



# The RelA NF- $\kappa$ B subunit and the aryl hydrocarbon receptor (AhR) cooperate to transactivate the *c-myc* promoter in mammary cells

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**NF- $\kappa$ B/Rel transcription factors regulate many genes involved in control of cellular proliferation, neoplastic transformation, and apoptosis, including the *c-myc* oncogene. Recently, we have observed that levels of NF- $\kappa$ B and aryl hydrocarbon receptor (AhR), which mediates malignant transformation by environmental carcinogens, are highly elevated and appear constitutively active in breast cancer cells. Rel factors have been found to functionally interact with other transcription factors. Here we demonstrate a physical and functional association between the RelA subunit of NF- $\kappa$ B and AhR resulting in the activation of *c-myc* gene transcription in breast cancer cells. RelA and AhR proteins were co-immunoprecipitated from cytoplasmic and nuclear extracts of non-malignant MCF-10F breast epithelial and malignant Hs578T breast cancer cells. In transient co-transfection, RelA and AhR gene products demonstrated cooperation in transactivation of the *c-myc* promoter, which was dependent on the NF- $\kappa$ B elements, and in induction of endogenous c-Myc protein levels. A novel AhR/RelA-containing NF- $\kappa$ B element binding complex was identified by electrophoretic mobility shift analysis of nuclear extracts from RelA and AhR co-transfected Hs578T cells. Thus, the RelA and AhR proteins functionally cooperate to bind to NF- $\kappa$ B elements and induce *c-myc* gene expression. These findings suggest a novel signaling mechanism whereby the Ah receptor can stimulate proliferation and tumorigenesis of mammary cells. *Oncogene* (2000) 19, 5498–5506.**

**Keywords:** NF- $\kappa$ B; RelA; AhR; *c-myc* oncogene; breast cancer

## Introduction

NF- $\kappa$ B/Rel is a family of dimeric transcription factors characterized by the presence of a Rel homology region (RHR) of about 300 amino acids in length, which controls multiple functions including dimerization, DNA binding, and nuclear localization. Classical NF- $\kappa$ B is a heterodimer composed of p65 (or RelA) and p50 (or NF $\kappa$ B1) subunits (Grimm and Baeuerle, 1993).

The RelA subunit has potent transactivation potential, while the p50 subunit has only modest transactivation ability *in vivo* (Grimm and Baeuerle, 1993; Ballard *et al.*, 1992; La Rosa *et al.*, 1994). Many genes are regulated by NF- $\kappa$ B (Grilli *et al.*, 1991; Grimm and Baeuerle, 1993). For example, we demonstrated that the *c-myc* oncogene is potently transactivated by NF- $\kappa$ B/Rel factors (La Rosa *et al.*, 1994). In most cells, other than B lymphocytes, NF- $\kappa$ B/Rel proteins are sequestered in the cytoplasm bound to one of the specific inhibitory proteins termed I $\kappa$ Bs of which I $\kappa$ B- $\alpha$  is the paradigm. A variety of agents can induce NF- $\kappa$ B/Rel, including oxidative stress (Grimm and Baeuerle, 1993; Verma *et al.*, 1995). Activation of NF- $\kappa$ B involves phosphorylation and degradation of I $\kappa$ B, which allows for translocation of an active NF- $\kappa$ B complex into the nucleus where it can bind to NF- $\kappa$ B responsive elements (Verma *et al.*, 1995). However, we recently demonstrated that breast cancer cell lines and primary breast cancer specimens are typified by aberrant constitutive activation of NF- $\kappa$ B (Sovak *et al.*, 1997).

We have postulated that one mechanism leading to constitutive NF- $\kappa$ B activation may be oxidative stress induced by activation of cytochrome P450 enzymes, some of which are regulated by the aryl hydrocarbon receptor (AhR) (Nebert *et al.*, 1990, 1991). The AhR is a cytosolic protein complexed with heat shock protein (Hsp90) and an immunophilin-like molecule, ARA-9/XAP-2/AIP (Carver and Bradfield, 1997; Jain *et al.*, 1994; Perdew and Bradfield, 1996; Ma and Whitlock, 1996; Meyer *et al.*, 1998), and c-Src (Enan and Matsumura, 1996). Acute activation can occur with multiple agents, including classes of carcinogenic environmental chemicals (e.g. dioxins, polycyclic aromatic hydrocarbons (PAH), and planar polychlorinated biphenyls (PCBs)). Upon activation, the receptor translocates to the nucleus, binds specific response elements (XREs), and induces transcription of a number of genes, including those encoding the P450 enzymes CYP1A1, CYP1A2, and CYP1B1. As predicted from the working model, we have recently found high levels of constitutively active AhR in PAH-induced rat mammary tumors (Trombino *et al.*, 2000), that coincide with constitutively active NF- $\kappa$ B (Sovak *et al.*, 1997).

The recent work of Tian *et al.* (1999) suggests a second pathway linking AhR and NF- $\kappa$ B activities. In particular, they demonstrated a physical association between the AhR and the RelA subunit of NF- $\kappa$ B in murine hepatoma cells, and transcriptional down-

