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The c-*rel* protooncogene product c-Rel but not NF-κB binds to the intronic region of the human interferon-γ gene at a site related to an interferon-stimulable response element

ANTONIO SICA*, TSE-HUA TAN†, NANCY RICE‡, MARCUS KRETZSCHMAR§, PARITOSH GHOSH*, AND HOWARD A. YOUNG*¶

*Laboratory of Experimental Immunology, †Biological Carcinogenesis and Development Program, Program Resources, Inc./DynCorp, and ‡Advanced BioScience Laboratories-Basic Research Program, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD 21702-1201; and §Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, New York, NY 10021

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ABSTRACT **Interferon-γ (IFN-γ) is an important immunoregulatory protein that is expressed usually only in large granular lymphocytes and T cells. The gene encoding IFN-γ was previously found to contain an intronic enhancer element that was not tissue-specific in its activity, despite the restricted expression of the intact IFN-γ-encoding gene. Using nuclear extracts from the human T-cell line Jurkat, we have now identified two protein-binding regions in this intronic enhancer element. One of the protected regions has strong partial identity to the NF-κB site present in the promoter region of the human interleukin 2-encoding gene. Based on this observation and recent reports of the interaction of the c-*rel* protooncogene product (c-Rel) with NF-κB sites, we determined whether c-Rel could interact with the intronic enhancer element in the human IFN-γ genomic DNA. Most surprisingly, gel-shift analysis, using c-Rel expressed in *Escherichia coli* established that c-Rel binds specifically to the IFN-γ intronic DNA but not to the interleukin 2-like NF-κB site. Additional studies with antibodies prepared against c-Rel peptides verified specificity of the interaction of c-Rel with this binding site. In addition, using an affinity-purified p50 subunit of the NF-κB complex, we observed that the p50 protein did not bind to this additional c-Rel-binding site. Furthermore, nucleotide sequence analysis of this DNA region revealed a strong similarity of the additional c-Rel-binding site to a previously identified IFN-stimulable response element. These data show that c-Rel can interact with DNA regions distinct from that recognized by NF-κB and may, in fact, be involved in transcriptional regulation of the IFN-stimulable genes via the IFN-stimulable response element.**

Interferon-γ (IFN-γ) has been shown to play a major role in promoting specific mechanisms of host defense (for review, see ref. 1). This protein has numerous immunomodulatory effects including induction of Fc receptor, major histocompatibility complex class I and class II antigen expression (2), regulation of cytokine gene expression [e.g., interleukin (IL) 1, IL-6, and tumor necrosis factor] (3), and promotion of activation of numerous immune effector cells, including monocytes and B cells. IFN-γ also has demonstrated promise as a therapeutic agent for immunodeficiency states, infections, and neoplastic states and has often been evoked in the pathogenesis of such disorders (1).

In contrast to its diverse biological effects, IFN-γ production *in vivo* is tightly regulated, as synthesis of IFN-γ mRNA has been detected predominantly in activated T cells and large granular lymphocytes (LGL) (1, 4–6). This pattern of gene expression is unique among cytokines, and the molecular mechanisms that regulate IFN-γ gene expression have not yet been fully defined. Recent reports, however, have identified DNA sequences in the 5′ promoter region of the human gene that respond to phytohemagglutinin/phorbol 12-myristate 13-acetate (7–9) and human T-lymphotropic virus type I tax protein (9), and ConA and estrogen in the 5′ promoter region of the mouse gene (10).

This laboratory has identified enhancer activity present in the first intron of human IFN-γ genomic DNA (nucleotides 405–674), which is active in both T cells and fibroblasts and is phorbol 12-myristate 13-acetate-inducible in T cells but constitutive in fibroblasts (7). This region previously has been found to contain a strong DNase I hypersensitive site (11, 12), to have homology to DNA sequences upstream of the IL-2 gene (11), and also to contain a possible Z-DNA region. Based on these observations, analysis of the interaction of this intronic DNA with DNA-binding proteins may provide important information about the control of IFN-γ gene expression. In this report, we show that this intronic DNA contains a binding site for the c-*rel* protooncogene product c-Rel that is distinct from the NF-κB site and does not interact with NF-κB. Furthermore, this nucleotide region has partial identity to an IFN-stimulable response element (ISRE), suggesting that c-Rel might play a role in IFN gene induction.

