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Hormone-regulated v-*rel* estrogen receptor fusion protein: reversible induction of cell transformation and cellular gene expression

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We describe the construction of a v-*rel* estrogen receptor fusion protein (v-*rel*ER) which allows the regulation of v-*rel* oncoprotein activity by hormone. In the presence of estrogen, v-*rel*ER readily transformed primary chicken fibroblasts and bone marrow cells *in vitro*. In both cell types, v-*rel*-specific transformation was critically dependent on the presence of estrogen or the estrogen agonist 4-hydroxytamoxifen (OHT). Withdrawal of estrogen or application of an estrogen antagonist, ICI164,384 (ICI) caused a reversal of the transformed phenotype. We also demonstrate that the v-*rel*ER protein binds to NF-κB sites in an estrogen-dependent manner, thereby showing that sequence-specific DNA binding of v-*rel*ER is critical for the activation of its transforming capacity. In transient transfection experiments, we failed to demonstrate a clear repressor or activator function of the v-*rel* moiety in v-*rel*ER. However, in v-*rel*ER-transformed bone marrow cells, estrogen and OHT induced elevated mRNA levels of two cellular genes whose expression is constitutive and high in v-*rel*-transformed cells. These results suggest that v-*rel* might exert part of its activity as an activator of *rel*-responsive genes.

Key words: estrogen receptor/NF-κB/oncogene/transformation/v-*rel*

Introduction

The v-*rel* oncogene was first identified as the transforming component of the avian retrovirus REV-T (for review see Rice and Gilden, 1988; Gilmore, 1991). The gene encodes a protein of 59 000 Daltons which localizes in both the cytoplasm and the nucleus and forms complexes with several other cellular proteins including $p68^{c\text{-}rel}$ (Morrison *et al.*, 1989; Gilmore, 1991 and references therein). The function of v-*rel* was unknown until the demonstration that it was homologous in part to members of the transcription factor family NF-κB (for reviews see Gilmore, 1990, 1991; Baeuerle, 1991; Blank *et al.*, 1992). This growing family of proteins includes the p50, p49 and p65 subunits of NF-κB, the *Drosophila* morphogen dorsal, and the *Lyt-10* gene product (Neri *et al.*, 1991). Each of these proteins has been shown to bind DNA and appears to be involved in positive and negative regulation of gene expression (for reviews see Baeuerle, 1991; Gilmore, 1991; Blank *et al.*, 1992).

While a variant of the p65 subunit of NF-κB has been shown to transform rat embryo fibroblasts and induce tumors in athymic nude mice when overexpressed (Narayanan *et al.*, 1992), v-*rel* remains the sole naturally occurring oncogenic member of this family. We and others have shown previously that v-*rel* induces a set of characteristic transformation-specific changes in chicken embryo fibroblasts (CEFs; Morrison *et al.*, 1991 and references therein). In addition, we have determined that the hematopoietic target cell transformed by v-*rel* exhibits properties of an early progenitor cell that expresses markers of the myeloid, lymphoid and erythroid lineages (Morrison *et al.*, 1991).

The mechanism by which v-*rel* brings about oncogenic transformation remains unclear, but was suggested to be the result of aberrant gene expression induced by v-*rel*. It has been suggested that v-*rel* transforms by inhibiting the normal activity of c-*rel* (or of other members of the NF-κB/*rel*/dorsal transcription factor family) in a dominant negative fashion (reviewed in Gilmore, 1991 and Forrest and Curran, 1992). Additionally, several groups have demonstrated that v-*rel* represses gene expression in transient transactivation assays (Ballard *et al.*, 1990; Inoue *et al.*, 1991; Richardson and Gilmore, 1991; McDonnell *et al.*, 1992) leading again to the hypothesis that v-*rel* represses the transcription of genes involved in the regulation of growth control. However, these genes have yet to be identified.

We have chosen to approach the identification of *rel*-regulated genes by constructing an inducible form of v-*rel* whose activity can be modulated by hormone. In this approach, first described by Picard *et al.* (1988), the human estrogen receptor hormone binding domain (ER) is fused in frame to the protein of interest, rendering that protein's activity hormone-inducible. Several proteins, including the *myc*, *fos* and *myb* oncogene products (Eilers *et al.*, 1989; Superti-Furga *et al.*, 1991; Burk and Klempnauer, 1991) have been fused to this ER domain and shown to function in a hormone-inducible fashion. Thus, this approach should allow one to control and examine the biological and biochemical activities of the v-*rel* oncoprotein and in addition to search for *rel*-regulated genes.

In this report, we describe the construction and characterization of a v-*rel*ER fusion protein. In the presence of estrogen, v-*rel*ER transforms both avian fibroblasts and primary bone marrow cells, while in the absence of hormone v-*rel*ER is nontransforming in both cell types. Estrogen also induces v-*rel*ER to bind to DNA in a sequence-specific fashion, thereby correlating DNA binding with the activation of its transforming potential. Finally, we have identified two cellular genes whose transcription is upregulated in a hormone dependent fashion. NF-κB/*rel* sites present in the promoter regions of these two genes are bound by v-*rel*ER in a hormone-dependent manner in electrophoretic mobility shift assays. It is therefore possible that v-*rel*ER (and perhaps also v-*rel*) may exert its specific effects on gene expression

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and transformation at least in part as a transcriptional activator rather than as a repressor of transcription.

Results

Fusion of v-rel to the estrogen receptor hormone binding domain generates a hormone-dependent v-rel

To generate a conditional v-*rel* oncoprotein, a chimeric v-*rel*ER gene was constructed by fusion of v-*rel* to the hormone binding domain of the human estrogen receptor (ER). This v-*rel*ER fusion was cloned into the replication competent avian retrovirus vector RCAS (Hughes *et al.*, 1987), thereby generating RCASv-*rel*ER (Figure 1A).

Hormone-dependent transformation of chicken embryo fibroblasts (CEFs). To demonstrate the integrity of the retroviral construct, RCASv-*rel*ER DNA was transfected into CEFs which were then assayed by Western blotting using a polyclonal antibody directed against v-*rel* (Morrison *et al.*, 1989). Figure 1B shows that these RCASv-*rel*ER-transfected fibroblasts (in the following referred to as v-*rel*ER fibroblasts) did indeed express the v-*rel*ER fusion protein of the predicted size (90 kDa; Figure 1B). As expected, fibroblasts transfected with the identical retroviral vector containing v-*rel*ER in an antisense orientation did not express $p90^{v\text{-}relER}$ (data not shown). Figure 1B also shows that $p90^{v\text{-}relER}$ is expressed at higher levels than the endogenous $p68^{c\text{-}rel}$ and comparable to $p59^{v\text{-}rel}$ in v-*rel*-transformed control fibroblasts.

In order to determine if v-*rel*ER induced the characteristic phenotype associated with v-*rel* expression in fibroblasts (Morrison *et al.*, 1991), v-*rel*ER fibroblasts were grown in the presence or absence of estrogen. While v-*rel*ER fibroblasts grown in the presence of hormone exhibited the transformed morphology characteristic of v-*rel*-transformed CEFs, they were phenotypically very similar to normal CEFs in the absence of hormone (Figure 2A). In addition, following administration of hormone, untreated v-*rel*ER fibroblasts acquired the characteristic morphology of v-*rel*-transformed cells within 2 days. This v-*rel* specific phenotype could be 'switched' on and off upon addition or withdrawal of estrogen, respectively (data not shown).

In addition, the phenotype of fibroblasts expressing v-*rel*ER was analyzed in response to the 'anti-estrogens' 4-hydroxytamoxifen (OHT) and ICI164,384 (ICI). It has been demonstrated previously that these compounds behave as an estrogen agonist and an antagonist, respectively, for the estrogen receptor (Jordan *et al*, 1984; Wakeling and Bowler, 1988a,b; Berry *et al.*, 1990; Green and Chambon, 1991; Pham *et al.*, 1991; Dauvois *et al.*, 1992). OHT is believed to promote high affinity DNA binding of the receptor but does not induce the transcriptional activation function TAF-2 contained in the estrogen receptor hormone binding domain (see Discussion). ICI interferes with receptor dimerization and acts as a pure antagonist (Dauvois *et al.*, 1992).

Therefore, fibroblasts expressing v-*rel*ER (initially grown in the absence of estrogen) were treated with estrogen, OHT or ICI at concentrations from 10^{-9} M to 10^{-6} M. As shown in Figure 2A, both estrogen and OHT induced transformation-specific changes in the morphology of v-*rel*ER fibroblasts at concentrations higher than 10^{-8} M, whereas ICI treated cells exhibited a normal morphology at all concentrations tested (Figure 2A, and data not shown). At a concentration of 10^{-6} M, OHT was cytotoxic (data not shown; see also below). As expected, neither estrogen, OHT nor ICI affected the morphology of v-*rel*-transformed CEFs.

