Stable expression of the avian retroviral oncoprotein v-Rel in avian, mouse, and dog cell lines

Thomas D. Gilmore,* Jims Jean-Jacques, Rebecca Richards, Catherine Cormier,† Juhee Kim, and Demetrios Kalaitzidis

Biology Department, Boston University, 5 Cummington Street, Boston, MA 02215, USA

Received 29 May 2003; returned to author for revision 11 July 2003; accepted 23 July 2003

Abstract

Overexpression of the retroviral oncoprotein v-Rel can rapidly transform and immortalize a variety of avian cells in culture. However, mammalian models for v-Rel-mediated oncogenesis have been compromised by the fact that high-level expression of v-Rel has been reported to be toxic in many mammalian cell types, including mouse 3T3 cells, Rat-1 cells, and mouse bone marrow cells. In this article, we demonstrate that 3T3 cells can support expression of v-Rel for at least 24 days when infected with a mouse stem cell virus (MSCV) retroviral vector containing \textit{v-rel}. In retrovirus-infected 3T3 cells, v-Rel is located in the nucleus and can bind to DNA, but does not transform the cells. On the other hand, 3T3 and Rat-2 cells do not express v-Rel after stable transfection with a pcDNA-based v-Rel expression vector. We also show that infection of the IL3-dependent mouse B cell line BaF3 with the MSCV-\textit{v-rel} vector results in expression of v-Rel, but does not convert these cells to growth factor independence. In contrast to 3T3 cells, the dog osteosarcoma D17 cell line can support a high level of v-Rel expression, after either transfection or infection with a retroviral vector. That is, v-Rel can be stably expressed as a nuclear, DNA-binding protein in D17 cells to approximately the same level as in chicken embryo fibroblasts. These results suggest that the restriction to v-Rel expression in rodent fibroblasts is generally absent in D17 cells and that the type of \textit{v-rel} expression vector determines whether 3T3 cells can support stable expression of v-Rel. The findings reported here are an essential first step in the development of mammalian systems to study Rel-mediated oncogenesis.

© 2003 Elsevier Inc. All rights reserved.

Keywords: v-Rel; Rel; NF-κB; Rel; Transcription factor; Cell toxicity; D17 cells; 3T3; BaF3; Retroviral oncoprotein

Introduction

The retroviral oncoprotein v-Rel, encoded by the highly oncogenic avian Rev-T retrovirus, is a transcription factor in the Rel/NF-κB family (reviewed in Gilmore, 1999b). v-Rel causes rapidly fatal lymphoid cell malignancies in young birds and efficiently transforms and immortalizes a variety of avian lymphoid cells in tissue culture. The oncogenicity of v-Rel is dependent on its ability to form homodimers, bind to DNA, and activate transcription. That is, mutations in v-Rel that abolish or diminish any one of these activities correspondingly affect the transforming activity of v-Rel.

The vertebrate cellular Rel/NF-κB family includes p50/p105, p52/p100, c-Rel, RelA (p65), and RelB (Gilmore, 1999a). These proteins can, with few exceptions, form homodimers and heterodimers, which bind to specific target DNA sites to influence the expression of a wide variety of genes involved in many cellular processes (Pahl, 1999; see also www.nf-κb.org). In most normal cell types, Rel/NF-κB dimers reside primarily in the cytoplasm in a latent, inactive state, by virtue of their interaction with IκB inhibitor proteins (reviewed in Silverman and Maniatis, 2001).

In several settings, cellular Rel/NF-κB transcription factors have also been associated with human oncogenesis (reviewed in Gilmore et al., 2002). First, in many tumor cell types, there is constitutively nuclear and active NF-κB.
Second, in several human lymphoid cancers, amplifications, chromosomal rearrangements, and point mutations have been detected in genes encoding Rel/NF-κB or IκB factors. Third, overexpression of wild-type human c-Rel can malignantly transform primary chicken spleen cells (Gilmore et al., 2001).

