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SHORT REPORTS

Characterization of a human REL-estrogen receptor fusion protein with a reverse conditional transforming activity in chicken spleen cells

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Overexpression of the human REL transcription factor can malignantly transform chicken spleen cells *in vitro*. In this report, we have created and characterized a cDNA encoding a chimeric protein (RELΔ424–490-ER) in which sequences of a highly transforming REL mutant (RELΔ424–490) are fused to the ligand-binding domain of the human estrogen receptor (ER). Surprisingly, RELΔ424–490-ER is constitutively nuclear in A293 cells, and RELΔ424–490-ER activates transcription in the absence, but not in the presence, of estrogen in κB-site reporter gene assays. Furthermore, RELΔ424–490-ER transforms chicken spleen cells in the absence of estrogen, but the addition of estrogen blocks the ability of RELΔ424–490-ER-transformed cells to form colonies in soft agar, even though estrogen induces increased nuclear translocation of RELΔ424–490-ER in these cells. ERα can also inhibit REL-dependent transactivation *in trans* in an estrogen-dependent manner, and ERα can interact with REL *in vitro*. Thus, the RELΔ424–490-ER fusion protein shows an unusual, reverse hormone regulation, in that its most prominent biological activities (transformation and transactivation) are inhibited by estrogen, probably due to an estrogen-induced interaction between the ER sequences and sequences in the Rel homology domain. Nevertheless, these results indicate that the continual activity of REL is required to sustain the transformed state of chicken spleen cells in culture, suggesting that direct and specific inhibitors of REL may have therapeutic efficacy in certain human lymphoid cancers.

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The *REL* gene (i.e. human c-*rel*) encodes a transcription factor in the Rel/NF-κB family. The *REL* gene is frequently amplified in human B-cell cancers, including Hodgkin's lymphomas and diffuse large B-cell lymphomas (reviewed in Gilmore *et al*., 2004). Moreover, overexpression of *REL* can transform primary chicken lymphoid cells *in vitro* (Gilmore *et al*., 2001; Starczynowski *et al*., 2003; Fan *et al*., 2004). Thus, REL may serve as a target for intervention in certain human B-cell malignancies.

Like other members of the Rel/NF-κB family, REL has an N-terminal DNA-binding/dimerization domain, called the Rel homology domain, and a C-terminal transactivation domain. Numerous studies have determined that the avian retroviral oncoprotein v-Rel and human REL must bind to DNA and activate transcription in order to transform chicken spleen cells *in vitro* (reviewed in Gilmore, 1999; Gilmore *et al*., 2004). These results suggest that Rel proteins induce oncogenesis through the chronic, low-level activation of gene expression in target lymphoid cells.

Fusion of the hormone-binding domain of the human estrogen receptor (ER) to v-Rel (Boehmelt *et al*., 1992; Capobianco and Gilmore, 1993), chicken c-Rel (Zurovec *et al*., 1998), and mouse c-Rel (Grumont *et al*., 1999) has been shown to confer estrogen-dependent transcriptional activation activity onto these proteins. Therefore, in an effort to create a conditional human c-Rel (REL) protein, we fused human ER sequences at the C terminus of a highly transforming mutant of REL, RELΔ424–490 (Figure 1a). RELΔ424–490 has a 66 amino-acid deletion that removes one of the two C-terminal transactivation subdomains, and RELΔ424–490 transforms chicken spleen cells more efficiently than wild-type REL (Starczynowski *et al*., 2003). Thus, RELΔ424–490-ER contains the intact REL DNA-binding/dimerization domain, one of the C-terminal REL transactivation subdomains, a three amino-acid linker, and the hormone-binding domain of human ERα (Figure 1a).

To express this fusion protein in avian cells, the RELΔ424–490-ER cDNA was subcloned into a spleen necrosis virus retroviral vector. Chicken embryo fibroblasts (CEF) were transfected with retroviral vectors for RELΔ424–490 or RELΔ424–490-ER in the presence of replication-competent helper virus plasmid DNA, and anti-REL Western blotting was performed (Figure 1b). CEF expressed an immunoreactive protein of the appropriate size for RELΔ424–490-ER, which was larger than RELΔ424–490 and was not detected in control CEF.

To assess the effect of estrogen on activities of RELΔ424–490-ER, CEF expressing RELΔ424–490-ER were treated with 2 μM 17-β-estradiol (estrogen) for 20 h.

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CEF were then subjected to indirect immunofluorescence using a primary anti-REL antiserum. As shown in Figure 1c, RELΔ424–490-ER was primarily a cytoplasmic protein in the absence of estrogen, but localized to the nucleus upon addition of estrogen. Moreover, nuclear extracts from RELΔ424–490-ER-expressing CEF treated with estrogen had increased nuclear κB site-binding activity, which was supershifted by anti-REL antiserum, as compared to the same cells grown in the absence of estrogen (data not shown). As controls, we show that the nuclear localization of nonfused RELΔ424–490 and the cytoplasmic localization of wild-type REL were not altered by the addition of estrogen (Figure 1c). Moreover, as we have shown previously (Capobianco and Gilmore, 1993), a v-Rel-ER protein also showed estrogen-dependent nuclear localization in CEF. Therefore, estrogen induces the RELΔ424–490-ER fusion protein to enter the nucleus and show increased DNA-binding activity in CEF.