The morphological alterations of v-*rel*ER fibroblasts in response to estrogen or OHT also correlated with specific changes in two parameters of fibroblast transformation, namely actin bundle breakdown and reduction of fibronectin network expression (Royer-Pokora *et al.*, 1978; Morrison *et al.*, 1991). As shown in Figure 2B, indirect double

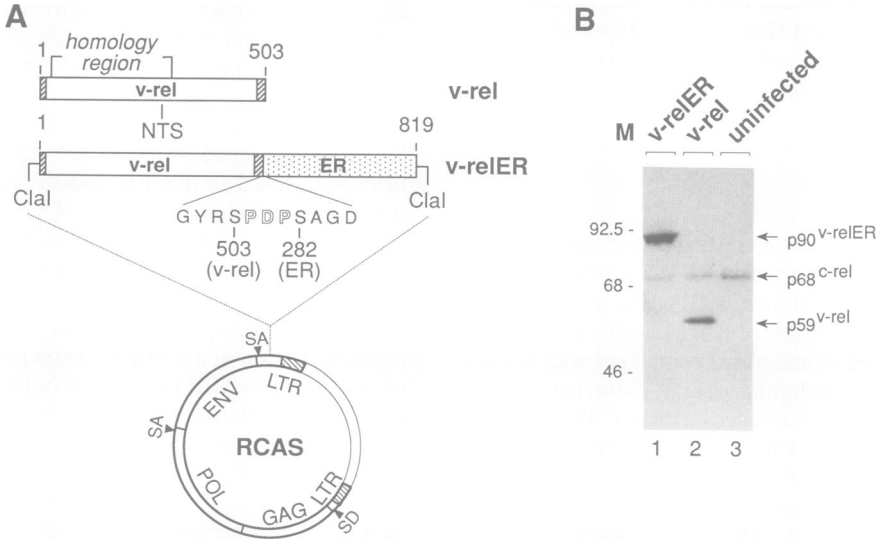

Fig. 1. Construction of a hormone-dependent v-*rel*ER fusion gene. (**A**) A fusion of v-*rel* with the hormone binding domain (ER) of the human estrogen receptor was cloned into the *Cla*I site of the recombinant avian retrovirus vector RCAS (see Materials and methods). The amino acid sequence of the v-*rel*ER junction is shown. The retroviral long terminal repeat (LTR) sequences, splice donor and acceptor sites (SD and SA, respectively), the nuclear translocation signal (NTS) and the region of homology to other members of the NF-κB/*rel*/dorsal family (homology region) are indicated. (**B**) Expression of $p90^{v\text{-}relER}$ in CEFs. RCASv-*rel*ER-transformed CEFs were lysed and subjected to Western blot analysis using a *rel*-specific antibody (SB66; see Materials and methods). V-*rel*-transformed and uninfected CEFs were used as controls (lanes 2 and 3, respectively). The positions of $p59^{v\text{-}rel}$, $p68^{c\text{-}rel}$ and $p90^{v\text{-}relER}$ and of the molecular weight markers (M) are indicated.

immunofluorescence for actin and fibronectin revealed the breakdown of actin bundles in the presence of both estrogen and OHT. ICI treated fibroblasts, however, formed stronger and more distinct actin bundles. Similarly, the extracellular fibronectin network partially disappeared in the presence of estrogen and OHT, but not upon ICI treatment (Figure 2B).

Thus, expression of these characteristic transformation parameters correlates well with the morphological observations described above, supporting the notion that transformation of v-*rel*ER fibroblasts was hormone-inducible. The observation that OHT induced the same v-*rel*-specific phenotype as estrogen, while ICI did not, indicates that transformation does not involve the ER domain of the v-*rel*ER fusion protein (see Discussion).

V-relER transforms primary chicken bone marrow cells in vitro in a hormone-dependent manner. To investigate whether v-*rel*ER would also transform chicken bone marrow cells in a hormone-dependent fashion, the v-*rel*ER-transformed fibroblasts described above were used as a source of virus for infection of primary chicken bone marrow cells (see Materials and methods). V-*rel*-transformed bone marrow cells generated similarly were used as a positive control. After infection, the cultures were split and kept either in the absence or presence of estrogen.

After ~12–16 days an outgrowth of v-*rel*ER-transformed cells was readily obtained only in the presence of estrogen, whereas the untreated culture underwent crisis, accompanied by a progressive loss of dividing cells and cell death. In contrast, outgrowth of transformed cells from the v-*rel* cultures was unaffected by application of hormone (Figure 3A), consistent with the observation that endogenous estrogen receptor is undetectable by Northern blot hybridization in v-*rel*ER- and v-*rel*-transformed bone marrow cells (data not shown).

We next assayed the ability of v-*rel*ER-transformed bone marrow cells to form colonies in semisolid media. Cells from v-*rel*ER-transformed cultures were seeded into methocel in either the absence or presence of estrogen, or the presence of the estrogen antagonist ICI. V-*rel*-transformed cells derived from the same bone marrow preparation were used as a control. The appearance of transformed colonies was scored 5 days after seeding. V-*rel*ER-transformed cells grew into macroscopic colonies in the presence of estrogen, but failed to do so in the absence of hormone or in the presence of ICI (Figure 3B). V-*rel*-transformed cells formed similar

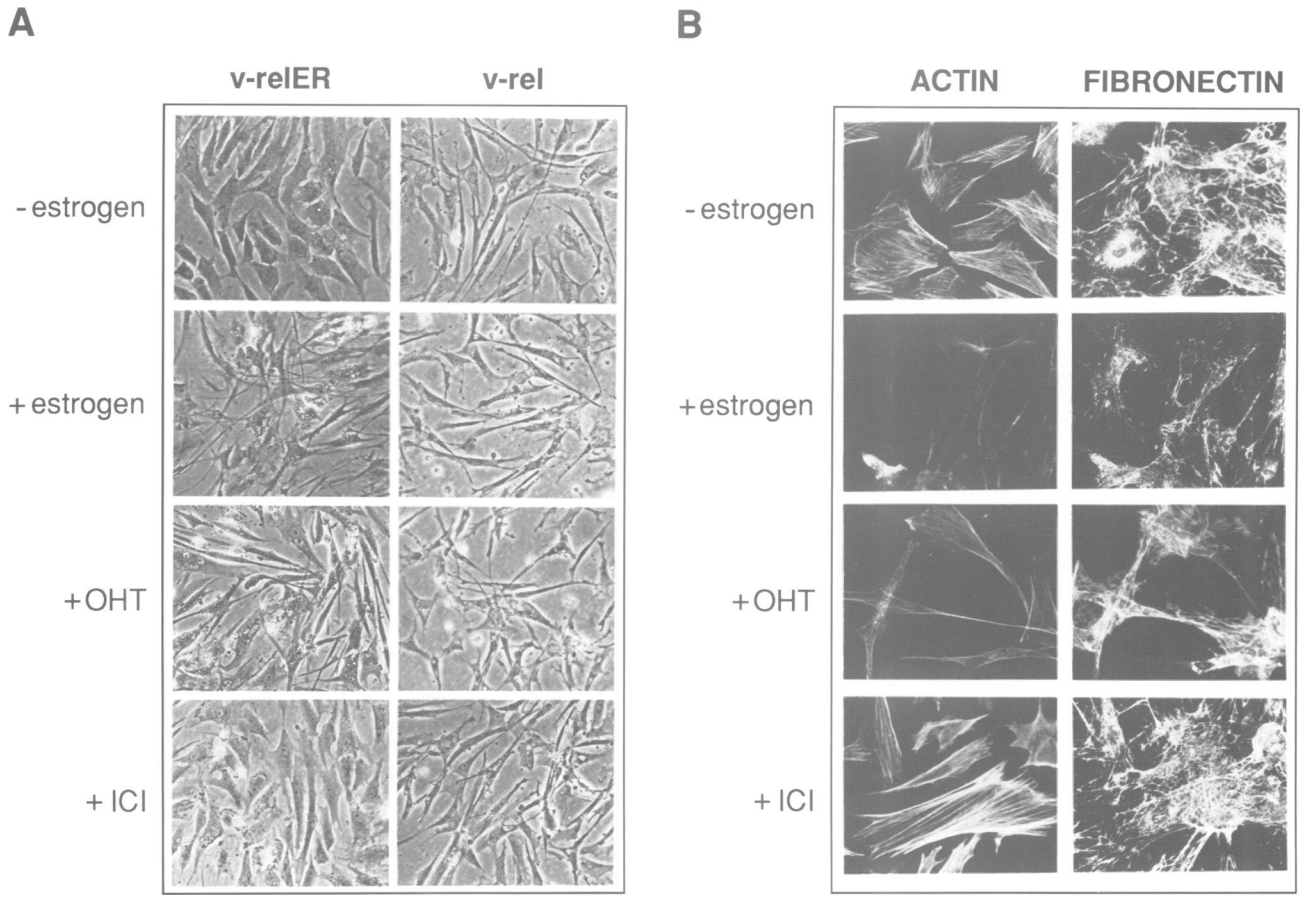

Fig. 2. Estrogen and OHT, but not ICI induce transformation-specific changes in fibroblasts expressing v-*rel*ER. (**A**) The morphology of v-*rel*ER CEFs grown for 3 days in the presence of 10^{-6} M estrogen (+estrogen), 10^{-7} M OHT (+OHT) or 10^{-6} M ICI (+ICI), or in the absence of drug (−estrogen) is shown. V-*rel*-transformed CEFs used as a control were treated similarly. Note the v-*rel*-specific morphology of v-*rel*ER fibroblasts after addition of estrogen and OHT. (**B**) Actin and fibronectin expression in v-*rel*ER CEFs in response to estrogen, OHT or ICI. V-*rel*ER CEFs were treated with estrogen, OHT or ICI, or left untreated as described in (A). Following fixation, permeabilized cells were incubated with rhodamine-conjugated phalloidin to reveal specifically the actin filaments (ACTIN). Indirect immunofluorescence (anti-fibronectin antibody followed by a fluorescein-conjugated goat anti-rabbit immunoglobulin) was used to stain extracellular fibronectin (FIBRONECTIN). Photographs of identical areas are shown. Note absence of actin cables and reduced fibronectin network for estrogen and OHT treated v-*rel*ER cells.

