

SHORT REPORTS

Deletion of either C-terminal transactivation subdomain enhances the *in vitro* transforming activity of human transcription factor REL in chicken spleen cellsDaniel T Starczynowski¹, Joseph G Reynolds¹ and Thomas D Gilmore^{*,1}¹Department of Biology, Boston University, 5 Cummington Street, Boston, MA 02215, USA

The *REL* gene is amplified in many human B-cell lymphomas and we have previously shown that expression of *REL* from a retroviral vector can malignantly transform chicken spleen cells *in vitro*. To identify *REL* protein functions necessary for malignant transformation, we have performed deletion analysis on *REL* sequences encoding residues of two C-terminal subdomains that are involved in transcriptional activation. We find that deletion of both C-terminal transactivation subdomains abolishes the ability of *REL* to transform chicken spleen cells *in vitro*. In contrast, deletion of either transactivation subdomain alone, which reduces the transactivation ability of *REL*, enhances the transforming activity of *REL*. Transforming *REL* mutants missing C-terminal sequences can also be selected at a low frequency *in vitro*. The *REL* transactivation domain can be functionally replaced in transformation assays by a portion of the VP16 transactivation domain that activates at a level similar to *REL*-transforming mutants. We also find that deletion of 29 C-terminal amino acids causes the subcellular localization of *REL* to change from cytoplasmic to nuclear in chicken embryo fibroblasts. In contrast, wild-type *REL* and all transforming *REL* mutants are located primarily in the cytoplasm of transformed spleen cells. Nevertheless, treatment of transformed spleen cells with leptomycin B causes wild-type *REL* and two *REL* mutants to relocalize to the nucleus, and nuclear extracts from these transformed cells contain *REL* DNA-binding activity. Taken together, these results suggest the following: (1) that *REL* must activate transcription to transform cells *in vitro*; (2) that a reduced level of transactivation enhances the oncogenicity of *REL*; (3) that *REL* shuttles from the cytoplasm to the nucleus in transformed chicken spleen cells; and (4) that mutations in *REL*, in addition to amplifications, could activate its oncogenicity in human lymphomas.

Oncogene (2003) 22, 6928–6936. doi:10.1038/sj.onc.1206801**Keywords:** c-Rel; Rel; NF- κ B; malignant transformation; lymphoma**Introduction**

The Rel transcription factor has been directly implicated in animal and human lymphoid cell cancers in several ways. First, v-Rel encoded by the avian Rev-T retrovirus causes rapidly fatal lymphoid cell tumors in young birds, transforms a variety of avian cell types *in vitro*, and can cause T-cell lymphomas in transgenic mice (reviewed in Gilmore, 1999). Second, a retroviral insertion upstream of *c-rel* has been found in one chicken B-cell lymphoma (Kabrun *et al.*, 1990). Third, the human *REL* gene is frequently amplified in human B-cell lymphomas, including Hodgkin's lymphomas (Joos *et al.*, 2002; Martín-Subero *et al.*, 2002), diffuse large-cell B-cell lymphomas (Lu *et al.*, 1991; Houldsworth *et al.*, 1996; Joos *et al.*, 1996; Barth *et al.*, 1998, 2001; Rao *et al.*, 1998; Palanisamy *et al.*, 2002; Rosenwald *et al.*, 2002), and follicular lymphomas (Goff *et al.*, 2000; Nagy *et al.*, 2000; Neat *et al.*, 2001). Fourth, the *REL* locus has undergone genetic alterations in one diffuse large B-cell lymphoma cell line (Kalaitzidis and Gilmore, 2002; Lu *et al.*, 1991) and one primary Hodgkin's lymphoma (Barth *et al.*, 2003), which result in the production of C-terminally truncated *REL* proteins. Finally, chicken, mouse, and human c-Rel can transform chicken lymphoid cells *in vitro* (reviewed in Gilmore, 1999; Gilmore *et al.*, 2001).

Like other Rel/NF- κ B family members, c-Rel has an N-terminal domain (the Rel homology domain) that mediates DNA binding, dimerization, nuclear translocation, and inhibitor (I κ B) binding. c-Rel can form homodimers or heterodimers with other cellular Rel/NF- κ B family members, such as p50, p52, and RelA (p65). The C-terminal half of human c-Rel (*REL*) has a transactivation domain, with two distinct regions: an extreme C-terminal subdomain (domain II, amino acids (aa) 540–587) that shows basal activity and a subdomain from approximately aa 422 to 497 (domain I) that shows both basal activity and enhanced transactivation in response to external stimuli such as tumor necrosis factor or phorbol ester (Martin *et al.*, 2001). Thus, c-Rel complexes generally activate target gene expression; however, at least one study has indicated that c-Rel can also repress transcription (Grigoriadis *et al.*, 1996). *c-rel* knockout mice are viable, but have defects in B-cell function, especially in the

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mitogen-induced proliferative response (Köntgen *et al.*, 1995). Thus, c-Rel appears to play a key role in the growth and survival of B cells by modulating target gene expression.

Extensive mutational studies on the retroviral oncoprotein v-Rel have indicated that v-Rel must form homodimers, bind to DNA, and activate transcription to transform chicken lymphoid cells *in vitro* (reviewed in Gilmore, 1999). That is, mutations that disrupt any of these three functions abolish the transforming activity of v-Rel. Although the N-terminal Rel homology domains are highly conserved among human, mouse, and avian c-Rel, residues of C-terminal to the Rel homology domain are much less conserved. Indeed, the aa sequence identity between the C-terminal halves of chicken and human c-Rel is only about 10% (Gilmore *et al.*, 2001). Nevertheless, all these C-terminal c-Rel sequences contain transcription activation domains (Bull *et al.*, 1990; Kamens *et al.*, 1990; Martin *et al.*, 2001), and avian and mammalian c-Rel proteins can transform chicken lymphoid cells in culture (Gilmore *et al.*, 2001).

In this report, we have performed deletion analysis to identify sequences in the C-terminal half of human REL that are important for its transforming activity in chicken spleen cells. Our results indicate that REL must activate transcription in order to transform chicken lymphoid cells *in vitro*, but that certain deletions that reduce the transactivation function of REL enhance its transforming activity.