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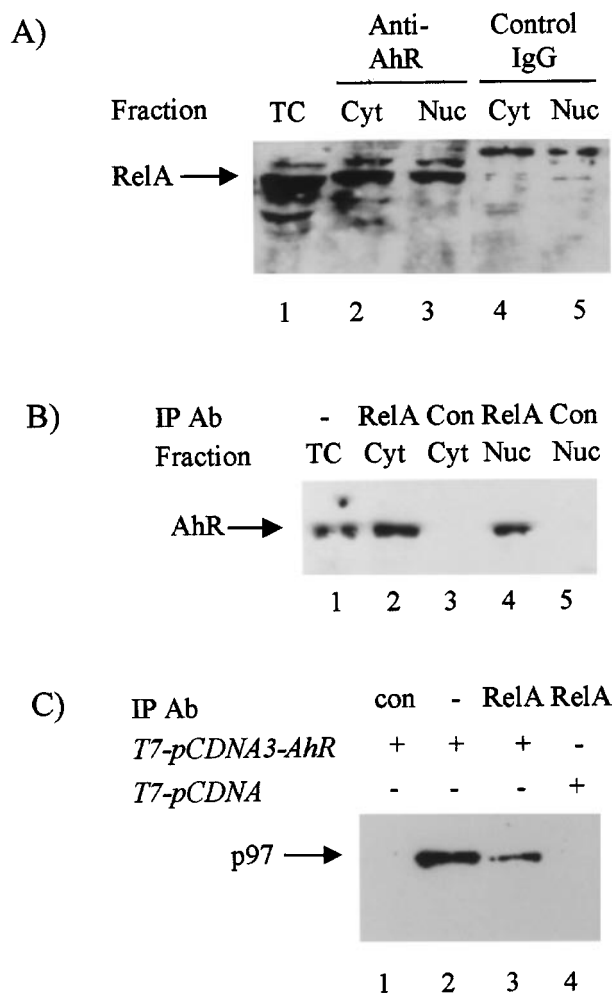
regulation by AhR-NF- $\kappa$ B subunit complexes of an NF- $\kappa$ B element driven construct. To test for this additional regulatory mechanism in human breast epithelial and tumor cell lines, the potential for AhR and NF- $\kappa$ B subunits to associate and regulate an NF- $\kappa$ B-regulated gene promoter, i.e. *c-myc*, was examined. We report that the RelA and AhR proteins co-precipitate in untransfected or *RelA*- and *AhR*-transfected human mammary epithelial cell (HMEC) lines. In contrast to the previous report by Tian and coworkers (1999), however, we find RelA and AhR cooperate to positively transactivate the *c-myc* gene, apparently via direct binding to NF- $\kappa$ B elements. These findings suggest a new mechanism whereby aberrant constitutive NF- $\kappa$ B/AhR expression can promote activation of the *c-myc* gene and thereby proliferation and neoplastic transformation.

## Results

### *RelA and AhR are associated in HMECs*

To assess the association of the RelA subunit of NF- $\kappa$ B with AhR in breast epithelial cells, co-immunoprecipitation studies were performed. Total cell, nuclear, and cytoplasmic extracts were prepared from malignant Hs578T breast cancer cells, which have been found to express both RelA (Sovak *et al.*, 1997), and AhR proteins. Samples of the nuclear (50  $\mu$ g) and cytoplasmic (100  $\mu$ g) fractions were treated with either a goat antibody against AhR (Figure 1a, lanes 2,3) or a goat IgG fraction, as control (Figure 1a, lanes 4,5). Immune complexes were isolated using protein A-Sepharose and subjected to electrophoresis, along with a sample of total cell lysate (Figure 1a, lane 1). The resulting immunoblot was probed with a rabbit polyclonal antibody for expression of the 65 kDa RelA subunit. In the total cell lysate, RelA-specific antibody recognized a protein of the expected molecular weight (65 kDa). The AhR antibody co-precipitated RelA protein from either cytoplasmic or nuclear extracts (Figure 1a), with somewhat greater amounts seen with the cytoplasmic sample. In contrast, the control goat IgG failed to co-precipitate detectable levels of RelA protein. To confirm this association we performed the inverse experiment of immunoprecipitating nuclear (100  $\mu$ g protein) or cytoplasmic (200  $\mu$ g protein) extracts with a rabbit antibody against RelA and then immunoblotting for AhR using a goat antibody. The RelA antibody co-precipitated AhR protein from either cytoplasmic or nuclear extracts (Figure 1b). In contrast, the control rabbit IgG failed to co-precipitate detectable levels of AhR protein. As seen above, somewhat greater amounts of complexes were detected in the cytoplasm. These findings suggest that endogenous AhR is associated with RelA in both the nucleus and the cytoplasm of Hs578T cells; although, the majority of the complexes, as judged from this and two duplicate experiments ( $0.80 \pm 0.07$ ), are present in the cytoplasm.

We next sought to assess the ability of RelA to associate with the AhR in non-malignant MCF-10F HMECs. To this end, MCF-10F cells were transfected with a vector expressing the *T7-pcDNA3-AhR* vector encoding T7-tagged AhR. Alternatively, cells were



**Figure 1** AhR and RelA are associated in Hs578T and MCF-10F cells. **(a)** Cytosolic (100  $\mu$ g from 1 mg total) or nuclear (50  $\mu$ g from 128  $\mu$ g total) proteins from Hs578T cells were immunoprecipitated using 5  $\mu$ g/ml of either a polyclonal goat anti-AhR antibody (lanes 2,3) or a control goat IgG (lanes 4,5). Of 30  $\mu$ l of resulting antibody-protein A-sepharose eluates, 20  $\mu$ l were subjected to immunoblot analysis with RelA-specific antibody (Cyt: cytosolic immunoprecipitate; Nuc: nuclear immunoprecipitate). Total cell lysate (30  $\mu$ g protein) was analysed as a positive control (lane 1) (TC: total cell lysate). The position of the 65 kDa RelA protein is indicated. **(b)** Cytosolic (200  $\mu$ g from 1.5 mg total) or nuclear (100  $\mu$ g from 330  $\mu$ g total) proteins from Hs578T cells were immunoprecipitated using 5  $\mu$ g/ml of either a polyclonal rabbit RelA-specific antibody (sc-372) (lanes 2,4) or a control rabbit IgG (lanes 3,5). Of 50  $\mu$ l of resulting antibody-protein A-sepharose eluates, 30  $\mu$ l were subjected to immunoblot analysis with polyclonal goat anti-AhR antibody (sc-8088) (Cyt: cytosolic immunoprecipitate; Nuc: nuclear immunoprecipitate). Total cell lysate (40  $\mu$ g protein) was analysed as a positive control (lane 1) (TC: total cell lysate). The position of the AhR protein is indicated. **(c)** Total cell proteins (100  $\mu$ g) from *T7-pcDNA3-AhR*- or *T7-pcDNA3*-transfected MCF-10F cells were immunoprecipitated using 5  $\mu$ g/ml of either normal rabbit IgG as a negative control (lane 1) or RelA-specific antibody (lanes 3,4). The resulting antibody-protein A-sepharose eluates were subjected to immunoblot analysis with T7 epitope-specific antibody, as described above. Total cell lysate (30  $\mu$ g protein) was analysed as a positive control (lane 2). The 97–100 kDa T7-AhR product is indicated

transfected with the parental *T7-pcDNA3* DNA, as control. Total cell lysates were prepared, and either immunoprecipitated with a RelA-specific antibody or an aliquot run directly on the gel. The resulting

immunoblot was probed with a T7 epitope-specific antibody. A protein of the size expected for T7-tagged AhR, i.e. 97–100 kDa, was recognized in total cell extracts from cells transfected with the *T7-pcDNA3-AhR* vector (Figure 1c, lane 2). Similarly a 97 kDa AhR protein was detected following co-precipitation with RelA-specific antibody (lane 3), whereas no protein was detected following ‘immunoprecipitation’ with control rabbit IgG (lane 1). No anti-T7 antibody-reactive protein was detected in extracts from cells transfected with the parental vector (lane 4). Furthermore, AhR protein was not detected in T7-AhR-specific immunoblots of *T7-pcDNA3*-transfected MCF-10F cell extracts precipitated with a p50-specific antibody (data not shown). Similar results were obtained with transfected Hs578T cells (not shown). Overall, these results indicate that the RelA, but not p50, and the AhR are physically associated within Hs578T and MCF-10F cells.