MATERIALS AND METHODS

Plasmids. The plasmid pKKrel contained the complete human c-Rel-coding sequence without the *Alu* exon (13) under the transcriptional control of the bacterial *trp–lac* (*trc*) fusion promoter (T.-H.T., unpublished work). The c-Rel mammalian cell expression vector, pRSVc-Rel, was constructed with the human c-Rel gene under the control of the Rous sarcoma virus long terminal repeat (T.-H.T., unpublished work). The parental pK-RSPA control vector (ref. 14, from D. Derse, National Cancer Institute-Frederick Cancer Research and Development Center) contained multiple cloning sites in between the Rous sarcoma virus promoter and the simian virus 40 polyadenylylation signal.

DNA-Binding Assay. Bacterial extract containing the human c-Rel protein was prepared as described by Kadonaga *et al.* (15). *Escherichia coli* DH5 [F^-, *endA1*, *hsdR17* (r_k^-, m_k^+), *supE44*, *thi-1*, λ^-, *recA1*, *gyrA96*, *relA1*] was used as the bacterial host. Gel-mobility-shift assays were done as described by Tan *et al.* (16). Two different antisera, α-27 recognizing the amino-terminal region of the human c-Rel protein (antiserum II; ref. 17) and α-1135, recognizing the

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Abbreviations: IFN, interferon; ISRE, IFN-stimulable response element; IL, interleukin; IL-2Rα, interleukin 2 receptor α; HIV-1, human immunodeficiency virus type 1.

¶To whom reprint requests should be addressed at: National Cancer Institute-Frederick Cancer Research and Development Center, Building 560, Room 31-93, Frederick, MD 21702-1201.

carboxyl-terminal region of the c-Rel protein (N.R., unpublished results), were preincubated for 10 min at room temperature with *E. coli* extract containing the c-Rel protein. The mixtures were further incubated at room temperature with ^{32}P-labeled probe, in a total vol of 20 μl, for 20 min before gel-shift analysis. For peptide competition assay, peptide and antiserum were preincubated together for 5 min at room temperature, followed by sequential incubation with the c-Rel-containing bacterial extract for 5 min and then with the ^{32}P-labeled probe for 20 min. DNase I footprint analysis was done essentially as described by Angel *et al.* (18). The sequence of the protected region E1 was obtained by Maxam–Gilbert chemical DNA sequencing (19).

Oligonucleotides. Oligonucleotides were synthesized by the phosphoramidite method on a Cyclone automated DNA synthesizer (MilliGen/Biosearch, Burlington, MA). The synthetic oligonucleotides were purified through Pure Pak cartridge columns (Perkin–Elmer). Complimentary strands were denatured at 85°C for 10 min and annealed at room temperature overnight. Sequences for the plus strand of the oligonucleotides used are as follows: C1, GAGACTTAAAAGGGA; C2, TTAAAAGGGATTTATGAAT; C3, TTTATGAATTTTCCAAAA; $\kappa B_{IL\text{-}2R\alpha}$ [κB site of the IL-2 receptor α (IL-2Rα) gene] (20), GATCAGGGGAATCTCCC; mutant $\kappa B_{IL\text{-}2R\alpha}$, GATCAGctcAATCTCCC (lowercase letters refer to mutated oligonucleotides); $\kappa B_{IL\text{-}2}$ (κB site of the IL-2 gene) (21), GATCGACCAAGAGGGATTTCACCTAAATC; κB_{IgK} (κB site of the immunoglobulin κ light-chain gene) (22), GATCAGAGGGGACTTTCCGAGAGG; 6-16 ISRE (23), GGGAAAATGAAACT; mutant 6-16 ISRE, GATAAAATGAAACT.

