Avian I κ B α Is Transcriptionally Induced by c-Rel and v-Rel with Different Kinetics

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Received 13 March 1995/Accepted 13 May 1995

The Rel/NF- κ B family of transcription factors participates in the regulation of genes involved in defense responses, inflammation, healing and regeneration processes, and embryogenesis. The control of the transcriptional activation potential of the Rel/NF- κ B proteins is mediated, in part, by their association with inhibitory proteins of the I κ B family. This association results in the cytoplasmic retention of these factors until the cell receives a proper stimulatory signal. The I κ B α gene is a target for regulation by the Rel/NF- κ B proteins and is in fact upregulated in response to Rel/NF- κ B activation. A naturally occurring oncogenic variant of the Rel/NF- κ B family, *v-rel*, transforms avian lymphocytes, bone marrow cells, monocytes, and fibroblasts. Avian I κ B α expression is upregulated in cells transformed by v-Rel. Avian I κ B α is also upregulated in fibroblasts overexpressing c-Rel and oncogenic variants of c-Rel. c-Rel, a carboxy-terminally truncated variant of c-Rel, and v-Rel are all able to directly transactivate the expression of the avian I κ B α gene. However, c-Rel was the most potent activator of this gene, and the induction of I κ B α expression showed faster kinetics in cells overexpressing c-Rel than in those overexpressing v-Rel. The regulation of I κ B α induction by the Rel proteins was shown to be dependent on a 362-bp region of the I κ B α promoter that contains two potential NF- κ B binding sites and one AP-1-like binding site. Results of electrophoretic mobility shift assays using these NF- κ B binding sites indicate that major changes in the profile of DNA binding complexes in fibroblasts overexpressing v-Rel correlated temporally with the kinetic changes in v-Rel's ability to activate the expression of the I κ B α gene.

Reticuloendotheliosis virus type T induces fatal lymphomas in infected chickens (4, 18). This oncogenic retrovirus encodes a 59-kDa oncoprotein, v-Rel, which itself is able to transform avian lymphoid cells and fibroblasts (4, 18, 39, 41). v-Rel is a variant of the product of the cellular proto-oncogene *c-rel* and is missing two amino-terminal and 118 carboxy-terminal amino acids that are normally present in c-Rel (10, 50, 56). In addition, v-Rel contains multiple amino acid substitutions and small in-frame deletions that contribute to its oncogenic potential (56). This is evidenced by the fact that while wild-type c-Rel and carboxy-terminally truncated variants of c-Rel are able to transform avian cells, they do so with a reduced efficiency compared with v-Rel (33).

c-Rel and v-Rel are members of the Rel/NF- κ B family of transcription factors. These factors regulate the expression of genes involved in defense responses, healing and regeneration processes, and embryogenesis (17, 21). The Rel/NF- κ B family includes c-Rel, v-Rel, RelA, RelB, NF- κ B1, NF- κ B2, and the *Drosophila melanogaster* proteins Dorsal and Dif. These factors are identified by a conserved amino-terminal region defined as the Rel homology domain. This domain contains regions important in DNA binding, hetero- and homodimerization, and nuclear translocation (6, 17, 36, 43). Various members of the Rel/NF- κ B family are able to heterodimerize with other family members as well as associate with other transcription factors (Fos/Jun, C/EBP, Sp1, high-mobility-group protein I, cyclic AMP-independent ATF factors, and BZLF1) and components of the general transcription machinery (TATA-binding protein and TFIIB) (15, 22, 29, 47–49, 54, 57). These associations ultimately determine the transactivation potential and define

the target gene specificity of Rel/NF- κ B proteins in a given cell.

Subcellular localization also plays a role in the activity of Rel/NF- κ B proteins. This is regulated, in part, by sequences located in the proteins' carboxy-terminal regions. For example, c-Rel, NF- κ B1, NF- κ B2, and Dorsal contain carboxy-terminal cytoplasmic retention signals (9, 16, 33, 51, 59). In addition, association with a family of inhibitory molecules (I κ B proteins) results in the retention of Rel/NF- κ B proteins in the cytoplasm. I κ B proteins are characterized by the presence of multiple ankyrin repeats and include I κ B α , I κ B β , I κ B γ , Bcl-3, Cactus, and the carboxy-terminal regions of the NF- κ B1 and NF- κ B2 precursors (26, 31, 32, 58, 60). Response to stimuli that result in the activation of Rel/NF- κ B family members causes their release from bound I κ B proteins (following the proteolysis of I κ B molecules) and thereby allows for the subsequent nuclear translocation of Rel/NF- κ B factors (23, 30, 55). However, the genes encoding many of the I κ B proteins are in turn upregulated by nuclear Rel/NF- κ B factors, thereby establishing an autoregulatory loop (11, 12, 27, 35, 45, 52, 53). This ensures a controlled, transient response of Rel/NF- κ B-dependent transcription upon cellular stimulation. This regulatory mechanism has been shown to be conserved among flies, birds, and mammals (1, 11, 12, 27, 31, 40, 45).