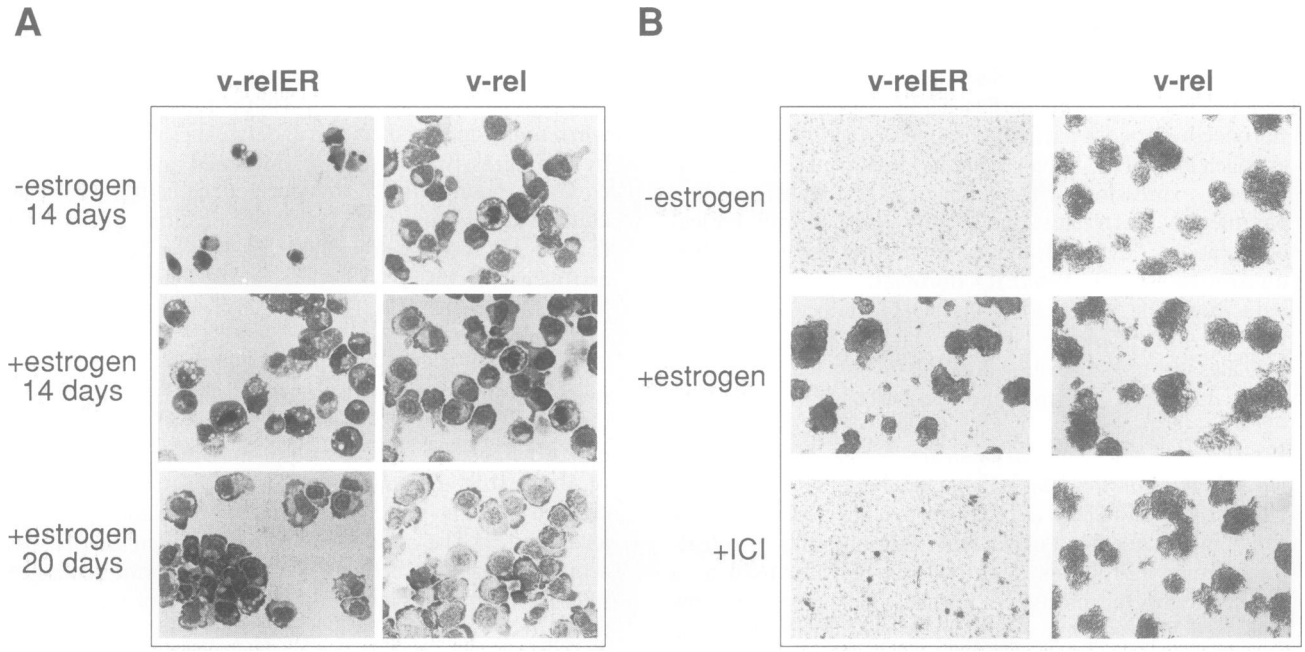

Fig. 3. V-*rel*ER transforms primary chicken bone marrow cells *in vitro* in an estrogen-dependent manner. (**A**) Chicken bone marrow cells were infected with v-*rel*ER or v-*rel*-containing retroviruses and grown in the absence or presence of estrogen. 14 and 20 days after infection, cells were cytocentrifuged onto slides, fixed, stained with May–Grünwald/Giemsa and photographed under green light. The outgrowth of v-*rel*ER-transformed bone marrow cells was estrogen-dependent, starting at day 14 after infection (+estrogen, 14 days). In the absence of hormone no outgrowth of transformed cells occurred (−estrogen, 14 days), whereas transformation of bone marrow cells by v-*rel* was independent of the application of hormone. (**B**) Colony formation of v-*rel*ER-transformed bone marrow cells is hormone-dependent. 5×10^4 v-*rel*ER- or v-*rel*-transformed bone marrow cells were seeded into 1 ml semisolid methocel medium in the absence or presence of 10^{-6} M estrogen (−, + estrogen, respectively) or in the presence of 10^{-6} M ICI (+ICI) as described in Materials and methods. Colonies were photographed 5 days after seeding.

colonies regardless of the presence or absence of hormone (Figure 3B).

V-*rel*ER colonies could be grown into clonal suspension cultures in the presence of estrogen, exhibiting a long lifespan similar to that of v-*rel*-transformed clones (Morrison *et al.*, 1991). As confirmed by Western blot analysis, these v-*rel*ER-transformed bone marrow clones expressed $p90^{v\text{-}relER}$ protein of the expected size at high levels (data not shown).

Proliferation of v-relER-transformed bone marrow cells is maintained by application of estrogen or OHT

Since the transformed phenotype of v-*rel*ER fibroblasts is induced by either estrogen or OHT but not by the estrogen antagonist ICI, we wanted to test whether or not v-*rel*ER-transformed bone marrow cells would respond similarly to these compounds. Therefore, we compared their influence on proliferation of v-*rel*ER-transformed bone marrow cells using a [^{3}H]thymidine incorporation assay (see Materials and methods).

As shown in Figure 4A, estrogen at concentrations of $>10^{-8}$ M induced growth of v-*rel*ER-transformed cells in a concentration dependent fashion. OHT at concentrations up to 10^{-8} M also induced proliferation of these cells, but was deleterious to cell growth at higher concentrations. The inhibitory effect of OHT at concentrations of $>10^{-7}$ M is most probably due to nonspecific cytotoxicity, since a similar phenomenon was also observed in the v-*rel* control culture (Figure 4A). This is clearly demonstrated in Figure 4B which shows histological staining of v-*rel*ER- and v-*rel*-transformed bone marrow cells treated with OHT at various concentrations. Survival of v-*rel*ER cells was critically dependent on OHT, whereas v-*rel* cells grew independently of OHT. Both v-*rel*ER- and v-*rel*-transformed bone marrow cells died, however, at higher OHT concentrations (10^{-6} M). As expected the pure estrogen antagonist ICI did not maintain growth of v-*rel*ER bone marrow cells at any concentration tested (Figure 4A).

In conclusion, the v-*rel*ER protein appears to represent a conditional version of v-*rel* whose transforming potential can be activated by hormone. In the presence of estrogen or of the estrogen agonist OHT, v-*rel*ER-expressing fibroblasts and bone marrow cells exhibit properties largely identical to those of v-*rel*-transformed cells. In the absence of estrogen or in the presence of the estrogen antagonist ICI, v-*rel*ER is biologically inactive.

Biochemical and functional properties of the v-relER protein are modulated by hormone

Estrogen, OHT and ICI cause v-relER to translocate to the nucleus. Two techniques were used to analyze the subcellular localization of the v-*rel*ER protein in response to hormone. First, v-*rel*ER CEFs kept in the presence of estrogen, OHT or ICI, or in the absence of hormone were fixed and stained with a polyclonal antibody directed against v-*rel*. Figure 5A shows that in the presence of estrogen, OHT or ICI $p90^{v\text{-}relER}$ is found predominantly in the nucleus, while in the absence of hormone it is also found in the cytoplasm.

We then tried to confirm and extend the immunofluorescence analysis by cell fractionation studies using v-*rel*ER-transformed bone marrow cells. [^{35}S]methionine-labeled cells cultivated in the presence of estrogen or in the absence of drug were fractionated and subjected to immunoprecipitation analysis with a polyclonal antibody directed against v-*rel* as described previously (Morrison