The radiolabeled double-stranded probes were labeled with [α-^{32}P]dCTP or dATP using the Klenow fragment of DNA polymerase I (Bethesda Research Laboratories) to a specific activity of ≈2 × 10^5 cpm/ng.

RESULTS

In an effort to identify the precise region of this intronic DNA responsible for the enhancer activity, we performed DNase I footprint analysis using a nuclear extract prepared from the human T-cell line Jurkat. From this analysis, we have identified two putative protein-binding regions, E1 and E2 (data not shown, E2 not further discussed). DNA sequencing permitted us to identify one of the protected regions (E1) as

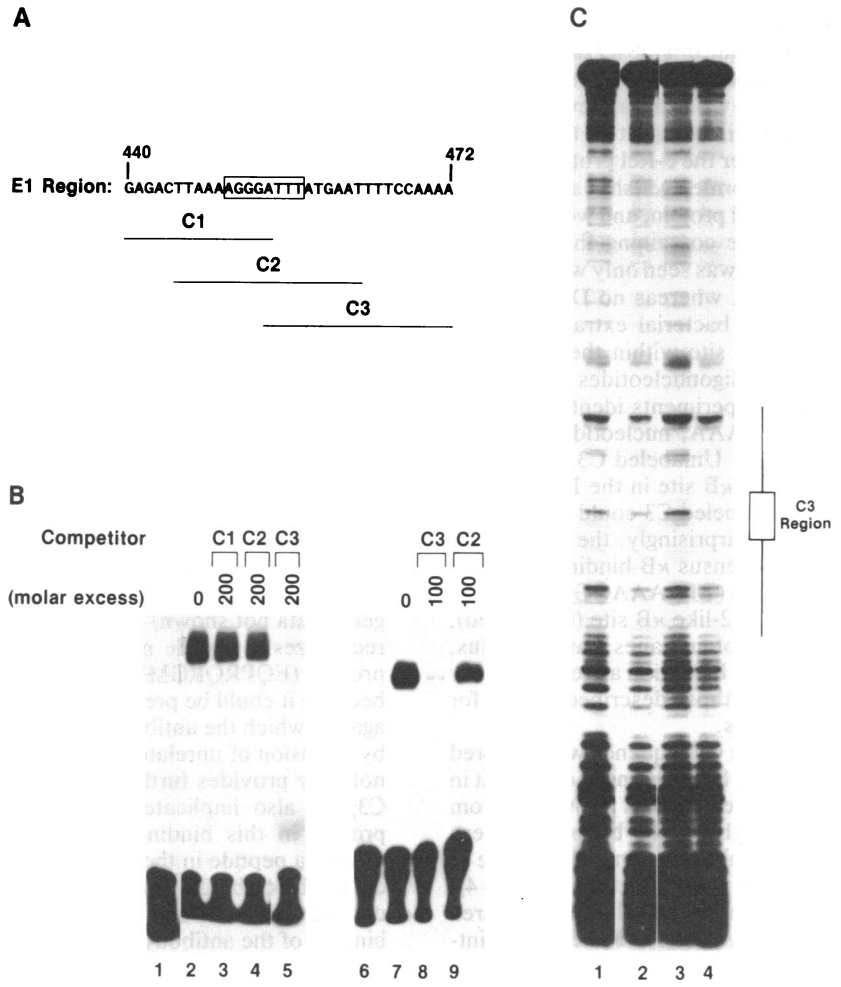

FIG. 1. (*A*) Sequence of IFN-γ intronic region E1 (nucleotides 440–472). The boxed region (*A*) is identical to a portion of the NF-κB site in the IL-2 gene. Oligonucleotides corresponding to subregions C1, C2, and C3 were synthesized and used in experiments reported in *B*. (*B*) Gel-shift analysis using the c-Rel protein. Lanes 1–5, labeled oligonucleotide containing the NF-κB sequence present in the IL-2Rα gene (20). Lanes: 1, control bacterial extract; 2–5, bacterial extract from *E. coli* transformed with the c-*rel* expression vector pKKrel; 3–5, 200 ng of unlabeled oligonucleotide C1, C2, or C3 were added to the binding reaction; 6–9, labeled C3 oligonucleotide; 6, control bacterial extract; 7–9, c-Rel-containing bacterial extract; 8 and 9, 100 ng of unlabeled oligonucleotide C3 or C2 were added to the binding reaction. (*C*) DNase I footprint analysis of the c-Rel protein binding to the human IFN-γ first intron. A DNA fragment containing nucleotides 405–674 from the human IFN-γ first intron was end-labeled with [γ-^{32}P]ATP and subjected to DNase I footprint analysis using an *E. coli* extract containing the c-Rel protein. Lanes: 1, no protein; 2, 18 μg of bacterial extract from control *E. coli* (c-Rel minus); 3 and 4, 18 μg of c-Rel containing bacterial extract plus 200 ng of unlabeled C3 and C2 oligonucleotides, respectively.

Table 1. Mutagenesis analysis of C3 region

Sequence	Oligonucleotide	Binding to c-Rel
455 472		
TTTATGAATTTTCCAAAA	C3	+
CGC.	C3–M1	+
. . . CGC.	C3–M9	+
