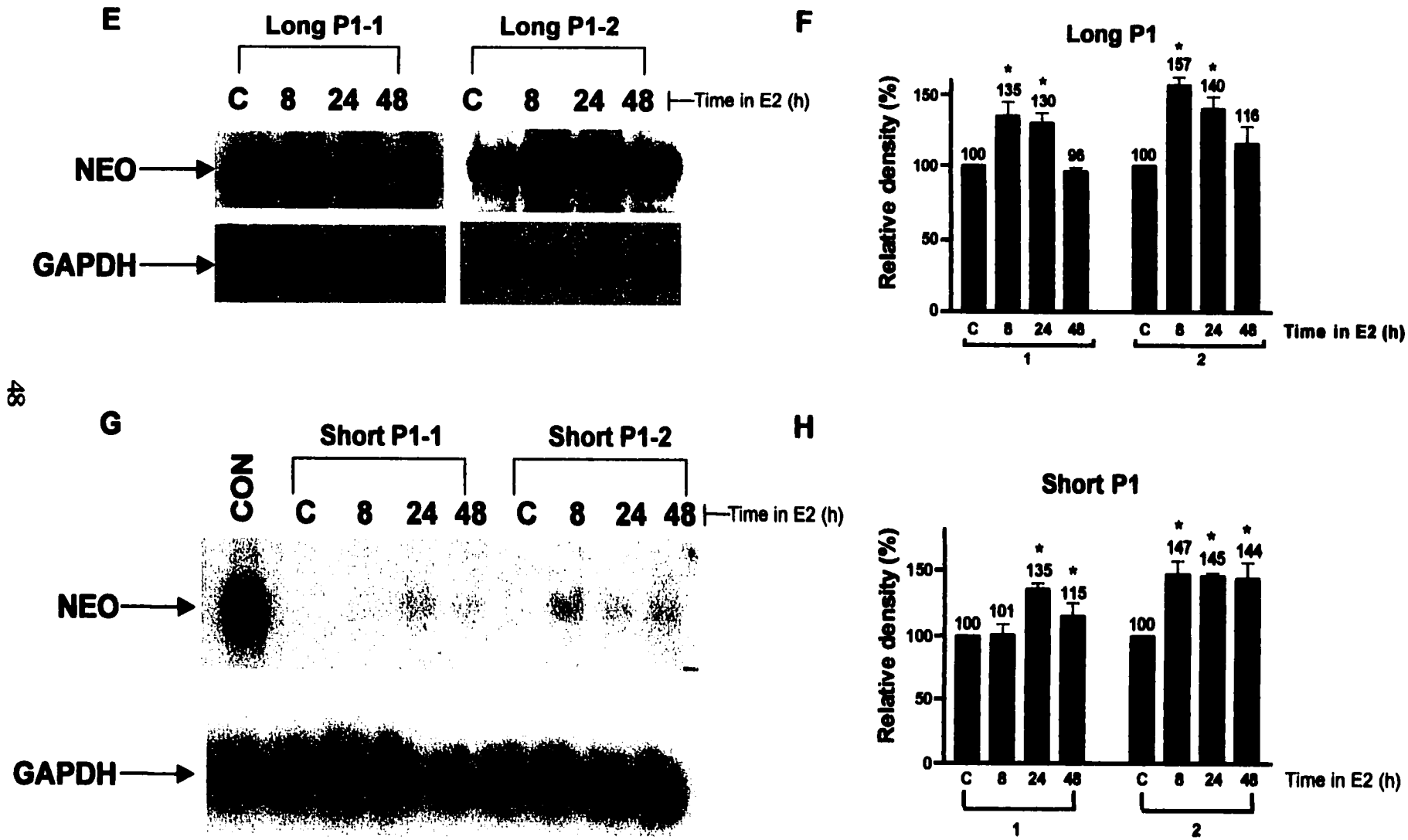

E
Long P1-1
Long P1-2
C 8 24 48 C 8 24 48
Time in E2 (h)
NEO
GAPDH
F
Long P1
Relative density (%)
150
100
50
0
100
135
130
96
100
157
140
116
C 8 24 48 C 8 24 48
Time in E2 (h)
1
2
G
CON
Short P1-1
Short P1-2
C 8 24 48 C 8 24 48
Time in E2 (h)
NEO
GAPDH
H
Short P1
Relative density (%)
150
100
50
0
100
101
135
115
100
147
145
144
C 8 24 48 C 8 24 48
Time in E2 (h)
1
2

Figure 7. Expression of Bcl-2 in MCF-7 cells requires P1

The effect of E2 on *Bcl-2* promoter activity was examined in MCF-7 cells stably transfected with the indicated Bcl-2Neo constructs. **(A)** Northern blot analysis of 20 μg of total RNA isolated from MCF-7 stable clones transfected with the Long P2 *Bcl-2* promoter construct, using a cDNA neo probe. *GAPDH* expression was used as an internal control for RNA loading. The MCF-7 clones, Long P2-1, Long P2-2 and Long P2-3 were E2-depleted by culture for 10 days in phenol red free DMEM before treatment for the indicated times (8, 24, 48 hours) with vehicle (C) or 10^{-8}M E2. **(B)** Densitometric analysis was performed separately on the three Long P2 MCF-7 clones and used to determine the percent induction of *Bcl-2* activity relative to *GAPDH* in control and E2 treated cells. **(C)** Northern blot analysis of RNA harvested from MCF-7 stable clones, Short P2-1, Short P2-2 and Short P2-3 that were E2-depleted by culture before treatment for the indicated times with vehicle (C) for 48 hours or 10^{-8}M E2. **(D)** Densitometric analysis was performed separately on the three Short P2 MCF-7 clones and used to determine the percent induction of *Bcl-2* activity relative to *GAPDH* in control and E2 treated cells. Values represent the mean ± standard error of at least three independent experiments.

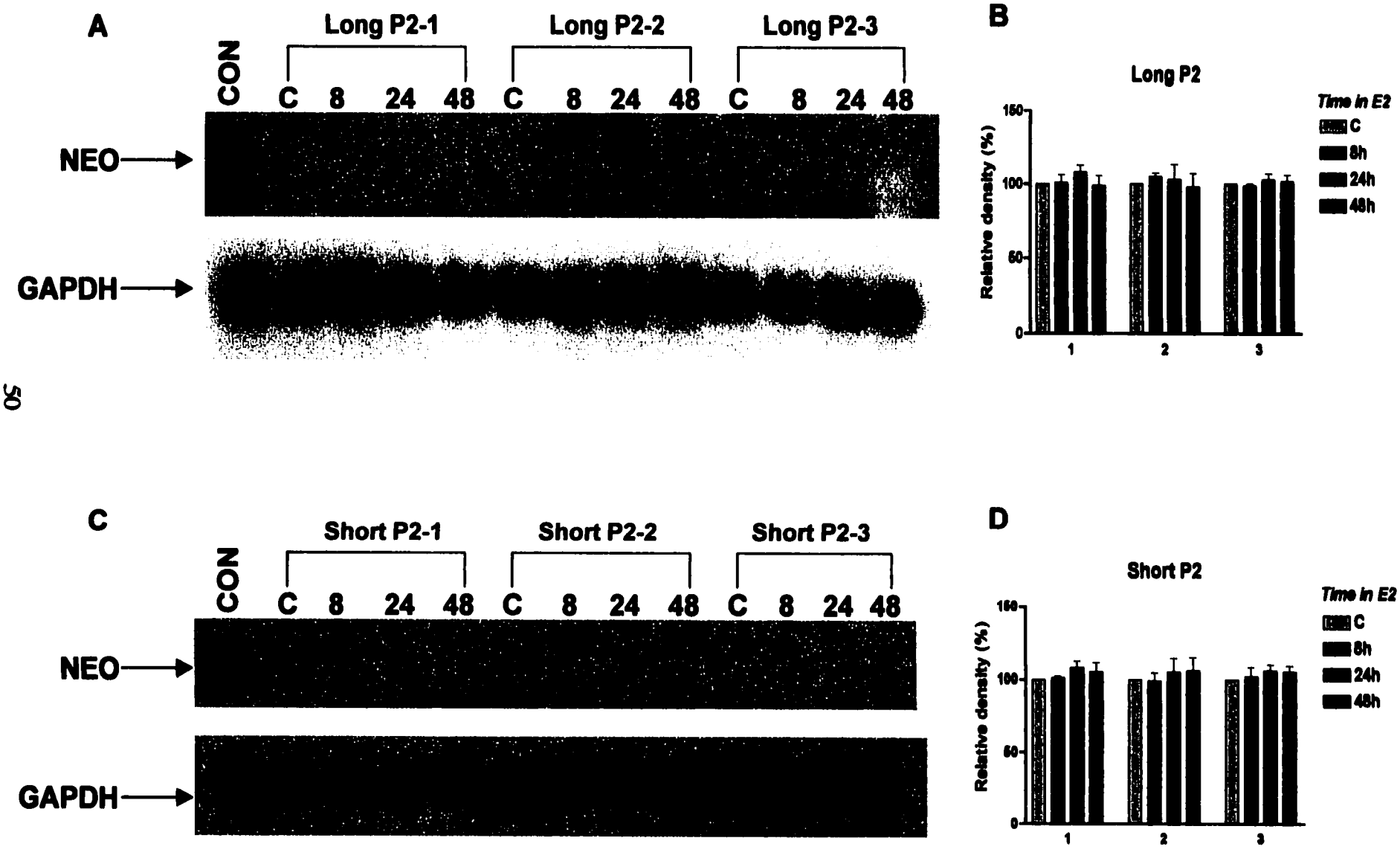

A
Long P2-1
Long P2-2
Long P2-3
CON
C 8 24 48 C 8 24 48 C 8 24 48
NEO
GAPDH
B
Long P2
Relative density (%)
150
100
50
0
1
2
3
Time in E2
C
8h
24h
48h
C
Short P2-1
Short P2-2
Short P2-3
CON
C 8 24 48 C 8 24 48 C 8 24 48
NEO
GAPDH
D
Short P2
Relative density (%)
150
100
50
0
1
2
3
Time in E2
C
8h
24h
48h

Figure 8. E2 effect on Bcl-2 promoter activity in MCF-7 cells

(A) Map of *Bcl-2* promoter luciferase constructs generated by ligating different *Bcl-2* fragments into a vector containing the luciferase reporter gene (see Materials and Methods). (B) Luciferase activity was measured in lysates from E2-depleted MCF-7 cells transiently transfected with Bcl-2Pluc constructs and treated with vehicle (C) or 10^{-8}M E2 for the indicated times. Luciferase values were normalized against β-galactosidase activities. Values represent the mean ± standard error of at least three independent experiments. ANOVA results: $^{*}p < 0.05$; $^{**}p < 0.01$.

