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Evidence for a non-α-helical DNA-binding motif in the Rel homology region

(photocrosslink/protein–DNA interactions/transcription factor NF-κB)

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ABSTRACT **The Rel family of transcription factors serve as terminal messengers in a variety of developmental and receptor-mediated signaling pathways. These proteins are related by a domain of ≈280 amino acids, the Rel homology region, which mediates dimerization and sequence-specific binding to DNA. Here we report the use of photocrosslinking and site-directed mutagenesis to identify specific contact partners in a Rel protein–DNA interface. Within the Rel homology region of NF-κB p50 (also known as KBF1), two amino acid residues were identified by photocrosslinking to adjacent bases in a β-interferon regulatory element. Secondary structure analysis suggests that the DNA-binding motif of the Rel homology region comprises a β-turn–β structure, in contrast to the α-helical motifs so commonly observed in transcription factors.**

Proteins of the Rel family of transcription factors, so designated for their homology to the *rel* oncogene product, serve important roles in transduction of developmental and mitogenic signals across the nuclear membrane (1, 2). The conserved structural feature of these proteins, the Rel homology region (RHR), is a domain of ≈280 amino acids that mediates dimerization, DNA binding, and interactions with accessory proteins (3–12). Unlike most transcription factors, which can typically be stripped down to a DNA-binding module containing <100 residues (13, 14), Rel proteins require the entire RHR in order to bind DNA; this difference suggests that the RHR may represent a unique solution to the architectural problem of DNA binding. Even though the small DNA-binding modules studied to date exhibit a great deal of structural diversity, most use an α-helix to make sequence-specific major-groove contacts. However, a growing number of sequence-specific DNA-binding proteins possessing non-α-helical elements have been implicated in direct interaction with DNA (15, 16). To gain information on the DNA-binding element of Rel proteins, we have used site-specific photocrosslinking (17, 18) to identify amino acid residues that lie close to the DNA interface. Analysis of the sequence surrounding these putative contact residues suggests that the RHR possesses a non-α-helical DNA-binding motif. The present experiments directly implicate residues in the N-terminal region of the RHR as making contacts with DNA, consistent with the results of experiments on mutant and chimeric Rel proteins (10–12, 19).

The present studies employed the Rel protein NF-κB, an inducible factor that activates transcription of a wide variety of genes involved in the inflammatory, immune, and acute-phase responses (2). NF-κB is also a target of subversion by numerous pathogenic viruses, including cytomegalovirus, human immunodeficiency virus type 1, and human T-lymphotropic virus type I, which recruit this host protein to activate transcription of viral genes. Each of the two subunits of NF-κB, p50 and p65, possesses an RHR, which mediates both hetero- and homodimerization (7, 20–22). The DNA sequence preferences of p50/p65, p50/p50, and p65/p65 are similar yet distinct (12, 22). To simplify the interpretation of crosslinking experiments, we have focused on the p50 homodimer, also known as KBF1 (7).

Urban *et al.* (20) showed that the NF-κB site of the β-interferon (β-IFN) promoter (β-IFN site, Fig. 1), when labeled at multiple sites with 5-bromo-2′-deoxyuridine (BrdUrd), underwent photocrosslinking to NF-κB, the p65 homodimer, and the p50 homodimer. In the present studies, we utilized β-IFN oligonucleotides containing BrdUrd at single positions within or flanking the NF-κB element to identify individual positions within the β-IFN site that underwent efficient crosslinking and to locate amino acid residues of p50 which are near these base pairs in the protein–DNA interface. Here we report the direct identification of two amino acids which are crosslinked to BrdUrd-substituted β-IFN oligonucleotides and the importance of these amino acids in DNA-binding properties of p50. Secondary structure analysis of the stretch of peptide which was crosslinked to DNA revealed a plausible DNA-binding motif in p50: β-strand–turn–β-strand.

MATERIALS AND METHODS

Materials. BrdUrd phosphoramidite was from Peninsula Laboratories. Oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer. The UV transilluminator (model 3-3000, four 15-W 300-nm bulbs, 21 cm × 38 cm UV filter glass) was from Fotodyne (New Berlin, WI). Trypsin was from Sigma, Pronase from Calbiochem, and lysyl endopeptidase from Waco BioProducts (Richmond, VA). The Mono Q HR 5/5 column was from Pharmacia. FPLC was performed on a Pharmacia system. The Muta-Gene phagemid kit was from Bio-Rad. Autoradiograms were quantified with a Fujix bio-imaging analyzer, BAS 2000, from Fuji Medical Systems (Stamford, CT).

