Oncogene (1998) 16, 3133–3142

http://www.stockton-press.co.uk/onc

A chicken c-Rel-estrogen receptor chimeric protein shows conditional nuclear localization, DNA binding, transformation and transcriptional activation

Michal Zurovec[1,2], Oleksi Petrenko[1,3], Richard Roll[1] and Paula J Enrietto[1]

[1]*Department of Microbiology State University of New York at Stony Brook, Stony Brook, New York, 11794, USA*

In this report, we characterize the biological and biochemical properties of a conditional protein containing chicken c-Rel fused to the hormone-binding domain of the human estrogen receptor. This chimeric c-RelER protein causes estrogen-dependent, but otherwise c-Rel-specific, transformation of avian fibroblasts *in vitro*. Our results demonstrate that c-RelER heterodimerizes with wild-type c-Rel and forms specific complexes with IκB-α. Estrogen causes translocation of c-RelER to the nucleus and stabilizes its binding to DNA. Hormone-activated c-RelER induces transcription of at least four cellular genes that are constitutively active in wild-type c-Rel-transformed fibroblasts. Two distinct cell populations were examined that differed with respect to their growth phenotypes. The growth of fibroblasts with moderate expression levels of c-RelER was stimulated by estrogen. In contrast, the addition of estrogen to cells with high c-RelER expression levels resulted in inhibition of cytokinesis and the arrest of growth. The carboxy terminal transactivation domain of c-Rel was required for the induction of these effects since neither v-Rel nor c-Rel deletion mutants were able to induce similar changes. Taken together, our results demonstrate that high levels of c-Rel expression can affect cell cycle control and/or cytokinesis. Furthermore, they also indicate that the biological properties of c-Rel in cell growth and differentiation will potentially differ depending on the level of expression.

Keywords: c-Rel; NF-κB; transformation; apoptosis

Introduction

The c-*rel* proto-oncogene encodes a sequence-specific transcription factor whose expression is largely restricted to hematopoietic organs. Recent studies indicate that c-Rel plays a critical role in regulation of B lymphocyte proliferation and T-cell activation (Kontgen *et al*., 1995, Gerondakis *et al*., 1996). Structurally, c-Rel belongs to a larger family of transcription factors, the Rel/NF-κB family, which also includes RelA, RelB, NF-κB1, NF-κB2, Dorsal, Dif, and Relish. All members of this family are related through a conserved amino-terminal region, the Rel homology domain. This region is responsible for several properties of the proteins, including the formation of homo- and heterodimers, nuclear localization, DNA binding, and interactions with the family of inhibitor proteins, the IκB family (reviewed in Siebenlist *et al*., 1994; Baeuerle and Henkel, 1994). A broad range of stimuli, such as cytokines, lipopolysaccharides or phorbol esters, promotes translocation of the Rel proteins from the cytoplasm to the nucleus by mechanisms involving phosphorylation and subsequent degradation of IκB-α (Ghosh and Baltimore, 1990; Brown *et al*., 1993; Henkel *et al*., 1993; Beg *et al*., 1993). Most members of the Rel/NF-κB family have been implicated in the transcriptional regulation of genes involved in immunoregulatory processes, cell growth, and differentiation (for a recent review see Baeuerle and Baltimore, 1996). Consistent with these findings, c-Rel has been implicated in the transcriptional regulation of genes encoding cell adhesion molecules, growth factors such as GM-CSF, interleukin-2 and 3, and their receptors (Kontgen *et al*., 1995; Gerondakis *et al*., 1996).

Alterations in several members of the Rel/NF-κB family have been associated with hematopoietic malignancies (Gilmore *et al*., 1996). However, c-Rel and its mutated viral homolog, v-Rel, are the only members of the family consistently shown to be oncogenic *in vivo* (Gilmore *et al*., 1996). Moreover, depending on the cell type, overexpression of c-Rel can lead either to transformation or programmed cell death (Abbadie *et al*., 1993). The mechanisms of these processes remain unclear, although most data available to-date indicate that they result from aberrant gene expression induced by Rel (Gilmore *et al*., 1996).

In order to more fully understand the mechanisms of c-Rel action and its transforming potential, we constructed an inducible form of c-Rel by fusing it to the hormone-binding domain of the human estrogen receptor (ER). Several functional properties of the protein were investigated, including subcellular localization, DNA binding, and activation of transcription of cellular genes. Using the inducible c-RelER system proliferation properties of cells were examined which correlated with high levels of c-Rel expression. We show that overexpression of c-Rel in fibroblasts results in the generation of cells with characteristic flattened morphology and dramatically decreased growth rates. Accumulation of these cells in the G1 phase and an extensive breakdown of cellular cytoskeleton suggest that high levels of c-Rel expression can affect cell cycle control and/or cytokinesis. The carboxy terminal transactivation domain of c-Rel is required for the

Correspondence: M Zurovec
Current addresses: [2]Biological Faculty, University of South Bohemia and Institute of Entomology, Czech Academy of Sciences, Branisovska 31, Ceske Budejovice 37005, Czech Republic [3]Department of Molecular Biology, Princeton University, Princeton, NJ 08544
Received 10 September 1997; revised 20 January 1998; accepted 21 January 1998

induction of the above effects since neither v-Rel nor a c-Rel carboxy terminal deletion mutant were able to inhibit cell growth and induce similar morphological changes. Taken together, these results indicate that the biological properties of c-Rel in cell growth and differentiation will potentially differ depending on the level of expression. Utilization of the conditional c-Rel variant described here may help in understanding the changes that accompany altered Rel expression in different cell types.

Results

Construction of c-RelER

To generate a conditional c-Rel protein, chicken c-*rel* sequences were fused in-frame to the hormone-binding domain of the human estrogen receptor (ER). The c-RelER construct was subcloned into the polylinker region of the pCRNCM avian retroviral vector, downstream of the CMV promoter (see Figure 1a). Other retroviral constructs used in this work encoded wild-type c-Rel; Δc-Rel, a truncated version of c-Rel lacking 103 amino acid residues of the carboxy-terminal transactivation domain; Δc-RelER, in which Δc-Rel sequences were fused to the hormone-binding domain of the human estrogen receptor; v-Rel and v-RelER.

Western blot analysis of protein lysates prepared from pCRNCM-c-RelER-transfected CEF (in the following referred to as c-RelER fibroblasts) showed that the cells expressed the c-RelER protein of the predicted size (100 kDa, Figure 1b). Similarly, CEF transfected with Δc-Rel, Δc-RelER, v-Rel and v-RelER expressed high levels of the corresponding proteins.

Expression of c-RelER in chicken embryo fibroblasts

Previous studies have shown that overexpression of c-Rel morphologically transforms primary avian fibroblasts and confers a distinctive phenotype on these cells, characterized by altered morphology, extensive disruption of cellular cytoskeleton, and an extended life span (Abbadie *et al*., 1993; Kralova *et al*., 1994). To determine if expression of c-RelER induces a similar phenotype, c-RelER fibroblasts were grown in the presence or absence of estrogen. The cells grown in the absence of estrogen were morphologically indistinguishable from control pCRNCM-transduced CEF (Figure 2a). In contrast, most c-RelER fibroblasts grown in the presence of estrogen displayed characteristic fusiform morphology, which became evident within 48 h after the addition of estrogen. This phenotype could be reversed upon addition or withdrawal of hormone. Fibroblasts expressing Δc-RelER exhibited similar estrogen-dependent morphological changes (Figure 2a).