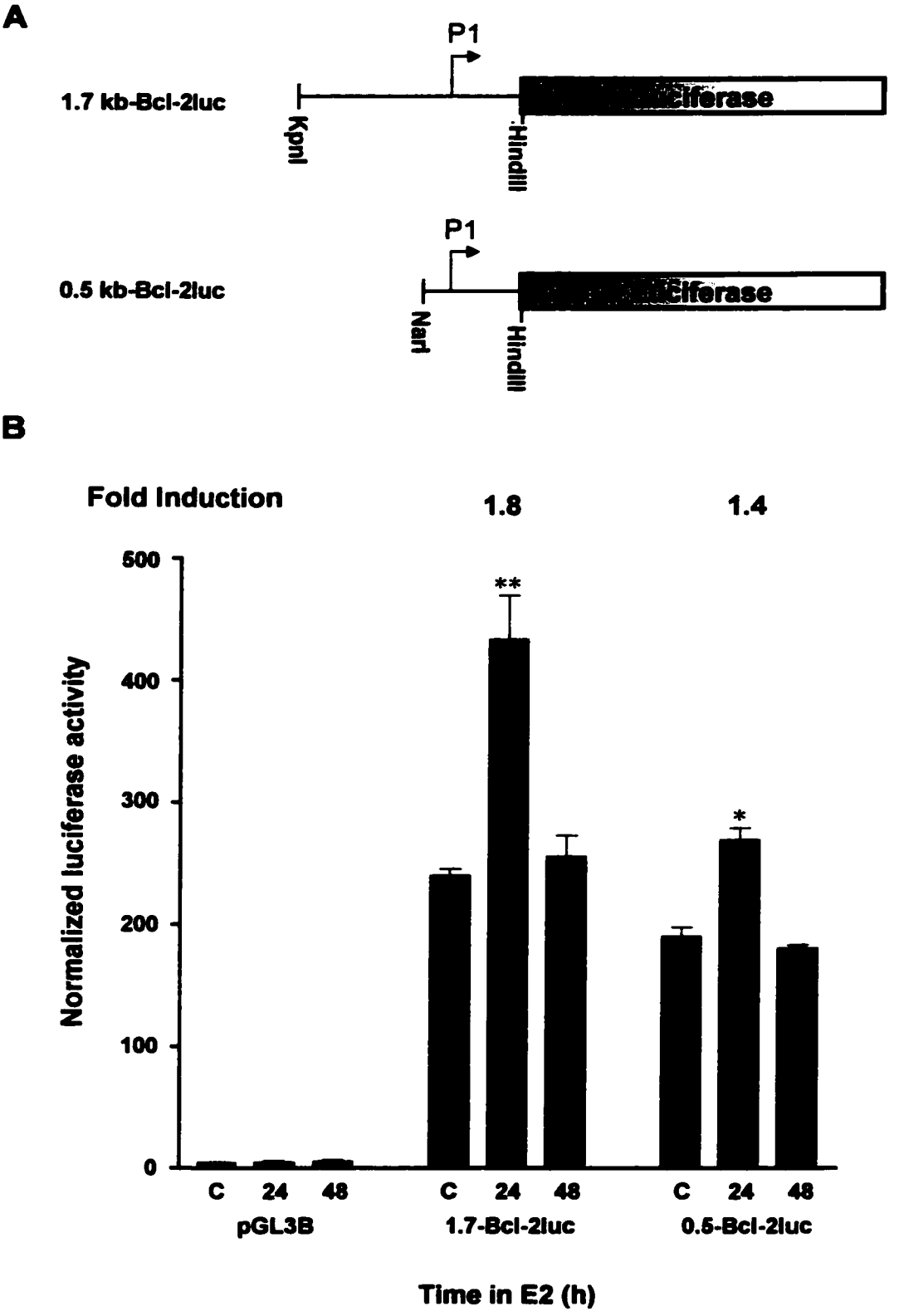
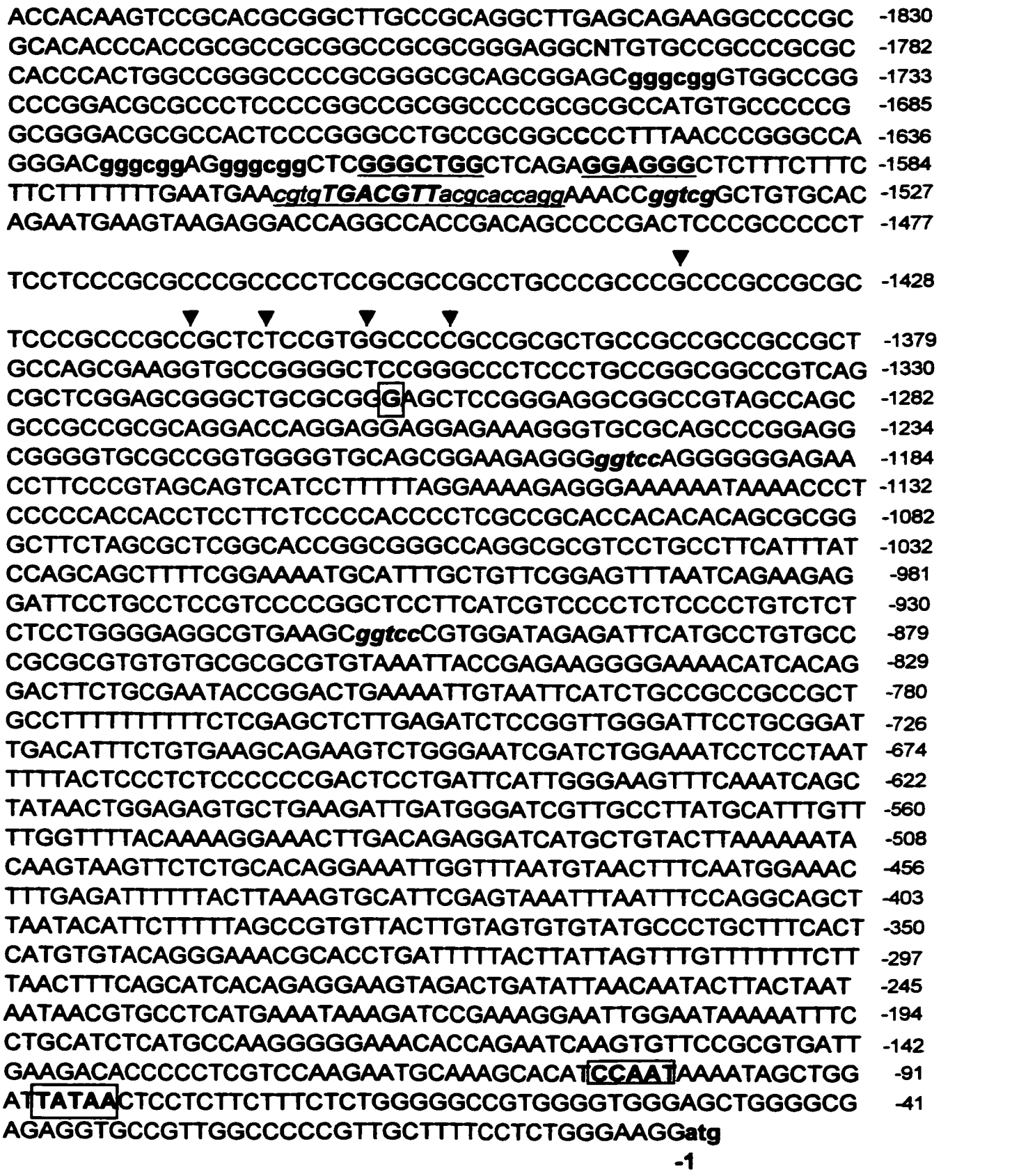

A
P1
1.7 kb-Bcl-2luc
KpnI
HindIII
P1
0.5 kb-Bcl-2luc
NarI
HindIII
B
Fold Induction
1.8
1.4
Normalized luciferase activity
500
400
300
200
100
0
**
*
C
24
48
C
24
48
C
24
48
pGL3B
1.7-Bcl-2luc
0.5-Bcl-2luc
Time in E2 (h)

Figure 9. Sequence analysis of the genomic 5' untranslated region (UTR) of the *Bcl-2* gene.

A schematic representation of the 5' UTR of the *Bcl-2* gene, much of which has been left unpublished, illustrating several important and putative regulatory regions. The P2 promoter region is marked by the presence of TATAA and CCAAT motifs (boxed areas). Several Sp1 binding sites (**gggcgg**) and arrowheads which indicate transcriptional initiation sites, mark the region of the principal promoter, P1. The GC-rich regions which bind Sp1 proteins and are allegedly responsible for E2-directed *Bcl-2* transcriptional upregulation are –1613 (5'-**GGGCTGG**) and –1600 (5'-**GGAGGG**). Another downstream E2-responsive region which contains a cAMP responsive element (CRE) begins at –1567 (5'-*cgtg**TGACGT**Tacgcaccagg*). The CRE motif is indicated in bold letters. Other possible enhancer sites include estrogen response element (ERE) half-sites. These sites are imperfect and are indicated as ***ggtcn***, where **n** can be any nucleotide. The boxed and bolded **G** at position –1300 marks the 3' end of the *Bcl-2* promoter fragment found in the Long P1 construct, and the 1.7 kb and 0.5 kb luciferase constructs.