In avian fibroblasts overexpressing v-Rel prior to transformation, v-Rel is predominately found in the nucleus (19, 20, 25, 33, 38, 39). However, upon cellular transformation, the majority of v-Rel is located in the cytoplasm (19, 25, 33, 38, 39). Cytoplasmic v-Rel is complexed with I κ B α and the precursors of NF- κ B1 and NF- κ B2 (7, 13, 14, 33, 46). In addition, this change in subcellular localization of v-Rel correlates with an increase in the expression of the genes encoding I κ B α and NF- κ B2 (25, 33). These genes along with *nfkbl* are also upregulated in fibroblasts transformed by c-Rel and carboxy-terminally truncated variants of c-Rel (33). These studies in-

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dicates that the Rel/NF- κ B/I κ B autoregulatory loop functions in transformed avian fibroblasts and suggests that v-Rel participates in the induction of avian I κ B expression. However, the exact role that v-Rel plays in gene expression has been controversial. While some studies point to the role of v-Rel as a dominant negative repressor of κ B-dependent transcription, other studies indicate that v-Rel functions as a transcriptional activator (2, 3, 8, 33, 37, 42, 47). Therefore, the function of v-Rel in the transformed cell remains to be determined.

In this study, we examined the role of c-Rel and v-Rel in the upregulation of the I κ B α gene in avian fibroblasts. Potential regulatory elements in the I κ B α promoter have been identified as responsive to transactivation by both c-Rel and v-Rel, although v-Rel was less potent of a transactivator than c-Rel. These regulatory elements contain putative κ B elements and were bound by c-Rel- and NF- κ B1(p50)-containing complexes in c-Rel-overexpressing fibroblasts. They were also bound by complexes containing c-Rel and v-Rel and complexes containing NF- κ B1(p50) and v-Rel in v-Rel-overexpressing cells. In addition, while both c-Rel and v-Rel overexpression resulted in the upregulation of the I κ B α gene, the kinetics in v-Rel-overexpressing cells were delayed compared with the kinetics in cells overexpressing c-Rel. This delay in v-Rel-dependent induction of I κ B α gene expression correlated with the accumulation of κ B binding complexes containing v-Rel and c-Rel or v-Rel and NF- κ B1(p50). Furthermore, while c-Rel plus NF- κ B1(p50) and c-Rel plus v-Rel were able to synergistically transactivate the I κ B α promoter, v-Rel and NF- κ B1(p50) could not. This finding indicates that the complex containing c-Rel and v-Rel may account for the transcriptional induction of the I κ B α gene in chicken embryo fibroblasts (CEFs) overexpressing v-Rel.

MATERIALS AND METHODS

Cells and tissue culture. CEFs were prepared from 11-day-old SPAFAS embryos (Norwich, Conn.). CEFs were maintained in culture in Eagle's minimal medium (JRH Biosciences) supplemented with 5% fetal calf serum (Summit Biotechnology), 3% chicken serum (GIBCO-BRL), and antibiotics as previously described (33). DT95 cells, an avian B-cell line derived from a chicken infected with avian leukosis virus, were maintained in culture as previously described (25).

Transfections and luciferase assays. Construction of the RCAS-rel expression vectors has been described previously (33). A 1.3-kb *HindIII-EcoRI* fragment of the genomic clone of avian I κ B α (containing the transcriptional start site and 900 bp of upstream sequence) was subcloned into the pGL-2 Basic luciferase reporter vector (Promega, Wis.) to generate p40-luc. The various deletion constructs were generated by using the 1.3-kb *HindIII-EcoRI* fragment as a template in PCRs with a downstream primer (flanking the *EcoRI* site) and various upstream primers (corresponding to the desired 5' deletion points). These PCR products were then subcloned into pBluescript (Stratagene, Calif.). These constructs were all sequenced for verification. These constructs were then digested with *KpnI* and *BamHI* (sites flanking the inserted PCR fragments), and inserts were purified and then subcloned into *KpnI-BglIII*-digested pGL-2 to generate the various deletion-reporter constructs.

Secondary cultures of CEFs were seeded at a density of 5×10^5 cells per 60-mm-diameter culture dish 1 day prior to transfection. Cells were transfected with CsCl-purified plasmids as previously described (33). Thirty-six hours post-transfection, cells were harvested and luciferase activity was determined as instructed by the manufacturer (Promega). Final values of luciferase activity were normalized to transfection efficiency as previously described (33). The adjusted luciferase activity obtained for cells transfected with the control vector (RCAS) was considered baseline activity, and the fold induction by cotransfection with the various RCAS-rel vectors was determined by reference to this baseline. All experiments were performed at least five times with a variation of less than 20%.

RNA isolation and Northern (RNA) blot analysis. RNA was isolated and Northern blot analysis was performed as previously described (33). Northern blots were hybridized with a random-primed, 32 P-labeled 1.8-kb *EcoRI* fragment of the avian I κ B α cDNA as previously described (33).

Isolation of nuclei and electrophoretic mobility shift assays (EMSAs). Nuclear extracts were prepared by using a modified version of the technique described by Schreiber et al. (44). Briefly, 10^7 cells were harvested, washed with phosphate-buffered saline, and resuspended in 400 μ l of cold hypotonic lysis buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM

phenylmethylsulfonyl fluoride 1 mM leupeptin, 1 mM aprotinin, 1 mM pepstatin). Cells were pelleted and resuspended in 400 μ l of solution A. Cells were allowed to swell on ice for 30 min. Then 25 μ l of a 10% solution of Nonidet P-40 was added, the tube was vortexed vigorously for 20 s, and nuclei were obtained by a 30-s spin. The nuclear pellet was washed twice with buffer A without Nonidet P-40 and resuspended in 100 μ l of cold solution C (20 mM HEPES [pH 7.9], 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, protease inhibitors as in solution A). The samples were then rocked at 4°C for 30 min. The nuclear extract was centrifuged for 10 min in a microcentrifuge, and the supernatant was saved and used to determine final protein concentrations.