Despite its acute oncogenicity in avian cells, v-Rel has not been reported to transform any mammalian cell type in tissue culture. Moreover, v-Rel and the closely related chicken c-Rel have been shown to be toxic in many mammalian cells, including mouse and chicken bone marrow cells (Abbaddie et al., 1993; Schwartz and Witte, 1988), 3T3 cells (Gélinas and Temin, 1988), Rat-1 cells (Gélinas and Temin, 1988; Hannink and Temin, 1989), and HeLa cells (Bash et al., 1997). Transgenic mice in which v-rel is under the control of the T cell specific lek promoter do develop T cell leukemias (Carrasco et al., 1996). However, these malignancies take a long time to arise, suggesting that multiple mutagenic events have occurred in the cells; furthermore, malignant T cells from these mice grow poorly in cell culture and cannot be established (Carrasco et al., 1996). Thus, mammalian cell-culture model systems for studying the role of Rel/NF-κB factors in oncogenesis are lacking, and essentially nothing is known about the basis or potential for v-Rel-mediated toxicity in mammalian cells.

In this article, we show that mouse 3T3 cells can support expression of nuclear v-Rel when its expression is directed by a retroviral vector, but not by a plasmid vector. In contrast, the dog osteosarcoma D17 cell line can tolerate high-level expression of nuclear v-Rel, whether introduced on plasmid or retroviral vectors. Although v-Rel does not transform mouse fibroblasts or a mouse pro-B cell line, the results presented here are an essential first step in analyzing the effect of v-Rel in rodent systems.

Results

v-Rel can be expressed in 3T3 cells after infection with a retroviral vector

Previous reports (Gélinas and Temin, 1988; Hannink and Temin, 1989) failed to detect v-Rel expression in rodent fibroblasts after stable transfection with cytomegalovirus (CMV) promoter plasmids or retroviral provirus plasmids. Consistent with those reports, we found that v-Rel could not be detected by Western blotting after stable transfection and G418 selection of pools of 3T3 or Rat-2 cells using a pcDNA3-based v-Rel expression plasmid (containing the CMV promoter driving v-rel and the selectable neo gene controlled by a second promoter) (Fig. 1). In contrast, v-Rel could be readily detected in extracts from stable pcDNA3-v-rel transfectants of chicken DF-1 fibroblasts (an immortalized chicken fibroblast cell line; Himly et al., 1998) or D17 dog osteosarcoma cells (Watanabe and Temin, 1983) (Fig. 1).

Because most studies on v-Rel in avian cells have been performed with retroviral expression vectors, we sought to determine whether v-Rel could be expressed in 3T3 cells by infection with a mouse retroviral vector carrying v-rel. For these experiments, we first created pMSCV-v-rel in which v-rel was subcloned into the mouse stem cell virus (MSCV) vector pMSCV-puro (Hawley et al., 1994). Thus, pMSCV-v-rel contains v-rel as the 5′ gene and the gene for puromycin resistance located 3′ to an internal ribosome entry sequence (IRES) (Fig. 2A).

Viral stocks were prepared for MSCV-v-rel and were used to infect 3T3 cells, which were then selected for puromycin resistance for various periods of time. Anti-v-Rel Western blotting was performed on extracts of pooled puromycin-resistant cells that had been maintained in culture for up to 24 days. As shown in Fig. 2B, MSCV-v-rel-infected 3T3 cells expressed similar amounts of v-Rel whether assayed after selection with puromycin for 2, 7, 14, or 24 days. The similar levels of expression of v-Rel in these cultures at day 2 and day 24 is in stark contrast to what is seen in rodent fibroblasts after transfection with a plasmid-based v-Rel expression vector, wherein expression of v-Rel can be detected shortly after transfection but is absent after 2 weeks of selection (see Fig. 1B above, our unpublished results, and Hannink and Temin, 1989). 3T3 cells expressing v-Rel from MSCV-v-rel were not morphologically transformed (data not shown).