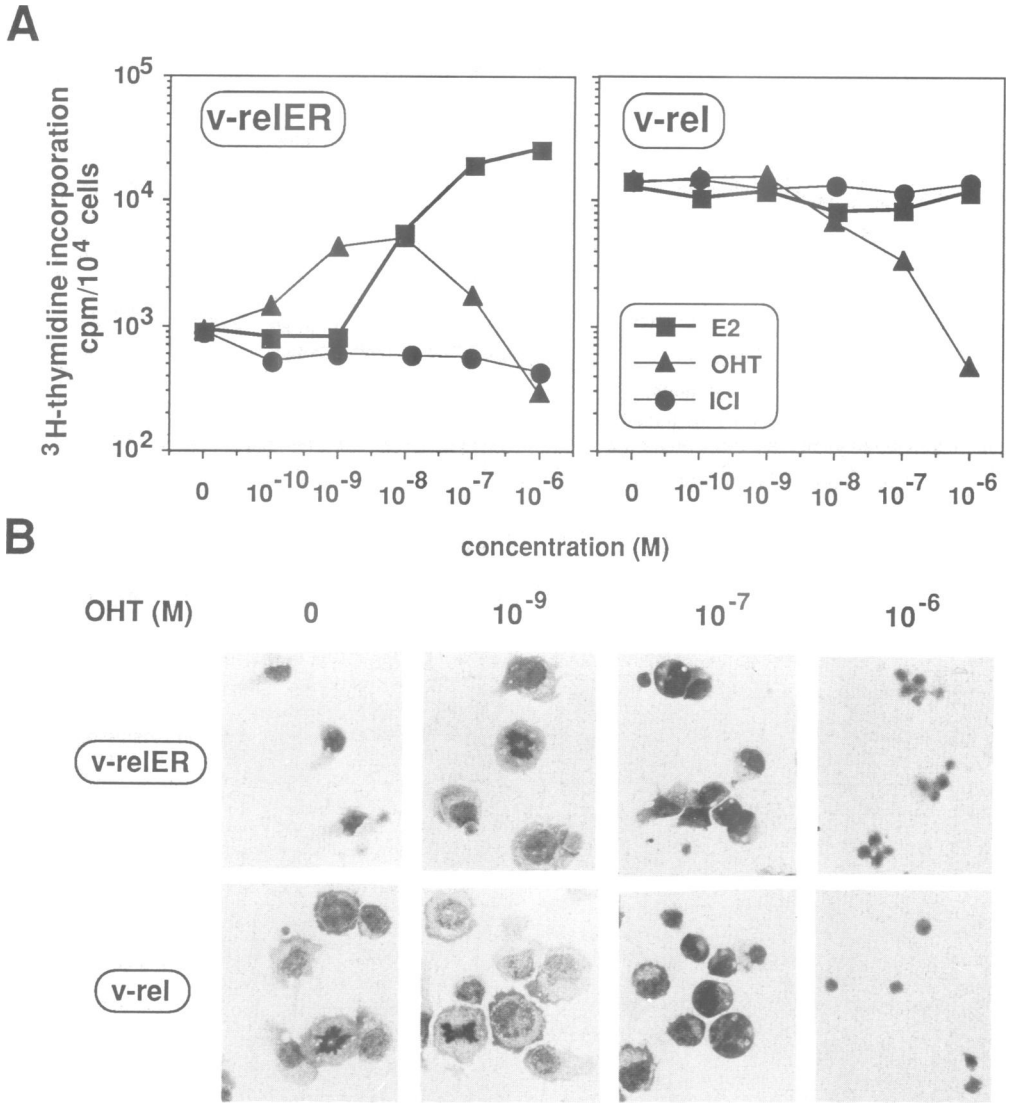

Fig. 4. Growth of v-*rel*ER-transformed bone marrow cells in response to different concentrations of estrogen, OHT and ICI. **(A)** 2.5×10^5 v-*rel*ER-transformed bone marrow cells (mass culture, see Figure 3A) were incubated in 1 ml of estrogen-depleted medium containing various concentrations of estrogen (E2), OHT or ICI as indicated. After 5 days the rate of DNA synthesis (measured by [^{3}H]thymidine incorporation, see Materials and methods) and cell numbers were determined. [^{3}H]thymidine incorporation per 10^4 cells is shown. V-*rel*-transformed bone marrow cells (mass culture, see Figure 3A) were treated identically and used as a control. When clones of v-*rel*- and v-*rel*ER-transformed bone marrow cells were used, a virtually identical result was obtained. **(B)** OHT supports growth of v-*rel*ER-transformed bone marrow cells at concentrations of $<10^{-7}$ M and is cytotoxic at higher concentrations. Aliquots from the experiment described in (A) were cytocentrifuged onto slides. Cells stained with May–Grünwald/Giemsa are shown. Note that OHT is cytotoxic at concentrations of $>10^{-7}$ M for both v-*rel*- and v-*rel*ER-transformed cells.

et al., 1991). Estrogen induced a clear shift of the v-*rel*ER protein to the nucleus while, in the absence of hormone, v-*rel*ER was found predominantly in the cytoplasmic fraction (Figure 5B). In addition, the subcellular localization of p68$^{c\text{-}rel}$ did not change upon addition of estrogen: this protein was found exclusively in the cytoplasmic fraction, as reported previously for v-*rel*-transformed cells (Morrison *et al.*, 1989).

Both v-rel and v-relER complex to the same cellular proteins in transformed cells. We and others have shown before that p59$^{v\text{-}rel}$ is complexed to several other cellular proteins of 124, 115, 68 (c-*rel*) and 36 kDa molecular weight (Simek and Rice, 1988; Morrison *et al.*, 1989; Davis *et al.*, 1990). The 36 kDa protein was recently identified as the avian equivalent of mammalian IκB β (Davis *et al.*, 1991; Kerr *et al.*, 1991). P124 appears to be homologous to mammalian p105—the precursor to the p50 subunit of NF-κB (Capobianco *et al.*, 1992). As our previous work suggested that the tertiary structure of v-*rel* is important in the correct binding of the associated proteins (Morrison *et al.*, 1992), it was important to determine if the addition of the ER domain to v-*rel* perturbed the structure of the protein and, consequently, the appropriate binding of p124, p115, p68$^{c\text{-}rel}$ and p36.

To this end, v-*rel*ER-transformed bone marrow cells were labeled with [^{35}S]methionine after incubation for 3 days in the presence of estrogen, OHT or ICI. They were then subjected to immunoprecipitation analysis employing a polyclonal antibody directed against v-*rel*. As can be seen in Figure 6, p90$^{v\text{-}rel\text{ER}}$ appears to be complexed to the same set of cellular proteins as p59$^{v\text{-}rel}$ (see Morrison *et al.*, 1991) both in the presence of estrogen, OHT or ICI, and in the absence of drugs. However, we reproducibly observed that

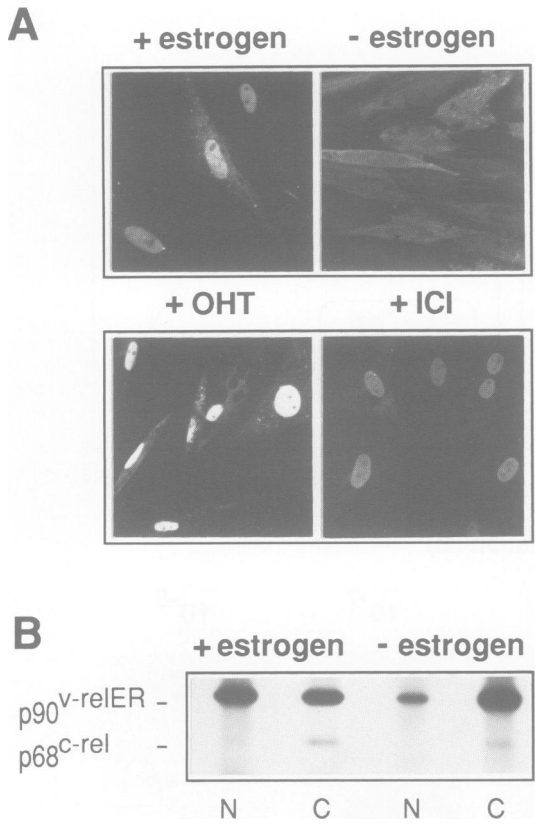

Fig. 5. Subcellular localization of the p90$^{v\text{-}relER}$ protein. (**A**) Immunofluorescence analysis of v-*rel*ER-transformed CEFs. V-*rel*ER CEFs were incubated with estrogen, OHT or ICI (+estrogen, +OHT, +ICI, respectively) or without drug (−estrogen) as described in Figure 2. Fixed and permeabilized cells were incubated with the anti-*rel* antibody SB66 followed by an incubation with FITC-conjugated goat anti-rabbit IgG to visualize v-*rel*ER essentially as described before for v-*rel* (Morrison *et al.*, 1991). (**B**) v-*rel*ER-transformed bone marrow cells (clone #6) were kept for 3 days in the presence or absence of estrogen (+estrogen and −estrogen, respectively). Following metabolic labeling with [^{35}S]methionine, nuclear (N) and cytoplasmic (C) extracts were prepared (see Materials and methods) and analyzed by immunoprecipitation employing the anti-*rel* antibody SB66. The positions of p90$^{v\text{-}relER}$ and p68$^{c\text{-}rel}$ are indicated.

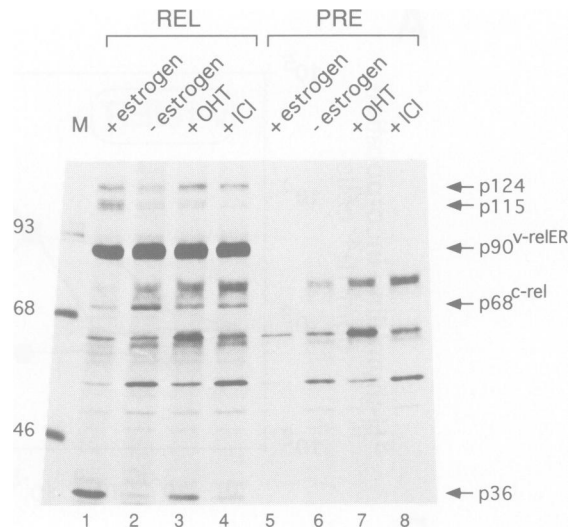

Fig. 6. p90$^{v\text{-}relER}$ forms specific complexes with cellular proteins. V-*rel*ER-transformed bone marrow cells (clone #23) were kept for 3 days in the presence of 10^{-6} M estrogen (+estrogen), 10^{-8} M OHT (+OHT) or 10^{-6} M ICI (+ICI) or left untreated (−estrogen). Cells were labeled with [^{35}S]methionine for 2 h, lysed and subjected to immunoprecipitation analysis using either the anti-*rel* antibody SB66 (Morrison *et al.*, 1991; lanes 1−4) or a preimmune control serum (pre, lanes 5−8). The positions of p124, p115, p90$^{v\text{-}relER}$, p68$^{c\text{-}rel}$, p36 and of the molecular weight markers (M) are indicated. The results depicted here for clone #23 were essentially identical to that observed for another v-*rel*ER-transformed bone marrow cell clone and were also obtained when the v-*rel*-specific monoclonal antibody #6 (Kabrun *et al.*, 1991) was used (data not shown).

p36 I$\varkappa$B β protein was less efficiently coimmunoprecipitated by anti-v-*rel* sera in the absence of estrogen or presence of ICI (Figure 6). Preliminary data suggest that in the case of ICI the reduced coimmunoprecipitation is due to a less stable complex, which could be more sensitive to detergent disruption.