ACCACAAGTCCGCACGCGGCTTGCCGCAGGCTTGAGCAGAAGGCCCCGC -1830
GCACACCCACCGCGCCGCGGCCGCGCGGGAGGCNTGTGCCGCCCGCGC -1782
CACCCACTGGCCGGGCCCCGCGGGCGCAGCGGAGCgggcggGTGGCCGG -1733
CCCGGACGCGCCCTCCCCGGCCGCGGCCCCGCGCGCCATGTGCCCCCG -1685
GCGGGACGCGCCACTCCCGGGCCTGCCGCGGCCCCTTTAACCCGGGCCA -1636
GGGACgggcggAGgggcggCTCGGGCTGGCTCAGAGGAGGGCTCTTTCTTTC -1584
TTCTTTTTTTGAATGAAcgtgTGACGTTacgcaccaggAAACCggtcgGCTGTGCAC -1527
AGAATGAAGTAAGAGGACCAGGCCACCGACAGCCCCGACTCCCGCCCCCT -1477
TCCTCCCGCGCCCGCCCCTCCGCGCCGCCTGCCCGCCCGCCCGCCGCGC -1428
TCCCGCCCGCCGCTCTCCGTGGCCCCGCCGCGCTGCCGCCGCCGCCGCT -1379
GCCAGCGAAGGTGCCGGGGCTCCGGGCCCTCCCTGCCGGCGGCCGTCAG -1330
CGCTCGGAGCGGGCTGCGCGGGAGCTCCGGGAGGCGGCCGTAGCCAGC -1282
GCCGCCGCGCAGGACCAGGAGGAGGAGAAAGGGTGCGCAGCCCGGAGG -1234
CGGGGTGCGCCGGTGGGGTGCAGCGGAAGAGGGggtccAGGGGGGAGAA -1184
CCTTCCCGTAGCAGTCATCCTTTTTAGGAAAAGAGGGAAAAAATAAAACCCT -1132
CCCCCACCACCTCCTTCTCCCCACCCCTCGCCGCACCACACACAGCGCGG -1082
GCTTCTAGCGCTCGGCACCGGCGGGCCAGGCGCGTCCTGCCTTCATTTAT -1032
CCAGCAGCTTTTCGGAAAATGCATTTGCTGTTCGGAGTTTAATCAGAAGAG -981
GATTCCTGCCTCCGTCCCCGGCTCCTTCATCGTCCCCTCTCCCCTGTCTCT -930
CTCCTGGGGAGGCGTGAAGCggtccCGTGGATAGAGATTCATGCCTGTGCC -879
CGCGCGTGTGTGCGCGCGTGTAAATTACCGAGAAGGGGAAAACATCACAG -829
GACTTCTGCGAATACCGGACTGAAAATTGTAATTCATCTGCCGCCGCCGCT -780
GCCTTTTTTTTTTCTCGAGCTCTTGAGATCTCCGGTTGGGATTCCTGCGGAT -726
TGACATTTCTGTGAAGCAGAAGTCTGGGAATCGATCTGGAAATCCTCCTAAT -674
TTTTACTCCCTCTCCCCCCGACTCCTGATTCATTGGGAAGTTTCAAATCAGC -622
TATAACTGGAGAGTGCTGAAGATTGATGGGATCGTTGCCTTATGCATTTGTT -560
TTGGTTTTACAAAAGGAAACTTGACAGAGGATCATGCTGTACTTAAAAAATA -508
CAAGTAAGTTCTCTGCACAGGAAATTGGTTTAATGTAACTTTCAATGGAAAC -456
TTTGAGATTTTTTACTTAAAGTGCATTCGAGTAAATTTAATTTCCAGGCAGCT -403
TAATACATTCTTTTTAGCCGTGTTACTTGTAGTGTGTATGCCCTGCTTTCACT -350
CATGTGTACAGGGAAACGCACCTGATTTTTACTTATTAGTTTGTTTTTTTCTT -297
TAACTTTCAGCATCACAGAGGAAGTAGACTGATATTAACAATACTTACTAAT -245
AATAACGTGCCTCATGAAATAAAGATCCGAAAGGAATTGGAATAAAAATTTC -194
CTGCATCTCATGCCAAGGGGGAAACACCAGAATCAAGTGTTCCGCGTGATT -142
GAAGACACCCCCTCGTCCAAGAATGCAAAGCACATCCAATAAAATAGCTGG -91
ATTATAACTCCTCTTCTTTCTCTGGGGGCCGTGGGGTGGGAGCTGGGGCG -41
AGAGGTGCCGTTGGCCCCCGTTGCTTTTCCTCTGGGAAGGatg
-1

maximal induction of *neo* expression occurring after 24 hour estrogen treatment (Fig. 6A, B). The Short P1+P2 construct, a 5' deletion mutant of the Long P1+P2, retained the hormone responsiveness as did the Long P1 construct, which lacks the P2 promoter region (Fig. 6C, D). Maximal induction by estrogen of the Long P1 occurs after 8 hours treatment and appears to decline at 48 hours (Fig. 6E, F). The Short P1 construct also exhibited neo induction although minimally (Fig. 6G, H). Therefore, all P1 containing plasmids were active in MCF-7 cells in contrast to the P2 containing constructs (Long P2, Short P2) which exhibited no activity either in the presence or absence of estrogen (Fig. 7A, B). It was concluded from these data that the E2-reponsiveness of the Bcl-2 promoter was retained in the 1.7 kb XhoI-SacI region of the Long P1 construct (see Fig. 4).

To further define the minimal E2 responsive element of this 1.7 kb sequence, restriction fragments of this region were ligated into an expression vector containing the luciferase reporter gene (pGL3-Basic; Promega) and used for transient transfection studies in MCF-7 cells. We turned to the luciferase system because it is an extremely sensitive assay, much more so than Northern blot analysis, and is time-efficient since it allows for the execution of transient transfections in place of generating stable clones. Luciferase activity was normalized for differences in transfection efficiency by co-transfection with a plasmid expressing the β-galactosidase gene driven by the CMV promoter. The 1.7 kb region that conferred E2-responsiveness was excised from its original plasmid and subcloned into pGL3-Basic (see Materials and Methods) to generate 1.7kb-Bcl-2luc (Fig. 8A). Exposure of MCF-7 cells to estrogen transiently transfected with 1.7kb-Bcl-2luc resulted in an

almost 2-fold increase in luciferase activity. The pattern of *Bcl-2* promoter-mediated gene transcription by E2 was similar to that observed in the Long P1 stable clones, however, in this context maximal induction of luciferase activity was seen after 24 hour hormone treatment (Fig. 8B). To further define the estrogen responsive region we made a smaller construct, a 5' truncated fragment of the 1.7 kb region, the 0.5kb-Bcl-2luc construct (Fig. 8A). Although estrogen stimulated a 1.4 fold-induction in luciferase activity in MCF-7 cells transiently transfected with the 0.5kb-Bcl-2luc construct (Fig. 8B), maximal induction of gene transcription probably requires the full 1.7 kb promoter fragment. In these studies this 500 bp sequence was identified as the minimal estrogen responsive element required for E2-directed transcriptional regulation of *Bcl-2* expression.

Regulation of Bcl-2 by p53 in the presence of estrogen

The involvement of p53 in mediating DNA damage induced cell cycle arrest or apoptosis in certain cell types is well documented (reviewed in Levine, 1997). Interestingly, it also appears to play a role in the regulation of *Bcl-2* expression in E2-dependent breast cancer cells. In many breast carcinomas there exists an inverse relationship between p53 and *Bcl-2* expression, which is enhanced in tumours with mutant p53 (Haldar et al., 1994). There is evidence that p53 can down-regulate *Bcl-2* expression both *in vivo* and *in vitro* (Miyashita et al., 1994a), but the mechanism by which this repression occurs remains to be elucidated. To further characterize this relationship we investigated

Figure 10. Isolation of MCF-7 (HPV16-E6) stable cell lines

MCF-7 cells were transfected with the human papilloma virus *HPV16-E6* oncogene to reduce or eliminate p53 protein. Stable clones were selected by co-transfection with a plasmid expressing the puromycin resistance gene driven by the CMV promoter and E6 expression was verified by Northern blot analysis. The MCF-7/E6 clones used for further experiments were labelled 3 and 9. Untransfected MCF-7 cells cultured in phenol red containing media (DMEM) were used as controls (CON).

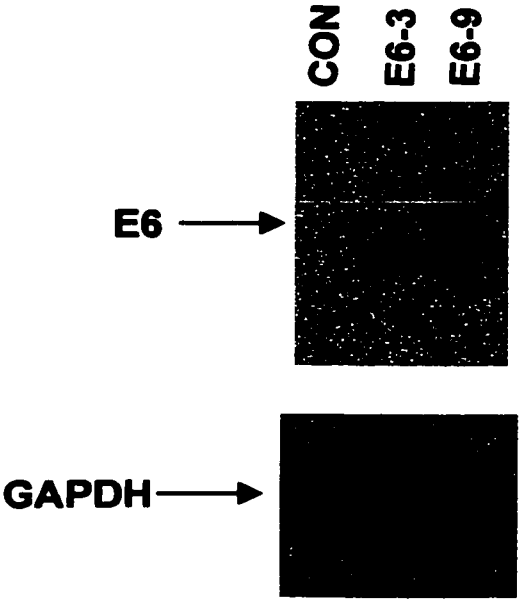

CON
E6-3
E6-9
E6
GAPDH

Figure 11. Effect of E2 on Bcl-2 expression in p53 deficient MCF-7 cells

(A) Lysates obtained from MCF-7/E6 expressing clones (3 and 9) were analyzed for immunoreactivity with anti-p53 by Western blot analysis. **(B)** Western blot analysis of Bcl-2 was performed on lysates depleted of E2 by culture for 10 days in phenol red free DMEM before treatment with either vehicle (-) or 10^{-8}M E2 (+) for 48 h. Control MCF-7 cells, wild-type (CON) or expressing E6 protein (phenol red, PR) were cultured continuously in complete DMEM. PR indicates that those cells were grown in phenol red containing media which possesses estrogenic activity. An α-actin antibody was used as a control for protein loading. **(C)** Densitometric analysis was performed separately on the two E6-expressing MCF-7 clones and used to determine the percent induction of Bcl-2 protein relative to α-actin in control and E2 treated cells. Values represent the mean ± standard error of at least three independent experiments. ANOVA results: $^{*}p<0.05$.