The morphological alterations of c-RelER CEF correlated with changes in other parameters characteristic of c-Rel-induced phenotype, namely actin bundle breakdown and altered cell growth. As shown in Figure 2b, well-defined actin bundles were observed in control pCRNCM-transduced cells and in c-RelER fibroblasts grown in the absence of estrogen. In contrast, c-RelER CEF grown in the presence of estrogen exhibited extensive breakdown of actin bundles. Similar disruption of cellular cytoskeleton was found in wild-type c-Rel or v-Rel-expressing fibroblasts.

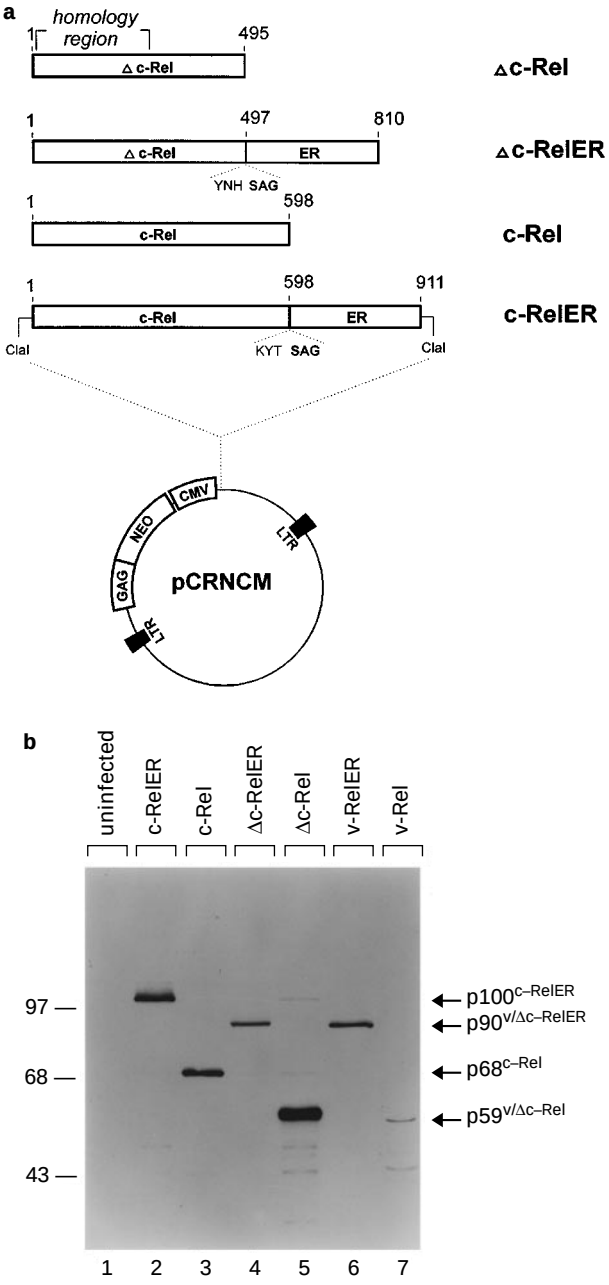

Figure 1 Construction and expression of c-RelER in CEF. (**a**) c-Rel, c-RelER, Δc-Rel, and Δc-RelER were subcloned into the polylinker region of the pCRNCM avian retroviral vector downstream of the CMV promoter. The amino acid sequences of the junctions in c-RelER and c-RelER are shown. Δc-Rel and Δc-RelER lack 103 and 101 amino acid residues of the c-Rel carboxy terminal transactivation domain, respectively. (**b**) Expression of the retroviral constructs in primary avian fibroblasts. CEF transfected with the corresponding retroviral constructs were subjected to Western blot analysis using a Rel-specific antibody as described in Materials and methods. The positions of the Rel proteins are indicated on the right. CEF expressing v-Rel and v-RelER are shown as controls. The position of v-Rel and Δc-Rel is indicated as p59v/Δc-Rel. Similarly, the position of v-RelER and Δc-RelER is indicated as p90 v/Δc-RelER. On the left-positions of relative molecular weight markers are indicated

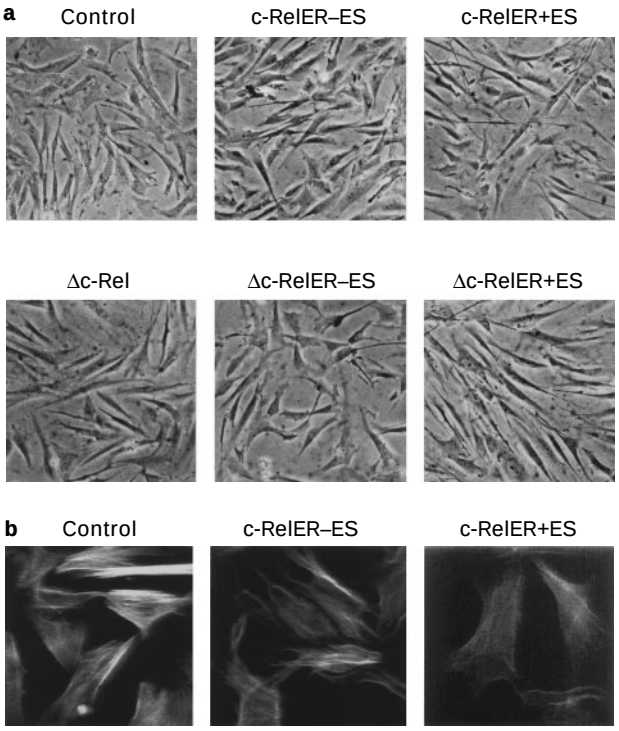

Figure 2 Induction of transformation-specific changes in transfected CEF. (**a**) The morphology of control CEF, Δc-Rel, c-RelER and Δc-RelER CEF is shown. c-RelER and Δc-RelER were grown 72 h in the absence of estrogen (−ES) or in the presence of estrogen (+ES). (**b**) Changes in actin organization in response to hormone activation of c-RelER. Untransfected control CEF and c-RelER CEF grown in the presence or absence of estrogen were incubated with rhodamine-conjugated phalloidin to reveal the actin filaments

Subcellular localization of c-RelER

Immunofluorescent staining of cells using a Rel-specific antibody revealed that in the absence of estrogen, both c-RelER and Δc-RelER were localized in the cytoplasm. Estrogen induced translocation of the chimeric proteins from the cytoplasm to the nucleus (see Figure 3). Similar hormone-dependent properties were previously reported for v-RelER and v-MybER (Burk and Klempnauer, 1991; Boehmelt *et al*., 1992; Capobianco and Gilmore, 1993). The phenotype of c-RelER fibroblasts was also analysed in response to two estrogen analogs, 4-hydroxytamoxifen (OHT) and ICI 164,384 (ICI). OHT and ICI behave as a partial estrogen agonist and antagonist, respectively, for the authentic estrogen receptor (Wakeling and Bowler, 1988). Both OHT and ICI induced a shift of the chimeric proteins to the nucleus, although ICI-mediated translocation was markedly weaker in all cells tested (see Figure 3g). Similar results were obtained when cytoplasmic and nuclear extracts were analysed by Western blotting (data not shown). In contrast to these results, the predominantly cytoplasmic localization of wild-type c-Rel did not change upon addition of estrogen. It was also unresponsive to OHT and ICI. These results indicate that fusion to the estrogen-binding domain may block the cytoplasmic retention signal contained in the carboxy terminal part of c-Rel. Thus, Δc-Rel which lacks this region, is constitutively nuclear (see Figure 3c). Since there is no nuclear localization signal in the hormone-binding domain of the estrogen receptor (Green and Chambon, 1991), perhaps the nuclear localization sequence present in Rel-homology domain is involved in the shift of chimeric proteins to the nucleus (Capobianco and Gilmore, 1993). It is likely that the addition of hormone initiates conformational changes and thus triggers subsequent translocation of both c-RelER and Δc-RelER to the nucleus.