#### *RelA and AhR cooperate to activate the c-myc promoter in non-malignant MCF-10F cells*

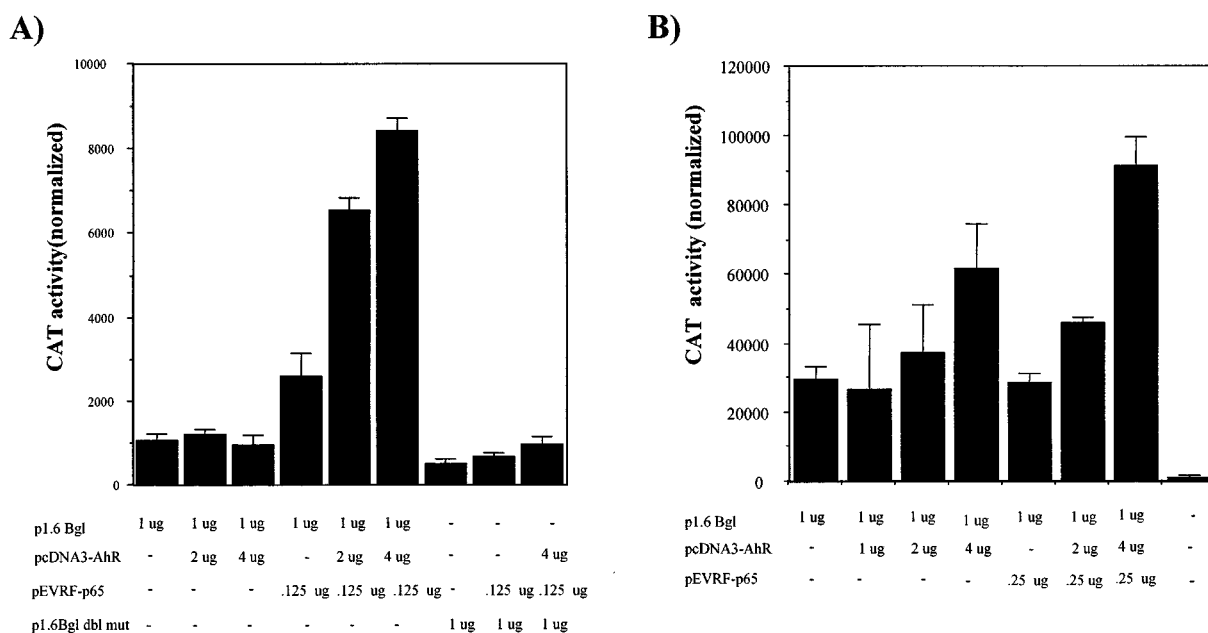
To determine whether AhR and NF- $\kappa$ B/Rel can function cooperatively, we examined the effects of *AhR* and *RelA* co-transfection on the *c-myc* promoter, a transcriptional target of classical NF- $\kappa$ B (La Rosa *et al.*, 1994). Since we have found that endogenous AhR levels decrease in cells as the cultures reach confluence (SAQ and DHS, unpublished observations), all transfections were performed with confluent cultures. In addition, in order to maximize conditions for observing RelA-AhR transcriptional cooperation, we first titrated the dose of expression vector transfected such that minimal augmentation of reporter activity would be observed. Addition of 1  $\mu$ g of the *pEVRF-p65* expression plasmid increased the transcriptional activity of the p1.6 Bgl promoter ~20-fold (data not shown), consistent with the potent role of RelA in transactivation of the *c-myc* promoter (La Rosa *et al.*, 1994). When the level of RelA expression plasmid was lowered eightfold to 0.125  $\mu$ g, a  $2.4 \pm 0.5$ -fold increase in transactivation of the murine *c-myc* promoter was observed (Figure 2a). This amount (0.125  $\mu$ g) was selected for future transfections. Minimal transcriptional activity was observed when MCF-10F cells were transfected with p1.6 Bgl alone (Figure 2a). This is consistent with the observation that *c-myc* gene transcription is minimal in cells at confluence. Transfection of 2 or 4  $\mu$ g of AhR alone had no effect on the transcriptional activity of the p1.6 Bgl promoter. However, when 0.125  $\mu$ g *pEVRF-p65* were co-transfected with 2  $\mu$ g *pcDNA3-AhR* expression plasmid, a  $6.2 \pm 0.3$ -fold induction of *c-myc* promoter activity was seen. Upon co-transfection of 0.125  $\mu$ g *pEVRF-p65* with 4  $\mu$ g *pcDNA3-AhR* expression plasmid the fold induction was  $8.0 \pm 0.2$ . Using the latter conditions, an average fold induction in three separate experiments of  $5.6 \pm 2.1$ -fold ( $P < 0.01$ ) was obtained. Thus, RelA and AhR cooperate to significantly increase *c-myc* promoter activity in MCF-10F cells.

We next asked whether the increase in transactivation of the *c-myc* promoter was mediated by the NF- $\kappa$ B elements located upstream of the promoter and/or within exon 1 (URE and IRE, respectively) (Duyao *et*

*al.*, 1990; Kessler *et al.*, 1992b). A transfection experiment, similar to that described above, was performed using the p1.6 Bgl double mutant (p1.6 Bgl dbl mut) reporter plasmid, in which the URE and IRE NF- $\kappa$ B sites have been mutated so that the promoter can no longer be transcriptionally activated by classic NF- $\kappa$ B (Duyao *et al.*, 1992; Kessler *et al.*, 1992a; La Rosa *et al.*, 1994). In the absence of exogenous RelA or AhR, the p1.6 bgl double mutant displayed about one half of the activity of the wild type p1.6 Bgl reporter (Figure 2a). This modest decrease in activity of the mutant *vs* wild type p1.6 Bgl reporter construct is consistent with the low levels of RelA/p50 complexes present in the MCF-10F cells (Sovak *et al.*, 1997). Ectopically expressed RelA in *pEVRF-p65* transfected cells was unable to transactivate the mutant construct, consistent with our previous findings (La Rosa *et al.*, 1994). Furthermore, co-transfection with 4  $\mu$ g *pcDNA3-AhR* and 0.125  $\mu$ g *pEVRF-p65* did not significantly affect the activity of the mutated *c-myc* promoter (Figure 2a). Taken together these findings indicate that RelA and AhR function cooperatively to transactivate the *c-myc* promoter via binding at the URE and/or IRE NF- $\kappa$ B elements.