. . . . . CCC.	C3–M2	−
. . . . . CGA.	C3–M8	−
. . . . . AT. . . .	C3–M3	−
. . . . . CGC	C3–M4	+
. . . . .	C3–M5	−
. . . . .	C3–M6	−
. . . . .	C3–M7	+

Dots indicate bases identical to that of top sequence (C3). Gel-shift analysis was done by using labeled C3 probe plus *E. coli* extract as the source of c-Rel protein and a number of mutated oligonucleotides (C3-M1 to C3-M9) as unlabeled competitors.

nucleotides 440–472 (position 1 being the cap site) of the human IFN-γ genomic DNA (Fig. 1*A*). Sequence analysis of this region demonstrated a DNA sequence (underlined nucleotides 451–457) (AAAGGGATTTATG) identical to the first 7 nucleotides of the NF-κB site in the IL-2 promoter (AGAGGGATTTCAC) (21). From this observation and recent reports on the interaction of c-Rel (for review, see ref. 24) with the NF-κB-binding site (25, 26) and evidence suggesting that the Rel family proteins can act as transcription factors (26–30), we tested whether the c-Rel protein can bind to the IFN-γ E1 region. We performed gel-shift analysis with an *E. coli* extract containing c-Rel protein, and we used as the labeled probe an oligonucleotide containing the IFN-γ E1 region. A DNA–protein complex was seen only with bacterial extract containing c-Rel protein, whereas no DNA–protein complex was seen with control bacterial extract (data not shown). To localize the binding site within the E1 region, gel-shift analysis was done with oligonucleotides spanning E1 (C1, C2, C3, Fig. 1*A*). These experiments identified the C3 region (TTTATGAATTTTCCAAAA, nucleotides 455–472) as a c-Rel-binding site (Fig. 1*B*). Unlabeled C3 was able to compete with the nuclear factor κB site in the IL-2Rα gene for c-Rel binding (lane 5), and labeled C3 could bind specifically to c-Rel (lanes 7 and 8). Surprisingly, the C3 oligonucleotide does not contain a consensus κB-binding site. Furthermore, the C2 oligonucleotide (TTAAAAGGGATTTATGAAT), which contained the IL-2-like κB site (underlined), was not able to bind the c-Rel protein (lanes 4 and 9). Thus, binding of c-Rel within the IFN-γ E1 region appears to occur at a particular site, unlike any of those described to date for the NF-κB–Rel family of proteins.