Complexes of c-RelER with other proteins

To determine if c-RelER forms complexes with the same set of proteins as wild-type c-Rel, ^{35}S-labeled lysates prepared from c-RelER fibroblasts were examined by direct immunoprecipitation using two types of antibodies: SB66 Rel-specific antibody, and HC20 antibody directed against the hormone-binding domain of the human estrogen receptor. This analysis revealed that c-RelER forms heterodimeric complexes with endogenous c-Rel (Figure 4). Similar amounts of c-Rel were coprecipitated from cells grown in the presence or absence of estrogen, indicating that dimerization of c-RelER with wild-type c-Rel does not depend on hormone-activation of c-RelER. In addition, a 36 kDa protein, which has been identified as IκB-α in previous works (Tung *et al*., 1988; Davis *et al*., 1991), is specifically complexed with both wild-type c-Rel and c-RelER (Figure 4, lanes 3, 7 and 9). Interestingly, proportional quantities of IκB-α were coprecipitated by anti-Rel sera from the cells over-expressing wild-type c-Rel or estrogen-activated c-RelER. However, the ability of c-RelER to form complexes that include IκB-α was reduced in the absence of estrogen or in the presence of its structural analogs.

c-RelER binds to NF-κB sites in vitro

To examine the DNA binding properties of c-RelER, nuclear and cytoplasmic extracts prepared from c-RelER CEF were analysed by mobility shift assays with a radiolabeled oligonucleotide that included an NF-κB binding site contained in the promoter of the chicken IκB-α gene. This region of the Iκ-α gene exhibits specific binding affinity with c-Rel (Schatzle *et al*., 1995). As can be seen in Figure 5, nuclear extracts prepared from control CEF showed low binding activity (Figure 5a, lane 2; Figure 5b, lane 2). In contrast, multiple protein-DNA complexes were observed in nuclear and cytoplasmic extracts prepared from c-RelER fibroblasts grown in the presence of estrogen (Figure 5a, lanes 4 and 6). The cytoplasmic extracts contained less DNA binding activity (Figure 5a, lanes 5 and 6), and protein-DNA complexes migrated more slowly in the gel, suggesting associations of c-RelER with other proteins present in the cytoplasm. A mutated NF-κB site was included in reactions as a control for the specificity of DNA binding. The specific bands disappeared when the mutated oligonucleotide was used (Figure 5a, lanes 7–10). Similar results were obtained when the DNA binding reactions were preincubated with the Rel-specific antibody. As expected, the intensity of Rel-specific bands was drastically reduced in extracts prepared from c-RelER cells grown in the absence of

estrogen. Similarly, the cytoplasmic and nuclear extracts prepared from cells maintained in OHT or ICI reproducibly showed less activity than the equivalent extracts from estrogen-treated cells.

Transcriptional activity of c-RelER

To test the ability of c-RelER protein to activate gene expression, transient transfection experiments were performed. The CMV-luc reporter plasmid used for this purpose contains four NF-κB-binding sites in its promoter region. The reporter plasmid and Rel constructs subcloned into the RSV-script expression vector (see Materials and methods) were cotransfected into QT6 quail fibroblasts. Following transfection, luciferase activity was measured in cells maintained in the presence or absence of estrogen. As shown in Table 1, the expression of wild-type c-Rel and estrogen-activated c-RelER and Δc-RelER resulted in threefold induction of luciferase activity, whereas expression of Δc-RelER resulted in about sixfold induction. While relatively low levels of induction were probably due to high basal levels of transcription from the reporter construct, OHT and ICI were less potent activators of both c-RelER and Δc-RelER under these experimental conditions.

Effect of c-RelER on expression of cellular genes

We next investigated the effect of c-RelER on transcriptional activation of cellular genes. Three genes were initially examined, those encoding the