To determine the effect of estrogen on the ability of RELΔ424–490-ER to activate transcription, we performed κB-site luciferase reporter gene assays in A293 cells, which have low levels of endogenous κB site-dependent transcription. In the absence of estrogen, RELΔ424–490-ER-expressing cells showed an approximately sevenfold increase in luciferase activity as compared to vector-transfected cells; in contrast, in the presence of estrogen, RELΔ424–490-ER-expressing cells showed low levels of luciferase activity that were similar to those seen in vector-transfected cells (Figure 2a). On the other hand, RELΔ424–490 activated transcription from the κB-site reporter plasmid in the absence or presence of estrogen and to approximately the same extent as RELΔ424–490-ER in the absence of estrogen.

Unlike what was seen by immunofluorescence in CEF (Figure 1c), nuclear extracts from A293 cells had similar amounts of RELΔ424–490-ER in the presence or absence of estrogen (Figure 2b). Moreover, the levels of nuclear RELΔ424–490-ER in A293 cells were similar to the nuclear levels of unfused RELΔ424–490 in these cells. As a control, we show that the cytoplasmic protein IκBβ is not present in these nuclear extracts (Figure 2b), indicating that the RELΔ424–490-ER seen in nuclear extracts from A293 cells is not due to cytoplasmic contamination. In addition, in the absence of estrogen, we detected increased nuclear κB-site DNA-binding activity in nuclear extracts from RELΔ424–490-ER-transfected cells as compared to vector-transfected cells,

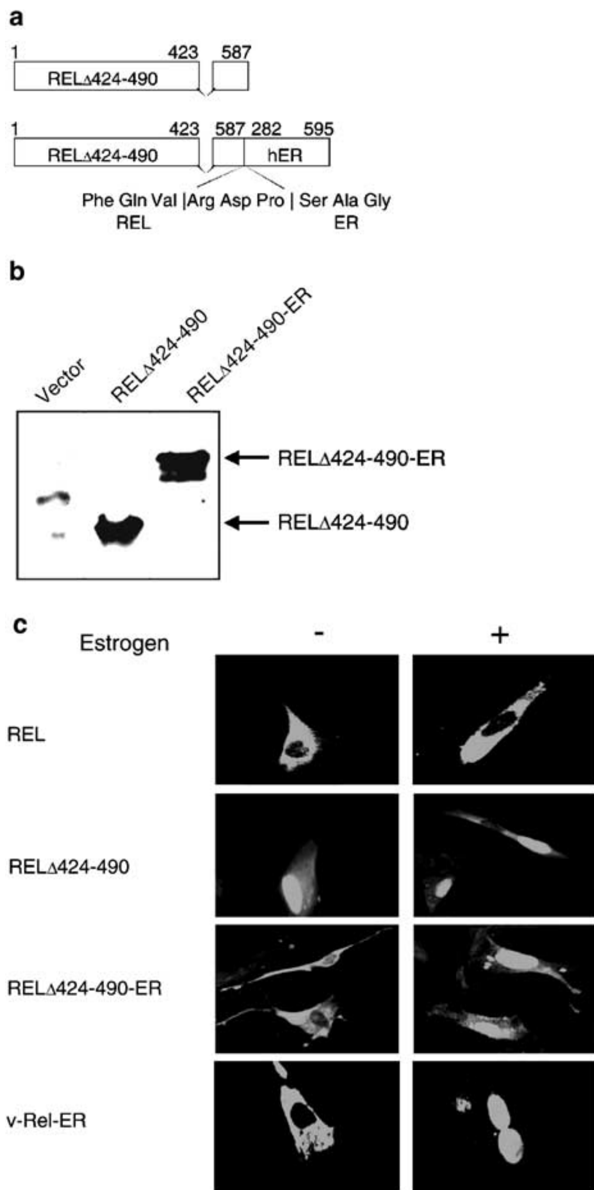
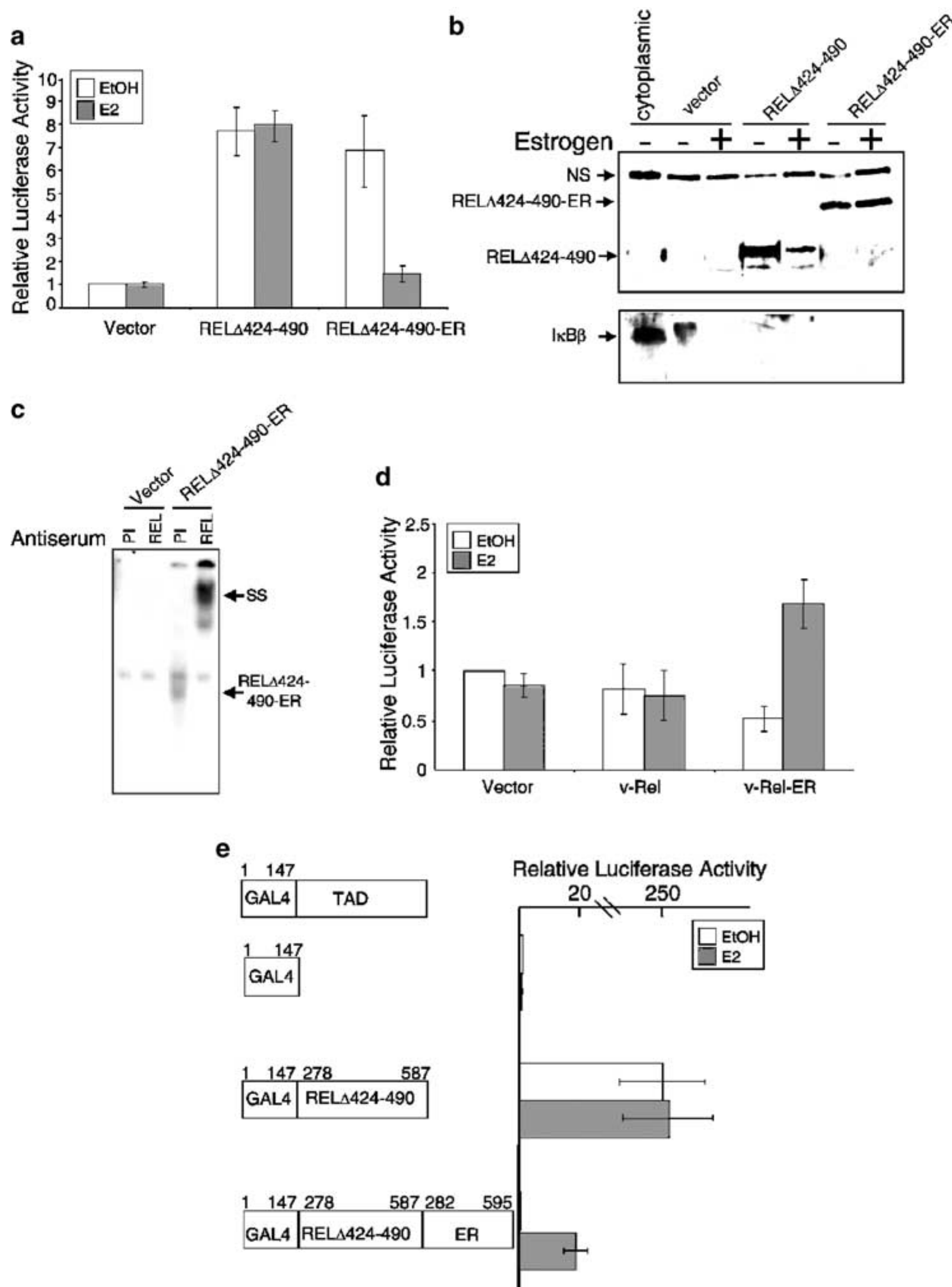