Thus, addition of the ER domain did not appear to alter significantly the tertiary structure of the v-*rel* moiety in v-*rel*ER. However, the formation or stability of complexes including p36 I$\varkappa$B β was reduced in the absence of estrogen or the presence of ICI, in correlation with the reversal of transformation.

V-relER binds to NF-$\varkappa$B sites in vitro. The transformation studies described above suggested that p90$^{v\text{-}relER}$ was behaving in a manner analogous to p59$^{v\text{-}rel}$ but in a hormone-inducible fashion. However, the estrogen antagonist ICI, while inducing translocation of v-*rel*ER into the nucleus, failed to activate its transforming potential. Thus properties of v-*rel*ER other than its nuclear localization must be responsible for the induction of transformation by hormone. This prompted us to test the ability of the v-*rel*ER protein to interact with specific DNA sequences *in vitro* in a mobility shift assay.

Cell extracts were prepared from v-*rel*ER-transformed bone marrow cells untreated or treated with estrogen, OHT or ICI (Dignam *et al.*, 1983) and analyzed by mobility shift assay using a NF-$\varkappa$B/*rel* binding site as described previously (Kabrun *et al.*, 1991). Cell equivalent amounts of cytoplasmic and nuclear extracts were used to measure the activity present in the cytoplasmic and nuclear fractions (Figure 7). As expected cytoplasmic and nuclear extracts of both estrogen and OHT treated cells yielded v-*rel*ER-specific band shifts (B_c and B_n in lanes 1, 2, 5 and 6). Most importantly, extracts from untreated or ICI-treated cells showed drastically reduced binding activity (Figure 7, lanes 3, 4, 7 and 8).

Protein−DNA complexes formed with cytoplasmic extracts migrated more slowly than the nuclear complexes, perhaps due to association with other proteins contained in the cytoplasmic fraction. Supershift experiments employing v-*rel*-specific polyclonal antibodies (Kabrun *et al.*, 1991) demonstrated the presence of v-*rel*ER in these complexes (data not shown). While the nuclear activity from estrogen- and OHT-treated cells appeared to bind similarly, the cytoplasmic extract from OHT-treated cells reproducibly showed less activity than the equivalent extract from estrogen-treated cells. This may reflect differences in the activities of estrogen and OHT on the estrogen receptor hormone binding domain of v-*rel*ER. It should be noted that multiple protein−DNA complexes of differing size are observed in cytoplasmic and nuclear extracts as we reported previously for v-*rel*-transformed cells (Kabrun *et al.*, 1991).

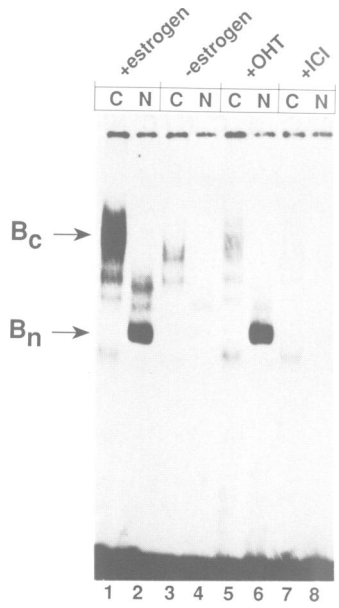

Fig. 7. V-*rel*ER binding to a consensus NF-κB/*rel* binding site *in vitro* is hormone-dependent. Cell extracts were prepared (see Materials and methods) from v-*rel*ER-transformed bone marrow cells (clone #23) following a 3 day incubation in the absence of hormone (−estrogen, lanes 3 and 4) or the presence of estrogen (+estrogen, lanes 1 and 2), OHT (+OHT, lanes 5 and 6) or ICI (+ICI, lanes 7 and 8) as described in Figure 6. Cell equivalent amounts of cytoplasmic (C) or nuclear (N) extracts were incubated with an oligonucleotide containing a consensus NF-κB site (Kabrun *et al.*, 1991) and analyzed by electrophoretic mobility shift assay (see Materials and methods). B_c indicates the band that shifted in the presence of cytoplasmic extract, whereas B_n indicates the band that shifted in the presence of nuclear extract. A virtually identical result was obtained with other v-*rel*ER clones.

The composition of these complexes, however, is not clear at present but is under analysis.

Our results indicate therefore that the ability of a hormone-activated v-*rel*ER to transform fibroblastic and bone marrow cells correlates with its ability to bind to specific DNA sequences.

Hormone-dependent transcriptional activity of v-relER

To determine whether the v-*rel*ER protein also regulated gene expression in a hormone-dependent fashion, transient transfection experiments were performed.

A CAT reporter gene construct containing three NF-κB/*rel* binding sites upstream of a minimal thymidine kinase promoter (see Materials and methods) was transiently transfected into the stably transformed v-*rel*ER fibroblasts described above, which showed the v-*rel*-specific morphological alterations in response to hormone (Figure 2A). Under these experimental conditions, estrogen but not OHT and ICI efficiently activated expression of the NF-κB/*rel*-dependent reporter gene (Figure 8). This induction was critically dependent on the NF-κB/*rel* binding sequences present in the reporter construct, since no CAT expression was observed in the absence of these sequences or for mutant NF-κB/*rel* sites (Figure 8 and data not shown). In addition, ethanol, the solvent used for estrogen, OHT and ICI, did not significantly activate expression of the CAT reporter gene (data not shown). Figure 8 also shows that in v-*rel*-transformed and uninfected CEFs, expression of the NF-κB/*rel* reporter gene construct was not affected by

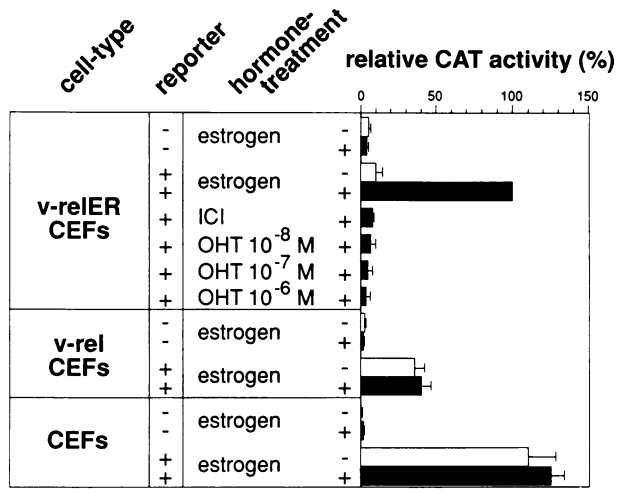

Fig. 8. V-*rel*ER activates expression of a transiently transfected reporter plasmid containing a trimerized NF-κB/*rel* binding site. V-*rel*ER-transformed fibroblasts (see Figure 2) were transiently transfected with a CAT reporter plasmid containing a trimerized NF-κB/*rel* binding site or a trimerized mutant NF-κB/*rel* binding site as indicated (reporter +/−, respectively). Untransformed and v-*rel*-transformed chicken embryo fibroblasts (CEFs) were used as controls. Following transfection, cells were treated with 10^{-6} M estrogen, 10^{-6} M ICI or 10^{-8}–10^{-6} M OHT for 48 h, or left untreated as shown. 2 days later cell extracts were prepared and assayed for CAT activity as described in Materials and methods. A constant amount of a luciferase-encoding reporter gene construct was cotransfected and used to correct for variations in the transfection efficiencies. The CAT activity measured and normalized for equal luciferase activity is shown with the CAT activity achieved by an estrogen-activated v-*rel*ER set arbitrarily to 100%.

estrogen. It is also obvious that the CAT signal in v-*rel*-transformed CEFs was 2- to 3-fold lower than that in uninfected CEFs. However, infection of CEFs with an empty vector (RCAS) also caused a significant reduction in the expression from the NF-κB/*rel* reporter plasmid, ultimately reducing the repressive effect in v-*rel* CEFs as compared with RCAS-infected cells to 1.5- to 2-fold (data not shown).

These results lead to several unexpected conclusions. First, transcriptional activation as measured by transient transfection experiments was only seen with estrogen but not with OHT, obviously failing to correlate with the transforming ability of v-*rel*ER. This also suggests that v-*rel*ER transactivates via the ER transactivation domain (TAF-2) in transient transfection assays, thus leaving it open whether or not the v-*rel* moiety in v-*rel*ER can act as a transactivator *in vivo*. Second, it remains unclear whether the rather moderate repression we observed for v-*rel* (as compared with uninfected CEFs) corresponds to the repression seen by other authors using different NF-κB recognition sites and experimental conditions (Ballard *et al.*, 1990; Inoue *et al.*, 1991; Richardson and Gilmore, 1991; Ballard *et al.*, 1992; McDonnell *et al.*, 1992 and references therein). The results obtained with CEFs containing empty vector would rather suggest that such a repression does not occur under our conditions.

