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# Temperature-Sensitive Transforming Mutants of the v-*rel* Oncogene

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**By making site-directed mutations in the avian retroviral oncogene v-*rel*, we created two temperature-sensitive (*ts*) transforming mutants; these changes were analogous to mutations previously shown to confer a *ts* function onto the Dorsal protein of *Drosophila melanogaster*. Chicken spleen cells infected with the *ts* v-*rel* mutants formed colonies in agar at 36.5°C but not at 41.5°C. In addition, spleen cells derived from the *ts* v-*rel*-transformed colonies could be propagated in liquid culture at 36.5°C but rapidly senesced at 41.5°C. Both mutant v-Rel proteins were also *ts* for DNA binding in vitro. These mutants may be valuable for identifying genes directly regulated by v-*rel*.**

The v-*rel* oncogene of the avian retrovirus Rev-T transforms chicken lymphoid cells in vivo and in vitro. The product of the v-*rel* oncogene, $p59^{v\text{-}rel}$ (or v-Rel), is a nuclear phosphoprotein that is a truncated and mutated version of the avian proto-oncoprotein c-Rel (reviewed in references 4 and 11).

c-Rel and v-Rel are members of a family of transcription factors that all contain a highly related N-terminal DNA-binding/dimerization domain of approximately 300 amino acids, termed the Rel homology (RH) domain (reviewed in references 1, 4, 10, and 12). This family also includes the vertebrate transcription factors RelA (p65), RelB, NF-κB p50/p105, and NF-κB p52/p100 and the *Drosophila melanogaster* embryonic ventral morphogen Dorsal. The C-terminal halves of Rel/NF-κB family proteins are generally unrelated but usually contain sequences involved in transcriptional activation.

Although the exact mechanism by which v-Rel transforms avian lymphoid cells is not known, the analysis of several v-Rel mutants indicates that v-Rel must form homodimers, bind to DNA, and weakly activate transcription in order to function as an active oncoprotein (2, 9, 19, 24–28, 30). As mentioned above, the dimerization and DNA-binding sequences of v-Rel reside within the conserved N-terminal RH domain, and the transcriptional activation sequences are located in the C-terminal half of v-Rel.

Isoda et al. (17) recently described two temperature-sensitive (*ts*) mutants of *dorsal* that contain point mutations that change single amino acids located within the RH domain of the Dorsal protein; one of these mutations is located toward the N terminus of the Dorsal RH domain near a sequence that has been shown to be important for DNA binding by Rel proteins (21, 23), and the second *ts* mutation in Dorsal is within a conserved protein kinase A consensus recognition sequence (Arg-Arg-Pro-Ser) that has been shown to be important for the formation of Rel homodimers (25, 30). Therefore, in an attempt to create a *ts* v-Rel mutant, we used site-directed mutagenesis to change the corresponding sequences in v-*rel*. Specifically, we changed codon 37 of v-*rel* from Gly to Glu (mutant v-G37E) and codon 273 of v-*rel* from Arg to His (mutant v-R273H) (Fig. 1).

Wild-type and mutant v-*rel* genes were subcloned into spleen necrosis virus vector JD214BS+ (29) and assayed for their transforming abilities by electroporating these plasmids into fresh avian spleen cells in the presence of Rev-A helper virus plasmid SW253 (31) as described previously (26). After 4 days in liquid culture at 37°C, cells were plated in soft agar and incubated at 36.5 or 41.5°C. Spleen cells electroporated with plasmid GM282 (7), expressing wild-type v-*rel*, formed colonies at both temperatures; spleen cells electroporated with either v-G37E or v-R273H formed colonies only at 36.5°C (Fig. 2A; data not shown for v-R273H). Generally, the efficiency of colony formation by v-R273H after electroporation was reduced at 36.5°C compared with that of wild-type v-Rel and v-G37E.