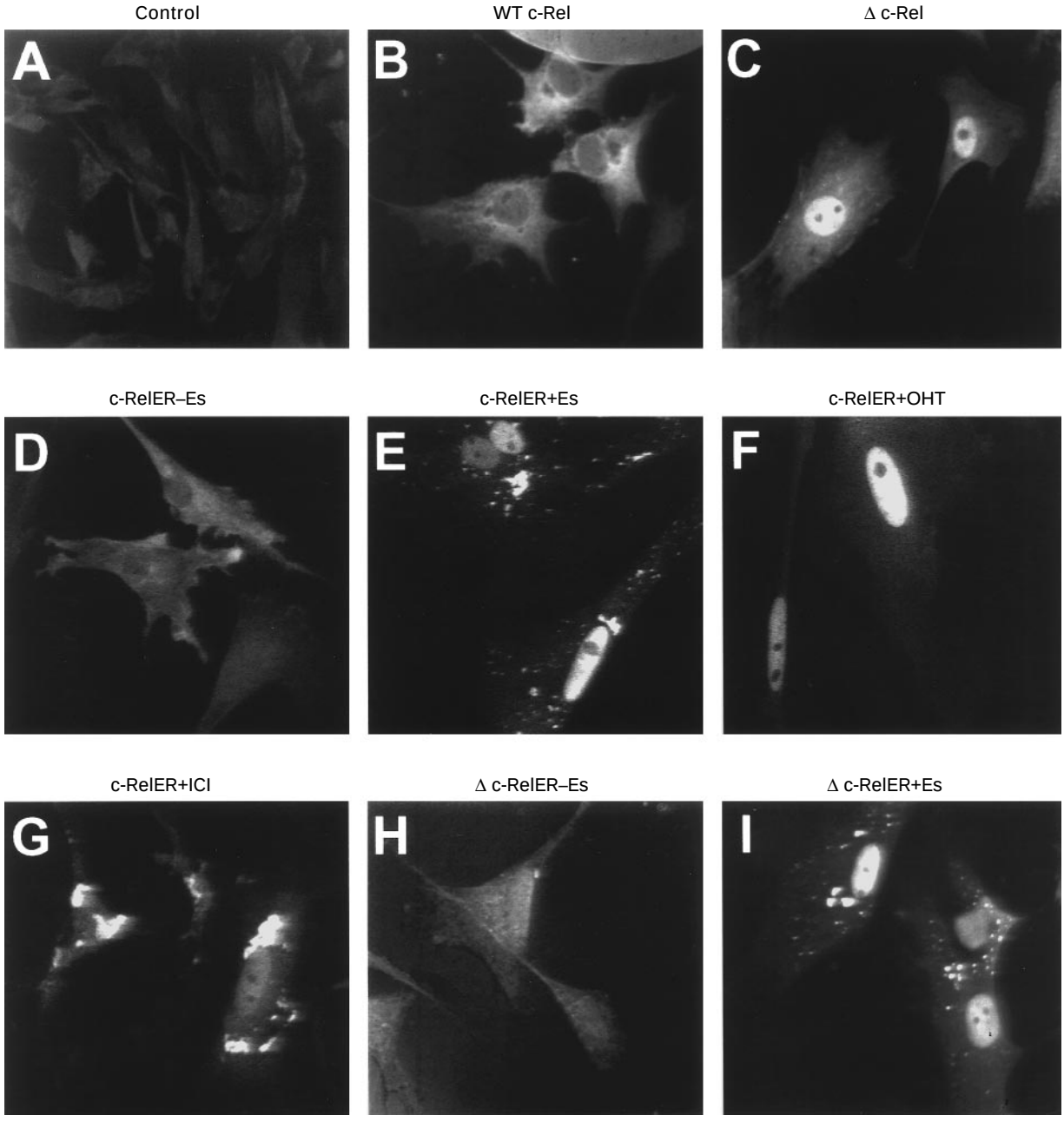

Figure 3 Subcellular localization of Rel proteins in control untransfected CEF (**a**), c-Rel CEF (**b**), Δc-Rel CEF (**c**), c-RelER CEF (**d**-**g**), and Δc-RelER CEF (**h** and **i**). c-RelER CEF were incubated with estrogen, OHT, or ICI (**e**, **f**, **g**, respectively), or without drug (**d**). Δc-RelER CEF were incubated with estrogen (**i**) or without (**h**). Fixed and permeabilized cells were incubated with the SB66 anti-Rel antibody, followed by incubation with fluorescein-conjugated anti-rabbit immunoglobulin

cytoplasmic inhibitor IκB-α, a cell surface antigen Sca-2, and a chicken homologue of the mammalian macrophage inflammatory protein-1 beta (Mip-1β). Previous studies established that these genes are constitutively activated in c-Rel-transformed fibroblasts and v-Rel-transformed hematopoietic cells (Kralova *et al*., 1994; Petrenko *et al*., 1997). This study allowed us to correlate the transformation-specific alterations of c-RelER fibroblasts with the transcriptional activity of the chimeric protein. As can be seen in Figure 6, estrogen-activated c-RelER induced the expression of all tested genes. In contrast, OHT and ICI failed to induce the expression of these genes. By examining several other genes with κB sites in their promoter regions, we found that the gene encoding 9E3/CEF4, an avian homolog of the human interleukin-8, was inducible by estrogen in c-RelER CEF. This induction was also found in c-Rel expressing CEF (lanes 3 and 9), but not in v-Rel-transformed fibroblasts and hematopoietic cells (lane 15 and data not shown). One aspect of the lack of CEF4 activation by v-Rel is that it was present at low levels in control CEF maintained in estrogen (lane 1), and activated to some extent by v-RelER in the presence of estrogen (lanes 15–17). This might suggest that the ER domain confers some type of gene-specific activation to v-Rel and c-Rel. However, examination of c-Rel CEF generated with the use of two different retroviral vectors, pCRNCM and RCAS, showed that they express 9E3/CEF4 irrespective of the presence of estrogen (data not shown). Thus, the expression of 9E3/CEF4 appears to be c-Rel but not v-Rel-specific.

Growth phenotypes of individual CEF clone lineages

Analysis of CEF clones expressing different levels of c-RelER revealed two distinct cell populations that

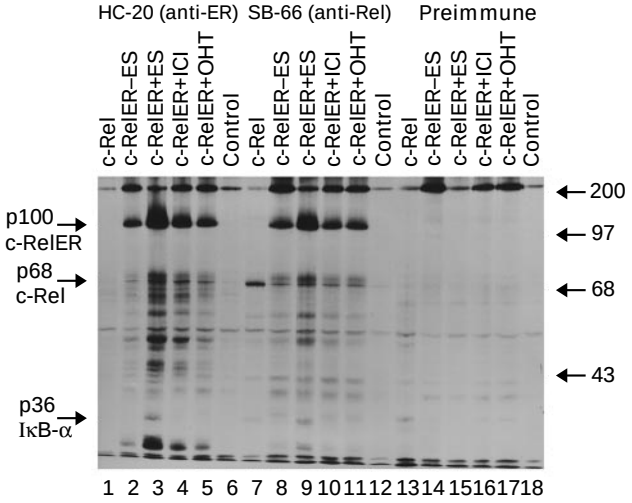

Figure 4 c-RelER forms specific complexes with cellular proteins. Protein extracts prepared from ^{35}S-methionine-labeled c-Rel CEF (lanes 1, 7 and 13), control untransfected CEF (lanes 6, 12 and 18), and c-RelER CEF grown in the presence of estrogen (lanes 3, 9 and 15), ICI (4, 10 and 16), OHT (lanes 5, 11 and 17), or mock treated cells (lanes 2, 8 and 14), were immunoprecipitated with the HC-20 anti-estrogen receptor antibody (lanes 1–6), SB66 anti-Rel antibody (lanes 7–11), or preimmune sera (lanes 13–18). The positions of the proteins which coprecipitate with c-RelER are indicated on the left

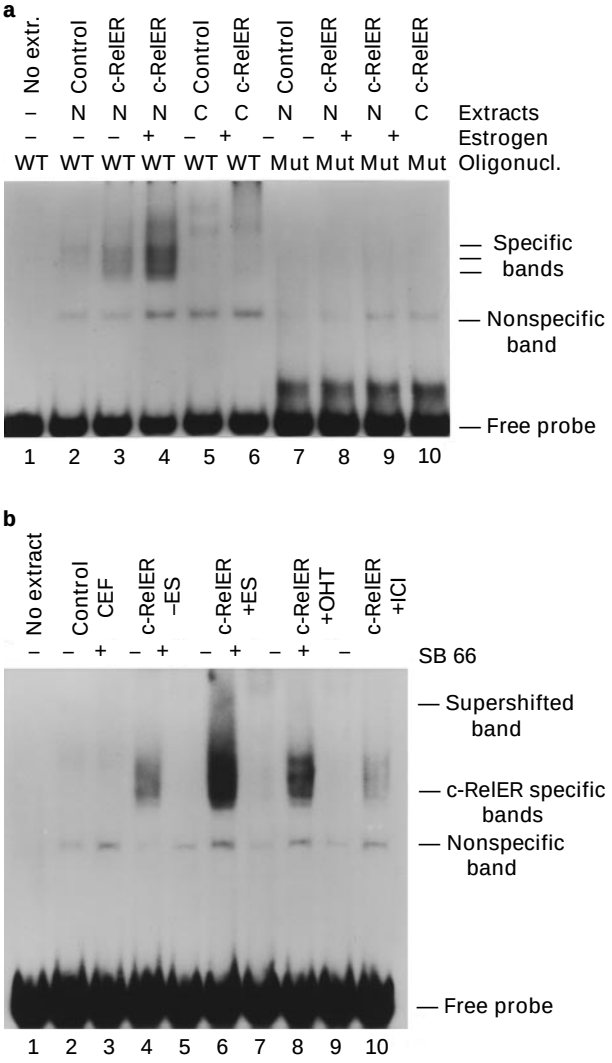

Figure 5 DNA-binding properties of c-RelER. (**a**) Nuclear (N) or cytoplasmic (C) extracts prepared from control untransfected CEF or c-RelER CEF that were treated (+) or untreated (−) with estrogen, were incubated with the oligonucleotide containing a wild-type NF-κB sequence present in the promoter region of the IκB-α gene (lanes 1–6) or a mutated NF-κB sequence (lanes 7–10). Lanes 4, 6, 9 and 10 contain extracts from estrogen treated CEF. (**b**) Nuclear extracts from untransfected CEF (lanes 2 and 3) or c-RelER CEF grown in the absence of estrogen (lanes 4 and 5), or in the presence of estrogen (lanes 6 and 7), ICI (lanes 8 and 9), or OHT (lane 10), were incubated with the oligonucleotide containing a wild-type NF-κB sequence in the presence of anti-Rel antibody (lanes 3, 5, 7, 9), or without antibody added (lanes 1, 2, 4, 6, 8 and 10). Lane 1 contains no extract