For EMSA reactions, 5 to 10 μ g of nuclear extracts was incubated in a total reaction volume of 30 μ l containing 20 mM HEPES (pH 7.9), 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 200 μ g of bovine serum albumin per ml, and 1 μ g of poly(dI-dC) for 10 min at room temperature. The appropriate double-stranded oligonucleotides (labeled with 32 P) were then added to the reaction mixture, which was incubated for 20 min at room temperature. The oligonucleotides for EMSAs were as follows: NF- κ B site 2, AATCTTAATGG GAATCCCCCGCAT; NF- κ B site 1, GCACCGGCGGGCTGTCCAGGCC TT; and mutant NF- κ B 2, AAATCTTAATGTTATCCCCCGCAT (only the top strand is shown). For competition analysis, a 100-fold molar excess of unlabeled oligonucleotide was also added. For supershift analysis, the appropriate antiserum was added, and the reaction mixture was incubated on ice for 45 min prior to the addition of labeled oligonucleotides. Antisera used in supershift analysis have been described previously (25). Samples were then analyzed by electrophoresis in a 5% polyacrylamide gel with 0.25 $\times$ Tris-borate-EDTA. Following electrophoresis, gels were dried and DNA protein complexes were visualized by autoradiography.

RESULTS

Identification of regulatory elements in the avian I κ B α promoter. Previous studies have shown that an autoregulatory network involving the Rel/NF- κ B family of transcription factors and their associated inhibitory molecules (I κ Bs) exists in murine, porcine, and human cells (11, 12, 27, 35, 45). This regulatory network involves the transcriptional activation of I κ B genes by various members of the Rel/NF- κ B family. We have previously demonstrated that in CEFs transformed by c-Rel, a carboxy-terminally truncated form of c-Rel (c-Rel Δ), or v-Rel, there is an induction of I κ B α mRNA (25, 33). This has also been shown for avian spleen cells and DT95 cells overexpressing both c-Rel and v-Rel (25). However, these studies demonstrated that v-Rel was less effective than c-Rel in the ability to induce the expression of the I κ B α gene. Nuclear run-on analysis has shown that the induction of I κ B α mRNA by both c-Rel and v-Rel is transcriptional in nature (data not shown).

The isolation and characterization of the avian I κ B α structural gene have been previously described (34). These studies identified many potential regulatory elements upstream of the transcriptional start site of this gene. These include potential NF- κ B binding sites as well as potential AP1, C/EBP and Sp1 binding sites (34). Figure 1 shows the locations of the transcriptional start site and potential regulatory elements of the I κ B α gene. To determine the mechanism of upregulation of I κ B α mRNA by c-Rel and v-Rel and to determine the role of the potential regulatory elements involved in that upregulation, a series of luciferase reporter constructs was generated. A 1.3-kb *HindIII-EcoRI* fragment containing the transcriptional start site and potential regulatory elements of the I κ B α gene was subcloned upstream of a promoterless luciferase gene (Fig. 1; described in Materials and Methods). This construct (p40-luc) was cotransfected into CEFs along with replication-competent retroviral vectors encoding either c-Rel (RCAS-c-rel), c-Rel Δ (RCAS-c-rel Δ), or v-Rel (RCAS-v-rel). Two days later, cells were harvested and luciferase activity was determined. As shown in Fig. 2, CEFs cotransfected with RCAS-c-rel, RCAS-c-rel Δ , or RCAS-v-rel exhibited 20-, 10-, and 2-fold increases in luciferase activity, respectively, compared with CEFs cotransfected with the control vector (RCAS) alone.

FIG. 1. Diagram of $I\kappa B\alpha$ promoter-luciferase reporter constructs and locations of regulatory elements. The 5' deletion point of each construct and the locations of various potential regulatory elements are indicated. The transcription start site (Tc) is indicated by an arrow, and the large boxed region represents transcribed sequences of the $I\kappa B\alpha$ gene that were subcloned upstream of the luciferase reporter.

Therefore, this 1.3-kb fragment of the $I\kappa B\alpha$ gene contains regulatory elements responsive to these Rel proteins. This responsiveness was consistent with the level of expression of the endogenous $I\kappa B\alpha$ gene. That is, both c-Rel and c-Rel Δ showed a more potent induction of this reporter construct than did v-Rel.

To define which potential regulatory elements in the $I\kappa B\alpha$ promoter are responsive to these factors, various deletion constructs were generated (described in Materials and Methods). The locations of these deletions are shown in Fig. 1. Deletion construct p40D1-luc removes a potential NF- κ B site, p40D2-luc removes a potential AP1 site as well, p40D3-luc removes an additional putative NF- κ B site, and p40D4-luc removes another potential NF- κ B binding site. These constructs were all separately subcloned upstream of a promoterless luciferase gene and cotransfected with the various Rel expression constructs as described above. Figure 2 shows that removal of the first potential NF- κ B binding site (p40D1-luc) resulted in a