Individual transformed colonies were picked for all three viruses, and cells were expanded in liquid culture at 36.5°C in Temin's modified Eagle's medium (Biologos) containing 20% fetal calf serum (Sigma). Cells transformed by wild-type v-*rel* grew vigorously and as large clumps of cells at both 36.5 and 41.5°C (Fig. 2B). Cells transformed by either v-G37E or v-R273H also grew rapidly as large clumps of cells at 36.5°C;

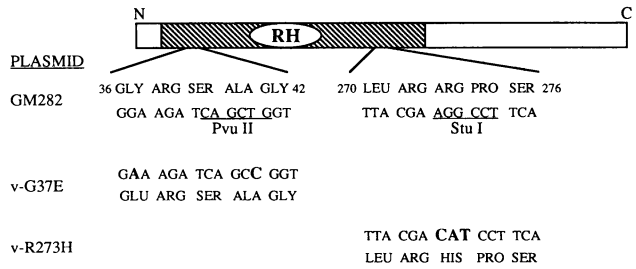


FIG. 1. Construction of *ts* mutants of v-*rel*. The general structure of v-*rel* is shown at the top. Site-directed mutagenesis, performed as described previously (22, 26), was used to create *ts* mutants v-G37E and v-R273H. The oligonucleotides used to make v-G37E and v-R273H were 5′TAAATGTGAAGAAAGATCAGCCGGTAGCAT TCC3′ and 5′AAGATGCAGTTACGACATCCTTCAGACCAGGC A3′, respectively. The relevant nucleotide and amino acid sequences in wild-type v-*rel* and the *ts* mutants are shown; the numbers next to the amino acids indicate their positions in wild-type v-Rel. The mutated nucleotides are indicated in a boldface for v-G37E and v-R273H. The *Pvu*II site and *Stu*I sites, which were destroyed in v-G37E and v-R273H, respectively, are indicated for wild-type v-*rel*. After site-directed mutagenesis in M13, the mutated v-*rel* genes were subcloned into avian retroviral vector JD214BS+ (29) by using *Xba*I.

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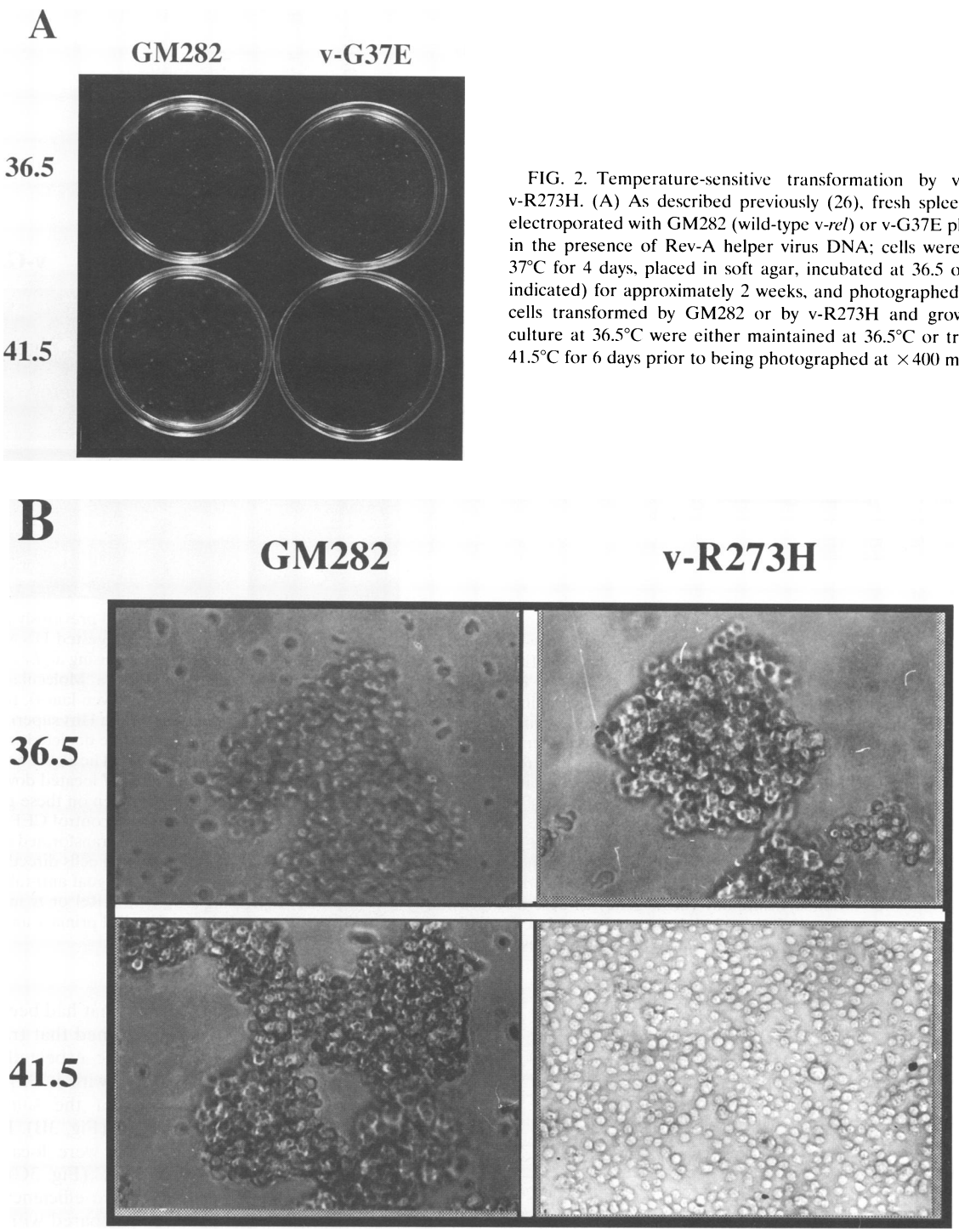