Table 1 Transient transactivation of CMV-Luc reporter plasmid

Transfected DNA	*Relative luciferase activity*			
	−Es	*+Es*	*+OHT*	*+ICI*
1. RSV-script	1.0	0.93	0.94	nd
2. RSV-script c-Rel	2.91	2.47	2.58	nd
3. RSV-script c-ReRelER	1.43	3.31	2.38	2.82
4. RSV-script Δc-Rel	5.81	6.36	5.34	5.72
5. RSV-script Δc-RelER	1.48	3.98	3.00	3.38
6. RSV-script v-Rel	5.57	4.98	nd	nd
7. RSV-script v-RelER	2.20	5.52	nd	nd

The CMV-luc reporter plasmid, RSV-script effector constructs, and SV40β-gal DNA were cotransfected into QT6 fibroblasts. Cells were maintained in the presence or absence of estrogen (Es), OHT, or ICI. Transfection efficiencies were normalized according to the levels of β-galactosidase activity. Values correspond to fold activation compared with the luciferase activity seen with empty vector in cells untreated with estrogen; nd, not determined

differed with respect to their growth phenotypes. While the growth of cells that exhibited low to moderate expression high levels of c-RelER resulted in retardation of their growth or complete growth arrest. Such cells were large with the morphology similar to senescing cells. In addition, at least 40% of estrogen treated CEF expressing high levels of c-RelER were binuclear (data not shown).

To determine if overexpression of wild-type c-Rel induced similar response, the growth phenotypes of pCRNCM-c-Rel CEF were analysed in more detail. Protein lysates prepared from RCAS-c-Rel CEF were used as controls for a comparison of c-Rel expression levels in pCRNCM-c-Rel clones. While the majority of pCRNCM-c-Rel CEF expressed c-Rel at levels equivalent or lower to those found in RCAS-c-Rel CEF, a proportion of clones expressed the protein at 2–3-fold higher levels (data not shown). The growth rates of such cells were extremely low compared to clones with lower levels of c-Rel expression (see Table 2). In addition, these cells were characterized by large size, flattened 'nonfusiform' morphology (Figure 7a), and significantly shortened life span. Again, at least 40% of such cells were binuclear (data not shown). These results indicate that high levels of c-Rel expression could interfere with cell cycle progression. To determine if this is the case, pCRNCM-c-Rel CEF were further analysed by flow cytometry. The results obtained with three representative clone lineages are shown in Figure 7b. Typically, the majority of control untransfected CEF contain a 2C DNA content as expected for cells in the G0/G1 phase, while the rest of the cells contain 4C amount of DNA, characteristic of G2/M cells. While CEF expressing low levels of c-Rel revealed a slight increase in cell number with 4C DNA content (Figure 7b), CEF expressing high levels of c-Rel showed a dramatic shift in DNA content distribution with the majority of cells having the 4C DNA content. As can be seen in Figure 7b, such cells were assigned by a curve fitting program to G2/M phase of the cell cycle. The presence of a large proportion of binuclear cells correlated with the number of cells with the 4C DNA and suggested that high expression levels of c-Rel inhibited cytokinesis, rather than mitosis, and that the examined cells were arrested in G1 phase. Interestingly, we did not observe a pre-G1 peak indicative of cells undergoing apoptosis. No cells expressing high c-Rel levels were assigned to S phase confirming that these cells were not replicating their DNA. Thus, the results indicate that the proportion of cells with the 4C DNA content, their morphology, and growth rates correlated with the levels of exogenous c-Rel expression.

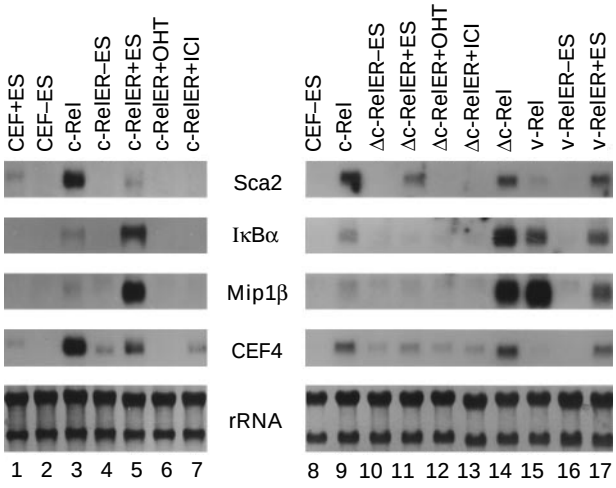

Figure 6 c-RelER and Δc-RelER modulate transcription of cellular genes. Total cellular RNA was prepared from CEF and subjected to Northern blot analysis. Untransfected CEF grown in the presence of estrogen (lane 1) or absence of estrogen (lanes 2 and 8) were used as controls. c-Rel CEF (lanes 3 and 9); c-RelER CEF grown without any drug (lane 4), in the presence of estrogen (lane 5), OHT (lane 6), or ICI (lane 7); Δc-RelER CEF grown without any drug (lane 10), in the presence of estrogen (lane 11), OHT (lane 12), or ICI (lane 13), Δc-Rel CEF (lane 14), v-Rel CEF (lane 15) and v-RelER CEF in the absence (lane 16) and presence (lane 17) of estrogen are shown. Hybridization probes Sca-2, IκB-α, MIP-1β, 9E3/CEF-4 are indicated in the middle. Ribosomal RNAs stained with methylene blue were used to control RNA integrity.

Table 2 Growth properties of CEF expressing exogenous c-Rel

Transfected DNA	*c-Rel expression*	*Cell division time (hrs)*	*Life span (passages)*
1. none	+	20–24	20–25
2. RCAS	+	24–28	20–25
3. pCRNCM	+	24–28	20–25
4. RCASc-Rel	+ +	22–24	40–50
5. pCRNCMc-Rel	+ +	24–28	35–40
6. pCRNCMc-Rel	+ + +	140–160	<15

Proliferation of CEF was measured in DMEM supplemented with 8% fetal calf serum and 2% chick serum. Relative levels of c-Rel expression were estimated by Western blot analyses of protein extracts using a Rel-specific antibody SB66. pCRNCMc-Rel CEF were selected, expressing c-Rel at levels equivalent to those in RCASc-Rel-infected CEF (+ +) or a two- to three-fold higher levels (+ + +)

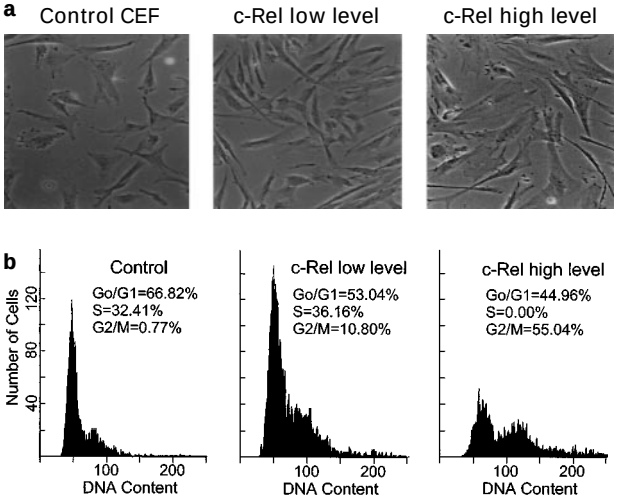

Figure 7 Characterization of CEF overexpressing c-Rel. (**a**) The morphology of control cells transfected with pCRNCM vector; pCRNCM-c-Rel CEF expressing low levels of c-Rel; pCRNCM-c-Rel CEF expressing high levels of c-Rel is shown. (**b**) Flow cytomety analysis of CEF expressing various levels of exogenous c-Rel. Nonsynchronized cells were stained with propidium iodide and DNA content was measured with FACScan. The percentages of cells in each cell cycle phase are indicated.