Analytical Photocrosslinking Reactions. A fragment of human p50 comprising residues 1–366 expressed in *Escherichia coli*, which retains the dimerization and DNA-binding activities of the wild-type protein, was used in all experiments; this is referred to as recombinant p50 (rp50) (23). Each crosslinking reaction mixture (20 μl) contained 120 fmol of ^{32}P-labeled DNA and 20 pmol of rp50 in binding buffer [0.015% bovine serum albumin/20 mM Hepes, pH 8.0/0.2 mM dithiothreitol/9% (vol/vol) glycerol/0.1 mM phenylmethanesulfonyl fluoride/100 mM NaCl/2.5 mM GTP]. After incubation at room temperature for 30 min, the samples were irradiated at 300 nm with a UV transilluminator for 1 hr. SDS/PAGE

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Abbreviations: RHR, Rel homology region; β-IFN, β-interferon; MHC, major histocompatibility complex; rp50, a recombinant fragment of human p50 comprising residues 1–366; BrdUrd, 5-bromo-2′-deoxyuridine.

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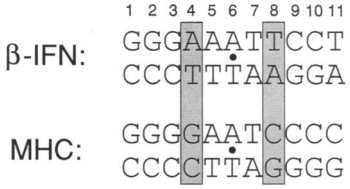

FIG. 1. Sequences of the NF-κB sites in the β-IFN promoter and *H-2K* major histocompatibility complex (MHC) class I promoter (2). The centers of dyad symmetry are indicated by dots. Positions 4 and 8, for which p50 and p65 show different sequence preferences, are highlighted; the β-IFN element is a p65 consensus site, whereas the MHC element is a p50 consensus site (12). Although NF-κB sites are often considered to be 10 bp long, base pair 11 is included here to emphasize the symmetry of the site.

loading buffer (5 μl) was added, and the samples were heated at 85°C for 10 min before SDS/15% PAGE. The autoradiogram of the gel was analyzed with a BAS 2000 analyzer, and the yield of crosslinked covalent protein–DNA complex from each reaction was determined by the relative intensities of the complex and free DNA bands.

Preparation of Photocrosslinked rp50–DNA Complexes. BrdUrd-containing DNA (15 nmol) was incubated with 50 nmol of rp50 in 8 ml of binding buffer at room temperature for 1 hr and then irradiated with 300-nm UV light for 2 hr. The reaction mixture was concentrated and washed extensively by ultrafiltration against 3.9 M urea/20 mM Tris·HCl, pH 7.5/0.5 mM EDTA. The sample (2 ml) was loaded onto an FPLC Mono Q HR 5/5 column and eluted with a two-step linear gradient from 0 to 0.5 M NaCl (24 ml) and then from 0.5 to 1.0 M NaCl (4 ml) at a rate of 1 ml/min in urea-free buffer (20 mM Tris·HCl, pH 7.5/0.5 mM EDTA). Fractions from 12–18 min, which contained the covalent rp50–DNA complex, were pooled and precipitated with acetone.

Preparation of Photocrosslinked Peptide–DNA Complexes. The rp50–DNA covalent complex was treated with 3.75 mM dithiothreitol/8.3 mM iodoacetamide/6.7 M urea/0.33 M NH_4HCO_3 for 20 min and then digested *in situ* with Pronase (300 μg), trypsin (300 μg), or lysyl endopeptidase (1.5 mg) in 2 M urea/0.1 M NH_4HCO_3 for 48 hr at 37°C. The resulting peptide–DNA complex was then purified by FPLC under the conditions described above. Fractions eluted at 0.4–0.5 M NaCl were pooled and precipitated with ethanol. Edman sequencing of peptide–DNA complexes was carried out as described (24) with an on-line model 120A HPLC system (Applied Biosystems).

Site-Directed Mutagenesis. Mutations of rp50 were generated with the Bio-Rad Muta-Gene kit by site-directed mutagenesis in the single-stranded form of the rp50-overproducing phagemid (25).