Figure 1 Expression and estrogen-dependent subcellular localization of a REL-ER fusion protein in CEF. (**a**) The structures of RELΔ424–490 and RELΔ424–490-ER are shown. RELΔ424–490-ER consists of full-length RELΔ424–490 (described in Starczynowski *et al*., 2003) (i.e. with aa 424–490 removed) fused to the hormone-binding domain of the human estrogen receptor (ER) (aa 282–595), separated by three linker amino acids (Arg-Asp-Pro). To create JD-RELΔ424–490-ER, an approximately 500 bp 3′ fragment of RELΔ424–490 (lacking the stop codon) was amplified by PCR with *Bam*HI restriction site-containing primers (forward, 5′CGCGGATCCGCGGCAGAATCCTACTATCCCTCACC 3′, and reverse, 5′CGGGATCCCGTACTTGAAAAAATTCATAT GGAAAGGAG 3′), the fragment was digested with *Bam*HI, and was fused to the estrogen-binding domain of ERα from pHE14 (Kumar *et al*., 1986). An *Apa*I/*Cla*I fragment containing the RELΔ424–490-ER fusion sequence was used to replace the 3′ sequence of *REL* in *Apa*I/*Cla*I-digested JD-REL (Gilmore *et al*., 2001). For more details, see http://people.bu.edu/gilmore/nf-kb/lab/publ.html. (**b**) CEF were transfected with retroviral expression vectors for the expression of no protein (Vector), RELΔ424–490, or RELΔ424–490-ER, and Rev-A helper virus as described previously (Kalaitzidis *et al*., 2002). Western blotting with an antiserum directed against a sequence in the Rel homology domain (1 : 2500) was then performed as described previously (Kalaitzidis and Gilmore, 2002; Kalaitzidis *et al*., 2002). The positions of the relevant proteins are indicated. (**c**) CEF were transfected with retroviral expression vectors for the expression of REL, RELΔ424–490, RELΔ424–490-ER, and v-Rel-ER, and cells were incubated in the absence (−) or presence (+) of 2 μM estrogen for 20 h. Indirect immunofluorescence was then performed as described previously using anti-REL and anti-v-Rel antiserum (Kalaitzidis and Gilmore, 2002)

and this activity could be supershifted by anti-REL antiserum (Figure 2c). These results indicate that, in A293 cells, the ER sequences modulate the transactivating activity of RELΔ424–490-ER, but do not affect nuclear localization or DNA binding.