Binding of the Rel protein to the C3 sequence was explored in two additional ways. (*i*) DNase footprinting showed that in the presence of c-Rel the C3 region was protected from digestion (Fig. 1*C*), confirming that c-Rel binds at or very close to C3. This protection was abolished in the presence of excess competitor C3 (lane 3) but not competitor C2 (lane 4). The region of DNase protection in Fig. 1*C* falls within an area of relative DNase I inaccessibility. Thus, DNase I footprinting is relatively insensitive in this area, possibly due to the presence of secondary structural effects in the DNA. Nevertheless, we observed colocalization of both the protected region and the C3 region. Nonspecific protection was observed in the area 3′ to the C3 region, which we attribute to a possible load artifact, as a long exposure of the gel did not show any protection in this region (data not shown). (*ii*) A series of mutant oligonucleotides were synthesized to identify more specifically those nucleotides in C3 important for c-Rel binding. As summarized in Table 1, the nucleotide sequence AATTTTCC appeared to contain the c-Rel-binding

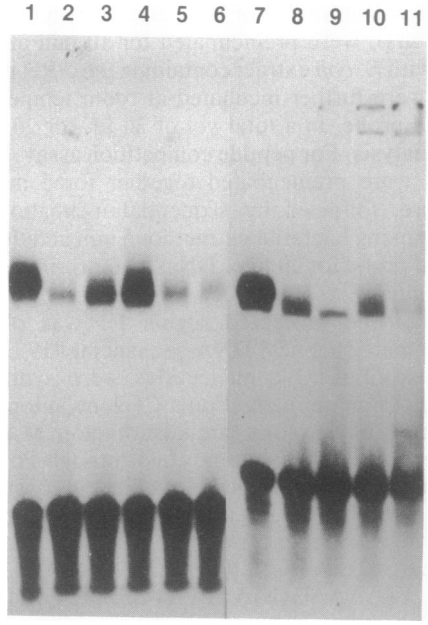

FIG. 2. Effect of c-Rel-specific peptide antisera on c-Rel binding. The ^{32}P-labeled c-Rel binding site (C3) was used as the labeled DNA probe. Lanes: 1, no antiserum; 2, 4 μg of antiserum α-27; 3 and 4, 4 μg of antiserum α-27 plus 3 and 9 μg of peptide 27, respectively; 5 and 6, 4 μg of antiserum α-27 plus 3 and 9 μg of peptide 1135; 7, no antiserum; 8 and 9, 4 and 8 μg of antiserum α-27, respectively; 10 and 11, 4 and 8 μg of antiserum α-1135, respectively.

site. Previously identified Rel-binding sites, including the NF-κB sites in the IL-2Rα chain (20), IL-2 gene (21), and IgK gene (22), could all specifically compete with the IFN-γ DNA for binding the c-Rel protein (data not shown), but as demonstrated above, the NF-κB-like C2 site in the IFN-γ gene failed to compete in gel-shift studies. Thus, the c-Rel protein expressed in bacteria can bind both to known NF-κB sites and to the specific C3 site.

The binding site for NF-κB sequence occurs within the amino-terminal half of the v-Rel protein (31, 32). To determine whether this region is also important in binding the specific C3 site, we performed gel-shift assays with antiserum raised against a Rel peptide (Fig. 2). These experiments demonstrated that interaction of the c-Rel protein with either the C3 site (lanes 2, 8, and 9) or the NF-κB site in the IL-2Rα gene (data not shown) could be blocked by an antibody that recognizes a peptide near the amino terminus of the c-Rel protein (EQPRQRGMRFRY). This inhibition was specific because it could be prevented by inclusion of excess peptide against which the antibody was raised (lanes 3 and 4) but not by inclusion of unrelated peptide (lanes 5 and 6). This result not only provides further evidence that c-Rel protein binds C3 but also implicates the amino-terminal region of the protein in this binding. In contrast, an antiserum raised against a peptide in the carboxyl-terminal half of the protein did not block binding but rather resulted in a supershift of the complex (lanes 10 and 11). This result is consistent with the binding of the antibody to the C3–Rel complex and decreasing its mobility. Thus, as with NF-κB sites, the amino-terminal portion of the c-Rel protein appears important for binding of the specific C3 site.