#### *RelA and AhR activate the c-myc promoter in Hs578T cells*

We next asked whether RelA and AhR can activate the *c-myc* promoter in a human malignant breast cancer cell line by performing similar co-transfection analyses with Hs578T cells. In these cells the basal activity of the p1.6 Bgl promoter was notably higher than observed in transfected MCF-10F cells (Figure 2b). This result likely reflects the higher transfection efficiency of Hs578T cells (20–30% *vs* 5% for MCF-10F cells), and potentially the higher endogenous levels of nuclear NF- $\kappa$ B/Rel proteins in these malignant cells (Sovak *et al.*, 1997). Interestingly, the activity of the p1.6 Bgl reporter plasmid increased in a dose-dependent fashion with transfection of increasing levels of AhR expression plasmid alone. Following transfection with 4  $\mu$ g of *pcDNA3-AhR*, CAT activity was  $2.1 \pm 0.4$ -fold higher than basal levels. This result may be due to the effect of relatively high levels of constitutively active endogenous RelA protein present in these cells (see below). When a suboptimal dose (0.25  $\mu$ g) of *pEVRF-p65* plasmid alone was added, no apparent change in p1.6 Bgl activity was seen. However, co-transfection of both the RelA and AhR expression plasmids resulted in induction of a higher level of *c-myc* promoter activity than was seen following transfection of either plasmid alone (Figure 2b). Specifically, a  $3.1 \pm 0.3$ -fold induction of the *c-myc* promoter activity was observed following co-transfection with 4  $\mu$ g *pcDNA3-AhR* and 0.25  $\mu$ g *pEVRF-p65* expression plasmids. The fact that the cooperative effects seen following AhR and RelA plasmid co-transfections in Hs578T cells were not as great as those seen in co-transfected MCF-10F cells may have been due to the higher level of background activity in the former cells, as well as the modest induction of reporter activity following transfection with 4  $\mu$ g AhR expression plasmid alone in Hs578T cells.



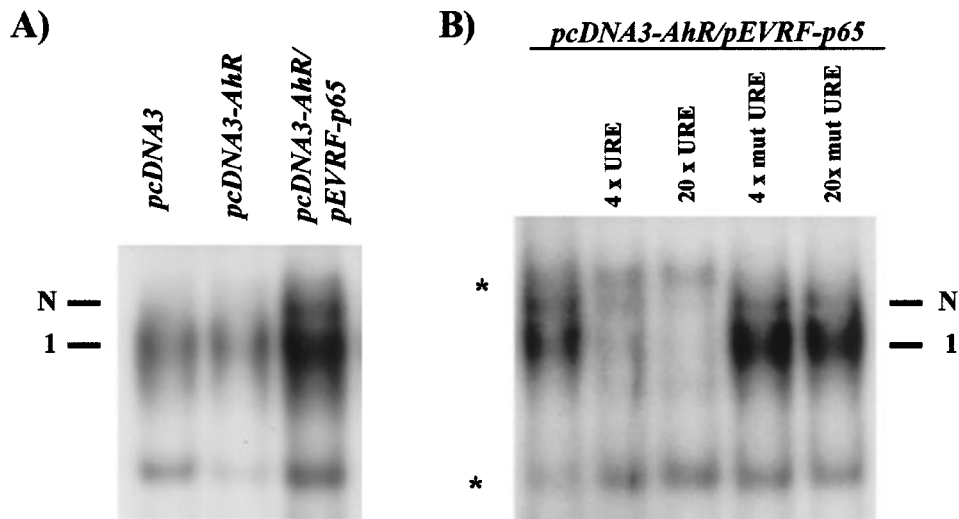
**Figure 2** RelA and AhR cooperate to transactivate the wildtype p1.6 Bgl, but not the p1.6 Bgl dbl mut, *c-myc* promoter construct. (a) Confluent MCF-10F cells (~200 000 cells in 35 mm<sup>2</sup> dishes) were transiently transfected, in duplicate, with either 1  $\mu$ g p1.6 Bgl or p1.6 Bgl dbl mut, and 0, 2, or 4  $\mu$ g *pcDNA3-AhR* (murine AhR) expression vector in the absence or presence of 0.125  $\mu$ g *pEVRF-p65* (RelA expression) plasmid using 7  $\mu$ l FUGENE reagent. In each transfection, 1  $\mu$ g of TK-luciferase plasmid was added as an internal control for normalization of transfection efficiency. Total DNA transfected was maintained at 6  $\mu$ g by addition *pcDNA3* plasmid (parent vector for *pcDNA3-AhR*). Transfected cells were harvested after 24 h in reporter lysis buffer, and analysed for CAT and luciferase activity. CAT activities are presented normalized for transfection efficiency, using the luciferase activity. (b) Confluent Hs578T breast cancer cells were transiently transfected, in duplicate, with 1  $\mu$ g of p1.6 Bgl plus 0, 1, 2, or 4  $\mu$ g of *pcDNA3-AhR*, in the absence or presence of 0.25  $\mu$ g of *pEVRF-p65* plasmid using 5  $\mu$ l of FUGENE reagent. In each transfection, 0.5  $\mu$ g of TK-luciferase plasmid was added and total DNA was maintained at 6  $\mu$ g by addition of the appropriate amounts of *pcDNA3* plasmid. After 24 h, cells were harvested and analysed for CAT and luciferase activities and protein levels. Values were normalized to protein levels because the TK-luciferase activity was not appreciable in these cells at confluence