FIG. 2. Temperature-sensitive transformation by v-G37E and v-R273H. (A) As described previously (26), fresh spleen cells were electroporated with GM282 (wild-type v-*rel*) or v-G37E plasmid DNA in the presence of Rev-A helper virus DNA; cells were cultured at 37°C for 4 days, placed in soft agar, incubated at 36.5 or 41.5°C (as indicated) for approximately 2 weeks, and photographed. (B) Spleen cells transformed by GM282 or by v-R273H and grown in liquid culture at 36.5°C were either maintained at 36.5°C or transferred to 41.5°C for 6 days prior to being photographed at ×400 magnification.

however, when these cultures were shifted to 41.5°C, the clumps disaggregated and the cells became smaller and stopped dividing (shown for v-R273H-transformed cells after 6 days at 41.5°C in Fig. 2B). The disaggregation of the clumps in *ts* mutant-transformed spleen cells became evident as early as 24 to 48 h at 41.5°C (data not shown).

To ensure that the spleen cells were infected with the proper v-*rel* genes, virus was harvested from spleen cell cultures transformed by mutant v-*rel* genes at 36.5°C, and this virus was used to infect cultures of chicken embryo fibroblasts (CEF), which were then grown at 37°C. Approximately 2 days after infection, CEF were lysed, Hirt supernatants containing double-stranded viral DNA were isolated, and samples were analyzed by Southern blotting with a v-*rel*-specific probe (8, 15). The site-directed mutations in v-G37E and v-R273H were both designed such that restriction enzyme sites were destroyed: in v-G37E, a *Pvu*II site was destroyed, and in v-R273H, a *Stu*I site was destroyed (Fig. 1 and 3A). Southern