Indirect immunofluorescence showed that v-Rel was expressed in the nucleus of MSCV-v-rel-infected 3T3 cells (Fig. 2C). To ensure that the nuclear v-Rel protein in these 3T3 cells was active, extracts from MSCV-v-rel-infected 3T3 cells were analyzed by an EMSA using a κB site probe from the chicken c-rel promoter that can be bound by v-Rel (Capobianco and Gilmore, 1991). As compared to control
cells, extracts from these v-Rel-expressing 3T3 cells contained increased kB site DNA-binding activity that could be supershifted by anti-v-Rel antiserum (Fig. 2D). Taken together, these results demonstrate that nuclear, DNA binding-competent v-Rel can be stably expressed in 3T3 cells when introduced by infection with a retroviral vector.

v-Rel does not convert the BaF3 mouse B cell line to IL3-independent growth

v-Rel was reported to be toxic in primary mouse bone marrow cells (Schwartz and Witte, 1988). However, avian cells of the B cell lineage are one of the primary target cells for transformation by v-Rel and the human c-rel gene is amplified in many B cell lymphomas (Gilmore, 1999b; Gilmore et al., 2002). Thus, we were interested in determining whether MSCV-v-rel could be used to express v-Rel in murine B cells and whether MSCV-directed expression of v-Rel would promote the growth of murine B cells. Growth of the murine pro-B cell line BaF3 is dependent on interleukin-3 (IL-3); however, BaF3 cells can be converted to IL-3-independent growth by a variety of oncogenes. To determine whether expression of v-Rel could transform BaF3 cells to IL-3 independence, BaF3 cells were infected with MSCV-v-rel in the presence of IL-3 and after 4 days IL-3 was removed. As a positive control, we also infected BaF3 cells with an expression vector for BCR-ABL, which has previously been shown to transform these cells to IL-3-independent growth (Daley and Baltimore, 1988). BaF3 cells infected with MSCV-v-rel did express readily detectable amounts of v-Rel when grown in the presence of IL-3 (Fig. 3). However, MSVC-v-rel-infected BaF3 cells did not convert to IL-3-independent growth, whereas BCR-ABL expression did transform these cells to IL-3 independence.

Fig. 2. v-Rel can be stably expressed in 3T3 cells by infection with a retroviral vector. (A) Bicistronic MSCV-based vector for the expression of v-rel and the puromycin resistance gene. IRES, internal ribosome entry sequence; LTR, long terminal repeat. (B) Anti-v-Rel and anti-actin Western blotting was performed on extracts from cells infected with the MSCV vector lacking v-rel (puro) or MSCV-v-rel (v-Rel). Cells were grown in the presence of puromycin for the indicated number of days. (C) 3T3 cells were infected with MSCV-v-rel and after 4 days were analyzed by indirect immunofluorescence using an anti-v-Rel primary antiserum. As shown, bright nuclear staining was detected. Uninfected cells in the culture did not visibly stain (not shown). (D) Extracts were prepared from uninfected (3T3) or MSCV-v-rel-infected 3T3 cells that had been selected with puromycin for 2 or 24 days. An EMSA was then performed using a kB site probe. Where indicated, preimmune (PI) or anti-v-Rel antiserum was also added. The v-Rel-DNA and supershifted complexes are indicated.

v-Rel can be stably expressed in the D17 dog osteosarcoma cell line either by transfection of a plasmid vector or by infection with a retroviral vector