Results

Deletion of 150 C-terminal residues from human REL abolishes its *in vitro* transforming activity

Previous results have shown that residues C-terminal to the Rel homology domain of v-Rel are required for transactivation and transformation (Sarkar and Gilmore, 1993; Smardova *et al.*, 1995; Chen *et al.*, 1999). To determine whether C-terminal sequences of human REL are also required for transformation, we constructed a series of C-terminal deletion mutants of REL, missing 29, 58, 90, 110, 132, and 150 aa (Figure 1b). All mutants contain an intact Rel homology DNA-binding domain, but are missing all or part of the two C-terminal transactivation subdomains (called I and II herein). All REL mutants were then transcribed and translated *in vitro* to confirm that they encoded proteins of the appropriate sizes (data not shown).

Previously, we found that coexpression of chicken Bcl-2 enhances the transforming activity of REL *in vitro* (Gilmore *et al.*, 2001). Therefore, as the most sensitive assessment of the transforming activity of these mutants, cDNAs encoding the C-terminal REL mutants were inserted into a bi-cistronic avian retroviral vector that also directs the expression of chicken Bcl-2 (Figure 1a). First, we showed that all mutants synthesized REL proteins of the appropriate sizes after transfection of the retroviral expression plasmids

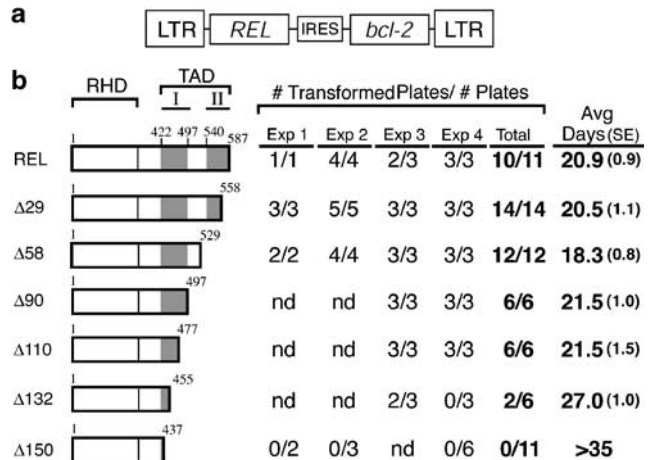


Figure 1 Transformation by C-terminal deletion mutants of REL. (a) The general structure of the bi-cistronic spleen necrosis virus vector used to express the C-terminal REL deletion mutants and chicken *bcl-2*. (b) The structures of the REL deletion mutants are shown on the left-hand side with the number of amino acids in each construct indicated above the boxes. RHD indicates the Rel homology domain; I and II indicate two C-terminal transactivation (TAD) subdomains, as described by Martin *et al.* (2001). The columns in the center list the number of plates that were transformed in liquid culture over the number of plates tested. The numbers on the right-hand side correspond to the average number of days (with standard error (S.E.)) that it took for the plates to become overgrown with transformed cells (see Gilmore *et al.*, 2001).

into chicken embryo fibroblasts (CEF) (Figure 2a). These REL mutants were then assayed for the ability to transform primary chicken spleen cells using a sensitive liquid outgrowth assay that we have developed (Gilmore *et al.*, 2001). Mutants Δ29, Δ58, Δ90, and Δ110 could all transform chicken spleen cells approximately as effectively as wild-type REL (Figure 1b). Mutant Δ132 was less transforming as judged by the reduced number of plates that were transformed, the increased time it took for transformed plates to emerge, and the failure of transformed plates to survive extended passage in culture (Figure 1b; data not shown). REL mutants missing 150 or 282 C-terminal residues (the entire C-terminal domain) were not transforming (Figure 1b; data not shown).

Western blotting on extracts from transformed spleen cells confirmed that all transforming REL proteins synthesized proteins of the expected sizes: that is, REL proteins from transformed spleen cells comigrated on SDS-polyacrylamide gels with the corresponding proteins synthesized in CEF (Figure 2b). This suggests that the REL deletion mutants were not consistently undergoing further mutations in the spleen cell transformation assays.

The retroviral oncoprotein v-Rel was previously shown to be primarily a cytoplasmic protein in transformed chicken spleen cells (Gilmore and Temin, 1986). Similarly, we find that all transforming REL mutants are primarily cytoplasmic proteins in transformed chicken spleen cells, as judged by indirect immunofluorescence (Figure 2c).