As the p50 subunit protein of the NF-κB complex has been conclusively demonstrated to bind to a number of different, but related, NF-κB sites (33), we tested the ability of the p50 protein to bind to the c-Rel-binding site. The p50 subunit protein, synthesized in bacteria, was purified by affinity-column chromatography (M.K. and R. Roeder, unpublished work) and was used in gel-shift analysis with a ^{32}P-labeled oligonucleotide containing the IL-2 receptor NF-κB site. As

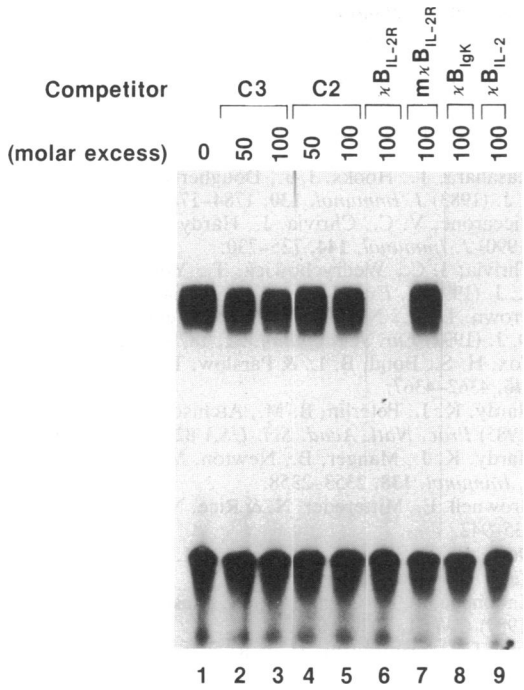

FIG. 3. Gel-shift analysis of the ^{32}P-labeled IL-2Rα chain NF-κB-binding site using the affinity-purified NF-κB p50 protein. Lanes: 1, 0 ng of unlabeled oligonucleotide; 2 and 3, 50 and 100 ng of unlabeled C3 oligonucleotide; 4 and 5, 50 and 100 ng of unlabeled oligonucleotide C2; 6 and 7, 100 ng of unlabeled wild type ($\kappa B_{IL\text{-}2R}$) and mutant ($m\kappa B_{IL\text{-}2R}$) receptor-α chain NF-κB site; 8, 100 ng of unlabeled IgK NF-κB-binding site κB_{IgK}; 9, 100 ng of unlabeled IL-2 NF-κB-binding site ($\kappa B_{IL\text{-}2}$).