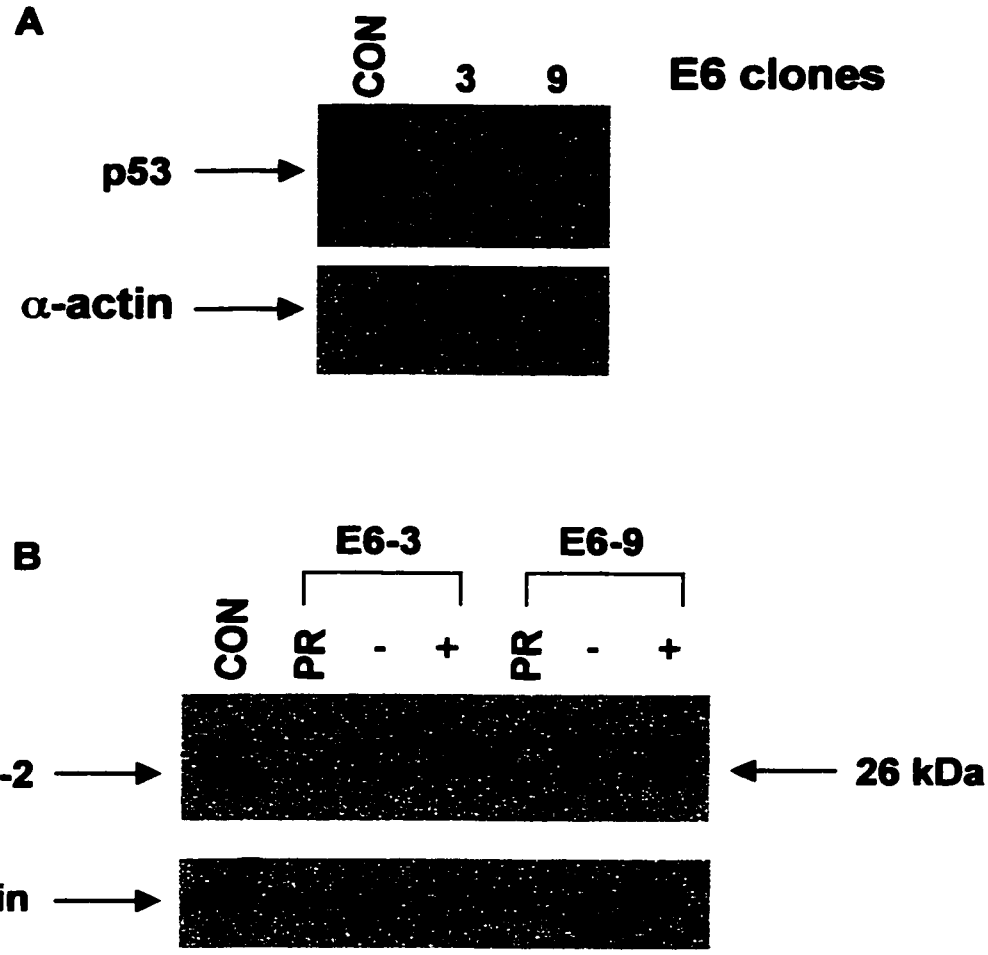
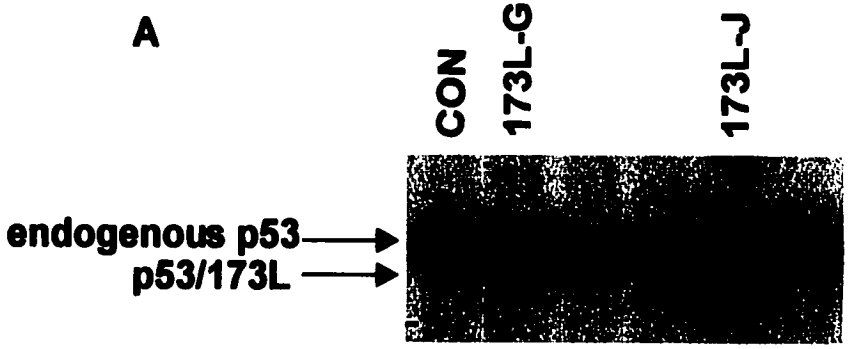
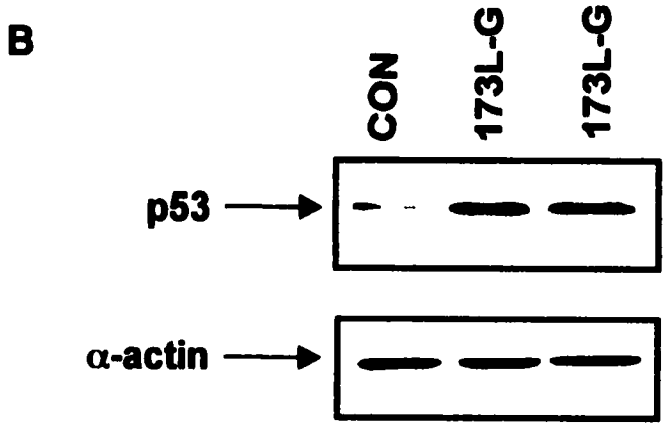
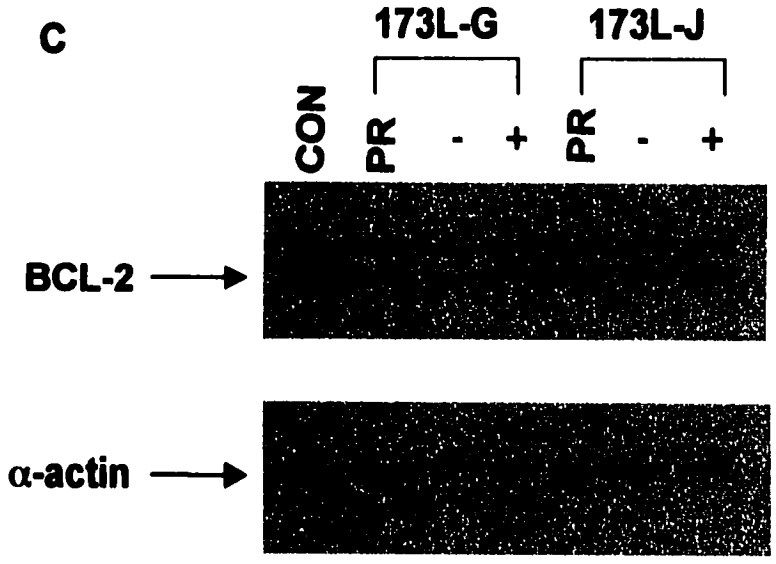

A
CON
3
9
E6 clones
p53
α-actin
B
E6-3
E6-9
CON
PR
-
+
PR
-
+
BCL-2
26 kDa
α-actin
C
Relative density (%)
200
100
0
*
*
E6-3
E6-9
CON
PR
-E2
+E2

Figure 12. Effect of E2 on Bcl-2 regulation in mutant p53-173L expressing MCF-7 cells

(A) Northern blot analysis of MCF-7 cells stably expressing mutant p53-173L construct. G and J were selected as positive clones as their RNA is slightly smaller than endogenous p53 mRNA. **(B)** Protein extracts obtained from MCF-7/p53-173L cell lines were analyzed by immunoblotting with anti-p53. **(C)** Western blot analysis of Bcl-2 was performed on lysates from MCF-7/173L clones depleted of E2 by culture for 10 days in phenol red free DMEM before treatment with vehicle (-) or 10^{-8} M E2 (+) for 48 h. Control MCF-7 cells, wild-type (CON) or expressing mutant p53 (PR), were cultured continuously in complete DMEM. PR indicates that the cells were grown in phenol red containing media which possesses estrogenic activity. Reactivity with α-actin antibody was used as a control for protein loading. **(D)** Densitometric analysis was performed separately on the two 173L-expressing MCF-7 clones and used to determine the percent induction of Bcl-2 protein relative to α-actin in control and E2 treated cells. Values represent the mean ± standard error of at least three independent experiments. ANOVA results: $^{**}p < 0.01$.