Effects of v-relER on expression of cellular genes

The above results suggest that a clear picture of the biochemical and biological properties of v-*rel*ER could not be obtained in CEFs due to the limitations of the transient transfection assay. Furthermore, the transfection efficiency observed with v-*rel*- and v-*rel*ER-transformed bone marrow

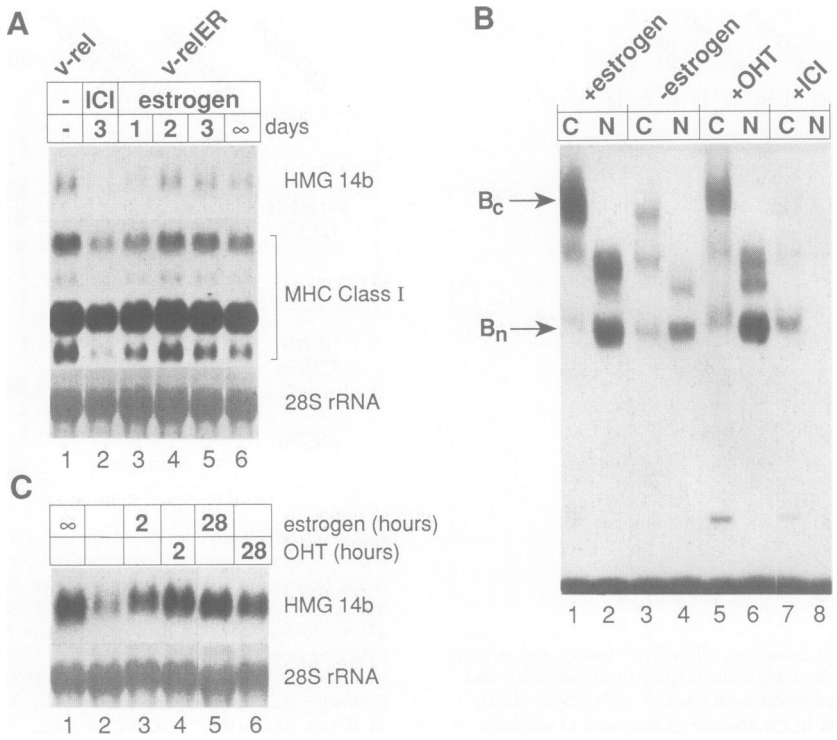

Fig. 9. V-*rel*ER modulates transcription of the HMG-14b and of a MHC class I-specific gene and binds to the MHC class I-responsive element *in vitro*. (**A**) V-*rel*ER-transformed bone marrow cells (clone #6) were kept in 10^{-6} M ICI for 3 days (lane 2) or treated with 10^{-6} M ICI for 3 days followed by an additional treatment with 10^{-6} M estrogen for 1, 2 or 3 days as indicated (lanes 3, 4 and 5). Alternatively, v-*rel*ER cells were kept continuously in 10^{-6} M estrogen (lane 6). V-*rel*-transformed cells grown in the absence of hormone were used as a control (lane 1). RNA was prepared and analyzed for HMG-14b (Srikantha *et al.*, 1990) and MHC class I-specific transcripts (clone B-F12α F10 in Kroemer *et al.*, 1990; B-F19α F3 in Kaufmann *et al.*, 1992) by Northern blot hybridization as described in Materials and methods. The 28S rRNA was stained with methylene blue (Khandjian, 1986) to show equal loading in all lanes. Two different exposures (6 and 48 h, respectively) of the MHC class I-specific transcripts are shown. (**B**) Electrophoretic mobility shift assay using the chicken MHC class I responsive element. Extracts were prepared from v-*rel*ER-transformed bone marrow cells grown in the absence of drug (− estrogen) or the presence of estrogen, OHT or ICI (+estrogen, +OHT, +ICI, respectively) as described in the legend to Figure 7. Cell equivalent amounts of cytoplasmic (C) or nuclear (N) extracts were incubated with an oligonucleotide containing the class I-responsive element from the chicken MHC locus (see Materials and methods). B_c refers to the band that shifted in the presence of cytoplasmic extract and B_n to the band that shifted with nuclear extract. (**C**) V-*rel*ER-transformed bone marrow cells (clone #6) were kept in 10^{-6} M ICI for 2 days, followed by treatment with 10^{-6} M estrogen or 10^{-8} M OHT for 2 and 28 h (lanes 3−6), or left untreated (lane 2) as indicated. As a control, cells were kept continuously in 10^{-6} M estrogen (lane 1). HMG-14b-specific transcripts and the 28S rRNA are detected as decribed in (A). Note that estrogen and OHT induce a rapid increase of HMG-14b mRNA levels.

cells was too low to allow similar experiments in this more relevant cell type. Therefore, we next investigated the effects of estrogen- or OHT-activated v-*rel*ER on the expression of cellular genes which are regulated by members of the NF-κB/*rel* family (for review see Baeuerle, 1991). This type of study should ultimately allow us to correlate the transformation-specific alterations of v-*rel*ER-expressing cells with specific changes in gene expression.

The mouse MHC class I gene H-2K^b has been shown to contain an NF-κB site in its promoter that is important for its regulation by members of the NF-κB/*rel* transcription factor family (Israël *et al.*, 1987, 1989; Baldwin and Sharp, 1988). Therefore, we analyzed the expression of the MHC class I gene cluster in v-*rel*ER-transformed bone marrow cells. A chicken MHC class I-specific probe (B-F12α F10 in Kroemer *et al.*, 1990; B-F19α F3 in Kaufmann *et al.*, 1992) detected increased levels of two transcripts (1.2 and 1.7 kb) when the cells were treated with estrogen and OHT, while ICI had no such effect (Figure 9A and data not shown). In v-*rel*-transformed cells their expression was high and constitutive (Figure 9A).

Inspection of the corresponding promoter sequence of this MHC class I gene identifies a potential NF-κB/*rel* binding site at position −200 (referred to as the class I-responsive element in Kroemer *et al.*, 1990). Thus, we examined the ability of the v-*rel*ER protein to bind to a respective oligonucleotide in electrophoretic mobility shift assays. Specific protein−DNA complexes (B_c and B_n in Figure 9B) were detected when the oligonucleotide was incubated with cytoplasmic or nuclear extracts from estrogen- or OHT-treated cells. In contrast, extracts from untreated or ICI-treated cells contained much reduced binding activity (Figure 9B). These results correspond to those obtained with the NF-κB element used in Figure 7. They suggest that v-*rel*ER is involved in the regulation of MHC class I gene expression although a direct interaction with the promoter *in vivo* remains to be demonstrated.

In the 5′ untranslated region of the chicken gene encoding the high mobility group protein 14b (HMG-14b, Srikantha *et al.*, 1990), several NF-κB binding sites are obvious. It was therefore of interest to test this gene for regulation by v-*rel*ER. HMG-14b mRNA expression was high and constitutive in v-*rel*-transformed cells (Figure 9A) and rapidly induced by estrogen and OHT but not by ICI in v-*rel*ER-transformed bone marrow cells (Figure 9C). This observation is in line with the finding that an NF-κB site present in the HMG-14b promoter binds v-*rel* and v-*rel*ER *in vitro* (A.Walker and P.Enrietto, unpublished), strongly

suggesting that regulation of HMG-14b transcription might occur through binding of *rel* to this sequence. However, the relevance of HMG-14b gene regulation by v-*rel* (and v-*rel*ER) and its potential contribution to the v-*rel*-induced transformed phenotype remains to be shown.

In summary, the regulation of at least two cellular genes occurs in a hormone-responsive fashion in v-*rel*ER-transformed cells. Their expression levels in the presence of estrogen were close to those observed for v-*rel*, confirming our notion that v-*rel*ER is a conditional version of v-*rel* that closely mimics v-*rel* action in its ligand-activated state, but is essentially inactive in the absence of hormone. These data leave open the possibility that v-*rel*ER (and perhaps v-*rel*) may function in part as a transcriptional activator. Experiments are in progress to demonstrate a direct effect of v-*rel*ER on the expression of these genes.

Discussion

In this report we describe the biological and biochemical activities of a chimeric protein containing v-*rel* fused to the hormone binding domain of the human estrogen receptor. This v-*rel*ER protein causes estrogen-dependent, but otherwise unaltered v-*rel*-specific transformation of avian fibroblasts and bone marrow cells. Hormone-activated v-*rel*ER also elevated transcription of two cellular genes that are constitutively activated in v-*rel*-transformed bone marrow cells. Our results demonstrate that the ER−oncogene fusion protein approach (Picard *et al.*, 1988; Eilers *et al.*, 1989; Burk and Klempnauer, 1991) can be successfully used in primary chicken hematopoietic cells.

V-relER as a means to identify the hematopoietic target cell for v-rel transformation. Conditional oncogenes (e.g. temperature-sensitive mutants) have been useful for studying the mechanisms of oncogene function and for identifying the type of hematopoietic cell they transform (Graf *et al.*, 1978; Beug *et al.*, 1984; Knight *et al.*, 1988; and references therein). Thus, a conditional v-*rel* protein such as the v-*rel*ER described here should be very useful in elucidating the type of progenitor transformed by v-*rel*, a question that we have recently attempted to address (Morrison *et al.*, 1991).

Cells transformed by the v-*rel*-containing virus REV-A/REV-T were initially classified as early preB/preT lymphoid progenitor cells (Beug *et al.*, 1981; Lewis *et al.*, 1981). However, immunosuppressive and cytopathic effects of the non-transforming associated helper virus REV-A hampered a detailed analysis. Recent *in vivo* studies suggested that the helper virus had a dramatic effect on target cell specificity: REV-A/REV-T-transformed cells were of both the T-cell and the myeloid lineage whereas infection with REV-T together with less cytotoxic helper viruses transformed cells of various B-lymphoid differentiation stages (Barth *et al.*, 1990). The construction of a replication-competent virus containing v-*rel* (Morrison *et al.*, 1991) circumvented this problem and allowed v-*rel*-transformed cells to be analyzed in the absence of interfering activities of the associated helper virus. Using this system, Morrison *et al.* (1991) showed that v-*rel*-transformed bone marrow cells coexpressed surface antigens specific to both the lymphoid and myeloid lineage. Sometimes a few cells even expressed erythroid-specific surface markers. We thus speculated that v-*rel* transforms cells which exhibit properties of an early, multipotent hematopoietic progenitor cell.