FIG. 2. Responsiveness of the $I\kappa B\alpha$ promoter-luciferase reporter constructs to Rel proteins. CEFs were transfected with 2 μ g of the various $I\kappa B\alpha$ promoter-luciferase reporter constructs indicated along with 8 μ g of either RCAS, RCAS-c-rel, RCAS-c-rel Δ , or RCAS-v-rel. Two days later, cells were harvested and luciferase activity was determined. Fold stimulation of luciferase activity was determined by comparison with the activity obtained when the RCAS vector was cotransfected with the various reporter constructs.

decrease in reporter activity and that removal of the potential AP1 binding site (p40D2-luc) resulted in a dramatic decrease in reporter activity in response to c-Rel. Removal of the first NF- κ B binding site as well as the potential AP1 and second NF- κ B binding sites (p40D3-luc) rendered the $I\kappa B\alpha$ promoter virtually unresponsive to either c-Rel, c-Rel Δ , or v-Rel. However, the construct p40D4-luc restored some responsiveness, indicating that some negative regulatory elements may be located between D3 and D4. The downstream NF- κ B sites may play a role in the activation of the p40D4-luc construct; however, the role of these sites in $I\kappa B\alpha$ induction remains to be determined. Removal of the first NF- κ B site and the AP1 site had the most dramatic effect on expression of the reporter construct, indicating that these sites may play a major role in $I\kappa B\alpha$ expression. In addition, the removal of the AP1 site (located at nucleotide -664) had the most dramatic effect on the reporter construct in response to c-Rel overexpression, indicating that factors binding to the AP1 (-664) and NF- κ B (-615) sites may cooperate in the activation of the $I\kappa B\alpha$ gene. Since removal of the AP1 binding site had a much more dramatic effect on the response of the $I\kappa B\alpha$ promoter to c-Rel than v-Rel, these studies also imply that c-Rel and AP1 factors may functionally interact with different consequences than v-Rel and AP1 factors. However, future studies using site-directed mutagenesis will be required to fully define the roles that these specific sites and factors play in the transcriptional regulation of the $I\kappa B\alpha$ gene. These studies were also performed with DT95 cells, an avian B-cell line, with the same results (data not shown), indicating that these regulatory elements are not cell type specific in their function.

Induction of $I\kappa B\alpha$ mRNA is delayed in v-Rel-overexpressing CEFs. An interesting observation was that v-Rel did not activate the $I\kappa B\alpha$ promoter as strongly as it induced $I\kappa B\alpha$ mRNA levels in transformed CEFs (relative to c-Rel) (25, 33). c-Rel induces $I\kappa B\alpha$ mRNA in CEFs approximately five times more strongly than v-Rel, but the cotransfection studies represented in Fig. 2 indicate that v-Rel expression resulted in a 10-fold less induction of transcription from the $I\kappa B\alpha$ promoter than did c-Rel expression. A possible explanation for this discrepancy is that the experiments examining the levels of $I\kappa B\alpha$ mRNA were done at 3 to 4 weeks posttransfection with RCAS-v-rel, while

FIG. 3. Kinetics of endogenous $\text{I}\kappa\text{B}\alpha$ mRNA induction. (A) CEFs were transfected with RCAS (lane 1), RCAS-c-rel (lane 2), or RCAS-v-rel (lane 3). Two days posttransfection, cells were harvested, RNA was isolated, and Northern blot analysis (using a radiolabeled probe specific for avian $\text{I}\kappa\text{B}\alpha$) was performed. The top portion of the panel corresponds to the Northern blot, and the bottom portion represents the same blot stained with methylene blue showing the 28S rRNA to verify equal loading of samples. (B) CEFs were transfected with RCAS-v-rel and harvested at the time of transfection (lane 1), 1 week posttransfection (lane 2), 2 weeks posttransfection (lane 3), or 3 weeks posttransfection (lane 4). RNA was isolated, and Northern blot analysis was performed as described above. The top portion of the panel represents the Northern blot, and the bottom portion represents the same blot stained with methylene blue as described above.

the studies evaluating the activation of the $\text{I}\kappa\text{B}\alpha$ promoter were performed 2 days posttransfection. Therefore, to determine if these differences were also seen in endogenous $\text{I}\kappa\text{B}\alpha$ mRNA levels, Northern blot analysis was performed. Figure 3A shows that at 2 days posttransfection, RCAS-c-rel resulted in a dramatic increase in $\text{I}\kappa\text{B}\alpha$ mRNA levels compared with levels in cells transfected with RCAS alone (compare lanes 1 and 2). However, CEFs transfected with RCAS-v-rel showed very low levels of $\text{I}\kappa\text{B}\alpha$ mRNA 2 days posttransfection (compare lanes 2 and 3). The levels of c-Rel and v-Rel were comparable in transfected cells 2 days posttransfection, as determined by Western blot (immunoblot) analysis (data not shown). To determine when the expression of v-Rel resulted in an increase in $\text{I}\kappa\text{B}\alpha$ mRNA, a kinetic experiment was performed. CEFs were transfected with RCAS-v-rel and harvested at the time of transfection and 1, 2, and 3 weeks later. RNA was isolated, and Northern blot analysis was performed. Figure 3B shows that maximal induction of $\text{I}\kappa\text{B}\alpha$ mRNA levels occurred at 2 to 3 weeks posttransfection. No increase in $\text{I}\kappa\text{B}\alpha$ mRNA levels was seen in CEFs transfected with RCAS alone over this same time period (33). When CEFs 3 weeks after transfection with RCAS-v-rel were subsequently transfected with the p40-luc construct, luciferase activity was four times higher than in cells cotransfected with the control vector (RCAS). This increase in $\text{I}\kappa\text{B}\alpha$ promoter activity was therefore fivefold less than seen for CEFs transfected with RCAS-c-rel (data not shown). This finding is in accordance with the results obtained for endogenous $\text{I}\kappa\text{B}\alpha$ mRNA levels. Therefore, v-Rel induces $\text{I}\kappa\text{B}\alpha$ mRNA to a lower level and with delayed kinetics compared with c-Rel in CEFs. Similar results have been found for avian spleen cells and DT95 cells (25).