#### AhR/RelA complexes bind to the URE NF- $\kappa$ B element

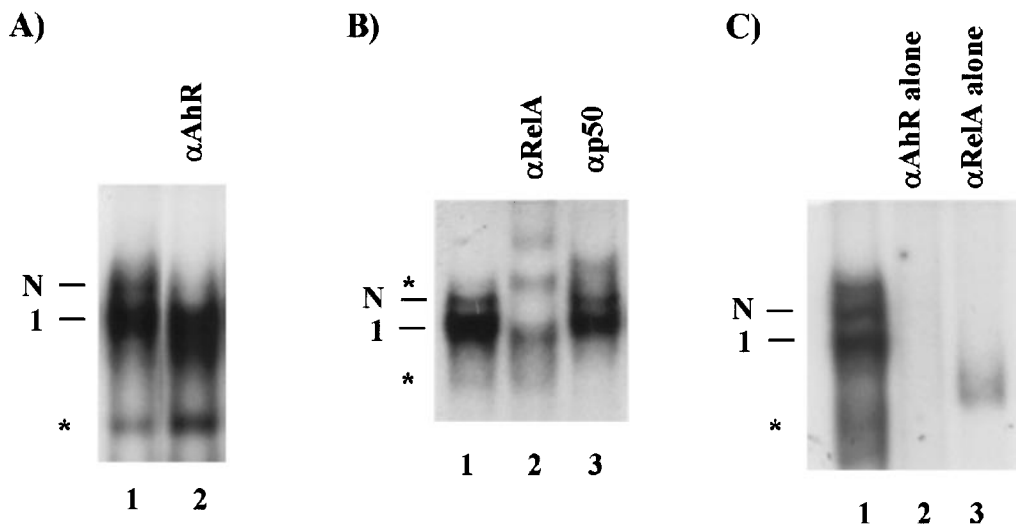
To determine whether the AhR and RelA proteins are able to associate with the NF- $\kappa$ B binding elements in the *c-myc* gene, electrophoretic mobility shift assays (EMSA) were performed using an oligonucleotide containing the NF- $\kappa$ B upstream regulatory element (URE) as probe. To enhance AhR and RelA expression, nuclear extracts from transfected Hs578T cells were used. (Hs578T cells were selected rather than MCF-10F cells because of the better transfection efficiency obtained with this line.) Confluent cultures of Hs578T cells were transfected using FUGENE with *pcDNA3-AhR* vector DNA in the absence or presence of *pEVRF-p65* RelA expression plasmid. As an additional control, cells were transfected with empty parental *pcDNA3* vector alone. Nuclear extracts, prepared 24 h post-transfection, were then used in EMSA. Since an AhR/RelA complex might not bind DNA with the same affinity as typical NF- $\kappa$ B complexes, a lower dI:dC concentration was used to reduce the likelihood of competing away a specific binding complex. In control cells transfected with only parental *pcDNA3*, a major band migrating with the mobility of classic NF- $\kappa$ B was detected (labeled as band 1 in Figure 3a). No change in the binding pattern was seen upon transfection with *pcDNA3-AhR*. When nuclear extracts from cells co-transfected with *pEVRF-p65* and *pcDNA3-AhR* expression plasmids were used, both the putative classic NF- $\kappa$ B band

and a novel upper band (labeled 'N') were seen (Figure 3a). The intensity of band 1 increased. Equal loading of the lanes was confirmed in EMSA for an Oct-1 probe (data not shown). Addition of fourfold or 20-fold molar excess wild type URE oligonucleotide successfully competed away complexes represented in both bands, whereas addition of similar amounts of mutant URE oligonucleotide, having the same two G to C conversions as in the p1.6 Bgl dbl mut construct (Duyao *et al.*, 1992), failed to compete (Figure 3b).

To determine the identity of the subunits found in the two specific binding complexes, supershift EMSA was performed using polyclonal rabbit antibodies raised against either RelA or the AhR. Addition of the AhR antibody specifically ablated band N without significantly changing the migration pattern of band 1 (Figure 4a). Antibody alone plus probe did not yield a similar complex (Figure 4c). Furthermore, the AhR antibody had no effect on binding of nuclear proteins to an Oct-1 sequence (data not shown). Addition of the RelA-specific antibody (sc-372X) clearly ablated formation of both band 1 and band N (Figure 4b,c). Addition of a second RelA-specific antibody #1226 (kindly provided by N Rice) similarly reduced formation of both band 1 and band N (data not shown). In contrast, addition of an antibody against the p50 subunit reduced band 1 and ablated a minor band below (Figure 4b). An equivalent amount of a rabbit polyclonal antibody against an irrelevant protein



**Figure 3** Expression of RelA and AhR yields a novel URE NF- $\kappa$ B element binding complex. (a) Co-transfection with AhR and RelA expression vectors leads to formation of a novel complex. Confluent cultures (100 mm<sup>2</sup> dishes) of Hs578T cells were transfected with either 52  $\mu$ g *pcDNA3* empty vector, or 50  $\mu$ g *pcDNA3-AhR* in the absence or presence of 2  $\mu$ g *pEVRF-p65* expression plasmid using 70  $\mu$ l FUGENE reagent. After 24 h, nuclear proteins were isolated using the method of Dignam *et al.* (1983), and subjected to EMSA for NF- $\kappa$ B binding. N indicates position of a new complex; 1, indicates position of a previously observed major complex. (b) Competition EMSA confirms the specificity of the major bands. Nuclear extracts of Hs578T cells co-transfected with *pcDNA3-AhR* and *pEVRF-p65* were pre-incubated with either 4- or 20-fold molar excess unlabeled wildtype (URE) or mutant (mt URE) URE prior to the 30 min incubation reaction with the radiolabeled URE. Two nonspecific bands were identified and marked with an asterisk (\*)



**Figure 4** Novel NF- $\kappa$ B binding complex contains AhR and RelA protein. Nuclear extracts from the AhR and RelA expression vector co-transfected cells, prepared as described above in Figure 3, were incubated with the URE probe. Following a 30 min binding reaction, antibodies were added as indicated, the reactions incubated for an additional 1 h, and subjected to EMSA. Alternatively as control, antibodies were added to the probe in the absence of extract, and the mixture incubated as above, and subjected to EMSA. (a) Extracts were incubated in the absence (lane 1) or presence of 1  $\mu$ l AhR-specific antibody (BioMol #SA-210) (lane 2) and subjected to EMSA. Specific binding complexes are indicated as band 1 and band N, as above; nonspecific bands are marked with an asterisk (\*). (b) Extracts were incubated in the absence (lane 1) or presence of either 1  $\mu$ l RelA-specific antibody (sc-372X) (lane 2) or 1  $\mu$ l p50-specific antibody (sc-114) (lane 3), and processed as above. (c) Extract was incubated in the absence of antibody and EMSA performed, as above (lane 1). Alternatively either 1  $\mu$ l AhR-specific antibody (BioMol #SA-210) (lane 2) or 1  $\mu$ l RelA-specific antibody (sc-372X) (lane 3) was incubated with the probe alone, and subjected to EMSA

(YY1, sc-281-X) failed to alter binding to the URE (data not shown). Thus, band 1 contains RelA and p50 proteins, and most likely represents binding of classical NF- $\kappa$ B heterodimers (RelA/p50). Based on its migration, the minor lower band likely consists of p50 homodimers. Finally, band N contains both RelA and AhR proteins.