In contrast to 3T3 and Rat-2 cells, we found that the D17 dog osteosarcoma cell line could tolerate pcDNA plasmid-driven expression of v-Rel approximately as well as the DF-1 chicken fibroblast cell line (see Fig. 1B, and Gilmore, 1999b). As a second means of comparing the levels of expression of v-Rel between avian fibroblasts and D17 cells, we infected primary chicken embryo fibroblasts (CEF) and D17 cells with spleen necrosis virus (SNV) vector MH105 (White and Gilmore, 1996) that contains v-rel as the 5’ gene, followed by an IRES and a 3’ neo resistance gene (see Fig. 4A). D17 cells are permissive for SNV replication and the promoter in the SNV long terminal repeat has been
shown to work efficiently in D17 cells (Embreton and Temin, 1987). MH105-infected D17 cultures were selected with G418 for approximately 3 weeks, and equalized protein extracts were subjected to Western blotting with anti-v-Rel antiserum (Fig. 4B). In two separate experiments, we found that v-Rel was expressed to approximately the same extent in infected CEF and in D17 cells. For each cell type, uninfected cells did not express v-Rel. Thus, by infection with a retroviral vector (Fig. 4B) and by transfection with a plasmid expression vector (Fig. 1B), v-Rel can be expressed to approximately the same level in D17 cells and in primary (CEF) and immortalized (DF-1) chicken fibroblasts.

As shown by indirect immunofluorescence, v-Rel is also primarily a nuclear protein in DF-1 and CEF chicken cells and in D17 dog cells (Fig. 4C). In addition, an EMSA using a κB site probe was performed on extracts from MH105-infected D17 cells. As shown in Fig. 4D, extracts from D17 cells show high levels of a κB site-binding activity, which is not present in control uninfected cells and which can be super-shifted with anti-v-Rel antiserum. Taken together, these results show that v-Rel localizes to the nucleus as an active DNA-binding protein in D17 cells.

Expression of v-Rel does not affect the growth rate of D17 cells

Expression of chicken c-Rel has been shown to arrest HeLa cells at the G1/S-phase transition point of the cell cycle (Bash et al., 1997). To determine whether v-Rel affected the growth of D17 cells, we compared the growth rates of control D17 cells and D17 cells expressing v-Rel (after transfection with pcDNA3-v-rel and selection with G418, as for Fig. 1). As shown in Fig. 5, control and v-Rel-expressing D17 cells grow at essentially identical rates. Therefore, v-Rel does not appear to affect the growth of D17 cells, after transfection with a v-Rel expression vector.
Discussion

In this article, we show that mouse 3T3 and D17 dog osteosarcoma cells can sustain high-level expression of v-Rel, which has previously been reported to be generally toxic in rodent cell lines (Geлинas and Temin, 1988; Hannink and Temin, 1989; Schwartz and Witte, 1988). Whereas D17 cells can support expression of v-Rel after either transfection with a plasmid expression vector or infection with a retroviral vector, 3T3 cells support v-Rel expression only after infection with an MSCV-based mouse retroviral vector.

The mechanism by which v-Rel expression is silenced in mouse and rat fibroblasts after transfection with v-Rel expression vectors (Hannink and Temin, 1989; Fig. 1, herein) is not known. Based on cotransfection experiments, it has been suggested that the lack of sustained v-Rel expression in these cells is due to a toxicity of v-Rel when its expression is directed from certain strong promoters (Geлинas and Temin, 1988; Hannink and Temin, 1989; Schwartz and Witte, 1988). Whereas D17 cells can support expression of v-Rel after either transfection with a plasmid expression vector or infection with a retroviral vector, 3T3 cells support v-Rel expression only after infection with an MSCV-based mouse retroviral vector.

In contrast to mouse 3T3 cells and rat fibroblasts, we show that D17 osteosarcoma dog cells can tolerate sustained expression of v-Rel after transfection with pcDNA-v-rel; moreover, D17 cells expressing v-Rel grow at the same rate as control D17 cells (Fig. 5). D17 cells may have a mutation(s) that allows them to tolerate high-level expression of v-Rel from these vectors. Alternatively, rodent cells, but not D17 cells, may have a mechanism(s) to silence expression from the plasmid-based transfection vectors. That this may, at least in part, account for the differences between transfected 3T3 and D17 cells is suggested by the observation that expression of β-galactosidase is stable in D17 cells but not 3T3 cells after transfection and selection with G418 (Fig. 6). Similarly, Hannink and Temin (1989) showed that v-Rel expression could be detected in the nucleus of Rat-1 cells 2 days after transfection with a CMV promoter-based vector, but not after 2 weeks of selection. In a cotransfection assay, the results of Geлинas and Temin (1988) suggested that v-rel expression was toxic in Rat-1 cells when expressed from a strong (MLV) viral promoter but not from a weak (SNV) viral promoter, whereas D17 cells appeared to be able to tolerate v-rel expression from either type of promoter. However, those authors did not directly assess v-Rel expression in their experiments. In any event, D17