A
CON
173L-G
173L-J
endogenous p53
p53/173L


B
CON
173L-G
173L-G
p53
α-actin


C
173L-G
173L-J
CON
PR
-
+
PR
-
+
BCL-2
α-actin

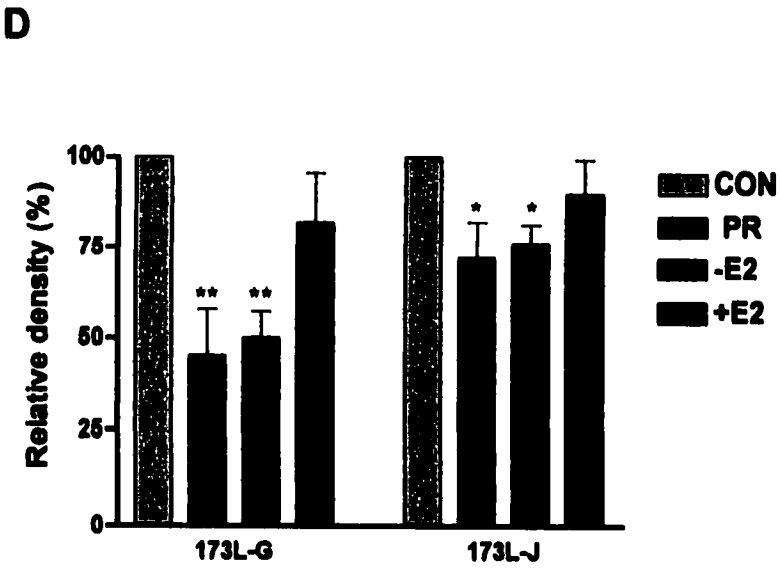
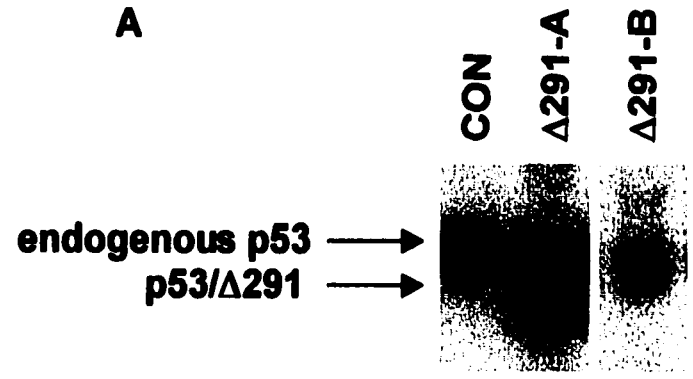
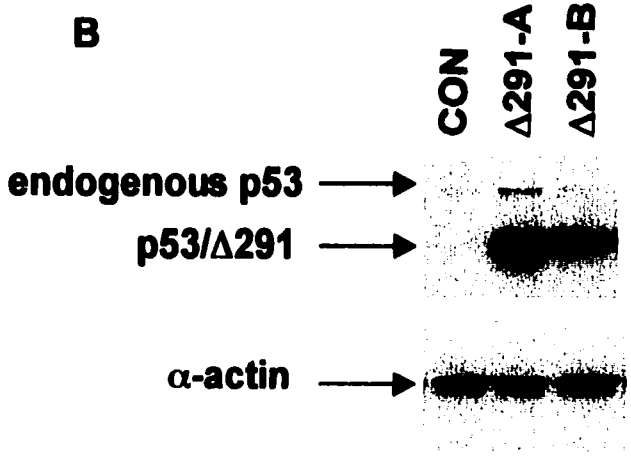
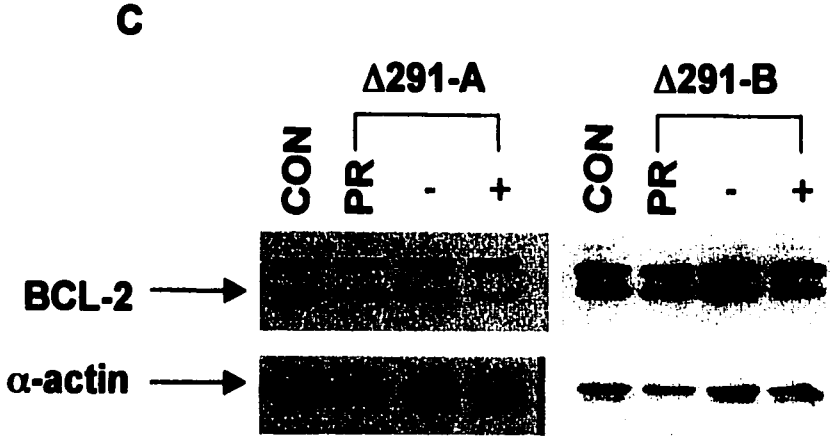

D
Relative density (%)
100
75
50
25
0
**
**
*
*
173L-G
173L-J
CON
PR
-E2
+E2

Figure 13. E2 inhibits Bcl-2 levels in mutant p53-Δ291 expressing MCF-7 cells

(A) Northern blot analysis of MCF-7 cells stably expressing mutant p53-Δ291 construct. The selected positive clones Δ291-A and Δ291-B possess a smaller p53 mRNA than does untransfected MCF-7 control cells (CON) which express endogenous, wild-type p53. **(B)** Cellular extracts obtained from MCF-7/p53-Δ291 cell lines were analyzed by immunoblotting with anti-p53. Δ291-A and Δ291-B express truncated p53 protein. **(C)** Western blot analysis of Bcl-2 was performed on lysates from MCF-7/Δ291 clones depleted of E2 and treated with vehicle (-) or 10^{-8}M E2 (+) for 48 h. Control MCF-7 cells, wild-type (CON) or expressing mutant p53 (PR), were cultured continuously in complete DMEM. PR indicates that the cells were grown in phenol red containing media which possesses estrogenic activity. Reactivity with α-actin antibody was used as a protein loading control. **(D)** Densitometric analysis was performed separately on the two Δ291-expressing MCF-7 clones and used to determine the percent induction of Bcl-2 protein relative to α-actin in control and E2 treated cells. Values represent the mean ± standard error of at least three independent experiments. ANOVA results: $^{**}p < 0.01$.


A
CON
Δ291-A
Δ291-B
endogenous p53
p53/Δ291


B
CON
Δ291-A
Δ291-B
endogenous p53
p53/Δ291
α-actin


C
Δ291-A
Δ291-B
CON
PR
-
+
CON
PR
-
+
BCL-2
α-actin

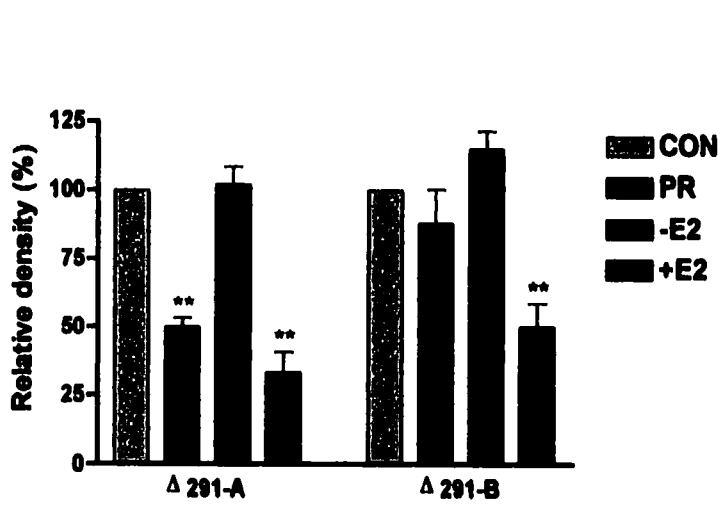
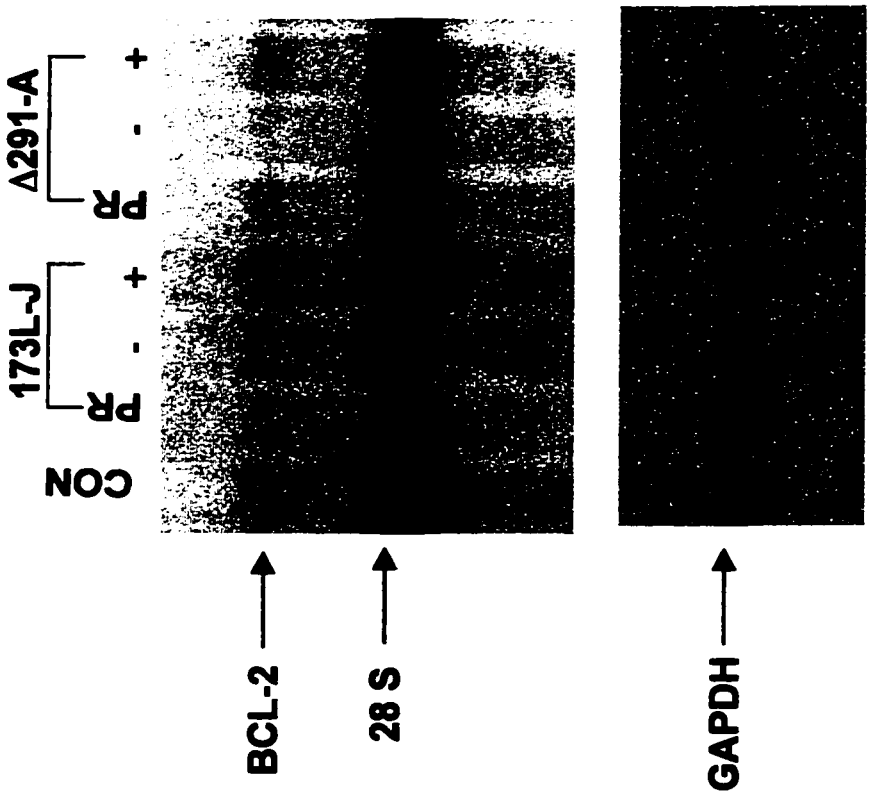

D
Relative density (%)
125
100
75
50
25
0
**
**
**
Δ 291-A
Δ 291-B
CON
PR
-E2
+E2

Figure 14. Effect of E2 on *bcl-2* mRNA regulation in MCF-7 cells expressing mutant forms of p53

Northern blot analysis of *bcl-2* was performed on total RNA extracted from representative MCF-7 clones expressing either one of the mutant p53 proteins (173L, Δ291). Clones were depleted of E2 and treated with vehicle (-) or 10^{-8}M E2 (+) for 48 h. Control MCF-7 cells, wild-type (CON) or expressing mutant p53 (grown in phenol red, PR), were cultured continuously in complete DMEM. GAPDH was used as an internal loading control.


CON
173L-J
PR
-
+
Δ291-A
PR
-
+
BCL-2
28 S
GAPDH

the effects of overexpression of various mutant p53 proteins and the absence of p53 on *Bcl-2* expression in MCF-7 cells.