Since the transactivation activity of RELΔ424–490-ER in A293 cells was the opposite of what we anticipated, that is, RELΔ424–490-ER-directed transactivation was inhibited by estrogen, we performed two additional control experiments. Unlike RELΔ424–490-ER, v-Rel-ER has previously been shown to be activated by estrogen in CEF (Boehmelt *et al*., 1992; Capobianco and Gilmore, 1993; Walker and Enrietto, 1996), and indeed, the κB-site transactivating ability of v-Rel-ER was also enhanced by estrogen in A293 cells (Figure 2d). In a second experiment, we replaced the REL DNA-binding domain by creating expression vectors for GAL4 DNA-binding domain fusion proteins that contained only the C-terminal transactivation domain sequences of RELΔ424–490 or RELΔ424–490-ER. We then conducted GAL4-site reporter gene assays in A293 cells in the presence and absence of estrogen (Figure 2e). GAL4-RELΔ424–490 activated transcription, as compared to GAL4 alone, to approximately the same extent with and without estrogen. On the other hand, GAL4-RELΔ424–490-ER activated transcription above control levels only in the presence of estrogen. Taken together, the results in Figures 2a and e indicate



that the ability of the fused ER sequences to inhibit transactivation by C-terminal sequences of RELΔ424–490 is dependent on the specific DNA-binding domain sequence to which these sequences are fused (i.e. REL *vs* GAL4).

To determine whether RELΔ424–490-ER could transform chicken spleen cells, *in vitro* liquid transformation assays (see Gilmore *et al*., 2001) were performed with the retroviral expression vector for RELΔ424–490-ER. Based on previous results wherein v-Rel-ER showed an estrogen-dependent ability to transform chicken lymphoid cells (Boehmelt *et al*., 1992; Capobianco and Gilmore, 1993), we initially attempted to establish transformed cultures with RELΔ424–490-ER in the presence of estrogen. In several frustrating attempts, we were only infrequently able to obtain transformed cultures in the presence of estrogen, and these cultures invariably expressed truncated RELΔ424–490-ER proteins missing ER sequences (see Figure 3c). Subsequently, we found that transformed cultures that expressed full-length RELΔ424–490-ER could be developed in the absence of estrogen (Figures 3a–c). As expected, RELΔ424–490 by itself efficiently transformed chicken spleen cells in the absence (Figure 3a) or presence of estrogen (data not shown). Although RELΔ424–490-ER transformed chicken spleen cell cultures slower and less efficiently than RELΔ424–490 (Figure 3a), the transformed cells that emerged from RELΔ424–490-ER liquid outgrowth assays looked quite similar to RELΔ424–490-transformed cultures (Figure 3b). Nevertheless, it was quite difficult to maintain RELΔ424–490-ER-transformed cultures for longer than about 2 months in culture, unlike RELΔ424–490-transformed cells that can be maintained for long periods of time. Thus, RELΔ424–490-ER appears to have a reduced transforming ability as compared to RELΔ424–490.

Among the 22 independent liquid outgrowth assays that we performed with RELΔ424–490-ER, six cultures were transformed and expressed a full-length RELΔ424–490-ER protein that could be detected by Western blotting with both anti-REL and anti-ER antiserum (Figure 3c). Two additional transformed cultures, however, expressed truncated RELΔ424–490-ER proteins, which were only slightly larger than RELΔ424–490 and could not be detected by anti-ER antiserum (data not shown). Thus, the cultures expressing the smaller RELΔ424–490-ER-derived proteins were likely transformed by deletion mutants that had lost ER sequences.

To assess the effect of estrogen on the growth of RELΔ424–490-ER-transformed cells, we placed RELΔ424–490-ER-transformed cells in soft agar in the presence or absence of 2 μM estrogen. Estrogen inhibited the ability of RELΔ424–490-ER-transformed cells to form colonies in soft agar (Figures 3d,e), whereas the addition of estrogen did not affect the ability of RELΔ424–490-transformed cells to form colonies in soft agar. Moreover, cells from a transformed culture that expressed a truncated RELΔ424–490-ER protein could form colonies in soft agar in the presence or absence of estrogen (Figure 3d).

Cells transformed by RELΔ424–490 and by full-length RELΔ424–490-ER (in the absence of estrogen) synthesized proteins of the correct sizes (Figure 3c). Nuclear extracts from RELΔ424–490- and RELΔ424–490-ER-transformed cells contained each of these proteins, as judged both by Western blotting (Figure 4a) and by κB site-binding activity (Figure 4b). However, the amount of RELΔ424–490-ER in nuclear