Fig. 5. v-Rel does not affect the growth of D17 cells. Control D17 cells and v-Rel-expressing D17 cells (from Fig. 1) were seeded at equal densities in several plates, and the number of cells in independent dishes was then monitored each day for 13 days. Cell number versus day after plating is plotted.

Fig. 6. β-Galactosidase can be stably expressed in D17 cells but not in 3T3 cells after transfection with a CMV promoter-based vector. Mouse 3T3 and dog D17 cells were transfected with a control vector (pcDNA3.1) or a pcDNA-lacZ vector for the expression of β-galactosidase. Cells were then selected with G418, and pools of cells were analyzed by Western blotting with an anti-β-galactosidase antiserum (bottom). The β-galactosidase protein is indicated by the arrow. The top panel shows a Coomassie blue stained gel of the same amounts of each extract used in the Western blot, as a loading control.
cells in general appear to be more similar to avian cells, such as CEF and DF-1 cells, which can also support high-level v-Rel expression from either plasmid or retrovirus vectors. Moreover, D17 cells, similar to avian cells, can efficiently support the replication of spleen necrosis virus retroviral vectors, whereas rodent cells generally cannot (Embretson and Temin, 1986, 1987).

Although v-Rel is potently transforming in chicken lymphoid cells, expression of v-Rel did not transform the mouse BaF3 pro-B cell line to IL-3-independent growth (Fig. 3). In addition, Schwartz and Witte (1988) were unable to malignantly transform primary mouse bone marrow cells after infection with an MLV-based retroviral expression vector. Therefore, either v-Rel simply cannot transform mouse lymphoid cells or another factor (e.g., an additional complementing oncprotein or tumor suppressor) is required for v-Rel to show transforming activity in mouse lymphoid cells. Consistent with the latter hypothesis, transgenic mice in which v-rel is expressed under the control of the T cell specific lck promoter develop T cell lymphomas only after approximately 6–10 months, suggesting that other mutations have occurred in these cells (Carrasco et al., 1996). Indeed, when these lck-v-rel transgenic mice are crossed to NF-κB p50 knockout mice, the T cell lymphomas develop much quicker (Carrasco et al., 1996), demonstrating that cellular mutations can enhance the transforming activity of v-Rel in mice.

In summary, we have created a retroviral vector that can be used to stably express high levels of v-Rel in mouse cells. Moreover, we have shown that the restriction to v-Rel expression that is sometimes seen in rodent cells is not present in all mammalian cell lines, in that the D17 dog cell line can support high-level v-Rel expression after transfection or infection. These studies may be useful in the development of mammalian models of oncogenesis induced by v-Rel or cellular Rel.

Materials and methods

Plasmid constructions

pcDNA-v-rel was created by subcloning an XbaI fragment containing v-rel into the XbaI site of pcDNA3.1. To construct pMSCV-v-rel, a Pmel fragment containing v-rel was subcloned at the unique HpaI site of pMSCV-puro (Hawley et al., 1994; a kind gift of Naomi Rosenberg). pMH105 has been described previously (White and Gilmore, 1996).

Cell maintenance and cell growth

All cells (CEF, DF-1, 3T3, D17, Rat-2, BaF3) were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). For routine maintenance of BaF3 cells, 0.5 ng/ml of IL-3 (R&D Systems, Minneapolis, MN) was included.

To compare the growth rates of v-Rel-expressing and uninfected D17 cells, approximately 10,000 cells of each cell type were passed into thirteen 60-mm plates. On each day for 13 days thereafter, the growth was monitored by counting the number of cells in a single plate after removing the cells with trypsin-EDTA.