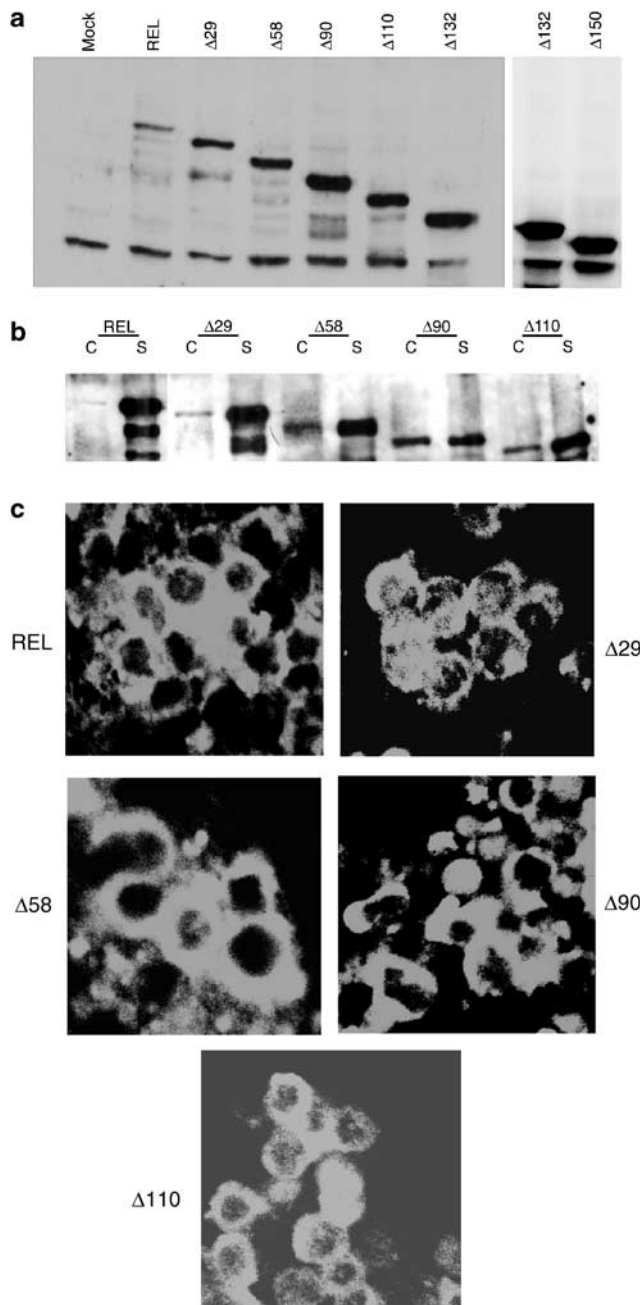


Figure 2 Expression of REL C-terminal deletion mutants *in vivo*. (a) Bi-cistronic retroviral expression vectors for the indicated proteins (see Figure 1) were transfected into CEF in the presence of helper virus. After 4 days, cell extracts were analysed by anti-REL Western blotting. The expression plasmids used are indicated above each lane. (b) Extracts from transfected CEF (C) (as in (a)) and transformed spleen cells (S) were analysed by anti-REL Western blotting. (c) Indirect immunofluorescence using an anti-REL antiserum was performed on spleen cells transformed by the indicated REL proteins

Deletion of 29 aa converts human REL from a cytoplasmic protein into a nuclear protein in CEFs

v-Rel is a nuclear protein in CEF, whereas chicken c-Rel is largely a cytoplasmic protein in these cells; moreover, deletion of C-terminal residues of chicken c-Rel, as

found in v-Rel, confers nuclear localization onto c-Rel (Gilmore and Temin, 1986; Capobianco *et al.*, 1990). To investigate the effect of C-terminal deletions on the subcellular localization of REL in fibroblasts, CEF were infected with viruses expressing REL C-terminal deletion mutants and were then analysed by indirect immunofluorescence (Figure 3a). Consistent with previous results, wild-type chicken c-Rel (Capobianco *et al.*, 1990) and human REL (Kalaitzidis and Gilmore, 2002) were both primarily cytoplasmic proteins in CEF. In contrast, REL deletion mutants Δ29, Δ58, and Δ150 were located almost exclusively in the nucleus of CEF. As a control, we also show that v-Rel is located in the nucleus of CEF. Therefore, the removal of as few as 29 aa is sufficient to cause the subcellular localization of

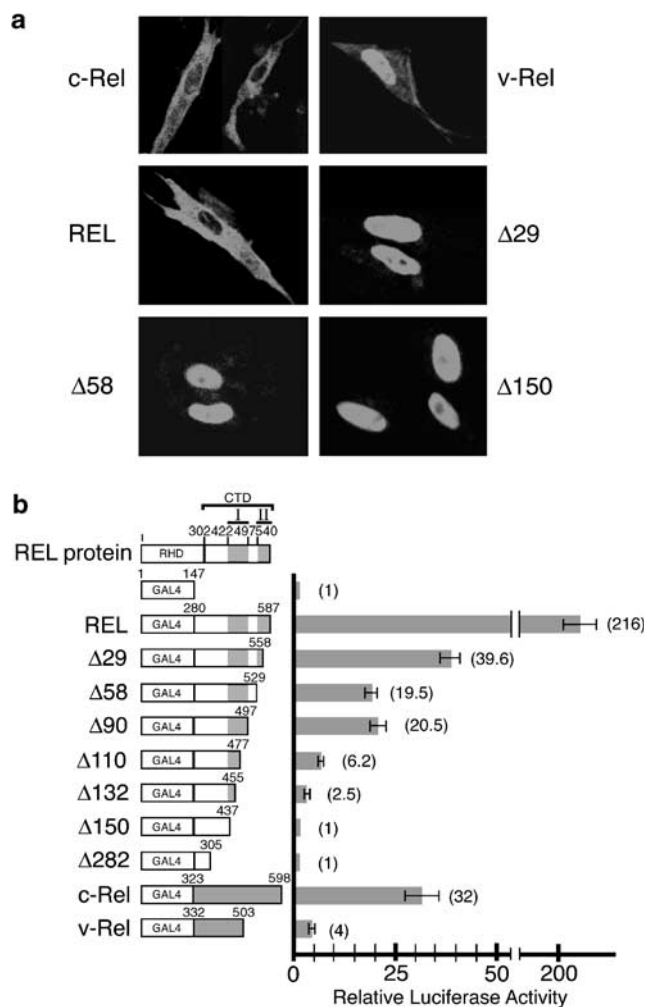


Figure 3 Effect of C-terminal deletions in REL on subcellular localization and transcriptional activation in CEF. (a) CEF transfected with expression vectors for the indicated proteins were analysed by indirect immunofluorescence with anti-v-Rel primary antiserum (v-Rel and chicken c-Rel) or anti-REL antiserum (human REL proteins). (b) GAL4 fusion proteins containing C-terminal domains (CTD) from the indicated proteins were analysed for their ability to activate transcription from a GAL4 site reporter plasmid in CEF. Values are relative to the activity seen with GAL4 alone (1.0) and in all cases, are the averages of at least three experiments performed with triplicate samples. c-Rel, chicken c-Rel; REL, human c-Rel

REL to switch from primarily cytoplasmic to largely nuclear in CEF.