Figure 2 RELΔ424–490-ER activates transcription in the absence of estrogen and this activity is inhibited by estrogen. (**a**) A293 cells were transfected with expression plasmids for the indicated proteins and a κB-site luciferase reporter plasmid along with a pgk-β-galactosidase internal control. Cultures were also incubated with ethanol (EtOH) or 2 μM estrogen (E2) for 48 h. Cells were then lysed and a luciferase assay was performed as described previously (Kalaitzidis *et al*., 2002). Values are relative to the value obtained for pcDNA3 vector in the absence of estrogen (1.0) and are the averages of three independent experiments performed in triplicate, $\pm$standard error (s.e.). Similar results were obtained in experiments performed with charcoal/dextran-stripped FBS (Hyclone, Logan, UT, USA) (data not shown). pcDNA-RELΔ424–490 was created by subcloning an *Xba*I/*Xho*I fragment into the corresponding sites of pcDNA3.1(−). pcDNA-RELΔ424–490-ER was created by subcloning an *Apa*I–*Hin*dIII fragment containing full-length RELΔ424–490-ER into pcDNA3.1. (**b**) A293 cells were transfected with the indicated pcDNA expression vectors, and cells were then treated with ethanol (−) or 2 μM estrogen (+) for 48 h. Nuclear extracts were then prepared as described (Kalaitzidis *et al*., 2002), and samples were subjected to Western blot analysis with either anti-REL (1 : 1000; top panel) or IκBβ (#sc-946, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1 : 350; bottom panel) antisera. Lane 1 contains 2% of the cytoplasmic extract from pcDNA vector-transfected cells in the top panel and 7% from pcDNA vector-transfected cells in the bottom panel. In all, 16% of each nuclear extract was loaded for both blots. NS, nonspecific band. (**c**) An EMSA was performed with nuclear extracts essentially as described previously (Kalaitzidis *et al*., 2002) using 40 000 c.p.m. of a ^{32}P-labeled κB-site probe and 5 μg of nuclear extract from A293 cells transfected with the indicated expression plasmids in the absence of estrogen. As indicated, either preimmune (PI) or anti-REL (REL) antiserum was also included. The positions of the RELΔ424–490-ER/DNA complex and the supershifted (SS) complex are indicted. (**d**) A κB-site reporter assay was performed as in (**a**) with pcDNA vectors encoding the indicated proteins in A293 cells treated with ethanol or 2 μM estrogen for 48 h. Values are the averages of three independent experiments performed in duplicate, are relative to ethanol-treated, vector-transfected cells (1.0). (**e**) Fusion of the 3′RELΔ424–490-ER transactivation domain (TAD) sequences to the DNA-binding domain of GAL4 (aa 1-147) restores estrogen-dependent transactivation. A293 cells were transfected with the indicated plasmids along with a GAL4-site luciferase reporter plasmid and a CMV-βgal plasmid as an internal control. Cells were treated for 24 h with ethanol or estrogen, lysed, and luciferase assays were performed as above. Values are relative to the value obtained for GAL4 alone minus estrogen (1.0) and are the averages of three independent experiments performed in triplicate. To create GAL4-3′RELΔ424–490-ER, an *Apa*I/*Kpn*I fragment was subcloned into *Apa*I/*Kpn*I-digested pSG424-RELΔ424–490. GAL4 plasmids and the amounts of DNA used for transfection of A293 cells in 35 mm plates (0.3 μg of GAL4-site luciferase reporter, 2 μg of GAL4-fusion protein expression plasmid, and 0.1 μg CMV-βgal transfection efficiency plasmid) were as described previously (Starczynowski *et al*., 2003)

extracts of transformed spleen cells in the absence of estrogen was much lower than the amount of RELΔ424–490 in nuclear extracts. Nevertheless, nuclear extracts from RELΔ424–490-ER-transformed spleen cells grown in the absence of estrogen do contain κB-site DNA-binding activity that is detectable and can be specifically supershifted with REL antiserum (Figure 4b). The addition of estrogen to RELΔ424–490-ER-transformed spleen cells caused the RELΔ424–490-ER protein to accumulate in the nucleus and resulted in an increase in nuclear κB site-binding activity (Figures 4a,b).

The ER has been shown to undergo estrogen-induced proteolysis (Horigome *et al.*, 1988). Thus, we were concerned that the failure of RELΔ424–490-ER to transform cells in the presence of estrogen could be simply due to reduced stability of the ER fusion protein. However, cytoplasmic extracts from RELΔ424–490-ER-transformed spleen cells treated with estrogen did not show any decrease in the amount of RELΔ424–490-ER as compared to extracts from nontreated cells (Figure 4c).

We postulated that the negative regulation of REL activity by the ER sequences in RELΔ424–490-ER was due to an estrogen-induced intramolecular interaction. To determine whether ER sequences could inhibit REL-induced transactivation, we performed a κB-site reporter assay in A293 cells wherein either RELΔ424–490 or wild-type REL and full-length ERα were coexpressed from separate plasmids (Figures 4d,e). As expected, we found that both REL and RELΔ424–490 by themselves activate transcription from the κB-site reporter plasmid, and that this transactivating ability was not affected by estrogen or the estrogen receptor antagonist 4-hydroxy-tamoxifen. However, even in the absence of estrogen, coexpression of ERα slightly inhibited the ability of both REL proteins to activate reporter gene expression, and the addition of estrogen further enhanced the ability of ERα to suppress REL- and RELΔ424–490-induced transactivation. In contrast, the addition of 4-hydroxy-tamoxifen alleviated ERα-mediated inhibition of REL and RELΔ424–490-induced transactivation. Taken