The profile of NF- κB binding complexes changes temporally in CEFs overexpressing v-Rel. Since the studies described above and the work of other laboratories have implicated Rel/NF- κB factors as being important in the regulation of $\text{I}\kappa\text{B}\alpha$ genes, we chose to examine the profile of nuclear NF- κB -binding proteins of CEFs in which expression of the $\text{I}\kappa\text{B}\alpha$ gene is induced. A possible explanation for the delayed kinetics of $\text{I}\kappa\text{B}\alpha$ induction in CEFs transfected with RCAS-v-rel may be that there is a change in the profile of NF- κB -binding factors in the nuclei of these cells at different times after transfection. In fact, a kinetic change in NF- κB binding complexes has been shown in avian spleen cells and DT95 cells following the transfection of a v-Rel expression construct (25). Therefore, we

examined the profiles of factors that were able to bind to the NF- κB binding sites located at -615 and -860 in the $\text{I}\kappa\text{B}\alpha$ promoter following transfection of either RCAS-c-rel or RCAS-v-rel (since these sites were located in the region required for full promoter activity in response to the overexpressed Rel proteins). No differences were seen in the profiles of NF- κB -binding factors regardless of which NF- κB binding site was used for analysis; therefore, EMSAs using a radiolabeled oligonucleotide containing the NF- κB binding site located at -615 were used for the following studies. EMSA using this NF- κB site (Fig. 4A) showed that the profile of proteins binding to this site did not change appreciably in CEFs transfected with RCAS-c-rel 2 days posttransfection compared with 3 weeks posttransfection. CEFs transfected with the RCAS vector alone exhibit virtually no binding activity to this κB site (Fig. 4A, lane 1). Nuclear extracts of CEFs 2 days after transfection with RCAS-c-rel contained three major binding complexes (Fig. 4A, lane 2). While the nuclear extracts of CEFs 3 weeks after transfection with RCAS-c-rel contained three complexes that comigrated with the complexes seen 2 days posttransfection, the amount of the two faster-migrating complexes increased dramatically (Fig. 4A; compare lanes 2 and 3). However, these differences may not be just quantitative in nature. It is possible that they represent changes in composition as well, but these newly formed complexes simply comigrate with the complexes seen 2 days following transfection. Additional studies using UV cross-linking and further supershift analysis may determine if qualitative differences are seen in these complexes with time. These complexes were shown to be specific by competition analysis with wild-type and mutant oligonucleotides (data not shown). Supershift analysis using an antiserum specific for either c-Rel or NF- $\kappa\text{B}1$ (p50) (Fig. 5A, lanes 2 and 3) indicated that all three complexes found in CEFs 2 days after transfection with RCAS-c-rel contain both c-Rel and NF- $\kappa\text{B}1$ proteins. The two major κB binding complexes found in CEFs 3 weeks after transfection with RCAS-c-rel also contain both c-Rel and NF- $\kappa\text{B}1$ (Fig. 6A, lanes 2 and 3). An antiserum to NF- $\kappa\text{B}1$ resulted in a complete supershift of complexes found in the nuclei of CEFs 3 weeks after transfection with RCAS-c-rel. However, this antiserum did not result in a complete

FIG. 4. Comparison of the profiles of nuclear κB -DNA-binding proteins following overexpression of c-Rel and v-Rel. (A) CEFs were transfected with RCAS (lane 1) or RCAS-c-rel (lanes 2 and 3). Two days (lanes 1 and 2) and 3 weeks (lane 3) posttransfection, cells were harvested, nuclear extracts were prepared and used in EMSA with the radiolabeled κB site found in the $\text{I}\kappa\text{B}\alpha$ promoter (located at -615). (B) CEFs were transfected with RCAS (lane 1) or RCAS-v-rel (lanes 2 and 3). Two days (lanes 1 and 2) and 3 weeks (lane 3) posttransfection, cells were harvested, nuclear extracts were prepared and used in EMSA as described above.

FIG. 5. Identification of nuclear κB DNA-binding factors 2 days posttransfection with Rel expression vectors. (A) CEFs were transfected with RCAS-c-rel and harvested 2 days posttransfection, and nuclear extracts were prepared and used in EMSA. The following antisera were preincubated with the nuclear extracts prior to analysis: lane 1, no antibody; lane 2, antibody to the C terminus of c-Rel; lane 3, antibody to the N terminus of NF- $\kappa B1$. (B) CEFs were transfected with RCAS-v-rel and harvested 2 days posttransfection, and nuclear extracts were prepared and used in EMSA. The following antisera were preincubated with the nuclear extracts prior to analysis: lane 1, no antibody; lane 2, antibody to the C terminus of c-Rel; lane 3, antibody to the envelope region of v-Rel; lane 4, antibody to the N terminus of NF- $\kappa B1$.

supershift of complexes found in the nuclei of CEFs 2 days after transfection with RCAS-c-rel; therefore, the presence of other Rel/NF- κB family members (or other factors) in these complexes remains to be determined. We are currently in the process of developing antisera specific for avian RelA and NF- $\kappa B2$ proteins.