Discussion

In this report, we characterize the biological and biochemical properties of a conditional protein containing c-Rel fused to the hormone-binding domain of the human estrogen receptor (c-RelER). This chimeric protein causes estrogen-dependent, but otherwise c-Rel-specific, transformation of avian fibroblasts *in vitro*. In order to understand the mechanisms of c-RelER action, different functional properties of the protein were investigated, including subcellular localization, DNA binding, and activation of transcription of cellular genes.

Our analysis revealed that in the absence of estrogen, c-RelER was found predominantly in the cytoplasm, while the hormone and its structural analogs, OHT and ICI, induced a shift of the c-RelER protein to the nucleus. Hormone activation of c-RelER had little effect on its ability to form homo- or heterodimers. Thus, c-RelER coimmunoprecipitated with c-Rel in the presence or absence of estrogen. In contrast, association of c-RelER with IκB-α, the inhibitor molecule, was stronger in the presence of estrogen. Similar amounts of IκB-α were coprecipitated by anti-Rel sera from the cells overexpressing estrogen-activated c-RelER or wild-type c-Rel. Furthermore, c-RelER exhibited the same target sequence specificity as c-Rel. The DNA-binding activity of c-RelER was significantly higher in extracts prepared from cells grown in the presence of estrogen. Although this difference may be ascribed to higher levels of c-RelER in the nuclei of hormone-stimulated cells, the addition of estrogen directly to the DNA-binding reaction resulted in a corresponding increase of binding activity (data not shown). Thus, in the absence of estrogen, c-RelER remains inactive, while in the presence of estrogen, hormone-activated protein forms stable complexes with DNA.

In correlation with these results, transient transactivation of CMV-luc reporter constructs revealed increased luciferase activity in cells transfected with wild-type c-Rel or hormone-activated c-RelER. In addition, at least four cellular genes that are constitutively active in c-Rel-transformed cells, Sca-2, MIP-1β, IκB-α and 9E3/CEF4, were up-regulated in c-RelER fibroblasts in a hormone-responsive fashion. This finding supports the conclusion that c-RelER is a conditional version of c-Rel. We previously reported that three of these genes, Sca-2, MIP-1β, and IκB-α, are also up-regulated in v-Rel-transformed fibroblasts and hematopoietic cells (Petrenko *et al*., 1997). Interestingly, most genes identified so far as v-Rel targets are also up-regulated in cells transformed by c-Rel. However, the kinetics and relative levels of their expression differ in each case. Thus, v-Rel induces higher levels of MHC class I and II, IL-2R, DM-GRASP and p75 in lymphoid cells (Hrdličková *et al*., 1994 1995; Zhang *et al*., 1995; Zhang and Humphries, 1996), while c-Rel induces higher levels of NF-κB1, IκB-α, and c-myb in lymphoid cells and fibroblasts (Hrdličková *et al*., 1995; Petrenko *et al*., 1997). Consistent with these results, v-Rel and Δc-Rel appear to be stronger activators of IκB-α and Mip-1β expression in the transformed fibroblasts, while c-Rel induced higher levels of Sca-2 and 9E3/CEF4.

Using the inducible c-RelER system, proliferation properties of cells were examined that correlated with high levels of c-Rel expression. Previous works have shown that overexpression of c-Rel confers a distinctive phenotype on primary avian fibroblasts, characterized by altered morphology, disruption of cellular cytoskeleton, and a remarkably extended life span (Abbadie *et al*., 1993; Kralova *et al*., 1994). CEF expressing c-RelER normally also displayed altered morphology, decreased amounts of actin bundles, and life span characteristic of c-Rel-transformed CEF. Two distinct cell populations were analysed which differed with respect to their growth phenotypes. While the growth of fibroblasts that exhibited moderate expression levels of c-RelER was stimulated by estrogen, the addition of estrogen to cells with high levels of c-RelER expression resulted in decreased cell growth or ultimate growth arrest. Such cells adopted large, flattened morphology typical of senescing cells. In addition, about 40% of them were binuclear and had no F-actin detectable. The carboxy terminal transactivation domain of c-Rel was required for the induction of these effects. Thus, neither Δc-Rel nor v-Rel were able to inhibit cell growth and induce similar morphological changes in fibroblasts. In contrast, high levels of c-Rel expression reproducibly resulted in the generation of cells with flattened morphology and dramatically decreased growth rates. Cell division did not occur and a large proportion of c-Rel-expressing cells were binuclear and had a 4C DNA content. Our preliminary analysis did not reveal the characteristic apoptotic DNA ladder typical of cells undergoing programmed cell death. Accumulation of these cells in G1 phase and an extensive breakdown of cellular cytoskeleton rather suggested that overexpression of c-Rel affected cell cycle control and/or cytokinesis. Our results are in agreement with previous studies which showed that high levels of c-Rel expression can affect cell cycle in bone marrow and HeLa cells (Abbadie *et al*., 1993; Baeuerle and Baltimore, 1996), or have a cytotoxic/cytostatic effect in transformed B-cell lines (Nehyba *et al*., 1994). Thus, these results suggest a critical role for c-Rel not only in the control of cell proliferation but also in the induction of cell death. Furthermore, indicated by these findings the biological properties of c-Rel in cell growth and differentiation potentially differ depending on the level of expression. Utilization of the conditional c-Rel variant described here may help in understanding the changes that accompany altered Rel expression in different cell types.

Materials and methods

Plasmids and constructs

Construction of c-RelER The chicken c-*rel* cDNA (amino acid residues 1–598, Capobianco *et al*., 1990), and the hormone-binding domain of the human estrogen receptor (ER, amino acid residues 282–595) contained in the *Bam*HI–*Sac*I fragment of HE14 (Kumar *et al*., 1986), were subcloned in-frame into the pBluescript SK$^+$ vector (Stratagene). The stop codon in c-*rel* and an internal *Cla*I-site of the chimeric construct were subsequently removed by site-directed mutagenesis without changing the amino acid coding to generate c-*rel*ER. The construct was

completely sequenced to ensure that no unexpected mutations were introduced. A truncated version of c-*rel*, Δc-*rel*, was generated by deleting 103 carboxy-terminal codons from the c-*rel* cDNA. Δc*rel*ER contains the ER domain of the human estrogen receptor fused to Δc-*Rel* (which has a deletion of 101 carboxy-terminal amino acids from wild-type c-Rel). v-Rel and v-RelER constructs were described earlier (Morrison *et al*., 1991, Boehmelt *et al*., 1992). All constructs were subcloned into the *Cla*I site of the pCRNCM-neo replication-defective avian retroviral vector (Metz *et al*., 1991), downstream of the immediate early CMV promoter. In addition RCAS-c-Rel construct (Abbadie *et al*., 1993) containing full-length c-Rel sequence subcloned into pRCAS (Hughes *et al*., 1987) was used as a control.