Using the conditional v-*rel*ER virus, it should now be possible to 'switch off' v-*rel* activity in transformed hematopoietic cells and determine whether these cells can differentiate into different lineages. Pilot studies along these lines have been hampered by the lack of avian hematopoietic growth factors such as IL-3, GM-CSF etc. By introduction of the respective mammalian growth factor receptors (P.Steinlein, E.Deiner, A.Leutz and H.Beug, in preparation) into v-*rel*ER-transformed cells, we eventually hope to understand better the nature of the hematopoietic target cell transformed by v-*rel*.

Possible mechanism of action of v-relER. In addition, in order to understand the biological properties of v-*rel*ER, we attempted to elucidate the mechanism of action of v-*rel*ER at the biochemical level. Several functional properties of v-*rel*ER were therefore examined. In the presence of estrogen, OHT or ICI, v-*rel*ER translocated to the nucleus. Like v-*rel*, v-*rel*ER was complexed to a specific set of cellular proteins of 124 kDa, 115 kDa, 68 kDa (c-*rel*) and 36 kDa molecular weight. However, in the absence of hormone and in the presence of ICI, the association of p36 I$\varkappa$B β to v-*rel*ER was found to be weaker. Furthermore, v-*rel*ER exhibited the same target sequence specificity as v-*rel*, as shown by mobility shift assay using both an NF-$\varkappa$B consensus binding site and NF-$\varkappa$B sites contained in a chicken MHC class I and the HMG-14b gene. Binding to these sites occurred in the presence of estrogen and OHT but not in the presence of ICI or the absence of hormone. A similar pattern was observed for transcriptional regulation of two cellular genes (MHC class I and HMG-14b) that are constitutively active in v-*rel*-transformed hematopoietic cells.

These results show that DNA binding, activation of cellular genes and perhaps stability of the p36/v-*rel*ER complex, but not nuclear localization, were correlated to the transformed phenotype of v-*rel*ER-expressing fibroblasts and bone marrow cells. However, further work will be required to determine which of these properties is responsible for transformation by v-*rel*.

Regarding the mechanism of v-*rel*ER action, the effects of the two 'anti-estrogens', OHT and ICI, were particularly revealing. OHT and ICI behave as an estrogen agonist and antagonist, respectively, for the authentic estrogen receptor. However, these activities were dependent on the cell-type and promoter context used (Jordan *et al.*, 1984; Wakeling and Bowler, 1988a,b; Berry *et al.*, 1990; Green and Chambon, 1991; Gronemeyer, 1991; Pham *et al.*, 1991; Dauvois *et al.*, 1992). While in these studies ICI acted as a pure estrogen antagonist, OHT retained the ability to activate both dimerization and DNA binding of the receptor. However, in contrast to estrogen, OHT did not activate TAF-2, the transcriptional activation function contained in the estrogen receptor hormone binding domain. OHT rather appeared to elicit its activity exclusively through TAF-1, the constitutive transcriptional activation function contained in the 5′ part of the receptor, which is replaced by v-*rel*-specific sequences in the v-*rel*ER chimeric protein.

By analogy, we speculate that both estrogen and OHT but not ICI activate the dimerization function contained in the hormone binding domain of the chimeric v-*rel*ER protein. Dimerization would then be required for v-*rel*ER binding to NF-$\varkappa$B sites via the v-*rel*-specific DNA binding domain. Since OHT, however, does not activate TAF-2, any transcriptional activity brought about by an OHT-bound v-*rel*ER should be exclusively ascribed to the v-*rel* moiety of

the v-*rel*ER protein. In contrast, an estrogen-activated v-*rel*ER should affect transcription also through TAF-2, since TAF-2 is activated by estrogen in the authentic estrogen receptor.

Does v-rel operate as a transcriptional activator or as a repressor? Based on transient transfection experiments it has been proposed that the v-*rel* oncoprotein efficiently suppresses gene expression via NF-κB/*rel* binding sites present in the reporter gene constructs used. These results have led to the notion that v-*rel* acts as a repressor of NF-κB/*rel*-dependent transcription in a dominant negative fashion and that this activity is important for its function as an oncoprotein. This idea is further strengthened by the observation that v-*rel* mutants which are active in transformation are efficient competitors of NF-κB/*rel*-mediated transcription whereas transformation-defective v-*rel* mutants are impaired in this activity (Ballard *et al.*, 1990; Richardson and Gilmore, 1991; McDonnell *et al.*, 1992; and references therein). However, repression of c-*rel*, the only avian gene known to be repressed by v-*rel*, is not mediated through the NF-κB/*rel* binding site contained in the c-*rel* promoter but rather appears to be indirect (Capobianco and Gilmore, 1991).

Surprisingly, we were unable to obtain clear evidence for such a repression. Rather, an estrogen-activated v-*rel*ER (which faithfully reproduced v-*rel*-specific properties in transformed cells) activated trancription of a NF-κB reporter construct in transient transfection assays. Since an OHT-activated v-*rel*ER consistently failed to stimulate reporter gene expression, these results suggest that transient transfection under our conditions only revealed transcriptional activation through TAF-2 contained in the ER domain of v-*rel*ER. Thus, the transient transfection assay provided little information regarding possible transcriptional regulation by v-*rel*ER.

Since the transcription of two cellular genes was upregulated in a hormone-inducible fashion which correlated with transformation, it is tempting to speculate that v-*rel*ER (and possibly also v-*rel*) might exert its specific effects on gene expression and transformation at least in part as a transcriptional activator rather than as a repressor of transcription. Our experiments do not rule out the possibility that v-*rel* may also repress the expression of certain genes, nor do they demonstrate a direct effect of v-*rel*ER on the transcription of the MHC class I or HMG-14b genes. However, the *rel* family member dorsal can both activate and repress, depending on the target gene (Thisse *et al.*, 1991; Ip *et al.*, 1991). It is possible that a hormone-activated v-*rel*ER acts via a completely different mechanism from v-*rel*, yet still induces transformation. Since many, if not all v-*rel*-specific properties are retained in hormone-activated v-*rel*ER, we consider this possibility rather unlikely.

Obviously, one of the important uses of the v-*rel*ER system is the search for genes whose expression is regulated by v-*rel* and v-*rel*ER. We hope that this approach might help to identify genes directly involved in transformation. Furthermore, the identification of such *rel*-regulated genes should provide the reagents for studying in more detail the mechanism by which v-*rel* interacts with specific target sequences and how it affects, as an activator or repressor, the expression of specific genes.

Materials and methods

Recombinant plasmid vectors

Construction of RCASv-relER. PCR technology was used to generate a *Bgl*II site at the v-*rel* 3′ end (adding 5′-CCAGATCT-3′ at amino acid position 503) of pBS*rel*CS (Morrison *et al.*, 1991). In v-*rel*ER this *Bgl*II site is fused to the estrogen receptor hormone binding domain (ER; amino acids 282–595) contained in a *Bam*HI–*Sac*I fragment of recombinant HE14 (Kumar *et al.*, 1986). The amino acid sequence generated at the v-*rel*/ER junction is shown in Figure 1A. Finally, the v-*rel*ER fusion gene was cloned as a *Cla*I fragment into the unique *Cla*I site of pRCAS (Hughes *et al.*, 1987) thereby generating pRCASv-*rel*ER and pRCAS-anti-v-*rel*ER, containing v-*rel*ER in the sense or antisense orientation, respectively.

Reporter plasmids. The CAT reporter constructs pTK-3κB and pTK-3mutκB contain three copies of the NF-κB consensus binding site and of the respective mutant (Kabrun *et al.*, 1991) at position −105 of the herpes simplex virus thymidine kinase promoter in recombinant pTK (Ibanez and Lipsick, 1990).

Cells and tissue culture

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal calf serum (FCS) and 2% chicken serum (ChS; referred to below as standard growth medium). Estrogen-depleted FCS and ChS were prepared as described before (Schroeder *et al.*, 1992).

Dilutions of 17-β-estradiol (Sigma), OHT and ICI were made using ethanol. These compounds were applied daily to cells grown in liquid culture and every third day in methocel assays. OHT at concentrations of $>10^{-7}$ M was deleterious to growth of both v-*rel*ER-transformed fibroblasts and bone marrow cells. Estrogen-depleted standard media were used in all titration experiments. Colony formation of v-*rel*ER-transformed bone marrow cells was monitored in CFU-E methocel (Radke *et al.*, 1982).

Transformation of fibroblasts

CEFs from 6–7 day embryos were prepared as described earlier (Vogt, 1969). pRCASv-*rel*ER DNA was introduced into CEFs using the calcium phosphate/DNA coprecipitation method (Graham and van der Eb, 1973). Briefly, 10^6 cells were transfected with 10 μg pRCASv-*rel*ER DNA and left in contact with the calcium phosphate precipitate for 20–24 h, followed by a complete medium change. 24 h later, cells were trypsinized and seeded at 0.5×10^6 cells/5 cm diameter culture dish. To select for transformed cells, the culture was overlaid with 0.72% agar in standard growth medium containing or not containing 10^{-6} M estrogen. Two weeks later, foci appeared in the sample supplemented with estrogen. Cells were then trypsinized and used for further experiments. V-*rel*ER-transformed cells obtained by this procedure showed the morphological changes in response to estrogen as depicted in Figure 2A. Alternatively, v-*rel*ER-transformed fibroblasts were also obtained simply by passaging v-*rel*ER-transfected fibroblasts in the presence of hormone.