There were major kinetic changes in the profiles of NF- κB binding factors in CEFs following the transfection of RCAS-v-rel. Nuclear extracts of CEFs 2 days after transfection with RCAS-v-rel contain five major NF- κB binding complexes (Fig. 4B, lane 2). However, 3 weeks following transfection of RCAS-v-rel, the nuclear extracts contain only three major NF- κB binding complexes, with the most prominent being the fastest-migrating complex (Fig. 4B, lane 3). These complexes were all shown to be specific by competition analysis (data not shown). Supershift analysis using a v-Rel-specific antiserum showed that the NF- κB binding complexes found in the nuclei of CEFs 2 days after transfection with RCAS-v-rel all contain v-Rel

FIG. 6. Identification of nuclear κB DNA-binding factors 3 weeks after transfection with Rel expression vectors. (A) CEFs were transfected with RCAS-c-rel and harvested 3 weeks posttransfection, and nuclear extracts were prepared and used in EMSA. The following antisera were preincubated with the nuclear extracts prior to analysis: lane 1, no antibody; lane 2, antibody to the C terminus of c-Rel; lane 3, antibody to the N terminus of NF- $\kappa B1$. (B) CEFs were transfected with RCAS-v-rel and harvested 3 weeks posttransfection, and nuclear extracts were prepared and used in EMSA. The following antisera were preincubated with the nuclear extracts prior to analysis: lane 1, no antibody; lane 2, antibody to the envelope region of v-Rel; lane 3, antibody to the C terminus of c-Rel; lane 4, antibody to the N terminus of NF- $\kappa B1$.

(Fig. 5B, lane 3). These studies also showed that antisera to both c-Rel and NF- $\kappa B1$ (p50) resulted in a supershift, indicating that both c-Rel and NF- $\kappa B1$ are also contained in some of these complexes (Fig. 5B, lanes 2 and 4). However, the complexity of the nuclear NF- κB binding profile in these cells made it difficult to determine exactly which complexes contained c-Rel or NF- $\kappa B1$. Supershift analysis of the nuclear NF- κB binding complexes in CEFs 3 weeks after transfection with RCAS-v-rel revealed that the most prominent complex in these cells is composed of two distinct complexes (Fig. 6B, lanes 3 and 4). One of these contains v-Rel and c-Rel (the top complex), and the other contains v-Rel and NF- $\kappa B1$ (p50) (the bottom complex). Again, all complexes in these cells contained v-Rel (Fig. 6B, lane 2). In addition, the two more slowly migrating complexes appeared to also contain some c-Rel and NF- $\kappa B1$ (p50), since they also shifted when antisera to these factors were included in the EMSA (Fig. 6B, lanes 3 and 4). Incubation of the various extracts described above with preimmune antisera failed to result in a supershift or decrease in NF- κB binding activity (data not shown).

Therefore, the qualitative profile of nuclear NF- κB binding complexes in CEFs overexpressing c-Rel does not change dramatically with time. However, CEFs overexpressing v-Rel do exhibit major qualitative changes in the profile of nuclear NF- κB binding complexes with time. Not only does the number of complexes change in these cells, but the major NF- κB binding complexes are composed of v-Rel and c-Rel or v-Rel and NF- $\kappa B1$ (p50). These changes occurred at the time that overexpression of v-Rel resulted in both an increase in the endogenous levels of $I\kappa B\alpha$ mRNA and an increase in v-Rel's ability to activate the p40-luc reporter construct.

v-Rel and c-Rel synergistically activate the $I\kappa B\alpha$ promoter, while v-Rel and NF- $\kappa B1$ do not. Since a correlation between the induction of $I\kappa B\alpha$ mRNA and the appearance of particular Rel/NF- κB complexes was observed, we wanted to determine if overexpression of these factors alone or in combination could result in the activation of the p40-luc reporter construct. Therefore, we first determined if there was a dose-dependent response of this reporter construct to individually expressed Rel/NF- κB factors in transfected CEFs. CEFs were transfected with increasing amount of RCAS-c-rel, RCAS-c-rel Δ , RCAS-v-rel, or RCAS-NF- $\kappa B1$ (p50), and the fold stimulation of the p40-luc reporter was determined (Fig. 7A). These experiments showed that induction of this reporter construct increased with a dose-dependent response for each of the Rel/NF- κB factors tested. As shown in Fig. 2, both c-Rel and c-Rel Δ resulted in higher induction of the reporter than v-Rel or NF- $\kappa B1$ (p50).