Reporter plasmids The CMV-luc reporter plasmid was constructed by subcloning of the CMV promoter region containing four putative NF-κB binding sites (nucleotides −671 to +77, Boshardt *et al*., 1985) derived from pCRNCM-neo into the *Bgl*II site of pGL2 basic vector (Promega). The SV40-gal plasmid was kindly provided by Dr D Bar-Sagi, Stony Brook, NY. To generate the RSV-script expression vector, the *Sal*I fragment of pREP7 (Invitrogen), containing the RSV promoter and the SV40 polyadenylation signal, was blunt-ended and subcloned into the *Kpn*I/*Sac*I-digested and blunt-ended pBluescript SK^+ DNA. *Cla*I restriction site was subsequently inserted into RSV-script by replacing the original 17 bp *Hind*III–*Xho*I fragment of pRep7 polylinker with 21 bp *Hind*III–*Xho*I fragment from pBluescript SK^+ polylinker. The Rel cDNAs were subcloned into *Cla*I site of the RSV-script vector using conventional techniques.

Cell culture and DNA transfection

Cells were maintained in DMEM supplemented with 8% fetal calf serum and 2% chicken serum. Estrogen (17-β-estradiol, Sigma) and ICI 146,384 (ICI Pharmaceuticals) were used at a final concentration 10^{-6} M. 4-hydroxy-tamoxifen (OHT, Aldrich) was used at a concentration 2×10^{-7} M. Chicken embryo fibroblasts (CEF) were prepared from 6- to 7-day-old embryos as described (Vogt, 1969). QT6 is a transformed quail fibroblast cell line (Moscovici *et al*., 1977). Avian fibroblasts expressing recombinant viruses were prepared using calcium-phosphate/DNA transfection protocol (Chen and Okayama, 1987). Transfected cells were selected in G418 (200 μg/ml), and characterized for Rel expression levels by Western blot analysis. Individual CEF clones were further used for the characterization of growth phenotypes. For other purposes, typically up to five clones were pooled and maintained in G418 (40 μg/ml).

Growth rate analysis and flow cytometry

Proliferation of fibroblasts was measured by direct counting of trypsinized cells using a Coulter counter. All values represent an average of three experiments. Standard deviation of estimates did not exceed 10%. For flow cytometry analysis, CEF were grown to 70% confluence. 5×10^5 nonsynchronized cells were collected, fixed in 70% ethanol, incubated with RNase A, and stained with propidium iodide as described (Vindelov *et al*., 1983). Fluorescence of nuclei was measured in FACScan (Becton Dickinson) and analysed with the MacCycle curve fitting program for DNA content and cell cycle analysis (Phoenix Flow Systems, Inc.).

Transient transfection assay

Semiconfluent QT6 cells were transfected with plasmid DNAs in 60 mm plates using the calcium phosphate transfection protocol. 5×10^5 cells were co-transfected with 2.5 μg of RSV-script-Rel together with 0.8 μg of CMV-Luc reporter plasmid and 1.5 μg of SV40 β-gal plasmid was used as an internal control. pBluescript plasmid DNA was added to each reaction to bring the total amount of DNA to 7 μg. The cells were incubated in contact with the calcium phosphate precipitate for 16 h, followed by a complete medium change. Three hours later, cells were trypsinized, split into three 60 mm plates, and incubated in the presence or absence of hormone. Forty-eight hours post-transfection cells were harvested, and luciferase activity was determined following the manufacturer's instructions (Promega). The values obtained were normalized with respect to the levels of β-galactosidase activity.

Immunoblot analysis

Protein lysates were prepared and analysed by Western blot analysis as previously described (Morrison *et al*., 1989). The following two antibodies were used, SB66, a rabbit polyclonal antibody raised against bacterially-expressed trpE-v-Rel fusion protein, is also able to recognize c-Rel (Schwarz and Witte, 1988; Morrison *et al*., 1989); HC-20, a rabbit polyclonal antibody raised against the hormone-binding domain of the human estrogen receptor (Santa Cruz Biotechnology).

Immunoprecipitations

CEF were preincubated in methionine- and cysteine-free medium and labeled with ^{35}S-trans Label (Amersham) for 2 h. Cells were lysed in 0.5% NP40, 0.5% deoxycholate, 20 mM Tris-HCl, pH 8.0, 50 mM NaCl and subjected to immunoprecipitation analysis using the Rel-specific SB66 antibody, and HC-20, a rabbit polyclonal antibody raised against the hormone-binding domain of the human estrogen receptor (Santa Cruz Biotechnology), as previously described (Morrison *et al*., 1989). Immunoprecipitates were analysed by SDS–PAGE.

Immunofluorescent staining

Fibroblasts were grown on coverslips, washed in PBS, and fixed in 3% paraformaldehyde for 15 min. Cells were subsequently permeabilized by incubation in 0.5% NP40 in phosphate-buffered saline (PBS) for 15 min and washed three times with 0.5% bovine serum albumin (BSA) in PBS. For immunofluorescence staining, cells were incubated with the SB66 antibody (diluted 1:20), for 1 h at 37°C. Cells were washed three times with 0.5% BSA in PBS and incubated with a 1:200 dilution of FITC-conjugated goat anti-rabbit IgG for 45 min at 37°C. Following extensive washing in PBS, cells were mounted in Gel/mount (Biomeda corp.). To stain actin cables, cells were incubated with rhodamine-conjugated phalloidin following the instructions of the manufacturer (Molecular Probes).

Gel-mobility shift assays

Nuclear or cytoplasmic extracts from c-Rel or c-RelER cells grown in the presence or absence of hormone were prepared as described by Dignam *et al*. (1983). Oligonucleotides contained the sequence present in the promoter region of the chicken IκB-α gene (Schatzle *et al*., 1995): an NF-κB-2 wild-type sequence, 5′-TGCAGTTAATGG-GAATCCCCCGCAT-3′, and a mutated NF-κB-2 sequence, 5′-TGCAGTTAATGAAGATCCCCCGCAT-3′. The double-stranded oligonucleotides were labeled using Klenow DNA polymerase and α^{32}P-dATP. 8 μg of the corresponding nuclear or cytoplasmic extracts were

incubated 15 min at room temperature with 10 000 c.p.m. of ^{32}P-labeled oligonucleotide in a final reaction volume of 30 μl. Incubation buffer contained 25 mM HEPES, pH 7.5, 1 mM DTT, 1 mM EDTA, 15% glycerol and 1 μg of poly [dA-dT].

For supershift analysis, the extracts were preincubated with the SB66 antibody for 15 min on ice prior to the addition of the labeled oligonucleotide. Reactions were then analysed by electrophoresis in 5% native polyacrylamide gels as described (Kabrun *et al*., 1991).