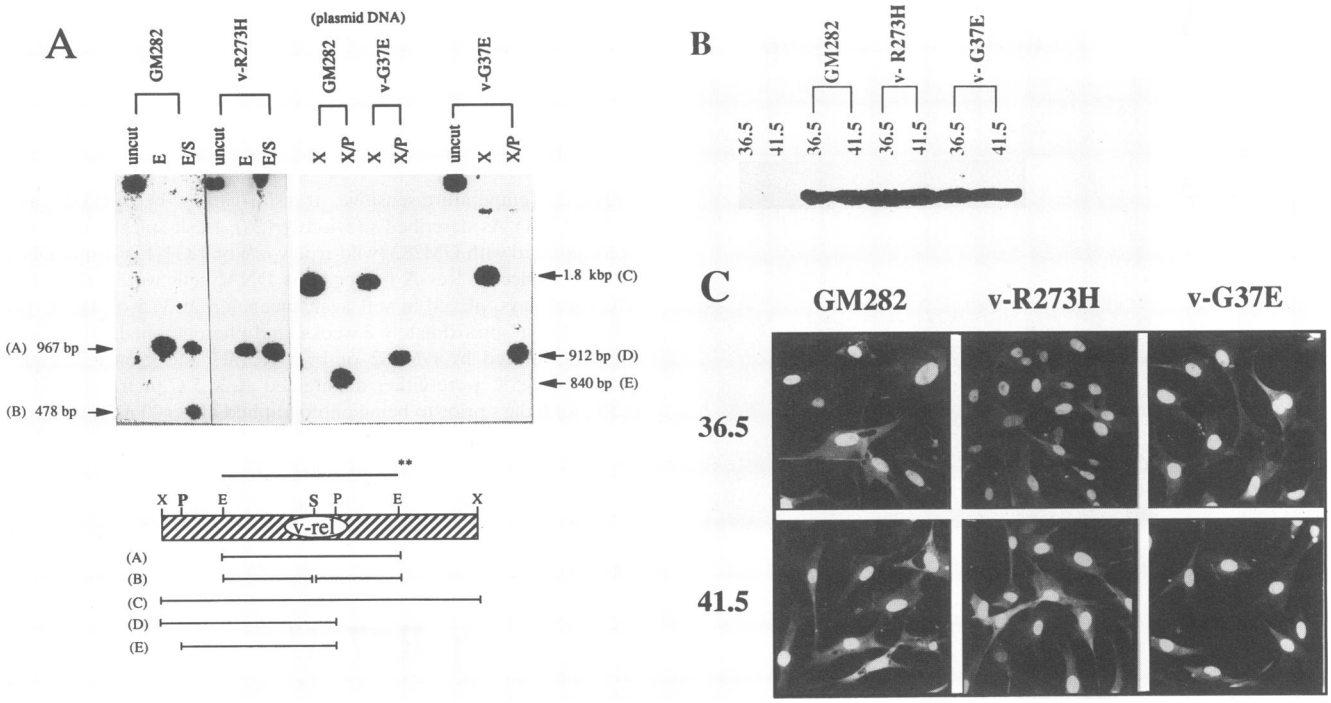



FIG. 3. Expression of wild-type and *ts* mutant v-*rel* genes in vivo. (A) Hirt supernatant DNA was prepared as described previously (8, 15) from CEF that had been infected with virus harvested from GM282-, v-G37E-, and v-R273H-transformed spleen cells. Undigested DNA (uncut) or DNA digested with relevant restriction enzymes (E, *Eco*RI; P, *Pvu*II; S, *Stu*I; X, *Xba*I) was analyzed by Southern blotting using an *Eco*RI-digested middle v-*rel* probe (indicated by the bar with asterisks above illustration), and images were generated by using a Molecular Dynamics PhosphorImager. In the first panel (first six lanes), all lanes contain Hirt supernatant DNA; in the second panel (last seven lanes), the first four lanes were prepared by using approximately 10 pg of digested plasmid DNA as size controls and the last three lanes contain Hirt supernatant DNA from v-G37E-infected CEF. Sizes of the relevant restriction fragments are indicated beside the blots, and the fragments are depicted schematically below the drawing of v-*rel*. The approximate positions of relevant restriction enzyme sites are indicated in the drawing. The *Pvu*II and *Stu*I sites that were mutated in v-G37E and v-R273H, respectively, are indicated in boldface in the drawing (two *Pvu*II sites in v-*rel* located downstream of the indicated 3′ *Pvu*II site, which are not relevant to the analysis shown here and generate fragments too small to be seen on these gels, are not indicated in the drawing). The digestion with *Stu*I in the third lane from the left was not complete. (B) Western blot of control CEF (unmarked lanes) and CEF expressing wild-type v-Rel, v-G37E, or v-R273H. CEF were infected at 37°C with virus harvested from transformed spleen cells, and 3 days later CEF were passed and maintained for 48 h at the indicated temperature. Lysates were prepared by boiling cells directly in sodium dodecyl sulfate sample buffer and samples were analyzed by Western blotting with an anti-v-Rel primary antiserum and a goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (Stratagene) as described previously (7). (C) CEF expressing wild-type v-Rel or *ts* mutant v-Rel proteins were grown at 36.5 or 41.5°C for 24 h before being analyzed by indirect immunofluorescence using an anti-v-Rel primary antiserum and a goat anti-rabbit fluorescein-conjugated secondary antibody as described previously (13).