Bone marrow cell transformation

Bone marrow from 4–7 day old chickens was isolated as described (Morrison *et al.*, 1991). For infection, bone marrow cells were cocultivated with virus-producing, v-*rel*- or v-*rel*ER-transformed, mitomycin C treated (5 μg/ml; 1–2 h) fibroblasts for 24 h, in either the presence or absence of 10^{-6} M estrogen. CFU-E medium (Radke *et al.*, 1982) supplemented with 1 μg/ml human recombinant insulin and 1% anemic chicken serum was used.

The outgrowth of v-*rel*ER-transformed bone marrow cells occurred between days 11 and 14 after infection. Cells were then adapted to standard medium conditions (see above). In rare instances, an outgrowth of v-*rel*ER-transformed bone marrow cells was obtained in the absence of estrogen. In such cells, v-*rel*ER had apparently undergone extensive deletions within or of the entire estrogen receptor hormone binding domain (data not shown). Such cells were not further analyzed.

For selection of clones of v-*rel*ER-transformed bone marrow cells, aliquots of a v-*rel*ER mass culture were seeded into CFU-E methocel. Individual colonies were isolated 7 days later and expanded in standard growth medium containing 10^{-6} M estrogen.

[^{3}H]thymidine incorporation assay

Cell growth and proliferation were measured as the rate of DNA synthesis by [^{3}H]thymidine incorporation as described by Leutz *et al.* (1984). Cells were grown in estrogen-depleted standard growth medium containing estrogen, OHT or ICI at various concentrations as indicated in the respective experiment, or without drug. At the end of the incubation period, 100 μl

aliquots of such cultures were incubated with 0.8 μCi [^{3}H]thymidine (specific activity 29 Ci/mmol; Amersham) for 2 h at 37°C, harvested onto filters (using a Skatron cell harvester) and subjected to liquid scintillation counting. Average values from triplicate samples were normalized to cell number.

Immunoprecipitation

RCASv-*rel*ER-transformed bone marrow cells were preincubated in methionine-free medium for 20 min and labeled with 250 μCi [^{35}S]methionine (1000 Ci/mmol, Amersham) for 1.5 h (Zenke *et al.*, 1988; Morrison *et al.*, 1989). Cells were lysed in 0.5% NP40, 0.5% sodium deoxycholate, 20 mM Tris−HCl, pH 8.0 and 50 mM NaCl and subjected to immunoprecipitation analysis as previously described (Morrison *et al.*, 1989). The antibodies used were a polyclonal antibody (SB66) raised against a bacterially expressed trpE−v-*rel* fusion protein (kindly provided by Drs Owen Witte and Richard Schwartz; Schwartz and Witte, 1988) and a monoclonal v-*rel* specific antibody (#6; Kabrun *et al.*, 1991). Immunoprecipitates were analyzed by SDS−PAGE (Laemmli, 1970).

Western blotting

1×10^7 cells were harvested, washed once in ice-cold PBS and lysed in 500 μl sample buffer (Ausubel *et al.*, 1991). Cell lysates were drawn several times through 25 G needles to reduce their viscosity. 40 μl aliquots of the lysates were incubated at 100°C for 5 min and subjected to SDS−PAGE (10% acrylamide) as described by Laemmli (1970).

For transfer of proteins to nitrocellulose filters (BA 85, Schleicher and Schüll, Germany) a semidry transfer system (LKB, Sweden) was used. All further incubations were performed at 37°C in a rotating glass tube. First, the nitrocellulose filter was incubated with 0.5% gelatin in PBS (1 h) to block nonspecific binding of the antibody. After two washing steps with washing buffer (0.02% gelatin, 0.25% Triton X-100 in PBS), the blot was incubated for 1 h with a 1:10^3 dilution of the polyclonal *rel* antibody SB66 (see above).

Subsequently, the blot was washed five times and then incubated for 45 min with 1 μCi of ^{125}I-labeled protein A (Amersham) in washing buffer. After five additional washing steps, the blot was analyzed by autoradiography.

Immunofluorescence

Fibroblasts expressing v-*rel*ER were washed twice with PBS and fixed for 15 min in 3% paraformaldehyde directly in the tissue culture dish. Cells were then permeabilized with 0.5% NP40 in PBS (15 min) and washed three times with 0.5% BSA in PBS.

For immunofluorescence analysis, cells were incubated with the polyclonal *rel* antibody SB66 diluted 1:100 (1 h at 37°C). Cells were washed three times with 0.5% BSA in PBS and incubated with a 1:50 dilution of FITC-conjugated goat anti-rabbit IgG F(ab')2 fragment (TAGO; 45 min at 37°C). After washing with PBS (three times) cells were mounted in Mowiol (Beug *et al.*, 1979).

TRITC-conjugated phalloidin (Sigma) was used for actin staining. The fibronectin-specific antibody has been described before (Morrison *et al.*, 1991).

RNA preparation and Northern blot hybridization

Isolation of RNA was as described in Chomczynski and Sacchi (1987) and the modification by Puissant and Houdebine (1990). 10 μg total RNA/sample were subjected to Northern blot analysis as described previously (Zenke *et al.*, 1988). Probes were prepared by random priming (Feinberg and Vogelstein, 1984) using an oligolabeling kit (Pharmacia, Sweden) according to the manufacturer's specification. The HMG-14b-specific probe used was a 0.76 kb *KpnI*−*BclI* fragment of the chicken HMG-14b cDNA (nucleotides 7004−7762; Srikantha *et al.*, 1990). The MHC class I cDNA (clone B-F19α F3) is described in Kaufmann *et al.* (1992) and is identical to clone B-F12α F10 in Kroemer *et al.* (1990).

Cell fractionation

Cells from v-*rel*ER-transformed bone marrow clones were incubated for 3 days in the presence of 10^{-6} M estrogen, 10^{-8} M OHT or 10^{-6} M ICI or without drug. The cells were fractionated essentially as described in Morrison *et al.* (1989), except that they were lysed in a buffer containing 50 mM Tris−HCl pH 8.0, 1.1 mM $MgCl_2$, 0.5% Triton X-100 and 5 mM KCl (lysis buffer). Nuclei were pelleted and washed twice with lysis buffer. Subsequently, both cytoplasmic and nuclear fractions were adjusted to RIPA conditions and immunoprecipitated with a polyclonal antibody directed against v-*rel* (SB66).

Electophoretic mobility shift assay

Nuclear or cytoplasmic (S100) extracts from v-*rel*ER cells grown in the presence of estrogen, OHT or ICI, or without drug were prepared as described by Dignam *et al.* (1983). Extracts were incubated on ice for 15 min in the presence of 0.5 μg salmon sperm DNA and incubation buffer (final concentrations after oligonucleotide addition: 10 mM Tris−HCl pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% (v/v) glycerol and 3 mM dGTP), followed by the addition of $1-2\times10^4$ c.p.m. of labeled oligonucleotide with incubation at room temperature for 20 min. The reactions were performed in a final volume of 25 μl and run on 6% native polyacrylamide gels at 200 V in TBE (25 mM Tris base, 22 mM boric acid, 0.25 mM EDTA, pH 8.0) at 4°C.

Oligonucleotides contained the following sequences:
NF-κB, 5′-AGC TTC AAC AGA GGG GAC TTT CCG AGA GGC TCG AG-3′ and the complementary strand, 5′-AAT TCT CGA GCC TCT CGG AAA GTC CCC TCT GTT GAA-3′; MHC class I responsive element, 5′-GTG CTC GAA GGG CCG GGG GTT CCC ACA CCA-3′ and the complementary strand 5′-TGG TGT GGG AAC CCC CGG CCC TTC GAG CAC-3′. The probes were labeled following standard protocols using [^{32}P]dNTPs.

Transient transfection assay

Plasmid DNA used for transfection was prepared as described earlier (Disela *et al.*, 1991). Semiconfluent v-*rel*ER and v-*rel*-transformed CEFs as well as RCAS infected and uninfected CEFs were transfected using the calcium phosphate/DNA coprecipitation method (Graham and van der Eb, 1973). 16−20 h before transfection, 2×10^6 CEFs were plated in tissue culture dishes (10 cm diameter) in standard growth medium containing estrogen-depleted FCS. Cells were transfected with 2 μg pTK-3κB or pTK-3mutκB CAT reporter plasmid DNA (see above), respectively, together with a constant amount of 1 μg pRSVluc internal control plasmid (deWet *et al.*, 1987; in order to correct for variations in the transfection efficiencies). pBluescript (Stratagene) plasmid DNA was used to achieve a final amount of 20 μg DNA.

The calcium phosphate precipitate was left in contact with the cells for 20−24 h, followed by a complete medium change. Cells were then treated with 10^{-6} M estrogen, $10^{-8}-10^{-6}$ M OHT or 10^{-6} M ICI for 20−24 h and harvested using a rubber policeman. Cell extracts were prepared by three cycles of freeze−thawing. Aliquots of such extracts were analyzed for their protein content, CAT activity (Gorman *et al.*, 1982) and luciferase activity [deWet *et al.* 1987; using a Clinilumat bioluminescence counter (Berthold, Wildbach, Germany)]. A Phosphor-Imager (Molecular Dynamics) was used to quantify CAT activity.

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