REL C-terminal mutants that transform cells can also activate transcription, whereas nontransforming REL mutants cannot activate transcription

REL has previously been shown to have two C-terminal transactivation subdomains that are essentially located in the C-terminal 165 aa (Martin *et al.*, 2001). To determine the effect of the C-terminal deletions on transactivation by REL, we measured the activity of GAL4 fusion proteins containing C-terminal sequences from wild-type REL or the C-terminally truncated mutants in GAL4 site reporter gene assays in CEF (Figure 3b). GAL4 fusion proteins containing wild-type REL sequences or sequences from $\Delta 29$, $\Delta 58$, $\Delta 90$, $\Delta 110$, and $\Delta 132$, all activated transcription above control (GAL4 alone) levels. Even though the C-terminal sequences from all REL mutants activated transcription much less strongly than wild-type REL sequences, they activated transcription in the same range as C-terminal sequences from the highly oncogenic v-Rel protein and from chicken c-Rel in these GAL4 reporter assays. In contrast, C-terminal sequences from the nontransforming mutants REL $\Delta 150$ and REL $\Delta 282$ did not activate transcription above the levels seen with GAL4 alone. These results show that C-terminal sequences of transforming REL proteins activate transcription, albeit across a broad range, whereas C-terminal sequences from nontransforming REL proteins do not activate transcription.

Deletion of either transactivation subdomain I (REL $\Delta 424$ –490) or II (REL $\Delta 58$) enhances the transforming activity of REL

We were next interested in analysing the transforming and transactivating activities of REL mutants that were missing either transactivation subdomain I (REL $\Delta 424$ –490) or II (REL $\Delta 58$) (Figure 4a). As compared to wild-type REL sequences, transactivation by GAL4 fusions of REL $\Delta 424$ –490 and REL $\Delta 58$ was reduced by about 6–10-fold (Figure 4a).

In preliminary experiments, we found that REL $\Delta 424$ –490 and REL $\Delta 58$ both transformed chicken spleen cell cultures more rapidly than wild-type REL, when both were expressed with *bcl-2* (Figure 1b; data not shown). In addition, we previously showed that wild-type REL can transform chicken lymphoid cells in the liquid outgrowth assay, but REL does not efficiently induce colony formation in soft agar, whereas the highly transforming v-Rel oncoprotein can readily induce colony formation in soft agar (Gilmore *et al.*, 2001). To determine whether the apparent increased transforming activity of REL $\Delta 424$ –490 and REL $\Delta 58$, when coexpressed with Bcl-2 in liquid transformation assays, was an inherent property of these REL mutants, we first created retroviral expression vectors for REL, REL $\Delta 424$ –490, and REL $\Delta 58$ by themselves. These vectors were then used to compare the transforming activities of REL, REL $\Delta 424$ –

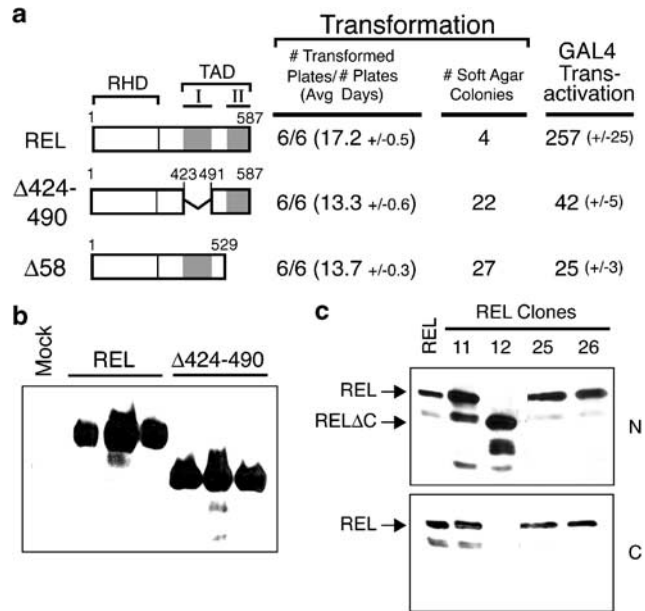


Figure 4 Effect of deletion of individual transactivation subdomains on REL protein activity. (a) The general structures of the REL proteins analysed are shown on the left-hand side. REL, REL $\Delta 424$ –490, and $\Delta 58$ were analysed in single gene retroviral vectors (JD214BS +, see Materials and methods). The activities of these proteins are then shown for the following: liquid outgrowth transformation (from two independent experiments performed in triplicate; average days to transform in parenthesis); soft agar colony formation (average number of colonies per plate from two independent assays performed in triplicate); and transcriptional activation as GAL4 fusion proteins (values are relative to GAL4 alone, as in Figure 3b). (b) Anti-REL Western blot (using an anti-C-terminal REL antiserum) on extracts of seven primary spleen cell cultures electroporated independently with no viral DNA (Mock), JD-REL, or JD-REL $\Delta 424$ –490, as indicated. Cell extracts were prepared 5 days after electroporation and equal amounts of protein were loaded in each lane. (c) Anti-REL Western blotting of extracts from transformed spleen cells established in the liquid outgrowth assay (REL) or from four independent REL-transformed cell clones established first in the soft agar assay and then expanded in liquid culture. N, anti-Rel homology domain REL antiserum; C, anti-C-terminal REL antiserum (see Materials and methods). Arrows indicate full-length REL and a C-terminally truncated form of REL (REL ΔC) present in cell clone 12

490, and REL $\Delta 58$ in both the liquid outgrowth assay and the more stringent soft agar colony assay. As shown in Figure 4a, REL $\Delta 424$ –490 and REL $\Delta 58$ transformed cultures more rapidly than REL in the liquid outgrowth assay and induced soft agar colony formation approximately 5–7 times more efficiently than wild-type REL. As a control, we performed Western blotting with an anti-C-terminal REL antiserum to show that primary spleen cell cultures electroporated with the retroviral vectors for REL or REL $\Delta 424$ –490 expressed similar amounts of protein shortly after electroporation (Figure 4b); REL $\Delta 58$ is also expressed at similar levels in such cultures, as judged by Western blotting with an anti-REL antiserum against residues within the RH domain (data not shown). Taken together, these results indicate that mutants REL $\Delta 424$ –490 and REL $\Delta 58$ are inherently more transforming than wild-type REL.