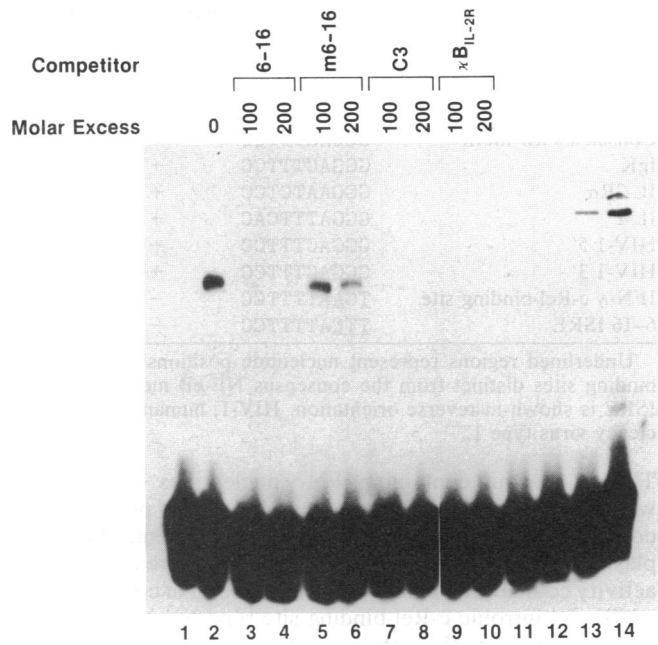

FIG. 4. Gel-shift analysis of the ^{32}P-labeled 6–16 ISRE oligonucleotide using the c-Rel-containing bacterial extract. Lanes: 1, control bacterial extract; 2, 0 ng of unlabeled oligonucleotide; 3 and 4, 100 and 200 ng of unlabeled 6–16 ISRE oligonucleotide, respectively; 5 and 6, 100 and 200 ng of unlabeled mutant 6–16 ISRE, respectively; 7 and 8, 100 and 200 ng of unlabeled C3 oligonucleotide, respectively; 9 and 10, 100 and 200 ng of unlabeled IL-2Rα NF-κB-binding site ($\kappa B_{IL\text{-}2R}$), respectively; 11 and 12, 4 and 8 μg of antiserum α-27, respectively; 13 and 14, 4 and 8 μg of antiserum α-1135, respectively.

expected, binding to this site was prevented by excess unlabeled NF-κB sequences from the IL-2Rα, IgK, and IL-2 genes (Fig. 3, lanes 6, 8, and 9). However, neither the c-Rel-binding C3 oligonucleotide nor the κB-like C2 sequence competed with the ^{32}P-labeled IL-2Rα site for p50 binding (lanes 2–5). Thus, while both p50 and c-Rel bind κB sites, only c-Rel and not p50 binds to the specific C3 sequence.

It has been reported (23) that the presence of an ISRE, homologous to ISREs of the 6–16, interferon-stimulated genes ISG15, and ISG54 genes can confer responsiveness to both α- and γ-IFNs when placed upstream of a reporter gene. A comparison between 6–16 ISRE (GGGAAAATGAAACT) and the reverse strand of the c-Rel-binding site (GGAAAATT) shows complete homology in a 7-base-pair (bp) region. To test whether the 6–16 IRSE was also a potential c-Rel-binding site, we performed gel-shift analysis using bacterial c-Rel and a labeled oligonucleotide containing the 6–16 IRSE sequence. Consistent with the above hypothesis, the 6–16 IRSE oligonucleotide bound specifically to c-Rel (Fig. 4, lane 2). This binding was prevented by excess unlabeled 6–16 IRSE (lanes 3 and 4) and also by C3 (lanes 7 and 8) and the IL-2Rα NF-κB site (lanes 9 and 10). As with C3, antibody that recognizes the amino-terminal region of c-Rel abolished binding to 6–16 IRSE (lanes 11 and 12), whereas carboxyl-terminal antibody resulted in supershifting (lanes 13 and 14). Thus, the 6–16 IRSE sequence, like C3 and NF-κB sites, binds c-Rel. The intensity of binding was lower than with C3, suggesting a somewhat lower affinity of the ISRE for c-Rel. No binding of the NF-κB p50 subunit to the ISRE was observed (data not shown). This result suggests the possibility that genes containing the 6–16 IRSE can be regulated by c-Rel.

DISCUSSION

The c-*rel* gene is a member of the early-response gene family and is induced by serum, phorbol ester, ConA, and lipopolysaccharide (34–36). The c-Rel protein shares extensive sequence homology with the p50 and p65 subunits of the κB-binding factors NF-κB (31, 32, 37). The experiments described here provide evidence that c-Rel protein expressed in bacteria can interact with a specific nucleotide sequence distinct from the NF-κB-binding sites. Mutations outside the sequence AATTTTCC did not affect binding, whereas mutations within this sequence (three mutants were tested) abolished binding to bacterial-derived c-Rel. This and the 6–16 ISRE sequence of TTTCATTTTCC (reverse orientation) are similar to the 3′ half site of the κB enhancer elements in the immunoglobulin κ light-chain gene (GGGGACTTTCC), human immunodeficiency virus 1 (HIV-1) 5′ long terminal repeat (AGGGACTTTCC), and HIV-1 3′ long terminal repeat (GGGGACTTTCC) (underlined regions). Given the high degree of similarity of c-Rel to p65, which displays a preference for this half of the binding site (38), c-Rel is probably also preferentially recognizing this half site. This finding is further substantiated by the fact that both the oligonucleotide C2 containing the IL-2-like κB site sequence TTAAAAGGGATTTATGAAT and the 6–16 ISRE mutant oligonucleotide sequence GTTCATTTTAT (reverse orientation), were not recognized by the bacterial c-Rel due to the absence of the CC nucleotides in the 3′ site. No c-Rel specific AATTTTCC-binding sites have been found near the classical NF-κB-binding site in the IL-2, IL-2Rα, IgK genes or in the HIV-1 long terminal repeat. This additional c-Rel-binding site differs from the consensus NF-κB site at three of the invariant positions (Table 2). Moreover, the NF-κB p50 protein doesn't bind to the c-Rel-binding site or the IFN-γ NF-κB-like site in the first intron of the IFN-γ genomic DNA. Thus, this nucleotide region appears to differentially bind different members of the Rel/NF-κB family of proteins.