Since the major nuclear NF- κB binding complexes in CEFs overexpressing c-Rel contained both c-Rel and NF- $\kappa B1$ (p50), we wanted to determine if coexpression of c-Rel and NF- $\kappa B1$ (p50) in CEFs would result in an increased induction of the p40-luc reporter construct. A constant amount of RCAS-c-rel was cotransfected along with the p40-luc construct and increasing amounts of RCAS-NF- $\kappa B1$ (p50) into CEFs, and the amount of luciferase activity was determined. Cotransfection of CEFs with a constant amount of RCAS-c-rel and increasing amounts of RCAS-NF- $\kappa B1$ (p50) resulted in a synergistic increase in the activity of the $I\kappa B\alpha$ promoter (Fig. 7B). To test the role in $I\kappa B\alpha$ induction of the two major NF- κB binding complexes (v-Rel-NF- $\kappa B1$ and v-Rel-c-Rel) found in the nuclei of CEFs transfected with RCAS-v-rel 3 weeks posttransfection, CEFs were transfected with a constant amount of RCAS-c-rel along with the p40-luc reporter construct and increasing amounts of RCAS-v-rel. In addition, CEFs were cotransfected with constant amounts of RCAS-v-rel along with the p40-luc reporter construct and increasing amounts of

FIG. 7. (A) Dose-dependent response of the IκBα promoter to Rel proteins. CEFs were cotransfected with a constant amount of the p40-luc reporter construct (2 μg) and increasing amounts of the various Rel expression vectors (RCAS-c-rel, RCAS-c-relΔ, RCAS-v-rel, and RCAS-p50). Thirty-six hours later, cells were harvested and luciferase activity was determined. The fold stimulation of luciferase activity was determined by comparing the obtained values with those for transfection with the RCAS vector. (B) Synergistic stimulation of the IκBα promoter by various Rel proteins. CEFs were cotransfected with the p40-luc reporter construct (2 μg) and the indicated Rel expression vectors. Two days posttransfection, cells were harvested and fold stimulation of luciferase activity was determined.

RCAS-NF-κB1(p50). Two days later, cells were harvested and luciferase activity was determined. While c-Rel and v-Rel synergistically activated the IκBα promoter, v-Rel and NF-κB1(p50) did not (Fig. 7B). This result indicates that the v-Rel-c-Rel complex in CEFs 3 weeks after transfection with RCAS-v-rel may account for the increase in transcription of the IκBα gene at that time. This result also indicates that the v-Rel-NF-κB1(p50) complexes seen in the nuclei of these cells do not function as a transactivator of the avian IκBα gene.

DISCUSSION

Previous studies have shown that porcine, murine, and human Rel/NF-κB transcription factors and their cognate inhibitors (IκBs) are part of an autoregulatory network (11, 12, 27, 35, 45). Activation of Rel/NF-κB factors and their subsequent release from cytoplasmic complexes with IκBs results in the nuclear translocation of these factors and increased expression of genes under their regulation. One of the target genes for Rel/NF-κB regulation is the gene encoding IκBα. Various members of the Rel/NF-κB family have been shown to transcriptionally activate the IκBα gene (11, 12, 27, 35, 45). The ability of certain Rel/NF-κB factors to regulate the expression of IκBα may be cell type specific, and a differential response to various external signals may also be seen. However, the mechanism of activation has been shown to be transcriptional and dependent on the presence of NF-κB binding sites located in the promoters of the various IκBα genes (11, 12, 27, 35, 45). Additional, potential regulatory elements have also been identified in IκBα promoters, but their role in expression has yet to

be determined. An additional level of regulation of IκBα is due to the association with Rel/NF-κB factors, which results in an increase in the half-life of IκBα (12, 25, 45). These two regulatory mechanisms thereby result in an increase in the steady-state levels of IκBα following activation of Rel/NF-κB transcription factors. This may serve to limit the induction of genes under control of Rel/NF-κB factors following stimulation.

We have previously shown that avian IκBα is subject to similar regulatory mechanisms. In both CEFs and avian lymphoid cells (DT95) overexpressing either c-Rel or v-Rel, the steady-state levels of IκBα mRNA and protein are increased (25, 33). However, while v-Rel is able to stabilize IκBα as efficiently as c-Rel, it is a less effective inducer of IκBα mRNA levels (25, 33). In fact, v-Rel is able to induce IκBα mRNA five times less efficiently than c-Rel and does so with delayed kinetics. These observations led to the hypothesis that v-Rel may not directly induce expression of the IκBα gene. In this study, we found that c-Rel and v-Rel are able to upregulate the avian IκBα promoter directly. However, v-Rel is a less potent activator than c-Rel. Activation of the IκBα promoter is dependent on a 362-bp region that contains two potential NF-κB binding sites and one potential AP1 binding site. Deletion of this region rendered the IκBα promoter unresponsive to either c-Rel, c-RelΔ, or v-Rel. Interestingly, while removal of the first NF-κB site (−860) resulted in a decrease in response to the overexpressed Rel proteins, the concomitant removal of the AP1 site (−664) resulted in a dramatic decrease in the activity of the IκBα promoter in response to c-Rel (compare construct p40D1-luc with p40D2-luc in Fig. 2). This same effect was not seen in response to v-Rel, implying that c-Rel and AP1 factors may functionally interact while v-Rel and AP1 factors may not. Recent evidence supporting this possibility indicates that while c-Rel and AP1 factors are able to synergistically activate the IκBα promoter, v-Rel and AP1 factors do not coactivate this promoter (32a). However, the exact roles of these individual sites and additional potential regulatory sites in the regulation of IκBα expression remain to be determined; these studies are in progress.