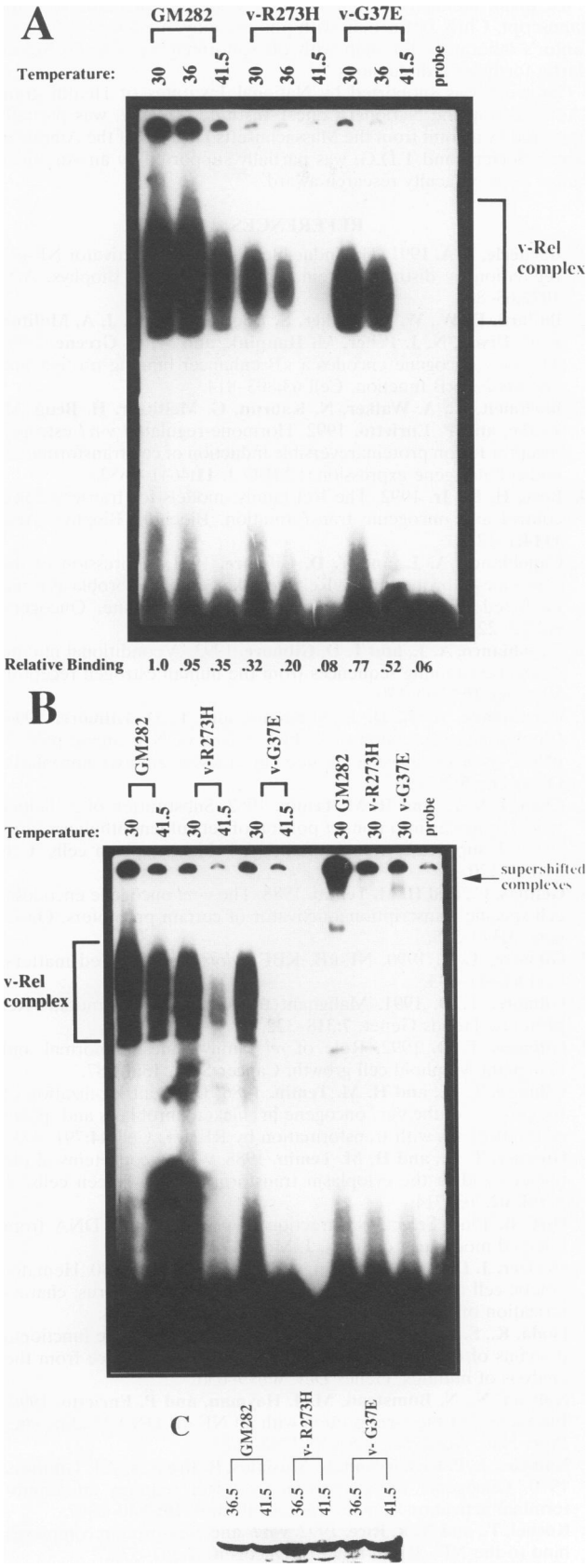
TABLE 1. Transformation of chicken spleen cells by *ts* mutant v-Rel proteins

| Virus | Spleen cell transformation (colonies/ml)[a] | |
|---|---|---|
| | 36.5°C | 41.5°C |
| GM282 | 180 | 320 |
| v-G37E | 370 | 0 |
| v-R273H | 35 | 0 |

[a] Virus was harvested from exponentially growing spleen cell lines at 36.5°C that had been transformed by the indicated viruses. One-milliliter aliquots of dilutions ($10^0$, $10^{-1}$, $10^{-2}$, and $10^{-3}$) of the freshly harvested virus were then used to infect approximately $3 \times 10^7$ fresh chicken spleen cells for 1 h at 37°C; cells were then plated in soft agar and maintained at the indicated temperatures (14, 16). Colonies were counted approximately 2 weeks later. In each case, titers were determined from two plates at different dilutions each containing 35 or fewer colonies, and values are the averages of two independent experiments.

blotting of Hirt supernatant viral DNA that had been digested with relevant restriction enzymes confirmed that transformed spleen cells were producing virus of the expected genotype (Fig. 3A). Furthermore, CEF infected with v-G37E or with v-R273H synthesized v-Rel proteins of the same size as wild-type v-Rel at both 36.5 and 41.5°C (Fig. 3B). Lastly, like wild-type v-Rel, both *ts* v-Rel proteins were located in the nucleus of CEF grown at 36.5 and 41.5°C (Fig. 3C).