Transfections and infections

To establish stable pools of D17, DF-1, Rat-2, or 3T3 transfectants, cells were transfected using DMSO/polybrene (Kawai and Nishizawa, 1984) in duplicate 60-mm dishes with 0.1–0.5 μg of pcDNA3.1 or pcDNA-v-rel. The next day, media was changed to DMEM/10% FBS containing 600 μg/ml G418 every 3–4 days thereafter. Fourteen days later, the G418-resistant cells from each cell type were passed (as pools) into two 100-mm tissue culture plates. Cells were allowed to grow until approximately confluent (~3–4 days) and were then processed for Western blotting or continued passage as described below.

MH105 virus was obtained by harvesting the supernatant from MH105-transformed chicken spleen cell cultures (White and Gilmore, 1996). MSCV virus stocks were prepared by transfecting 60-mm plates of 293T cells (DuBridge et al., 1987) with 15 μg of pMSCV-puro or pMSCV-v-rel plasmid DNA and 15 μg of ψEco helper virus plasmid DNA (Muller et al., 1991) by the calcium phosphate method, also including 50 μg/ml chloroquine (Pear et al., 1993). Twenty-four hours later the transfection mix was removed, and 3 ml DMEM/10% FBS was added. Over the following 2 days, media was harvested several times, and virus was pooled and stored at −80°C.

For infection of D17 and CEF, cells were each passed into 60-mm dishes at approximately 30% confluency. The next day, 1 ml of MH105 virus containing 10 μg of polybrene was added and cells were incubated for 1 h at 37°C. After 1 h, the media was removed and 3 ml DMEM/10% FBS was added. 3T3 cells were infected in 60-mm dishes with 1 ml of MSCV-puro (empty vector) or MSCV-v-rel virus and 15 μg/ml polybrene. Cells were incubated overnight. The next day media was removed and 3 ml DMEM/10% FBS was added. The cells were incubated for 2 days in normal media and then selected with media containing puromycin (2.5 μg/ml) as indicated in the text.

For infection of BaF3 cells, approximately 10⁶ cells were infected with 1 ml of MSCV-BCR-ABL or MSCV-v-rel virus in medium containing 8 μg of polybrene. Cells were then spin-infected by centrifugation at 1800 rpm for 45 min at room temperature. An additional 1 ml of DMEM/10% FBS was then added, including IL-3 to a final concentration of 0.5 ng/ml, and cells were grown at 37°C. To assay for conversion of BaF3 cells to IL-3-independent growth, 4 days after infection, cells were pelleted, washed with DMEM, and then resuspended in DMEM/10% FBS without
IL-3. IL-3-deprived cultures were then monitored for growth over the next 10 days (IL-3-independent growth was seen in BCR-ABL cultures within 5 days of removal of IL-3).

Western blotting

Cells were either lysed directly in SDS sample buffer or in AT buffer [20% glycerol; 1% Triton X-100; 20 mM HEPES (pH 7.0); 1 mM EDTA; 1 mM EGTA; 20 mM NaF; 1 mM Na2P2O7 · 10 H2O; 1 mM DTT; 1 mM Na3VO4; 1 µg/ml PMSF; 1 µg/ml leupeptin; 1 µg/ml pepstatin] as described (Gapuzan et al., 2002). Equal amounts of cellular protein, either judged by Bradford assays (for AT buffer), were incubated with 1/1000 H9262 and then probed by Coomassie blue staining (of extract aliquots described (Gapuzan et al., 2002). Equal amounts of cellular protein, either judged by Bradford assays (for AT buffer) as described previously (Gilmore and Temin, 1986). Cells were either lysed directly in SDS sample buffer or in AT buffer, and 20 µg of protein was used in the DNA-binding reactions. For supershifts, extracts were incubated with 1 µl of preimmunovection of the G1/S-phase transition. Mol. Cell. Biol. 17, 6526–6536.