The fact that a region of the IκBα promoter containing NF-κB binding sites was important in the regulation of IκBα gene expression coupled with the observation that v-Rel overexpression in CEFs resulted in a delayed activation of both endogenous IκBα mRNA levels and an IκBα promoter-luciferase reporter construct prompted us to examine the profiles of nuclear NF-κB binding factors before and after endogenous IκBα induction. Two days following the transfection of CEFs with RCAS-v-rel, the profile of nuclear NF-κB binding factors consisted of five major complexes present at approximately equivalent levels. However, 3 weeks following transfection, the profile of NF-κB binding complexes was less varied and the level of the two faster-migrating complexes increased. One of these two major complexes was shown to contain v-Rel and c-Rel, and the other contained v-Rel and NF-κB1(p50) (the presence of additional Rel/NF-κB factors or other factors in these complexes will be determined in future studies as antisera to these factors become available). Therefore, at a time when overexpression of v-Rel results in an increase in the levels of endogenous IκBα mRNA and increased expression of an IκBα promoter-luciferase reporter construct, there are changes in the profile of nuclear NF-κB binding factors. The time required to achieve these changes may account for the delayed kinetics of IκBα induction in v-Rel-overexpressing cells. Similar results have been found for DT95 cells overexpressing v-Rel, but the profile of NF-κB binding complexes is different from that seen in CEFs overexpressing v-Rel (25). These differences could be due to cell-type-specific responses.

We also observed that while v-Rel and c-Rel were able to function synergistically in the activation of the I κ B α promoter, v-Rel and NF- κ B1(p50) were not. Therefore, the transactivating complex in v-Rel-overexpressing cells may consist of v-Rel-c-Rel heterodimers, and the v-Rel-NF- κ B1(p50) complex may be inactive. This would also explain why the levels of I κ B α induction are less in CEFs overexpressing v-Rel than in those overexpressing c-Rel, since c-Rel and NF- κ B1(p50) are able to synergistically activate the I κ B α promoter. In fact, overexpression of c-Rel, NF- κ B1(p50), and v-Rel together resulted in an even higher activation of the I κ B α promoter indicating that the formation of both c-Rel-v-Rel and c-Rel-NF- κ B1(p50) complexes can overcome the weakly transactivating v-Rel-NF- κ B1(p50) complexes (data not shown).

The delay in the activation of I κ B α induction correlated with a delay in the accumulation of high levels of c-Rel-v-Rel complexes in the nuclei of CEFs overexpressing v-Rel. This delay could be due to a need for a threshold level of v-Rel to be reached, which could result in either the overexpression of c-Rel or an increase in the formation or nuclear translocation of c-Rel-v-Rel complexes. Interestingly, I κ B α induction by c-Rel was not subject to the same kinetic restraints. I κ B α expression is at high levels in CEFs 2 days following transfection with RCAS-c-rel even though c-Rel levels do not reach maximum until 2 to 3 weeks posttransfection (data not shown). We have previously demonstrated that overexpression of v-Rel in CEFs does not result in an increase in the steady-state levels of c-Rel (25, 33). Therefore, an increased expression of c-Rel does not account for the kinetic changes observed in nuclear NF- κ B binding complexes in CEFs overexpressing v-Rel. However, the increased expression of v-Rel could result in an increase in the formation of heterodimers with endogenous c-Rel, and this could lead to an increase in the presence of these complexes in the nuclei over time. An additional mechanism leading to the kinetic increase in the presence of these nuclear complexes may be that overexpression of v-Rel results in the sequestration of available pools of I κ B molecules, thereby resulting in increased nuclear translocation of cytoplasmic NF- κ B binding complexes.

Both c-Rel Δ and v-Rel were less effective in the ability to induce I κ B α expression than c-Rel, possibly because both c-Rel Δ and v-Rel are missing the most C-terminal transactivation domain that is present in c-Rel (33). Studies using c-Rel-v-Rel chimeras have revealed that the low activation of I κ B α gene expression can be mapped to mutations in the C terminus of v-Rel (24). Therefore, the decreased transactivation potential of c-Rel Δ and v-Rel could account for their lower induction of I κ B α expression. Through v-Rel's association with c-Rel, some transactivation potential would be regained, but association with NF- κ B1(p50) would not result in a DNA binding complex with high transactivation potential, since NF- κ B1(p50) has been implicated as being a weak transactivator (5, 21, 28). This is evidenced by the fact that while both c-Rel and NF- κ B1(p50) and c-Rel and v-Rel synergistically activate the I κ B α promoter, v-Rel and NF- κ B1(p50) do not.

There also appears to be an inverse correlation between the abilities of the various Rel proteins to induce I κ B α expression and cellular transformation. v-Rel is able to transform CEFs more efficiently than c-Rel Δ , which in turn transforms more efficiently than c-Rel (33). However, c-Rel transactivates the I κ B α promoter better than c-Rel Δ or v-Rel. Therefore, CEFs overexpressing c-Rel may show a transient elevation in the expression of Rel-regulated genes, and the high induction of I κ B α might serve to limit this response. However, CEFs overexpressing v-Rel may show a delayed, steady increase in Rel-regulated gene expression. This would be due to the fact that

v-Rel overexpression results in a delayed, lower-level induction of I κ B α , and v-Rel could also sequester the available pools of I κ B α , thereby releasing Rel/NF- κ B complexes to the nucleus to ensure a sustained increase in Rel-regulated gene expression. These differences between v-Rel and c-Rel could therefore account for their different transforming properties.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant CA33192 from the National Cancer Institute and grant 003658-470 from the Texas Advanced Research Program. J. Schatzle was supported by postdoctoral fellowship CA63767-02 from the National Cancer Institute.

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