To determine the approximate relative efficiency of transformation by mutant v-Rel proteins compared with wild-type v-Rel and to ensure that we had not selected for revertants of the *ts* mutant viruses in tissue culture, virus was harvested from transformed spleen cells grown at 36.5°C and was used to infect fresh spleen cells. Infected cells were then placed into soft agar at 36.5°C and at 41.5°C. Virus from GM282-transformed spleen cells induced colony formation at both 36.5 and 41.5°C (Table 1); as we have noted previously (14), viruses expressing wild-type v-Rel induce colony formation with greater efficiency at 40.5 to 41.5°C than at 36 to 37°C. Virus from v-G37E- and v-R273H-transformed spleen cells induced colony formation at 36.5°C, but no colonies were formed at




41.5°C in cells infected with v-G37E or v-R273H (Table 1). Once again, these results indicate that the spleen cells were transformed by unaltered v-G37E and v-R273H viruses that were displaying a *ts* phenotype for the induction of transformed colonies.

We and others have previously shown that v-Rel extracted from transformed spleen cells can bind to DNA containing κB sites (6, 18, 20) and that mutations that abolish or lessen the ability of v-Rel to bind DNA correspondingly affect its ability to transform chicken spleen cells (2, 24–26). To determine whether the ability of the mutant v-Rel proteins to bind DNA was thermolabile, an electrophoretic mobility shift assay (EMSA) using an oligonucleotide containing a binding site for v-Rel was performed on lysates from transformed spleen cells grown at 36.5°C. Whole cell lysates were prepared at 4°C in a detergent buffer (25 mM Tris-HCl [pH 7.4], 1.5 mM $MgCl_2$, 0.1 M KCl, 0.5% [vol/vol] Nonidet P-40, 0.5 mM dithiothreitol, 10% [wt/vol] glycerol, 1% [vol/vol] aprotinin [Sigma]) (6), and samples containing 40 μg of protein were incubated at 30, 36, or 41.5°C with a $^{32}$P-labeled oligonucleotide containing a consensus κB site from the chicken c-*rel* promoter (5). After 20 min at the given temperature, samples were placed on ice and were then immediately analyzed by polyacrylamide gel electrophoresis and autoradiography as described previously (5, 6). With lysates from cells transformed by wild-type v-Rel, roughly equal amounts of complex were detected at 30°C and at 36°C, and approximately one-third as much complex was detected when the assay was performed at 41.5°C (Fig. 4A). Generally, there was less complex seen with samples from v-G37E- and v-R273H-transformed spleen cells when the reaction was performed at 36°C compared with 30°C, and very little v-Rel complex was seen with the v-G37E and v-R273H samples when the binding assays were performed at 41.5°C (Fig. 4A). The major EMSA complex seen with lysates from all three infected cell lines could be supershifted by the inclusion of anti-v-Rel antiserum (Fig. 4B).

Although the ratios of DNA binding by the R273H and wild-type v-Rel proteins at 36°C and at 41.5°C are approximately equal (Fig. 4A), it is important to note that there is still approximately the same amount of complex with wild-type v-Rel at 41.5°C as with either of the *ts* mutant proteins when the DNA-binding assay is performed at 36°C (the permissive temperature for transformation by v-G37E and v-R273H).

FIG. 4. DNA binding by *ts* mutant v-Rel proteins in vitro. Whole cell lysates were prepared from infected spleen cell lines as described in the text, and EMSAs were performed with equalized amounts of cellular protein as described previously (6). (A) Lysates were prepared from spleen cells transformed by the indicated viruses and grown at 36.5°C, and samples containing 40 μg of protein were incubated for 20 min at the indicated temperatures with a $^{32}$P-labeled probe containing a v-Rel-binding site. Samples were then analyzed on a 5% polyacrylamide gel, and complexes were detected by autoradiography. The relative amounts of complex containing v-Rel proteins were determined by using a PhosphorImager (Molecular Dynamics). Values are expressed relative to the amount of probe bound by wild-type v-Rel at 30°C and were normalized by subtracting a background derived from the probe alone lane. (B) Lysates were prepared as described above from cells that had been shifted to 41.5°C for 12 h, and samples were incubated with radiolabeled probe at the indicated temperatures. In the three lanes before the probe control lane, anti-v-Rel antiserum was included in an EMSA that was performed at 30°C. The positions of the v-Rel-containing complexes and the supershifted complexes are indicated at the sides. (C) Spleen cells that had been maintained at 36.5°C or that had been shifted to 41.5°C for 12 h were analyzed by Western blotting with an anti-v-Rel antiserum as described for Fig. 2B.