The role of c-Rel in directly regulating IFN-γ expression *in vivo* remains to be fully elucidated. In preliminary experiments, cotransfection of a c-Rel expression vector with a

Table 2. Sequence homology between c-Rel-binding sites and NF-κB-binding sites

		p50 binding	c-Rel binding
Consensus κB motif	GGGANTYYCC		
IgK	GGGACTTTCC	+	+
IL-2Rα	GGGAATCTCC	+	+
IL-2	GGGATTTCAC	+	+
HIV-1 5′	GGGACTTTCC	+	+
HIV-1 3′	GGGACTTTCC	+	+
IFN-γ c-Rel-binding site	TGAATTTTCC	−	+
6–16 ISRE	TTCATTTTCC	−	+

Underlined regions represent nucleotide positions in the c-Rel-binding sites distinct from the consensus NF-κB motif (33). 6–16 ISRE is shown in reverse orientation. HIV-1, human immunodeficiency virus type 1.

TKCAT plasmid containing IFN-γ nucleotides +405–+2716 was able to increase the expression of the plasmid that contained the IFN-γ sequences but not the parental TK-CAT plasmid (data not shown). Whether the increase in CAT activity could be attributed to the interaction of c-Rel with the additional intronic c-Rel-binding site reported in this manuscript or whether other intronic sequences were also involved in this enhanced CAT expression needs to be determined. In contrast to those results, however, mutant full-length IFN-γ genomic DNA constructs lacking 750 bp of the first intron remain fully active upon transfection into a murine T-lymphoblastoid cell line (H.A.Y., unpublished observations), thus indicating that this DNA region is not absolutely required for transcription, at least in this murine T-cell line. However, additional studies in this laboratory suggest that c-Rel may also interact with IFN-γ 5′ regulatory regions and upregulate gene expression through 5′ regulatory elements (P.G., unpublished work). Experiments are currently underway to determine whether transfection of a v-Rel expression vector into cells that express IFN-γ can abrogate IFN-γ gene expression or IFN-γ-induced gene expression, as v-Rel has been demonstrated to inhibit κB enhancer activity (25, 26).

Most interestingly, DNA sequence analysis revealed homology of the c-Rel-binding site to an ISRE and suggests that c-Rel may be involved in the transcriptional regulation of the family of genes that contain this ISRE. Although proteins have been identified that interact with this DNA element (39), the relationship of these proteins to c-Rel remains to be determined. This study presents evidence that the c-Rel protooncogene product can potentially modulate gene activity by interacting with regions of DNA that are distinct from NF-κB sites. At the present time we cannot completely rule out the possibility that the bacterial-derived c-Rel used in these studies differs slightly in its DNA recognition sites from that of mammalian c-Rel. Nevertheless, while the c-Rel protein has clearly been shown to bind to the NF-κB site, there is no reason to believe that this site is the only DNA region with which c-Rel interacts. Thus, as additional c-Rel-binding sites in specific genes are identified, a more complete understanding of the role of c-Rel in regulating gene expression will emerge.

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