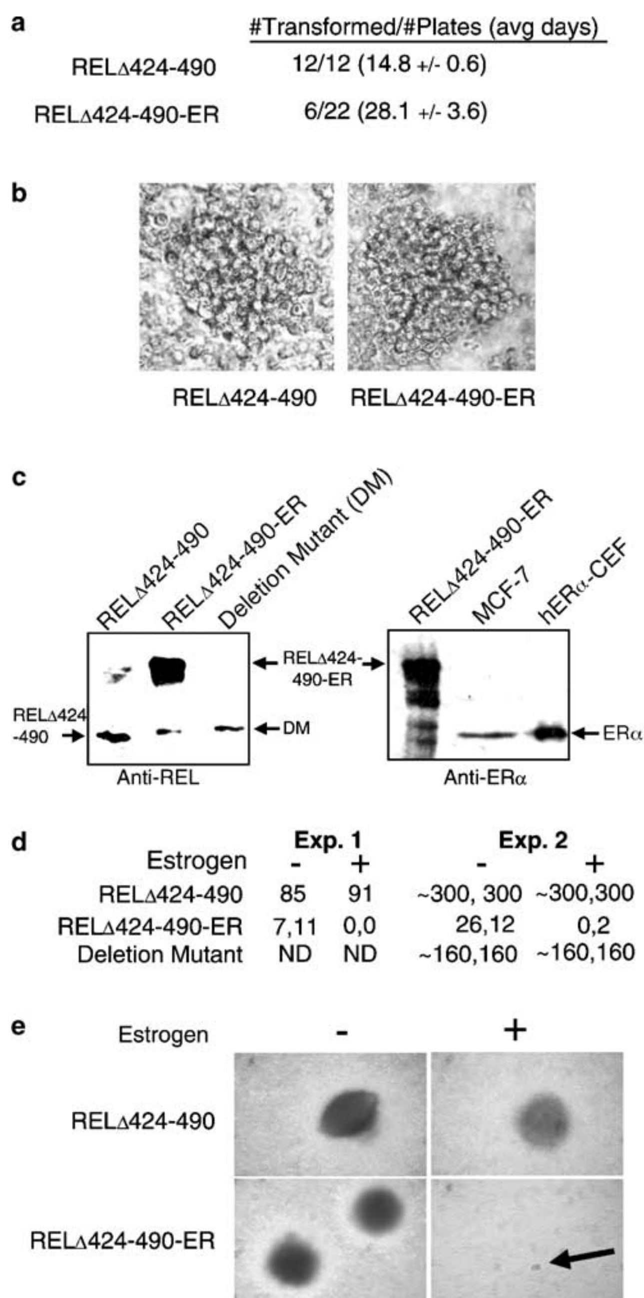


Figure 3 Transformation of chicken spleen cells in the absence of estrogen by RELΔ424–490-ER. (**a**) Primary chicken spleen cells were electroporated with retroviral expression vectors for RELΔ424–490 or RELΔ424–490-ER and Rev-A helper virus DNA. Cultures were then maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 20% fetal bovine serum (Biologos, Montgomery, IL, USA) without estrogen, until transformed cells grew out in liquid culture (as described in Gilmore *et al.*, 2001). The numbers of transformed plates, containing cells expressing the full-length indicated proteins, per number of plates electroporated are indicated. Number of days to transform in liquid culture is indicated in parentheses, ±s.e. The values are from five independent experiments. (As described in the text, two additional cultures electroporated with RELΔ424–490-ER became transformed, but expressed truncated RELΔ424–490-ER proteins.) (**b**) Cells from the indicated transformed cultures grown in the absence of estrogen were photographed. (**c**) Western blotting was performed on whole-cell extracts obtained from chicken spleen cells transformed by either RELΔ424–490, RELΔ424–490-ER, or a RELΔ424–490-ER spontaneous deletion mutant (DM) that appeared in cultures transformed in the presence of estrogen. In the left panel, anti-REL antiserum (1 : 1000) was used, and in the right panel, anti-ERα (Sigma, St Louis, MO, USA; cat. #E-0646; 1 : 2000) was used. Extracts from the MCF-7 human breast cancer cell line and CEF transfected with a pcDNA vector containing a full-length cDNA for human ERα were included in the right panel as positive controls. (**d**) Approximately, equivalent numbers of cells transformed by each of the indicated proteins were placed in soft agar, containing 20% FBS, DMEM, and 0.3% agar, in the presence or absence of 2 μM estrogen as indicated, and plates were incubated at 37°C for approximately 10 days. Numbers of soft agar colonies from each plate obtained in two experiments (Exp. 1 and 2) using independently derived transformed cultures are shown. ND, not done. (**e**) Photographs of representative soft agar colonies (from Figure 4d) are shown. The arrow points to a small, abortive colony