Thus, we believe that there is a threshold of v-Rel DNA-binding activity that is needed for transformation and that the *ts* mutant proteins fall below the required level at 41.5°C. In summary, these results show that the ability of v-Rel proteins to bind DNA is *ts* in vitro and that thermolabile DNA-binding activity is correlated with the ability of the *ts* proteins to transform chicken spleen cells in vitro.

To determine whether *ts* v-Rel proteins isolated from spleen cells grown at the nonpermissive temperature for transformation could bind DNA in vitro, lysates were prepared from wild-type v-Rel-, v-G37E-, and v-R273H-transformed spleen cell lines that had been incubated for approximately 12 h at 41.5°C. Lysates were prepared as described above, and an EMSA was performed with samples that had been incubated with labeled probe at 30°C and at 41.5°C. As seen with lysates prepared from cells grown at 36.5°C, wild-type v-Rel could bind DNA in vitro at both temperatures, whereas v-G37E and v-R273H proteins could bind DNA to a significant degree only when the assays were performed at 30°C (Fig. 4B). Wild-type v-Rel isolated from cells continuously maintained at 41.5°C showed binding properties in vitro identical to those seen with wild-type v-Rel isolated from cells shifted to 41.5°C 12 h prior to preparing detergent lysates (data not shown). As a control, Fig. 4C shows that spleen cells transformed by wild-type v-Rel or either *ts* mutant contain roughly equal amounts of v-Rel at 36.5°C or when shifted to 41.5°C for 12 h prior to preparing lysates for Western blotting (immunoblotting); furthermore, cells transformed by wild-type v-Rel contain approximately equal amounts of v-Rel when maintained continuously at 41.5°C (data not shown). These results show that the DNA-binding activity of the *ts* proteins from spleen cells can be restored by incubation at lower temperatures in vitro.

Although many *ts* mutants have been isolated for oncogenes encoding protein kinases, relatively few *ts* mutants have been described for the nuclear oncoproteins. We have used site-directed mutagenesis, based on *ts* mutations described in the *Drosophila dorsal* gene, to create two *ts* mutants (v-G37E and v-R273H) of the v-Rel oncoprotein. These mutant v-Rel proteins possess a conditional ability to transform avian spleen cells and to bind to DNA in vitro. It is likely that the two mutations affect distinct domains in v-Rel. That is, the G37E mutation probably directly affects DNA binding by v-Rel, since Gly-37 lies just C terminal to a conserved domain in Rel proteins that has been shown to be directly involved in contacting DNA (21, 23). On the other hand, the mutation in v-R273H is likely to affect the ability of v-Rel to form homodimers, a function necessary for DNA binding, since other mutations near Arg-273 have been shown to affect homodimer formation by v-Rel (25, 30).

Since most data indicate that v-Rel transforms avian spleen cells by affecting gene expression, the *ts* mutants may, as we have previously suggested (11), be useful for isolating genes that are directly controlled by v-Rel and that are relevant to the transformation process. Other conditional mutants of v-Rel have been created by fusing sequences from the human estrogen receptor (ER) to v-Rel (3, 6); however, the isolation, propagation, and maintenance of v-Rel–ER-transformed cells are less convenient than with cells transformed by the *ts* mutants described here, and experiments aimed at isolating v-Rel-regulated genes in v-Rel–ER-expressing cells are complicated by the fact that estrogen can itself affect gene expression. Finally, since the residues that we changed to create the *ts* mutants of v-Rel are conserved in all other Rel/NF-κB proteins, it may be possible to generate *ts* mutants of other Rel/NF-κB transcription factors by using the strategy outlined here.

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