Although wild-type REL induced colonies in soft agar at a low frequency (Figure 4a), we were able to pick and expand these colonies in liquid culture. Western blotting of extracts from cells derived from 41 individual soft agar colonies identified one cell clone that expressed a REL protein that was smaller than wild-type REL and that was not detected by a C-terminal anti-REL antiserum, indicating that this mutant REL is missing C-terminal sequences. An analysis of four such cell clones, including the one expressing the truncated REL protein, is shown in Figure 4c. Thus, the soft agar assay does appear to be able to select for REL C-terminal deletion mutants, as sometimes occurs with chicken c-REL (Hrdlicková *et al.*, 1994; Gilmore *et al.*, 1995). By SDS-polyacrylamide gel electrophoresis, we cannot exclude the possibility that the apparently full-length REL proteins that induce soft agar colony formation have single aa mutations or other small changes.

In line with the analysis of other REL mutants, we also analysed the expression and subcellular localization of REL Δ 424–490. By Western blotting, the appropriately sized REL Δ 424–490 protein was expressed in CEF and in transformed chicken spleen cells (Figure 5a). Moreover, REL Δ 424–490 was found in the cytoplasm of transformed spleen cells (see Figure 7a below), but was in the nucleus of CEF (Figure 5b).

An attenuated version of the VP16 transactivation domain, but not the full-length VP16 transactivation domain, can functionally substitute for the REL transactivation domain in transformation assays

The above results indicated that a C-terminal transactivation domain was required for transformation by REL. To determine whether a heterologous transactivation domain could functionally substitute for the REL transactivation domain, sequences encoding either the

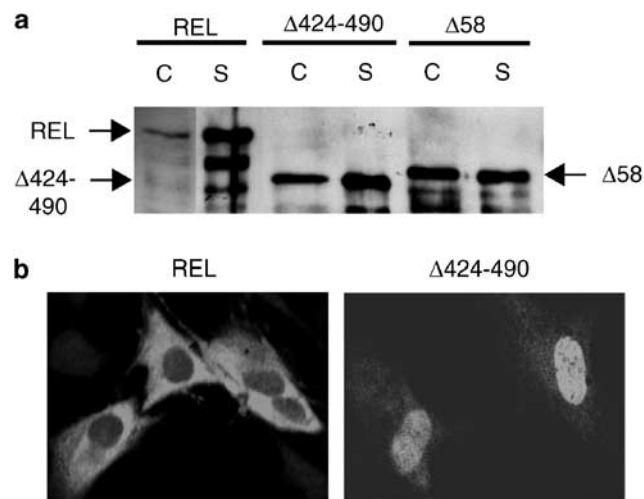


Figure 5 Expression and subcellular localization of REL Δ 424–490. (a) Anti-REL Western blotting of extracts from transfected CEF (C) or transformed spleen cells (S). (b) Indirect immunofluorescence using anti-REL primary antiserum of CEF expressing the indicated proteins

entire VP16 transactivation domain or the C-terminal portion of the VP16 transactivation domain were added to the C-terminus of a REL mutant missing the entire C-terminal transactivation domain (REL Δ 164) (Figure 6a). The addition of the entire VP16 domain conferred strong transactivating activity onto the chimeric REL Δ 164–VP16 protein (about 10-fold higher than wild-type REL), whereas the REL mutant containing the N-terminally deleted form of the VP16 domain (REL Δ 164–VP16 Δ N) activated about 4–5-fold less than REL (and at a level similar to highly transforming REL mutants described above, e.g., Δ 58 or Δ 424–490). Although neither REL Δ 164 nor REL Δ 164–VP16 could transform chicken spleen cells in liquid outgrowth assays, REL Δ 164–VP16 Δ N could readily transform cells (Figure 6a). Figure 6b shows that the appropriately sized REL Δ 164–VP16 Δ N protein is made in CEF and in transformed spleen cells. These results demonstrate that a heterologous transactivation domain (VP16 Δ N) can functionally substitute for the REL transactivation domain for transformation, and suggest that REL must transactivate within a certain range for transformation to proceed.

Treatment of transformed spleen cells with nuclear export inhibitor leptomycin B causes REL-transforming proteins to accumulate in the nucleus

One apparent discrepancy in the above-described experiments is that while our deletion analysis indicated

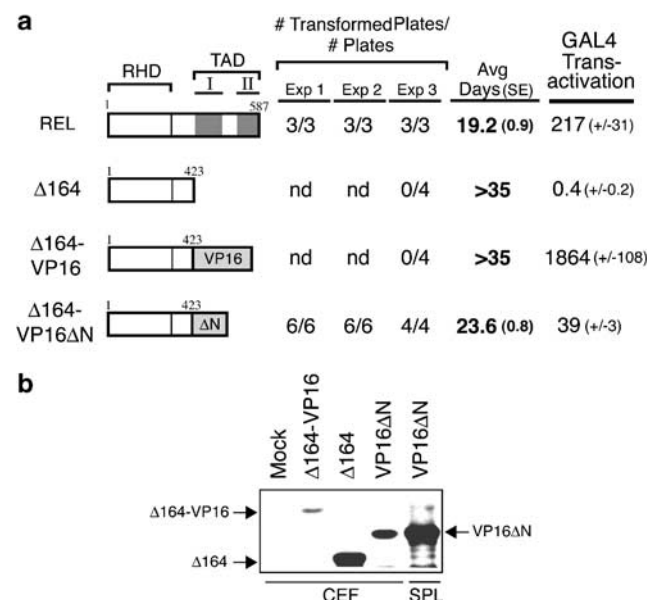


Figure 6 Effect of substitution of the VP16 transactivation domain on REL protein activity. (a) The structures and transforming and GAL4-fusion transactivation activities of wild-type REL, REL Δ 164, and the two REL Δ 164–VP16 fusion proteins are shown. Assays were conducted as described in Figure 4. nd, not done; SE, standard error. REL Δ 164 and REL Δ 164–VP16 were also assayed in combination with Bcl-2 (as in Figure 1b), and in each case, 0/6 plates were transformed over two assays (data not shown). (b) Anti-REL Western blotting of extracts from transfected CEF or transformed spleen cells (SPL)