To examine the role that wild-type p53 plays in the regulation of Bcl-2 expression, MCF-7 cells were stably transfected with the E6 oncoprotein (Fig. 10). The E6 protein, encoded by the oncogenic human papillomavirus types 16 and 18, has been shown to promote the degradation of p53 via ubiquitin-dependent proteolysis (Scheffner et al., 1993). Western blot analysis confirmed that these cells expressed very low levels of p53 (Fig. 11A). Subsequent analysis of Bcl-2 mRNA (not shown) and protein levels (Fig. 11B) failed to reveal any effect of p53 abrogation on E2-induced upregulation of Bcl-2.

Previous studies have divided the p53 protein into five functional domains; (i) a transcriptional activation domain that binds to TAF components of TFIID; (ii) a putative proline-rich signaling domain; (iii) a sequence specific DNA binding domain; (iv) a tetramerization domain; and (v) a domain that recognizes and binds to damaged DNA non-specifically (reviewed by Levine, 1997). Various p53 mutants were tested for their ability to regulate Bcl-2 expression in the presence and absence of estrogen. Deletion and point mutants specifically designed to target the conserved regions of the p53 protein, including a tumour derived DNA binding defective mutant (173L) whose cysteine residue at position 173 is mutated to a lysine (Crook et al., 1992) and a premature termination mutant that removed the C-terminal 102 amino acids, including the tetramerization domains and nuclear localization signals (Δ291) (Marston et al., 1994) were analyzed in parallel. MCF-7 cells were co-transfected with either p53 mutant construct and a puromycin

resistance plasmid. Stable cell lines were isolated by selection in puromycin and characterized by Northern blot (Figs. 12A, 13A). Western blot analysis for the levels of mutant p53 revealed that each of the mutants was efficiently expressed in MCF-7 cells (Figs. 12B, 13B). Overexpression of either of the transcriptionally inactive p53-173L or the C-terminal deletion mutant p53 Δ291, repressed basal Bcl-2 protein expression in MCF-7 cells as compared to untransfected control cells (Figs. 12C, 13C). Exposure of MCF-7 cells expressing mutant p53-173L to estrogen treatment did not prevent E2 stimulation of Bcl-2 expression (Fig. 12C). In complete contrast, E2 treatment of MCF-7 cells expressing mutant p53 Δ291 resulted in a decrease in Bcl-2 levels (Fig. 13C). Northern blot analysis of *Bcl-2* mRNA in these cells showed that *Bcl-2* transcription was induced upon E2 treatment (Fig. 14). Therefore, the observed E2-dependent decrease in Bcl-2 protein levels is mediated by a posttranscriptional mechanism.

CHAPTER IV

DISCUSSION

Transcriptional regulation of Bcl-2 expression by E2

Estrogens stimulate growth of hormone-responsive breast cancer cells through complex and still incompletely characterized mechanisms. A useful approach to understand tumour progression in mammary tissue is to identify estrogen-regulated genes and analyze their expression. Many studies have focused on hormone effects in modulating the activity of cell cycle factors that can directly affect the expression of genes important in the control of cell differentiation and proliferation (Musgrove et al., 1993). However, growing evidence has accumulated in recent years supporting the role of estrogen hormones in the prevention of PCD in human breast cancer (refs). For example, rapid regression of ER-positive MCF-7-derived breast cancers in nude mice, following estrogen ablation, was attributed either to arrest of cell proliferation or to PCD activation (Kyprianou et al., 1991). Studies from our lab have demonstrated that hormone protection from drug-induced apoptosis in MCF-7 cells was accompanied by an increase in *Bcl-2* mRNA and protein levels (Teixeira et al., 1995), and that Bcl-2 protects MCF-7 tumours from E2 withdrawal-induced regression (Pratt et al., 1998). Furthermore, it has been shown that 80% of the Bcl-2-positive mammary tumours express the ER compared to only 30% of the Bcl-2-negative group (Leek et al., 1994). Taken together, these results suggested that inhibition of PCD by estrogens was mediated by the *Bcl-2* gene, which is able to prevent apoptotic death in multiple contexts. We undertook this study to probe the molecular mechanisms of this observed E2-induced *Bcl-2* gene expression in MCF-7 breast cancer cells.

Initial analysis of constructs containing *Bcl-2* gene promoter fragments stably transfected into MCF-7 cells demonstrated that all P1 promoter containing plasmids driving the expression of the *neo* gene were active and could be further induced with E2 treatment. However, MCF-7 cells expressing constructs that contained only P2 promoter fragments, exhibited no activity and undetectable levels of *neo*. These results correspond to the observations which characterize P1 as the predominant promoter region where approximately 95% of transcripts initiate, whereas P2 is a minor promoter responsible for generating fewer than 5% of all transcripts in some lymphatic cell lines (Seto et al., 1988). Northern analysis of *Bcl-2* promoter-driven *neo* expression may not be a sensitive enough assay and could explain the low activity of P1 observed in MCF-7 cells transfected with the Short P1 construct. Another possibility may be that the shortened construct is missing necessary cis-elements required for full induction.

Bcl-2 promoter analysis also revealed a 1.7 kb E2-responsive region upstream of the principal promoter, P1. A further 5' deletion of the promoter inserts identified a minimal 500 bp NarI-HindIII region that retained hormone-inducibility in transient transfection assays in MCF-7 cells (Fig 8B). This 500 bp fragment includes the E2-responsive regions recently identified in the Bcl-2 5' UTR by Dong et al. (1999). In their studies hormone-responsive, ER-positive cells lines, MCF-7 and T47D, were transiently transfected with various *Bcl-2* promoter fragments ligated to a CAT reporter gene. The initial experiments were performed almost exclusively in T47D cells due to the poor transfection efficiencies reported with their MCF-7 cells. An estrogen-responsive region was identified at –1647 to –1289 according to their

Bcl-2 5' UTR sequence. Sequence comparison of E2-responsive regions and other potential enhancer elements between those reported by Dong et al., (1999) and sequence data obtained and generated by our laboratory revealed a slight discrepancy in nucleotide numbering. For example, the –1647 to –1298 hormone responsive region corresponds to –1659 to –1301 of our sequence in Fig. 9. This E2-responsive fragment generated a 6.4-fold induction compared to control cells when transiently transfected into T47D cells (Dong et al., 1999). Interestingly, when we transiently transfected our MCF-7 cells with either the 1.7 kb or 0.5 kb luciferase constructs (Fig. 8A), both which contain this E2-responsive region, upon treatment with E2, we observed a 1.8 and 1.4 fold induction, respectively. These induction values are somewhat lower than those reported by Dong et al., (1999), but perhaps they reflect the lower transcription efficiencies seen in MCF-7 cells or the cell-specific differences in *Bcl-2* promoter induction. Further promoter analysis using fragments of the –1647 to –1289 region identified a smaller hormone responsive element from –1603 to –1534 (or –1615 to –1546 in Fig. 9) that was active in both MCF-7 and T47D cells. Again, all of our E2-responsive constructs contained this element.

Subsequent analysis of the 70 bp sequence indicated that it did not contain perfect or imperfect EREs and that hormonal regulation was complex, requiring both upstream (-1603 to –1579) (-1615 to –1591 in Fig. 9) and downstream (-1554 to –1534) (-1566 to –1546 in Fig. 9) sequences for E2 responsiveness. The upstream region (-1603 to –1579) of the *Bcl-2* gene fails to bind ER in gel mobility shift assays, but does contain two G-rich sequences (Dong et al., 1999). G-rich elements are

known to be involved in the binding of the transcription factor, Sp1. The two potential Sp1 binding sites at –1601 (5'-GGGCTGG-3') and –1588 (3'-GGAGGG-5') (-1614 and –1600, respectively in Fig. 9) differ from the consensus GC-rich motif (GGGCGG), however, both G-rich motifs have previously been identified as Sp1 binding sites in the promoters of human CD14, rat luteinizing hormone, rabbit lung surfactant protein B, and rhesus growth hormone-variant genes (Zhang et al., 1994; Margana et al.,1997; Kaiser et al., 1998; Schanke et al., 1998). Interactions between Sp1 and ER proteins play a role in the regulation of at least several estrogen-inducible genes, including *c-myc*, *CKB*, *cathespin D*, *RARα*, *hsp27* and *TGFα* (Vyhlidal et al., 2000). ER/Sp1 action at these target genes requires the presence of Sp1 and ERE half-site motifs. Both ER and Sp1 proteins physically interact (Porter et al., 1997), however, transactivation is mediated by at least two different mechanisms. In one pathway the ER/Sp1 protein complex interacts with G-rich or GC-rich genomic sites and only the Sp1 protein binds DNA, whereas the other interaction of ER/Sp1 occurs with Sp1$(N)_x$ERE half-site motifs, where N is any nucleotide and 'x' is any number. In the latter case both the ER and Sp1 proteins bind DNA sites. The precise interaction between Sp1 and ER is not clear, however, one could speculate that both proteins form a complex with one another and to the downstream general transcriptional initiation machinery via interactions with nuclear coactivators. The ER has been shown to interact with several members of the p160/SRC-1 family as well as with CBP/p300 which can directly interact with TFII machinery (Hanstein et al., 1996; Kamei et al., 1996).

Gel mobility assays determined that the two putative Sp1 binding sites at –1601 and –1588 were found to bind Sp1 proteins and that this binding was enhanced in a dose-dependent manner with the addition of ER protein (Dong et al., 1999). Not surprisingly, mutation of both Sp1 binding sites inhibited E2-mediated gene induction confirming that transactivation by E2 was dependent on functional ER-Sp1 interactions with the two G-rich sequences in the *Bcl-2* promoter (Dong et al., 1999).