#### *AhR and RelA induce the endogenous c-myc gene*

To verify that the affects of AhR and RelA can be seen on chromosomal *c-myc* genes, co-transfection analysis was performed. Cultures of MCF-10F cells at 70% confluence were transfected with *pEVRF-p65* or *T7-pcDNA3-AhR* vector DNA alone or in combination.

Whole cell extracts were prepared and subjected to immunoblot analysis for c-Myc and  $\beta$ -actin protein, which confirmed equal loading (Figure 5). Using densitometry of this and a duplicate experiment, an increase in c-Myc level of  $3.1 \pm 0.00$ -fold and  $2.75 \pm 0.05$ -fold, respectively upon expression of RelA or AhR alone compared to control vector DNA was measured. An increase in c-Myc expression of  $9.5 \pm 3.2$ -fold was observed upon co-transfection of both *pEVRF-p65* and *T7-pcDNA3-AhR* vector DNAs into MCF-10F cells. Thus, while increases in the level of c-Myc protein were seen upon transfection of MCF-10F cells with either vector alone, a greater induction was seen upon transfection of the combination of *pEVRF-p65* and *T7-pcDNA3-AhR* vector DNAs. These results confirm the ability of RelA and AhR to cooperate in activation of the *c-myc* gene.

## Discussion

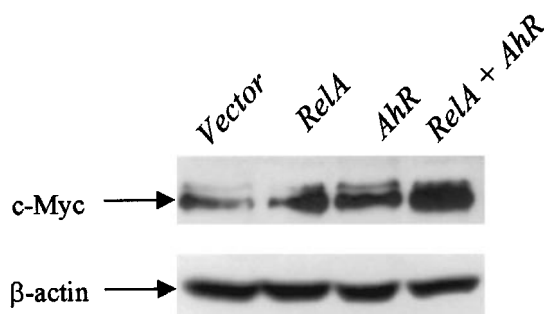
Here we show the physical and functional association of the RelA subunit of NF- $\kappa$ B and AhR in transactivation of the *c-myc* gene in breast epithelial cells. Specifically, RelA and AhR were physically associated in malignant Hs578T breast cancer. Using transfection analysis, RelA and AhR cooperated to transactivate the *c-myc* promoter in non-malignant MCF-10F mammary epithelial cells and to a lesser extent Hs578T cells. Furthermore, RelA and AhR enhanced endogenous c-Myc protein levels in MCF-10F cells. As judged by transfection and mobility shift analyses, the RelA and AhR proteins formed a novel complex that bound to the wild type but not mutant NF- $\kappa$ B element of the *c-myc* gene. We postulate it is this complex, binding via the NF- $\kappa$ B element, that transactivated the *c-myc* promoter. Co-transfection of vectors that express AhR and RelA proteins with a wild type *c-myc* promoter-reporter construct, but not with a promoter construct mutated in the NF- $\kappa$ B binding URE and IRE sites, led to increased levels of *c-myc* promoter transactivation. In contrast, cooperation between AhR and RelB or c-Rel subunits of NF- $\kappa$ B/Rel in transactivation of the *c-myc* gene was not

observed in similar transfection analysis (data not shown). Furthermore, the novel transcription factor complex did not appear to contain the p50 subunit. Consistent with these findings, RelA but not p50 was found to specifically interact with the AhR in murine hepatoma cells (Tian *et al.*, 1999). Thus, based on the relative mobility in EMSA, and the identified presence of both RelA and AhR in the novel complex, our results suggest that the RelA and AhR bind the URE as a heterotypic dimer composed of one subunit of each protein.

Recently, we demonstrated that rodent and human mammary tumors are typified by aberrant activation of NF- $\kappa$ B/Rel (Sovak *et al.*, 1997) and overexpression of AhR (Trombino *et al.*, 2000). These tumors are often also characterized by overexpression of *c-myc* (Berns *et al.*, 1992; Borg *et al.*, 1992; Pavelic *et al.*, 1991; DWK and GES, unpublished observations). While in some tumors *c-myc* genes were present in large copy numbers, in other cases overexpression of c-Myc protein was seen without gene amplification (Pavelic *et al.*, 1991). The ability of AhR to cooperate with RelA to transactivate promoters through NF- $\kappa$ B elements suggests a novel mechanism for regulation of *c-myc* gene expression.

RelA protein interactions with other transcription factors have been found to lead either to induction (Bassuk *et al.*, 1997; Shen and Stavnezer, 1998) or repression (Wissink *et al.*, 1997; Ferrier *et al.*, 1999) of gene transactivation. In most cases, transcription complex-DNA association involves binding sites for both NF- $\kappa$ B and the partner transcription factor, which are in close proximity to one another (Shen and Stavnezer, 1998; Dickinson *et al.*, 1999). However, neither a consensus XRE, which would bind AhR/ARNT complexes, nor an AhR binding half site (5'-CGTC-3') (Dickinson *et al.*, 1999) are present in close proximity to either the URE or the IRE NF- $\kappa$ B elements in the murine *c-myc* gene. In addition, a 10- or 50-fold molar excess of cold XRE did not compete successfully for AhR/RelA-URE binding (data not shown), suggesting that DNA domains typically bound by an AhR/ARNT complex are not required for AhR/RelA-URE binding. Consistent with this observation, competition EMSA with oligonucleotides mutated at additional bases within the core NF- $\kappa$ B element failed to successfully compete for binding (data not shown).