RNA isolation and Northern analysis

The CEF from 3–5 pooled clone lineages were grown 30–35 days after transfection in the culture and used for total RNA isolation by the lithium chloride-urea procedure of Auffray and Rougeon (1980). RNA aliquots (7 μg) were separated by formaldehyde-agarose gel electrophoresis and transferred to Hybond-N nylon membranes (Amersham). The membranes were stained in 1% methylene blue to verify the integrity of rRNA bands. Hybridization and washing conditions were as described in Church and Gilbert (1984). Hybridization probes were prepared by random priming of gel-purified c-DNA inserts. The cDNAs encoding chicken MIP-1β and Sca-2 were isolated in this laboratory (Petrenko *et al*., 1995, Petrenko *et al*., 1997). 1 kb 9E3/CEF-4 cDNA was kindly provided by Prof H Hanafusa and described in Sugano *et al*. (1987). Chicken IκB-α cDNA was kindly provided by Dr IM Verma.

Note added in proof

Recent studies on HeLa cells demonstrated that the expression of c-Rel leads to growth arrest at the G1/S-phase transition. See Bash *et al*. (1997). *Mol. Cell. Biol*. **17**, 6526–6536

Acknowledgements

We would like to thank Dr MJ Hayman for critical reading of the manuscript, Dr TD Gilmore for valuable discussion and advice. We thank Drs A Leutz, E Wimmer, D Bar-Sagi, H Hanafusa and IM Verma for recombinant plasmids. We thank David Colflesh from UMIC, University Medical Center Stony Brook for his help with Confocal Microscopy. We would also like to thank Kathlene Donnelly and Linda Whittaker for technical support. Part of this work was supported by the Grant from the Council for Tobacco Research #3196 (PJE). MZ was supported by a Fellowship from Fogarty Foundation (1FO TW04933–01).

References

Abbadie C, Kabrun N, Bouali F, Šmardová J, Stéhelin D, Vandenbunder B and Enrietto PJ.(1993). *Cell,* **75,** 899–912.

Auffray C and Rougeon O. (1980). *Eur. J. Biochem.,* **107,** 303–314.

Baeuerle PA and Baltimore D. (1996). *Cell,* **87,** 13–20.

Baeuerle PA and Henkel T. (1994). *Annu. Rev. Immunol.,* **12,** 141–179.

Beg AA, Finco TS, Nantermet PV and Baldwin AS. (1993). *Mol. Cell. Biol.,* **13,** 3301–3310.

Boehmelt G, Walker A, Kabrun N, Mellitzer G, Beug H, Zenke M and Enrietto PJ. (1992). *EMBO J.,* **11,** 4641–4652.

Boshardt M, Weber F, Jahn G, Dorsch-Hassler K, Flechenstein B and Schaffner W. (1985). *Cell,* **41,** 521–530.

Brown K, Park S, Kanno T, Franzoso G and Siebenlist U. (1993). *Proc. Natl Acad. Sci. USA,* **90,** 2532–2536.

Burk O and Klempnauer KH. (1991). *EMBO J.,* **10,** 3713–3719.

Capobianco AJ, Simmons DL and Gilmore TD. (1990). *Oncogene,* **5,** 257–265.

Capobianco AJ and Gilmore TD. (1993). *Virology,* **193,** 160–170.

Chen C and Okayama H. (1987). *Mol. Cell. Biol.,* **7,** 2745–2752.

Church GM and Gilbert W. (1984). *Proc. Natl Acad. Sci. USA,* **81,** 1991–1995.

Davis N, Ghosh S, Simmons DL, Tempst P, Liou HC, Baltimore D and Bose HR. (1991). *Science,* **253,** 1268–1271.

Dignam JD, Lebowitz RM and Roeder RG. (1983). *Nucleic Acids Res.,* **11,** 1475–1489.

Gerondakis S, Strasser A, Metcalf D, Grigoriadis G, Scheerlinck JY and Grumont RJ. (1996). *Proc. Natl. Acad. Sci. USA,* **93,** 3405–3409.

Gilmore TD, Koedood M, Piffat KA and White DW. (1996). *Oncogene,* **13,** 1367–1378.

Ghosh S and Baltimore D. (1990). *Nature,* **344,** 678–682.

Green S and Chambon P. (1991). In *The Oestrogen Receptor: From Perception to Mechanism*. Parker MG. (ed.) Academic Press: London. pp. 15–38.

Henkel T, Machleidt T, Alkalay I, Kronke M, Ben-Neriah Y and Baeuerle PA. (1993). *Nature,* **365,** 182–185.

Hrdličková R, Nehyba J and Humpries EH. (1994). *J. Virol.,* **68,** 2371–2382.

Hrdličková R., Nehyba J, Roy A, Humpries EH. and Bose HR. (1995). *J. Virol.,* **69,** 403–413.

Hughes SH, Greenhouse JJ, Petropoulos CJ and Sutrave P. (1987). *J. Virol.,* **61,** 3004–3012.

Kontgen F, Grumont RJ, Strasser A, Metcalf D, Li R, Tarlinton D and Gerondakis S. (1995). *Genes Dev.,* **9,** 1965–1977.

Kralova J, Schatzle JD, Bargmann W and Bose HR. (1994). *J. Virol.,* **68,** 2073–2083.

Kabrun N, Hodgson JW, Doemer M, Mak G, Franza BR and Enrietto PJ. (1991). *Proc. Natl Acad. Sci. USA,* **88,** 1783–1787.

Kumar V, Green S, Staub A and Chambon P. (1986). *EMBO J.,* **5,** 2331–2236.

Metz T, Graf T and Leutz A. (1991). *EMBO J.,* **10,** 837–844.

Moscovici C, Moscovici MG, Jimenez H, Lai MMC, Hayman MJ. and Vogt PK. (1977). *Cell,* **11,** 95–103.

Morrison LE, Kabrun N, Mudri S, Hayman MJ. and Enrietto PJ. (1989). *Oncogene,* **4,** 667–683.

Morrison LE, Boehmelt G, Beug H and Enrietto PJ. (1991). *Oncogene,* **6,** 1657–1666.

Nehyba J, Hrdličková R and Humpries EH. (1994). *J. Virol.,* **68,** 2039–2050.

Petrenko O, Ischenko I and Enrietto PJ. (1995). *Gene,* **160,** 305–306.

Petrenko O, Ischenko I. and Enrietto PJ. (1997). *Oncogene,* **15,** 1671–1680.

Schatzle JD, Kralova J and Bose HR. (1995). *J. Virol.,* **69,** 5383–5390.

Schwartz RC and Witte ON. (1988). *Virology,* **165,** 182–190.

Siebenlist U, Franzoso G and Brown K. (1994). *Annu. Rev. Cell Biol.,* **10,** 405–455.

Sugano S, Stoeckle MY and Hanafusa H. (1987). *Cell,* **49,** 321–328.

Tung HY, Bargmann WJ, Lim MY and Bose HR. (1988). *Proc. Natl Acad. Sci. USA,* **85,** 2479–2483.

Vindelov LL, Christensen IJ and Nissen NI. (1983). *Cytometry,* **3,** 323–327.

Vogt PK. (1969). In: *Fundamental Techniques in Virology,* Habel K and Salzman NP. (eds.) Academic Press: New York pp. 198–211.

Wakeling AE and Bowler J. (1988). *J. Steroid Biochem.,* **31,** 645–653.

Zhang G and Humphries EH. (1996). *Oncogene* **12,** 1153–1157.

Zhang G, Slaughter C and Humphries EH. (1995). *Mol. Cell. Biol.,* **15,** 1806–1816.