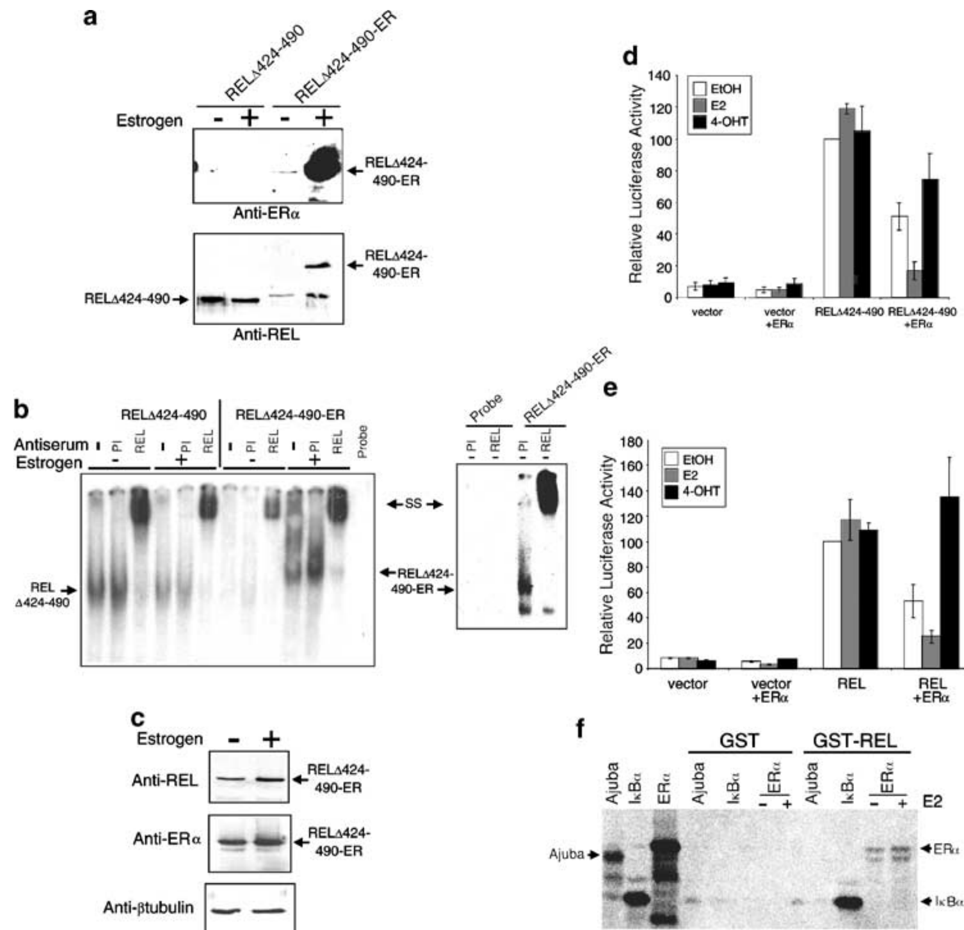


Figure 4 Estrogen-dependent regulation of RELΔ424–490 by ER sequences *in cis* and *in trans*. (**a**) Samples containing 5 μg of nuclear extracts from chicken spleen cells transformed by RELΔ424–490 or RELΔ424–490-ER and treated with either ethanol or 2 μM estrogen for 20 h were subjected to Western blotting with the indicated antisera. (**b**) An EMSA was performed using 20 000 c.p.m. of ^{32}P-labeled κB-site probe and 5 μg of nuclear extract from the indicated transformed cells treated with ethanol or 2 μM estrogen as in (**a**). Since the DNA-binding activity in nuclear extracts from RELΔ424–490-ER-transformed chicken spleen cells grown in the absence of estrogen is quite low, the last two lanes in the right panel used 10 μg of nuclear extract protein and 40 000 c.p.m. of ^{32}P-labeled κB-site probe. Where indicated, preimmune (PI) or anti-REL serum (REL) was added in a supershift assay. The relevant protein–DNA complexes and supershifted complexes (SS) are indicated. (**c**) Cytoplasmic extracts (15 μg) from cells transformed by RELΔ424–490-ER that had been treated with ethanol (−) or estrogen (+) for 20 h were subjected to Western blot analysis with the indicated antibodies. Anti-β tubulin antiserum (#sc-9104; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a concentration of 1 : 500. (**d**) κB-site reporter gene assays were performed in A293 cells as described for Figure 2a with the indicated expression plasmids. As indicated, cells were treated with either ethanol (EtOH), 2 μM estrogen (E2), or 1 μM 4-hydroxytamoxifen (4-OHT) (Sigma) for 48 h, and relative luciferase values were obtained. ERα was transfected at a 1 : 1 ratio with RELΔ424–490. Values are relative to the value obtained with ethanol-treated RELΔ424–490 (100), and are the averages of three experiments performed in duplicate for each condition, ±s.e. (**e**) κB-site reporter gene assays were performed as in (**d**), using wild-type REL in place of RELΔ424–490. (**f**) A GST pulldown assay was performed using GST alone or GST-REL (containing REL amino acids 1–323 from the Rel homology domain). The indicated *in vitro*-translated, ^{35}S-labeled proteins were challenged with the GST proteins and analysed essentially as described (Sif and Gilmore, 1994). The first three lanes contain 64% of the input for each *in vitro*-translated protein used in the GST pulldown experiment. Coomassie staining of the gel confirmed equivalent loading of GST proteins (data not shown). Where indicated, the GST pulldown experiments were performed in the presence of 10 nM β-estradiol. To create the GST-REL expression plasmid (pGEXkg-REL), an approximately 1 kbp fragment containing sequences of the Rel homology domain of REL was amplified by PCR from pGEM4-REL using a forward SP6 primer and a *Hin*dIII site-containing primer that included a stop codon (underlined) after *REL* codon 323 (reverse 5′CCCAAGCTT<u>TTA</u>TCCAATTGAACCGAGGAG 3′). The PCR product was then digested with *Nco*I/*Hin*dIII and the fragment was subcloned into *Nco*I/*Hin*dIII-digested pGEXkg to create an in-frame GST-REL fusion

together, these results indicate that the ability of ERα to inhibit REL-directed transactivation is enhanced by estrogen. Similar to our results, estrogen has previously been shown to enhance the ability of ERα to repress RelA-dependent transactivation (Valentine *et al*., 2000).

Finally, we sought to determine whether ERα could directly interact with REL sequences. Therefore, we performed a GST-pulldown assay with a GST-fusion protein (GST-REL) containing sequences from the N-terminal Rel homology domain of REL and *in vitro*-translated ^{35}S-labeled ERα (Figure 4f). In this

experiment, GST-REL could specifically interact with *in vitro*-translated ERα. That is, GST alone did not pulldown ERα nor did GST-REL interact with an unrelated protein Ajuba, and, as a positive control, we show that GST-REL interacts with *in vitro*-translated IκBα (Figure 4f).