Our findings differ significantly from those reported by Tian and coworkers (1999) who observed repression of RelA transactivation by AhR in a murine cell line. Several explanations may be given for these differences. Tian *et al.* (1999) used a multimerized consensus NF- $\kappa$ B element (5'-GGCAGGGGAATTCCCC-3') construct in their studies, while we employed a *c-myc* promoter construct. Of note, the core sequence of the consensus binding element differs significantly from that found in the two NF- $\kappa$ B elements within the *c-myc* gene (Duyao *et al.*, 1990; Kessler *et al.*, 1992b). Interestingly, no new binding complex was seen with the NF- $\kappa$ B consensus element (Tian *et al.*, 1999), whereas EMSA with the *c-myc* URE NF- $\kappa$ B element revealed a novel AhR/RelA-containing band, consistent with the functional cooperation in cells co-transfected with AhR and RelA expression vectors. If DNA binding is sequence specific, then only a subset of NF- $\kappa$ B element-containing genes may be affected by



**Figure 5** RelA and AhR cooperate to induce the endogenous *c-myc* gene in MCF-10F cells. Cultures of MCF-10F cells, at 70% confluence, were transiently transfected with 4  $\mu$ g *pEVRF-p65* or 20  $\mu$ g *T7-pcDNA3-AhR* DNA alone or in combination with 30  $\mu$ l FUGENE transfection reagent. Total transfected DNA was maintained at 24  $\mu$ g by addition of *pcDNA3* plasmid. Alternatively, cells were transfected with *pcDNA3* plasmid DNA alone (Vector). After 48 h, cells were harvested and samples of whole cell extracts (40  $\mu$ g) subjected to immunoblot analysis for c-Myc (786-4) and  $\beta$ -actin (AC-15) proteins

AhR/RelA binding. Furthermore, the *c-myc* promoter likely contains elements capable of binding other potential cooperating transcription factors. Important differences also reside within the cell types used, i.e. murine hepatoma and COS-7 cells *vs* human mammary cell lines. Interestingly, NF- $\kappa$ B failed to transactivate the *c-myc* promoter in normal mouse hepatocytes (Bellas and Sonenshein, 1999), while it effectively induced the promoter in breast cancer cells (Sovak *et al.*, 1997), suggesting that binding to overlapping elements within the *c-myc* gene ablated the ability of NF- $\kappa$ B to bind in hepatocytes.

Different subunits of the NF- $\kappa$ B/Rel family have been shown to interact with members of other protein families (Bassuk *et al.*, 1997; Shen and Stavnezer, 1998; Wissink *et al.*, 1997; Ferrier *et al.*, 1999; Dickinson *et al.*, 1999; Stein *et al.*, 1993; Raj *et al.*, 1996; Kalkoven *et al.*, 1996; Na *et al.*, 1999). In many of these cases, the associations are fairly specific for the RelA subunit, e.g., with glucocorticoid and progesterone receptors (Wissink *et al.*, 1997; Kalkoven *et al.*, 1996) and the YB-1 protein (Raj *et al.*, 1996). In contrast, Stat6 (Shen and Stavnezer, 1998), C/EBP (Stein *et al.*, 1993), and retinoid X receptor (Na *et al.*, 1999) functionally interact with both RelA and p50. Therefore, it is not unusual that only cooperation between AhR and RelA was detected in HMECs. Interestingly, a similar functional interaction of RelA with the progesterone receptor was noted in that p50 and c-Rel subunits failed to affect the transcriptional activity of the activated PR on a progesterone responsive element construct (Kalkoven *et al.*, 1996). The Rel homology region (RHR) is known to be important both for dimerization of NF- $\kappa$ B/Rel subunits (Grimm and Baeuerle, 1993), as well as for interaction with many of these other proteins (Wissink *et al.*, 1997; Stein *et al.*, 1993). While some AhR domains involved in AhR binding to proteins, such as ARNT, hsp90, and the immunophilin-like ARA-9 protein have been evaluated (Perdew and Bradfield, 1996; Meyer *et al.*, 1998; Okey *et al.*, 1994; Carver *et al.*, 1998), those required for AhR dimerization with other proteins, e.g. Rb (Ge and Elferink, 1998), have not been defined. The exact domains mediating the interactions between AhR/RelA and binding of the putative heterodimeric complex to the *c-myc* promoter are under investigation.

The human *c-myc* gene has been found to contain consensus XRE elements. Since our efforts were focused on the potential effects of AhR/RelA interactions, a *c-myc* promoter construct that does not contain these elements was used to reduce complications with effects of AhR alone. Finally, it should be noted that *c-myc* promoter activation following transfection with AhR- and RelA-encoding plasmids and AhR-RelA dimerization in the nuclei of non-transfected cells occurred in the absence of exogenous AhR ligands. These results suggest constitutive AhR activity in mammary tumor cell lines. This hypothesis is strongly supported by constitutive nuclear AhR expression (Chang and Puga, 1998; Singh *et al.*, 1996), and constitutive AhR-mediated transcriptional activity (Chang and Puga, 1998; Ma and Whitlock, 1996) in mouse hepatoma, monkey kidney, and human epithelial carcinoma cell lines. Furthermore, we have recently demonstrated constitutive nuclear AhR expression and high levels of an AhR-regulated gene, *CYP1B1*, in rat

mammary tumors (Trombino *et al.*, 2000). While these results support a role for constitutive AhR activation in tumorigenesis, organ defects observed in AhR<sup>-/-</sup> mice suggest a role for developmentally regulated AhR activation in organogenesis (Hushka *et al.*, 1998; Fernandez-Salguero *et al.*, 1995; Lahvis and Bradfield, 1998; Abbott *et al.*, 1999; Robles *et al.*, 2000). The endogenous signals that induce AhR activity, and the extent to which these AhR activities are modulated by exogenous AhR ligands remain to be elucidated.

## Materials and methods

### Cell growth and treatment conditions

MCF-10F is a human mammary epithelial cell line established from a patient with fibrocystic disease, which does not display malignant characteristics (Calaf and Russo, 1993). The Hs578T tumor cell line was derived from a mammary carcinosarcoma and is epithelial in origin (Hackett *et al.*, 1977).

### Synthesis of AhR expression construct

Full length AhR cDNA was PCR amplified using the pMu-AhR plasmid (kindly provided by Dr C Bradfield, University of Wisconsin, Madison, WI, USA) as template, with the following primers carrying *Xba*I restriction sites: sense 5'-CTA GTC TAG ACC ATG AGC AGC GGC GCC AAC-3'; anti-sense 5'-CTA GTC TAG AAA GCT TAG TAT CGA ATT-3'. The AhR cDNA was amplified with Pfu Turbo polymerase (Stratagene, La Jolla, CA, USA). The PCR product was gel purified and subcloned into the *Xba*I site of the *T7-pcDNA3* plasmid constructed by linking the DNA coding for the 11 amino acid leader peptide of the T7 major capsid protein (digested out from the pTOPE pET translation vector (Novagen, Madison, WI, USA) to the *Bam*HI site of *pcDNA3* (Invitrogen, Carlsbad, CA, USA). Proper AhR and *T7-pcDNA3* directional cloning was confirmed by restriction analysis and DNA sequencing.