With the finding that Sp1 bound to the two G-rich sites in the *Bcl-2* promoter region, Dong et al., (1999) attempted to see if ER proteins could form complexes with these Sp1 proteins. Gel mobility shift assays were performed on nuclear extracts from Drosophila SL2 cells, which do not express either transcription factor, transfected with ER and Sp1 expression plasmids using a labeled Sp1 oligonucleotide as a probe. They failed to observe supershifted ER/Sp1-DNA ternary complexes and from this concluded that the ER did not bind the *Bcl-2* promoter in the same region as Sp1. These indirect studies do not provide definitive answers as to whether or not ER can bind to the Bcl-2 promoter. Furthermore, although Dong et al., (1999) did not discover any EREs in the Bcl-2 5' UTR, consensus or otherwise, analysis of our own sequence data revealed several imperfect half-palindromic EREs (Fig. 9). The classical ERE sequence is GGTCAnnnTGACC and the sequence of the degenerate half-site ERE motif identified in the Bcl-2 5' UTR was ***ggtcn***, where n is any nucleotide. With respect to Sp1/ER interactions it has been shown that there can be considerable variability in the ERE half-site sequences, their orientation, and the number of intervening

nucleotides between Sp1 and ERE DNA-binding sites. The half-site most proximal to the Sp1 binding motifs thought to be responsible for driving E2-directed *Bcl-2* expression is approximately 60 bp from this region. The next closest is about 400 bp and the farthest is close to 1700 bp. Interestingly, all are located in very G-rich regions. It would be interesting to see if mutation of each ERE half-site would affect the induction of *Bcl-2* by E2. Even though the distances between the half-site EREs and the Sp1 binding sites appears great, ER and Sp1 interactions have been shown to occur in promoters with a Sp1$(N)_{30}$ERE (Vyhlidal et al., 2000). In addition, a recent report showed that transcriptional activation of the vitellogenen A1 gene promoter by E2 involved enhanced or synergistic interactions between ER and Sp1 bound to ERE and GC-rich sites that were separated by > 1000 bp (Batistuzzo de Medeiros et al., 1997).

In contrast, the downstream E2-responsive region at –1554 to –1534 (-1566 to –1546 in Fig. 9) was found not to bind Sp1 or ER proteins as indicated by gel shift mobility assays (Dong et al, 1999). The 21 bp sequence instead contained a cAMP response element (CRE) that bound the transcription factors ATF-1 and cAMP response element binding protein (CREB). Previous studies have demonstrated E2-dependent transcriptional activation of CAT reporter genes linked to an upstream CRE motif via induction of cAMP (Aronica et al., 1994). Similarly, transactivation by E2 of the –1554 to -1534 *Bcl-2* promoter region was dependent on induction of cAMP and subsequent activation though a cAMP response element.

To make matters even more complex, Perillo et al., (2000) have recently identified two imperfect estrogen-responsive elements located within the coding

region of the *Bcl-2* gene that can upregulate *Bcl-2* transcription and furthermore inhibit hydrogen peroxide-induced PCD in MCF-7 cells. Thus, it appears that transcriptional activation of *Bcl-2* by estrogen is intricately regulated, involving elements found both in the promoter and coding regions of the gene.

Regulation of Bcl-2 by p53 in the presence of E2

Other studies have shown that the tumour suppressor gene p53 can inhibit expression of Bcl-2 (Miyashita et al., 1994a), and this also correlates with an inverse relationship observed in breast tumours between Bcl-2 and p53 mRNA and protein levels (Haldar et al., 1994). This phenomenon seen in breast cancers suggests that perhaps p53 and Bcl-2 have some overlapping function. Perhaps Bcl-2 confers an initial survival advantage in the hormone-responsive stages of the disease whereas mutated p53 allows cells to survive in the later, more aggressive stages. Because of the crucial role p53 plays in tumour suppression, we explored the potential interaction between p53 and E2 in the regulation of the *Bcl-2* promoter.

Though p53 may have additional biochemical activities, this DNA-binding protein has been shown to function as a transcriptional regulator for a variety of genes (reviewed in Ko and Prives, 1996). For example, p53 can upregulate *Bax* expression, an activator of apoptosis which contains p53 consensus binding elements in its promoter (Miyashita et al., 1994b). Conversely, p53 has been shown to repress a number of genes with promoters lacking p53-binding sites, including, among others, *c-fos*, *c-jun*, *Rb*, *hsp70* and *Bcl-2* (Donehower and Bradley, 1993; Miyashita et al., 1994b). p53-mediated downregulation of *Bcl-2*, combined with an

increase in *Bax* expression may be one mechanism that contributes to p53-mediated apoptosis.

The 5' regulatory regions of the *Bcl-2* gene contain two promoters, P1 and P2. P1 is the predominant promoter and is TATA-less, GC-rich and contains several Sp1 binding sites. The second promoter, P2, is located downstream of the first and contains both a CCAAT box and a TATAA element. Recently, a 1.3 kb negative regulatory element (NRE) was identified in the 5'-UTR of the *Bcl-2* gene. This region, located between the two promoters and including P2, has been shown to inhibit expression from the homologous Bcl-2 P1 promoter, when positioned downstream of the promoter (Young and Korsmeyer, 1993). A shorter fragment of the 1.3 kb NRE located at –279 to –1 bp relative to the translation initiation site could also inhibit expression from the heterologous CMV immediate-early promoter at a transcriptional level (Young and Korsmeyer, 1993). Further analysis of this 1.3 kb NRE revealed a subfragment from –273 to –84 bp which contained two functionally separable cis-acting inhibitory elements (Miyashita et al., 1994b). One of these negative regulatory elements was p53-dependent and functioned in an orientation- and position-dependent fashion to downregulate expression of heterologous reporter constructs, thus having properties as a transcriptional silencer (Miyashita et al., 1994b). The other cis-acting element functioned in a p53-independent fashion to inhibit reporter gene expression in a position- and orientation-dependent manner. Within this second region of the *Bcl-2* gene exists a small open reading frame (ORF) located at –119 to –84 of which 11 amino acids can function as an inhibitor of translation but not of transcription of the Bcl-2 gene and heterologous genes (Harigai

et al., 1996). This upstream ORF (uORF) begins with an AUG start codon at –119 bp and potentially encodes an 11 amino acid peptide, ending with a TAA stop codon at –84 bp. Positioning of this small uORF between a heterologous promoter and CAT reporter gene inhibited reporter gene expression at the protein, but not mRNA level (Harigai et al., 1996). Mutating the initiation codon of the uORF abolished its inhibitory activity. Conversely, deletion of the same uORF from a construct containing the Bcl-2 P1 promoter and entire 5' UTR of the human Bcl-2 gene resulted in enhancement of reporter gene expression, again at the protein but not at the mRNA level (Harigai et al., 1996). Similarly, mutation of the initiation codon of the uORF produced the same effect. Taken together, these data support the idea that the uORF (-119/-84) is necessary as well as sufficient for translational regulation of the Bcl-2 gene. In fact, many proto-oncogenes, growth factor genes and growth factor receptor genes contain AUG start codons and associated upstream ORFs in their 5'-UTRs which may serve as devices for limiting the translation of potent proteins that would be harmful if overproduced (Kozak, 1991; Arrick et al., 1991).

Recent studies have provided evidence that suggests p53 can function not necessarily as a transcription factor, but in another way, perhaps by direct protein-protein signaling. It has been shown that p53 directly binds to and acts on several cellular proteins such as... (see Levine, 1997 and references therein). It has been postulated that p53 protein-protein interactions occur via binding of SH3 domains. The human p53 protein contains five copies of the amino acid motif proline-X-X-proline (where X is any amino acid) localized between residues 61-94, and this motif has been shown to bind SH3 domains (Levine, 1997). In fact, SH3 domains are

required for interaction of the p53 with the p53 binding protein 53BP2, which interestingly enough also associates with Bcl-2 (Naumovski and Cleary, 1996). Therefore, it is possible that p53 regulation of Bcl-2 expression might occur at both transcriptional and translational levels.

Consistent with the presence of the p53 negative response element, we have observed repression of Bcl-2 expression in T47-D (ER+) cells and MDA-MB-231 (ER-) cells both of which contain mutated p53. Furthermore expression of p53 mutant in the DNA binding domain (p53-173L) or a C-terminal mutant (p53Δ291) repressed basal Bcl-2 expression in MCF-7 cells. However, p53-173L did not prevent E2 stimulation of *Bcl-2*. In stark contrast, we have also shown that E2 treatment of MCF-7 cells expressing mutant p53Δ291, which is missing the entire C-terminus including tetramerization domains and nuclear localization signals, resulted in a decrease in Bcl-2 levels. Evaluation of *Bcl-2* mRNA in these cells showed that the mRNA was induced by E2 therefore the effects of Δ291 are post-transcriptional. Thus it appears that the carboxy-terminus of p53 is necessary for translation and/or stability of the Bcl-2 protein in the presence of estrogen. It is possible that following E2 induction of *Bcl-2* transcription the message is translated normally and p53 acts distally to govern the stability of the protein in the presence of the hormone. Lack of a carboxy-terminus may allow mutant p53 to destabilize or increase the rate of degradation of the Bcl-2 in the presence of E2, thus leading to lower protein levels. Another possibility may be that E2 and p53 cooperate to govern the translation rate of Bcl-2. Recently it has been demonstrated that p53 can selectively interact with the 5' UTR of *cdk4* mRNA and inhibit its translation (Miller et al., 2000). Interaction

with *Bcl-2* mRNA could provide a means for mutant p53 to regulate *Bcl-2* translation, possibly by enhancing translational repression of the uORF found in the *Bcl-2* 5'-UTR. As described above, the *Bcl-2* gene can be regulated by translational mechanisms which involve the 11 amino acid ORF contained within the NRE. Therefore, there is a basis in structure for potential regulation of the *Bcl-2* gene by p53 and E2.