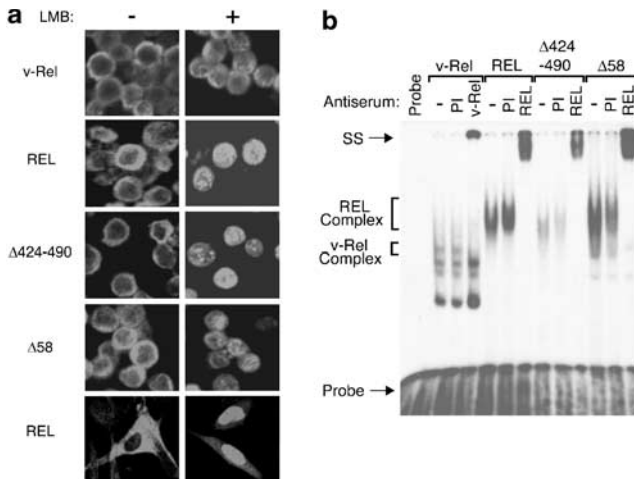


Figure 7 Active REL proteins appear to shuttle through the nucleus of transformed spleen cells. **(a)** Spleen cells transformed by the indicated proteins or CEF expressing wild-type REL (only bottom two panels) were untreated (–) or were treated with leptomycin B (LMB) (+) for 4 h, and were then analysed by indirect immunofluorescence using an anti-REL primary antiserum. **(b)** Nuclear extracts from cells transformed by the indicated proteins were analysed in a κ B site EMSA. Where indicated, supershifts were performed with preimmune (PI), anti-v-Rel, or anti-REL antiserum. The probe, DNA–protein complexes, and supershifted complexes (SS) are indicated

that REL proteins must be able to activate transcription in order to transform cells, all the transforming REL proteins appeared by immunofluorescence to be primarily in the cytoplasm of transformed spleen cells. To determine whether REL proteins might be shuttling through the nucleus, we treated spleen cells transformed by wild-type REL, REL Δ 58, REL Δ 424–490, and v-Rel with leptomycin B, an inhibitor of Crm1-mediated nuclear export. As shown in Figure 7a, treatment of transformed spleen cells with leptomycin B caused the REL proteins and v-Rel to accumulate in the nucleus. In addition, the subcellular localization of wild-type REL in CEF shifts from cytoplasmic to nuclear upon treatment with leptomycin B. Moreover, nuclear extracts from spleen cells transformed by these REL proteins contain a considerable amount of κ B site DNA-binding activity, which can be supershifted by anti-REL antiserum (Figure 7b). These results indicate that functional, DNA-binding REL proteins are in the nucleus of transformed chicken spleen cells, and suggest that overexpressed REL proteins are continually cycling from the cytoplasm to the nucleus.

Discussion

In this report, we show that REL must activate transcription in order to transform chicken spleen cells. However, deletion of either of the two C-terminal transactivation subdomains of REL enhances its transforming activity. Moreover, the REL transactivation domain can be functionally replaced by an attenuated

version of the VP16 transactivation domain, but not by the fully active VP16 transactivation domain. Taken together, these results suggest that there is an intermediate level of transactivation by REL that results in optimal transforming activity.

Overall, our results with REL are strikingly similar to what has been found with v-Rel and its progenitor avian c-Rel. That is, v-Rel has a deletion that removes a C-terminal transactivation domain of c-Rel (Richardson and Gilmore, 1991), and chicken c-Rel mutants that are missing some C-terminal transactivation sequences are more transforming *in vitro* and *in vivo* than wild-type c-Rel (Kamens *et al.*, 1990; Hrdlicková *et al.*, 1994; Nehyba *et al.*, 1994). Indeed, infection of chicken lymphoid cells with expression vectors encoding full-length chicken c-Rel often selects for transformed cells expressing c-Rel proteins that have deletions of C-terminal transactivating residues (Hrdlicková *et al.*, 1994; Gilmore *et al.*, 1995), similar to the truncated REL protein we have identified herein (Figure 4c). Nevertheless, extensive deletion analyses indicate that v-Rel, like human REL, must activate transcription at some level to transform chicken spleen cells *in vitro* (Sarkar and Gilmore, 1993; Smardova *et al.*, 1995; Chen *et al.*, 1999). Therefore, it appears that a threshold level of transactivation is required for transformation by both v-Rel and REL, but that an intermediate level of transactivation (as provided by partial C-terminal deletion mutants) enables avian and human c-Rel proteins to transform more efficiently than the high levels of transactivation provided by the wild-type chicken and human c-Rel proteins.

Why might reduced transactivation increase the transforming activity of chicken and human c-Rel proteins? The $I\kappa$ B α gene is a target gene for NF- κ B complexes, and the NF- κ B-induced accumulation of newly synthesized $I\kappa$ B α protein terminates the NF- κ B response (Verma *et al.*, 1995). Hrdlicková *et al.* (1995) have shown that chicken c-Rel is a more efficient inducer of the $I\kappa$ B α gene than v-Rel. Therefore, the level of target gene transactivation induced by overexpression of full-length REL is almost certainly countered by REL's efficient induction of $I\kappa$ B α gene expression, which then sequesters much REL in the cytoplasm (as in CEF). Of note, the strongly activating REL Δ 164–VP16 protein is located in the cytoplasm of CEF (data not shown), suggesting that it also is an efficient activator of the gene encoding $I\kappa$ B α . In contrast, a reduced ability of REL mutants Δ 58 and Δ 424–490 to induce $I\kappa$ B α gene expression may enable them to escape regulation by $I\kappa$ B α (as evidenced by their predominantly nuclear localization in CEF). Thus, the net long-term induction of transformation-specific target gene expression may actually be more efficient by REL mutants with reduced transactivating abilities. Consistent with this, v-Rel has recently been shown to induce a greater level of long-term expression of the IAP1 gene than chicken c-Rel, which initially induces higher levels of IAP1 transcription that subsequently decline due to regulation of c-Rel by newly synthesized $I\kappa$ B α (Kralova *et al.*, 2002). Nevertheless, overexpression of wild-type REL (or

chicken c-Rel) is likely to result in sufficient long-term induction of target gene expression for transformation due to overexpressed REL being present in excess to endogenous I κ B α .