In this report, we have characterized a conditionally active form of the human REL transcription factor in which sequences from the hormone-binding domain of ER are fused to the C terminus of a highly transforming REL protein, RELΔ424–490. Curiously, estrogen inhibits the transactivating and transforming activities of RELΔ424–490-ER, but estrogen induces the nuclear translocation of RELΔ424–490-ER in CEF and chicken spleen cells. Nevertheless, the results in this paper demonstrate that sustained activity of RELΔ424–490 is required for the maintenance of the transformed state in chicken spleen cells. Thus, these results suggest that REL is a suitable target in human cancers in which the *REL* gene is amplified or otherwise activated.

Several previous reports have demonstrated that full-length ERα can inhibit NF-κB-induced transactivation in an estrogen-dependent manner (Ray *et al*., 1994; Stein and Yang, 1995; Ray *et al*., 1997; Cerillo *et al*., 1998; Valentine *et al*., 2000; Evans *et al*., 2001; Sharma *et al*., 2001; Tzagarakis-Foster *et al*., 2002), but the mechanism by which this inhibition occurs is unclear. However, two studies have reported that ERα can bind preferentially to REL, as compared to other NF-κB subunits. In one study, REL activity was shown to be inhibited by ERα *in vitro* (Galien and Garcia, 1997). In a second study, van Gool *et al*. (2002) showed that the hormone-binding domain of ERα can interact with sequences in the Rel homology domain of REL in a two-hybrid assay in an estrogen-dependent manner, and, similar to our results, that estrogen-activated full-length ERα can inhibit *in trans* REL-induced transactivation in reporter gene assays. Moreover, we show here (Figure 4f) that REL can interact directly with ERα in a GST pulldown assay. Thus, the simplest explanation for the inhibitory effect of estrogen on RELΔ424–490-ER *in vivo* is that estrogen induces an intramolecular interaction between the hormone-binding domain of ER and sequences in the Rel homology domain of REL. This interaction between REL and ER sequences does not block the ability of RELΔ424–490-ER to bind to DNA (Figure 4b), but does block its ability to activate transcription (Figure 2a) and hence, transform cells. An alternative, but less likely, hypothesis for the reverse conditional transforming activity of RELΔ424–490-ER is that in the absence of estrogen it is a nuclear truncated form of RELΔ424–490-ER (as seen in Figure 4a) that causes transformation, whereas in the presence of estrogen, the induced nuclear localization of full-length RELΔ424–490-ER inhibits this truncated, transforming form of RELΔ424–490-ER, and hence the cells lose the transformed state.

We have previously shown that RELΔ424–490 is primarily a cytoplasmic protein in transformed chicken spleen cells, but that its nuclear transactivating activity, mediated by a small proportion of constitutively active and nuclear RELΔ424–490, is required for its transforming ability (Starczynowski *et al*., 2003). Therefore, in the absence of estrogen, RELΔ424–490-ER-transformed chicken spleen cells must contain sufficient nuclear transactivating activity to maintain the transformed state. This low-level nuclear activity in the absence of estrogen may be due to incomplete inhibition of the RELΔ424–490-ER fusion protein by heat-shock proteins, a phenomenon that has been reported for some heterologous hormone-binding domain fusion proteins (Picard, 2000).

Intriguingly, RELΔ424–490-ER and v-Rel-ER respond differently to estrogen. That is, the transforming and transactivating activities of v-Rel-ER are activated by estrogen (Boehmelt *et al*., 1992; Capobianco and Gilmore, 1993; Figure 2b), whereas both of these activities for RELΔ424–490-ER are inhibited by estrogen (Figures 2a and 3a,d). Thus, it is likely that in v-Rel-ER the ER sequences cannot form the intramolecular inhibitory interaction with the v-Rel sequences. Whether the contrasting effects of estrogen on v-Rel-ER *versus* RELΔ424–490-ER are due to a mutation that has occurred in v-Rel (for example, a mutation that abolishes its ability to be regulated by estrogen-ER), are due to a natural sequence difference between v-Rel and human REL, or are due to a peculiarity of the fusion design is not clear. Of note, there is an amino-acid sequence in human REL (LTTAL, aa 163–167) that is similar to a consensus nuclear receptor interacting motif (LXXLL) that can be bound by ERα (Gee *et al*., 1999; Robyr *et al*., 2000); the corresponding sequence is different in v-Rel (YTLAL, aa 171-175), chicken c-Rel (YTLAL, aa 162–166), and mouse c-Rel (FTTAV, aa 163–167), all of which are regulated in the conventional fashion when expressed as ER fusion proteins (Capobianco and Gilmore, 1993; Zurovec *et al*., 1998; Grumont *et al*., 1999).

Lastly, we would like to speculate that our results suggest that estrogen therapy could be used to treat lymphomas (or other tumors) that are dependent on continuous transcriptional activation by REL. That is, estrogen treatment may kill REL-dependent tumor cells that express ERα, either from the endogenous gene or an ectopic gene. Of note, some Hodgkin's lymphoma cells do express ER (Maia *et al*., 2000), and in at least one case, a patient with a Hodgkin's lymphoma (which commonly have amplified *REL*) showed a dramatic response after treatment with an estrogen analog (Stark *et al*., 1981).

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