Prior to initiation of translation, the ribosome is thought to progressively scan the 5' UTR for potential start codons (Kozak, 1991; Kozak, 1999). With p53 bound to the *Bcl-2* 5' UTR, either directly or indirectly via interaction with an associated molecule (eg. p53BP2), ribosomal scanning could be sterically hindered, resulting in 40S subunit stalling and the failure of translation to initiate. Since Δ291 p53 lacks its C-tail with the associated nuclear localization regions the protein is theoretically restricted to the cytoplasm. Interestingly cytoplasmic p53 has been found in association with ribosomes (Fontoura et al., 1997) and has been reported to regulate its own translation via its 5' leader sequence (Mosner et al., 1995). Being a DBD mutant, the potential interaction between *Bcl-2* mRNA and Δ291 p53 probably occurs through an associated protein and the resulting repression is initiated in the presence of E2.

The role of wild-type p53 in the regulation of Bcl-2 is not clear. Since MCF-7 cells are unable to survive overexpression of wild-type p53 we could not directly address the role of wild-type p53 in Bcl-2 regulation. Instead we examined the effects on the E2 regulation of Bcl-2 in the absence of p53 protein. The *HPV-E6* oncogene encodes a protein product that has been shown to recruit the cellular

ubiquitin-proteasome ligase E6-AP to target p53 for ubiquitin-mediated degradation (Scheffner et al., 1990, 1993). Accordingly, MCF-7 cells that overexpress E6 exhibit virtually undetectable levels of p53. However, low levels of p53 in MCF-7/E6 cells did not affect E2 induction of *Bcl-2* mRNA and protein levels. It is possible that the very low level of p53 that remained in the MCF-7/E6 expressing cells was enough to prevent any other effects of E6 protein. However, it should also be borne in mind that E6 is a multifunctional protein. This has perhaps been forgotten in numerous studies which have assumed that overexpression of E6 only affects p53 function; this is not the case.

Recent studies have shown an interaction between E6 and several cellular proteins, including: hDLG, the mammalian homologue of the *Drosophila* discs large tumour supressor protein (Lee at al., 1997); E6TP1, a putative regulator of mitogenic signalling (Gao et al., 1999); paxillin, a protein involved in transducing signals from the plasma membrane to the actin cytoskeleton (Tong and Howley, 1997); c-Myc, a well established oncoprotein which is associated with the promotion of both apoptosis and differentiation (Gross-Mesilaty et al., 1998); Bak, a pro-apoptotic Bcl-2 family member (Thomas and Banks 1998); E6BP, a calcium binding protein (Chen et al., 1995); Mcm7, a protein involved in DNA replication (Kühne and Banks, 1998); and telomerase (Klingelhutz et al., 1996).

In summary, HPV E6 has been shown to be capable of interfering with the cellular controls of DNA replication, through Mcm7; chromosomal structure, through telomerase; signal transduction, through E6TP1, paxillin, c-Myc, and E6BP; and apoptosis, through Bak and c-Myc. Since it is clear that many of these effects seen

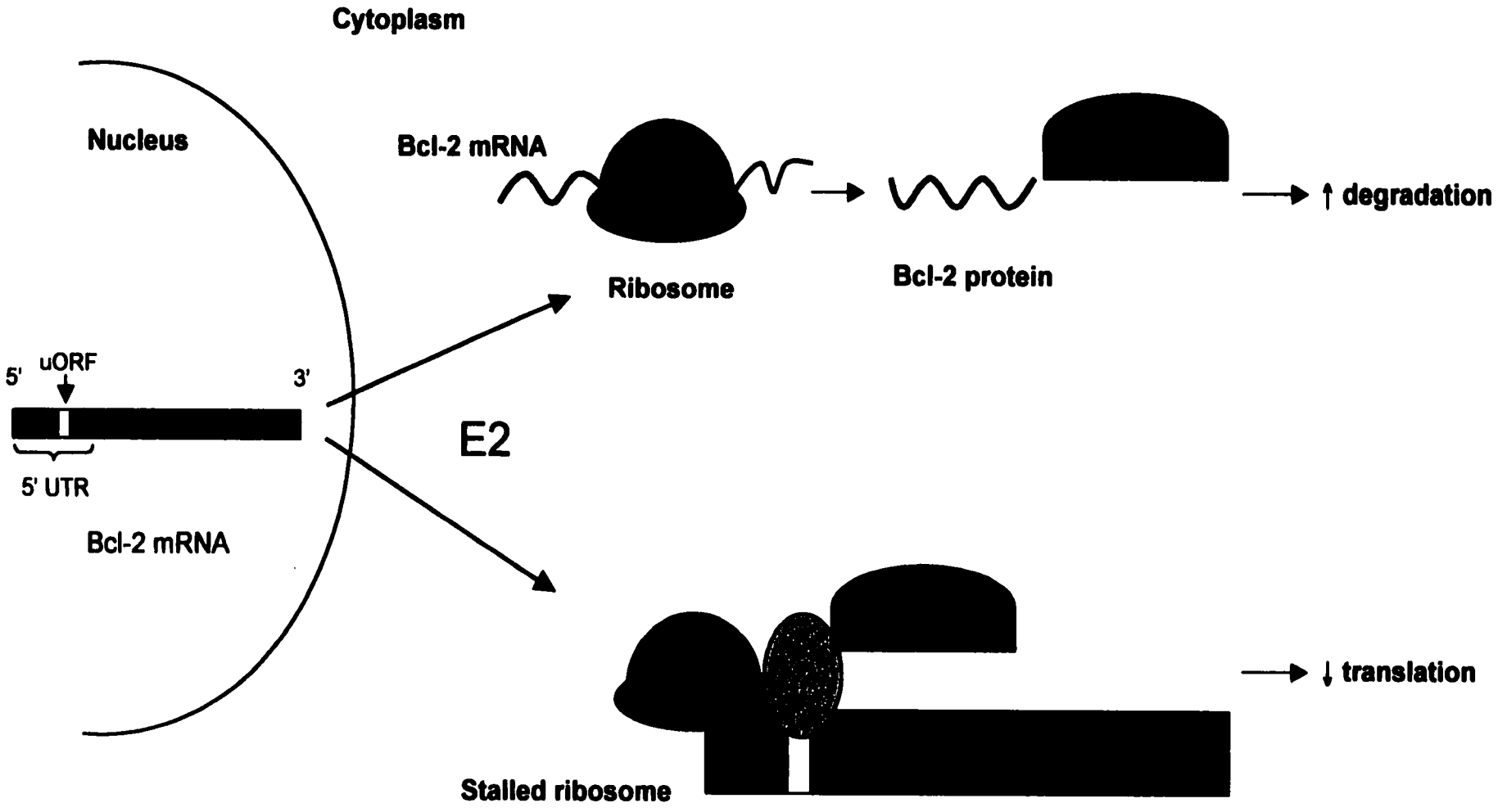
in cells upon overexpression of E6 are not related solely to the degradation of p53, care is required in interpreting the results of p53 studies performed in cells expressing HPV E6.

Despite the wide range of promoters that are inhibited by coexpression of p53 in transfected cells, it was reported that only those promoters containing TATA boxes, and not those containing an initiator element, are inhibited by p53 (Mack et al., 1993). The mechanism by which p53 represses these promoters is via its interaction with transcription binding protein (TBP). It is thought that p53 sequesters TBP and inhibits efficient initiation of transcription (i.e. squelching) (Mack et al., 1993).

In at least one case, transcriptional repression by p53 has been proposed to act through interaction with a transcriptional activator rather than the general transcriptional machinery. Repression of the *Hsp70* gene by p53 is mediated by an interaction between p53 and CCAAT binding factor (CBF), a transcriptional activator of the *Hsp70* promoter (Agoff et al., 1993). It is thought that perhaps a p53-CBF complex alters the ability of CBF to recognize the *Hsp70* promoter.

Figure 15. Proposed model of mutant Δ291 p53 action on Bcl-2 repression in the presence of E2.

Once transcribed, Bcl-2 mRNA is transported to the cytoplasm for translation into protein by ribosomes. In the presence of mutant Δ291 p53 and E2, the levels of Bcl-2 protein are inhibited. This decrease in Bcl-2 can be attributed to at least two mechanisms: (I) increased destabilization by increasing the rate of degradation of Bcl-2 by mutant p53 in the presence of E2; (II) E2 and p53 cooperate to govern the translation rate of Bcl-2. By interacting with the Bcl-2 mRNA, either directly or indirectly, mutant p53 could inhibit translation of Bcl-2 leading to decreased protein levels.


Cytoplasm
Nucleus
Bcl-2 mRNA
Ribosome
Bcl-2 protein
↑ degradation
5'
uORF
3'
5' UTR
Bcl-2 mRNA
E2
↓ translation
Stalled ribosome

SUMMARY

In summary, this study advances our understanding of the molecular processes occuring in estrogen-dependent human breast cancer cells. We have demonstrated that E2 mediates induction of an anti-apoptotic protein, Bcl-2, and it does so by a minimal 500 bp element identified in the promoter region of the gene. Our data provides a mechanism as to how E2 can prolong cellular survival which may subsequently lead to breast neoplasms.

Our other studies looking at the relationship between Bcl-2 and p53 in the presence of E2 revealed a novel observation of an E2-dependent decrease in Bcl-2 protein levels in the presence of a mutant p53 lacking its C-tail. E2 is still able to mediate Bcl-2 mRNA induction, suggesting that Bcl-2 protein downregulation is due to post-transcriptional mechanisms. This finding is extremely interesting because in many cases as breast carcinomas progress we see a reciprocal relationship between the two proteins, and this is amplified in the presence of mutant p53. Perhaps when the breast cancer cells are initially hormone-responsive E2 offer these particular cells an enhanced survival advantage by upregulating Bcl-2 levels. Once other changes occur in the cells (i.e. loss of hormone dependence, accumulation of mutant p53) Bcl-2 is no longer needed for survival and subsequent proliferation. One question that needs to be addressed is whether these proteins have overlapping function or do these changes occur in parallel. By elucidating other molecular mechanisms that may participate in mediating E2 action we can further increase our understanding of the underlying etiology of human breast cancers.

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