The accumulation of REL in the nucleus of transformed spleen cells upon treatment with leptomycin B suggests that REL is continually cycling from the cytoplasm to the nucleus. v-Rel has also been shown to cycle between the cytoplasm and the nucleus of transformed spleen cells (Sachdev and Hannink, 1998). Moreover, this continual nuclear localization of v-Rel seems to be required for its transforming activity in that the addition of a strong nuclear export signal onto v-Rel increases its rate of nuclear export and decreases its transforming activity (Sachdev *et al.*, 1997). Thus, with respect to nuclear-cytoplasmic shuttling, the transformation of chicken spleen cells by REL proteins and v-Rel is also likely to be mechanistically similar, and these results further support the hypothesis that REL must activate target genes in order to transform chicken spleen cells.

Replacement of the entire REL transactivation domain with the C-terminal portion of the VP16 transactivation domain created a protein (REL Δ 164–VP16 Δ N) that activated transcription at a level similar to REL Δ 58 and REL Δ 424–490. Strikingly, REL Δ 164–VP16 Δ N could also transform chicken spleen cells. In contrast, REL Δ 164–VP16, which contains the entire VP16 activation domain and activates transcription 10 times more strongly than the corresponding wild-type REL sequences in GAL4 site reporter gene assays, could not transform chicken spleen cells. Similarly, Gilmore *et al.* (1995) reported that a chimeric v-Rel–VP16 protein, which contained the intact VP16 transactivation domain, could not transform chicken spleen cells. Taken together, these results strongly suggest that the C-terminal sequences of Rel proteins (whether it be v-Rel or c-Rel proteins) contribute to transformation by supplying a transactivation function, and that it is the strength of the transactivation domain, but not the precise sequences of the activation domain, which is critical for optimal transforming activity. However, we cannot exclude an alternative hypothesis that it is reduced degradation of REL Δ 164–VP16 Δ N as compared to REL Δ 164–VP16 that is the key to their differences in transforming activity. That is, Molinari *et al.* (1999) have shown that the addition of strong transactivation sequences onto DNA-binding domains induces proteasome-mediated degradation more rapidly than weak activation sequences. Of note, the v-Myb protein also appears to activate transcription rather weakly, and the v-Myb transactivation domain cannot be functionally replaced in transformation assays by the strong VP16 transactivation domain (Engelke *et al.*, 1995).

REL gene amplifications have been found in approximately 15% of one subtype (called the germinal center subtype) of diffuse large B-cell lymphomas (Rosenwald *et al.*, 2002), in about 20–50% of classical Hodgkin's lymphomas (Joos *et al.*, 2002; Martín-Subero *et al.*, 2002), and at a lower frequency in other B-cell

lymphomas (Goff *et al.*, 2000). Moreover, increased levels of nuclear REL protein have been found in both diffuse large B-cell lymphomas and classical Hodgkin's lymphomas that have REL gene amplifications (Donnelly *et al.*, 2001; Barth *et al.*, 2003). Nevertheless, it has not been proven that REL contributes to the development of these cancers. However, we have shown previously that the expression of I κ B α in the RC-K8 diffuse B-cell lymphoma cell line kills these cells, which normally have constitutively high levels of nuclear REL DNA-binding activity (Kalaitzidis *et al.*, 2002). In addition, our demonstration of *in vitro* transforming activity of REL (Gilmore *et al.*, 2001, and herein) certainly suggests that REL is an oncogenic factor in some human B-cell cancers.

And what of human B-cell cancers that do not have REL gene amplification? Either the activity of genes other than REL causes these cancers or REL is activated by mechanisms other than amplification. For example, REL expression or activity may be enhanced by promoter/enhancer mutations or by the altered activity of proteins or pathways that control REL expression. Alternatively, the oncogenic activity of REL may be activated by mutations that directly affect REL protein structure or function, perhaps similar to those we have described in this report. Indeed, our demonstration that a C-terminal deletion mutant of REL can be generated in the stringent soft agar colony transformation assay (Figure 4c) demonstrates that such mutations can be selected under oncogenic pressure in cells. Interestingly, Barth *et al.* (2003) have recently identified two primary Hodgkin's lymphomas with REL alterations that may cause changes in REL activity by mechanisms other than gene amplification: namely, one that has a translocation of REL to a position near the immunoglobulin heavy-chain enhancer (overexpression) and one that expresses a C-terminally truncated REL protein (structural activation). Therefore, it seems warranted to screen for REL mutations in Hodgkin's lymphomas and diffuse large B-cell lymphomas.

Materials and methods

Plasmids

DNA manipulations were carried out by standard methods (Sambrook *et al.*, 1989). Further details of all subclones and primers used in this study can be obtained at www.nf-kb.org.

Spleen necrosis virus vectors pc-Rel/Bcl-2 and JD-REL have been described previously (Gilmore *et al.*, 2001). Retroviral vector pMH/Bcl-2 contains only chicken *bcl-2* as the 3' gene (White and Gilmore, 1996). REL C-terminal deletion mutants were generated by PCR using pGEM-Hu-c-Rel (Barkett *et al.*, 2001) as a template. In all cases, the 5' primer was within the Rel homology domain, and 3' primers were located at the appropriate positions and also contained a stop codon followed by a unique XhoI site. PCR products were then digested with EcoRV and XhoI, and these fragments were used to replace the corresponding wild-type REL sequences in pGEM-Hu-c-Rel. REL Δ 424–490 was created by introducing a

PCR-generated *SwaI* site in a *REL* cDNA at a position 1470 bp from the initiating ATG. *SwaI* digestion resulted in the removal of *REL* codons 424–490. *REL* Δ 164 was created by digesting pGEM-Hu-cRel with *SwaI* and then religating the plasmid, which results in the removal of codons 424–587. The *REL* Δ 164/VP16 fusion was created by replacing *REL* codons 424–587 with VP16 transactivation sequences (VP16 aa 413–490) by subcloning VP16 sequences as an *EcoRV*–*XhoI* fragment into *SwaI*/*XhoI*-digested pGEM-Hu-cRel. *REL* Δ 164–VP16 Δ N was created by digesting pGEM-*REL* Δ 164/VP16 with *XmaI* and then religating the plasmid, which results in the removal of codons 413–453 within VP16. Retroviral vectors for the expression of *REL* mutants and chicken Bcl-2 were created by subcloning *XbaI*–*XhoI* fragments from pGEM expression plasmids as replacements for wild-type *REL* in pc-Rel/Bcl-2 that had also been digested with *XbaI*–*XhoI*. JD-*REL* Δ 424–490, JD-*REL* Δ 58, JD-*REL* Δ 164, JD-*REL* Δ 164–VP16, and JD-*REL* Δ 164–VP16 Δ N were created by subcloning *XbaI*–*XhoI* fragments into JD214BS+ (Sif *et al.*, 1993) that had been digested with *XbaI* and *SalI*. Retroviral vector GM282BS+ containing wild-type *v-rel* has been described previously (Sif *et al.*, 1993).