### Transfection and immunoprecipitation analysis

Cells were transfected in 100 mm<sup>2</sup> culture plates with 5  $\mu$ g of *T7-pcDNA3-AhR* or *T7-pcDNA3* mixed with 6  $\mu$ l FUGENE transfection reagent (Boehringer Mannheim, Indianapolis, IN, USA), according to the manufacturer's instructions. After 36 h, cells were rinsed with cold PBS. For total cell lysates, transfected or untransfected cells were lysed in 1 ml immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 2  $\mu$ g/ml leupeptin; 2  $\mu$ g/ml aprotinin; 5  $\mu$ g/ml phenylmethylsulfonyl fluoride) containing 1% IGEPAL CA-630 detergent (Sigma Chemical Co., St. Louis, MO, USA) for 20 min on ice, and centrifuged at 14 000 r.p.m. for 10 min. Alternatively, cytosolic and nuclear fractions were prepared essentially as described (Pollenz *et al.*, 1994). Aliquots were incubated for 1 h with 5  $\mu$ g/ml polyclonal rabbit anti-RelA/p65 antibody, normal rabbit IgG, polyclonal goat anti-AhR antibody or normal goat IgG (all antibodies from Santa Cruz Biotechnology, Santa Cruz, CA, USA), and immunoprecipitates collected and washed using protein A-sepharose beads. Eluted proteins were subjected to electrophoresis and immunoblot analysis, as described previously (Yamaguchi *et al.*, 1997a,b). Blots were probed with HRP-anti-T7-epitope tag antibody (Novagen), anti-RelA antibody (sc-372, Santa Cruz Biotechnology), or polyclonal goat anti-AhR antibody (sc-8088, Santa Cruz Biotechnology) for 1 h at room temperature. After thorough washing, the membranes were treated for another 45 min with goat anti-rabbit-HRP antibody for RelA-specific

immunoblotting or with anti-goat IgG-HRP antibody for AhR-specific immunoblotting. Membranes were developed by chemiluminescence (Du Pont NEN Research Products Co., Boston, MA, USA) after washing three times with TBS containing 0.05% Tween (Sigma).

#### Transfection and immunoblot analysis

Cultures of MCF-10F cells, at 70% confluence, were transiently transfected with 4  $\mu$ g *pEVRF-p65* or 20  $\mu$ g *T7-pcDNA3-AhR* DNA alone or in combination with 30  $\mu$ l FUGENE transfection reagent. Total DNA transfected was maintained at 24  $\mu$ g by addition of *pcDNA3* plasmid. After 48 h, cells were rinsed with cold PBS, and harvested in lysis buffer (50 mM Tris-HCl, pH 8.0; 5 mM EDTA, pH 8.0; 150 mM NaCl; 0.5 mM DTT; 2  $\mu$ g/ml aprotinin; 2  $\mu$ g/ml leupeptin; 0.5 mM PMSF; 0.5% NP40). Whole cell extracts (WCE) were obtained by sonication, followed by centrifugation at 14 000 r.p.m. for 30 min. Samples (40  $\mu$ g) of WCEs were subjected to electrophoresis and immunoblot analysis, as above. Blots were probed with rabbit anti-c-Myc antibody (786-4, a gift from S Hann, Vanderbilt University, Memphis, TN, USA), and mouse anti- $\beta$ -actin monoclonal antibody (AC-15, Sigma).

#### Promoter activity analysis

Confluent cultures of MCF-10F or Hs578T cells were transiently transfected using FUGENE transfection, as above, with wild type p1.6 Bgl *c-myc* promoter CAT or a mutant *c-myc* promoter CAT reporter, termed p1.6 Bgl dbl mut vector, described previously (Duyao *et al.*, 1992). These constructs contain -1141 to +513 bp of the murine *c-myc* promoter/exon1/upstream sequences, including the two NF- $\kappa$ B elements in either wild type or mutant versions, driving a chloramphenicol acetyl transferase (CAT) reporter gene (Duyao *et al.*, 1992), and does not contain XREs. Vectors *pcDNA3-AhR*, encoding murine AhR (Dolwick *et al.*, 1993) and *pEVRF-p65*, encoding murine RelA protein (kindly provided by R Sen, Brandeis University, Waltham, MA, USA) were co-transfected, as indicated. In each transfection, 1  $\mu$ g of TK-luciferase plasmid was added as an internal control for normalization of transfection efficiency. Total DNA transfected was maintained at 6  $\mu$ g by addition of *pcDNA3* plasmid (parent vector for *pcDNA3-AhR*). Transfected cells were harvested after 24 h in reporter lysis buffer,

and analysed for CAT and luciferase activity, as described previously (Sovak *et al.*, 1997; Dolwick *et al.*, 1993).

#### EMSA

Nuclear extracts were prepared from breast cancer cells by a modification of the method of Dignam *et al.* (1983), and oligonucleotides probes radiolabeled essentially as we have described previously (Sovak *et al.*, 1997). The sequence of the URE NF- $\kappa$ B-containing oligonucleotide from the *c-myc* gene (Duyao *et al.*, 1990) is as follows: 5'-GATCCAAGTCCGG-GTTTTCCCAACC-3', where the underlined region indicates the core binding element. The mutant URE has a two G to C base pair conversion, indicated in bold, blocking the NF- $\kappa$ B/Rel binding (Duyao *et al.*, 1990): 5'-GATCCAA-GTCC**GCCTTTT**CCCAACC-3'. A slight modification of the usual NF- $\kappa$ B binding reaction (Sovak *et al.*, 1997) was used. <sup>32</sup>P-labeled oligonucleotide (20 000–25 000 c.p.m.) was incubated with 2.5  $\mu$ g of nuclear extract, 5  $\mu$ l sample buffer (10 mM HEPES, 4 mM DTT, 0.5% Triton X-100, and 2.5% glycerol), 0.1  $\mu$ g poly dI-dC as nonspecific competitor, and the salt concentration adjusted to 100 mM using buffer C. The reaction was carried out at room temperature for 30 min, and DNA/protein complexes were separated, as previously described (Sovak *et al.*, 1997). Where indicated, antibodies were added after the binding reaction and the mixture incubated for 1 additional hour. Antibodies used include: anti-RelA subunit, sc-372X from Santa Cruz Biotechnology and #1226, kindly provided by N Rice (NCI, Frederick, MD, USA); anti-p50 subunit, sc-114 from Santa Cruz Biotechnology; anti-AhR, #SA-210 from BioMol (Plymouth Meeting, PA, USA).

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