GAL4 expression plasmids pSG424 (aa 1–147 of GAL4), SG-*v-Rel*, SG-*ch-c-Rel*, and pSG-*REL* (*REL* aa 278–587), reporter plasmid GAL4-site luc (a kind gift from Joseph Lipsick), and transfection normalization plasmid pCMV- β gal have been described previously (Epinat *et al.*, 2000; Wang and Gilmore, 2001). Plasmids for the expression of GAL4 fusions to *REL* C-terminal deletion mutants were made by subcloning *BamHI*–*KpnI* fragments into *BamHI*/*KpnI*-digested pSG424.

Cells and spleen cell transformation assays

CEF and transformed spleen cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10–20% fetal bovine serum (FBS) (Biologos, Montgomery, IL, USA), 50 U/ml penicillin, and 50 μ g/ml streptomycin.

As described previously, spleen cell transformation was measured either in liquid outgrowth assays (Gilmore *et al.*, 2001) or by the formation of colonies in soft agar (Mosialos *et al.*, 1991). Transformed spleen cell cultures used for Western blotting and immunofluorescence were generated by continued passaging of cells from liquid outgrowth transformation assays. For the analysis of individual transformed cell clones (Figure 4c), colonies formed in soft agar by infection with JD-*REL* were picked and expanded in liquid culture in DMEM containing 20% FBS. Where indicated, cells were treated with 40 ng/ml leptomycin B for 4 h.

Cell transfection, Western blotting, and indirect immunofluorescence

CEF were cotransfected with 1 μ g of helper virus DNA (pSW253) and 10 μ g of retroviral vector DNA using the DMSO/polybrene method, as described previously (Kalaitzidis and Gilmore, 2002). Cells were maintained in DMEM containing 10% FBS for 3–4 days prior to obtaining extracts or performing indirect immunofluorescence.

For Western blotting, whole-cell extracts were obtained by resuspending cell pellets in 2 \times SDS sample buffer (125 mM Tris, pH 6.8, 4.6% w/v sodium dodecyl sulfate, 20% w/v glycerol, 10% v/v β -mercaptoethanol, and 0.2% w/v bromophenol blue), and heating samples at 95°C for 10 min. Proteins were then separated on SDS-polyacrylamide gels, transferred

onto nitrocellulose membranes (Osmonics, Westborough, MA, USA), probed, and visualized using the Pierce SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA). *REL* antibodies used for Western blotting were raised against either the epitope KAGINPFNVPEKQLNDIE (in the *Rel* homology domain) or NEQLSDSFPYEFFQV (C-terminal *REL* aa), and were kind gifts from Nancy Rice (see Kalaitzidis and Gilmore, 2002).

For immunofluorescence, CEF were passaged 4 days after transfection onto glass coverslips, or spleen cells were dried onto coverslips as described previously (White *et al.*, 1996). The subcellular localization of *Rel* proteins was determined by indirect immunofluorescence using anti-*REL* (1:50) or anti-*Rel* (1:40) primary antiserum and FITC-conjugated goat anti-rabbit IgG secondary antibody (1:80; Sigma, St Louis, MO, USA), as described previously (Gilmore and Temin, 1986; Kalaitzidis and Gilmore, 2002). Cells were visualized on an Olympus confocal microscope.

Luciferase reporter assays

CEF in 60 mm plates were transfected with 0.3 μ g of reporter plasmid GAL4 site luc, 0.5 μ g of normalization plasmid pCMV- β gal, and 2 μ g of SG424-based GAL4 expression plasmids using the DMSO/polybrene method, as described previously (Wang and Gilmore, 2001; Kalaitzidis *et al.*, 2002). Luciferase activity was measured using the Luciferase Assay System according to the manufacturer's instructions (Promega, Madison, WI, USA). Luciferase values were normalized to β -galactosidase values in all assays, as described previously (Kalaitzidis *et al.*, 2002).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSAs) were performed using nuclear extracts prepared essentially as described previously (Kalaitzidis *et al.*, 2002). Briefly, nuclear extracts were prepared as described previously (Schreiber *et al.*, 1989), with the inclusion of 1 mM DTT, 5 μ g/ml leupeptin, 62.5 μ g/ml aprotinin, 1 μ g/ml pepstatin, and 1 mM PMSF. Protein concentrations were determined using the Bio-Rad (Hercules, CA, USA) protein assay reagent. DNA-binding reactions were in a final volume of 50 μ l containing 10 mM Tris, pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 4% w/v glycerol, 2 μ g poly dI/dC, 10 000 cpm of ³²P end-labeled κ B probe (5'TCGAGAGGTCGGGAAATTCCCCCCC3'), and 5 μ g of nuclear extract. Samples were incubated at room temperature for 30 min. Complexes were resolved on 5% polyacrylamide Tris-glycine-EDTA gels, and were electrophoresed at 60 mA for approximately 1.5 h. Gels were dried and autoradiography was performed. For supershift assays, 1 μ l of antiserum was added after protein/DNA complex formation and the reactions were incubated on ice for 1 h. Antibodies were specific for *v-Rel* (Gilmore and Temin, 1986) or an epitope that includes the NLS of *REL* (a kind gift from Nancy Rice (Kalaitzidis *et al.*, 2002)).

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