Electrophoretic Mobility-Shift Assays. Proteins (10 nM) were incubated with a ^{32}P-end-labeled MHC probe, 5′-AGGGCTGGGGATTCCCCATCTCC·5′-GGAGATGGGGAATCCCCAGCCCT-3′ (1.5 nM), at room temperature for 30 min in the binding buffer described above. The mixtures were analyzed by nondenaturing 4% PAGE. In the mobility-shift specificity analysis, each binding reaction mixture contained 1.5 nM MHC probe, 100 nM protein, and where applicable, 20 μM nonspecific competitor DNA or 15 μM specific competitor DNA. In quantitative mobility-shift assays, ^{32}P-end-labeled MHC probe or β-IFN probe, 5′-AAAGTGGGAAATTCCTC·5′-TCAGAGGAATTTCCCAC-3′, (<25 pM << K_d) was titrated with proteins of various concentrations (determined in parallel by Bradford assay) in binding buffer. Each pair of proteins was analyzed in the same gel and their relative affinity was determined by the protein concentration at half-saturation of the probe (26).

RESULTS

Formation of Specific rp50–DNA Covalent Complex. Each β-IFN oligonucleotide containing BrdUrd at single positions was annealed to its unmodified complement to afford a duplex photoprobe. Electrophoretic mobility-shift assays (data not shown) revealed that substitution by BrdUrd exerted little effect on the strength or specificity of the protein–DNA complex, in agreement with prior work (27). The efficiency of crosslinking between rp50 and the BrdUrd-substituted oligonucleotides was examined as follows. Probes 5′ end-labeled with ^{32}P were incubated with rp50, and the respective protein–DNA complexes were then irradiated with UV light. Analysis of the reaction products by denaturing PAGE (Fig. 2) revealed that oligonucleotides having BrdUrd at positions 4, 5, and 8 underwent efficient crosslinking to rp50. The photoreaction proceeded with exquisite sequence selectivity, as judged by the low degree of crosslinking observed at positions −1, 6, 7, and 11.

To identify the amino acid residues of p50 that are in close proximity to the probe at position 4, a large-scale photocrosslinking reaction was carried out using the 4-(BrdUrd)-oligonucleotide probe. The protein–DNA complex was separated from unreacted DNA and protein by anion-exchange chromatography (FPLC Mono-Q) under denaturing conditions. The complex was then reduced, alkylated, and digested exhaustively with trypsin or Pronase. Fractionation of the digestion mixtures by anion-exchange FPLC yielded in each case a late-eluted peak with a UV spectrum characteristic of DNA ($\lambda_{max} \approx 260$ nm). Amino acid analysis of this peak, however, revealed that the material contained significant amounts of protein (data not shown). This material was then subjected to peptide sequence analysis.

Identification of Photocrosslinked Amino Acids. With the 4-(BrdUrd) probe, the crosslinked peptide obtained by Pronase digestion, RXVCEGPSHGGLPGAS, precisely matched residues 59–74 of p50; the unidentifiable residue X corresponded to Y60 (Fig. 3). The corresponding tryptic digestion product contained a 20-mer peptide, FRXVCEGPSHGGLPGASSEK, derived from residues 58–77; position X again corresponded to Y60. These sequence analyses were complicated by a background peak that was coeluted with arginine in the first two cycles. To eliminate this problem, it was necessary to obtain a version of the peptide–DNA complex having a longer amino terminus. Noting the presence of a lysine at position 52, we digested the covalent rp50–DNA complex with lysyl endopeptidase. In the resulting sequence, QRGFRFRXVCEGPSHGGLPGASSEK, the contaminant was absent, allowing us to identify unambiguously R59 as being unmodified and Y60 as being modified.

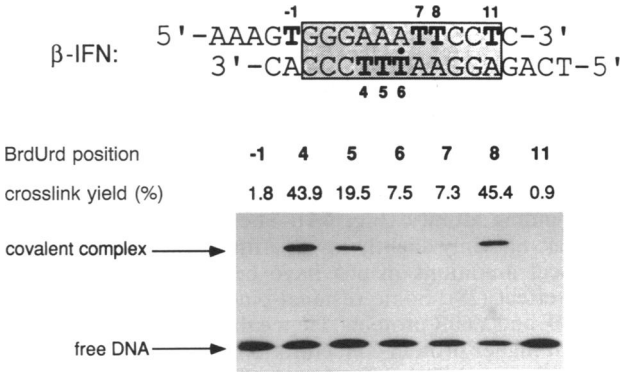

FIG. 2. SDS/PAGE analysis of photocrosslinking reactions. The sequence of the duplex oligonucleotide used in crosslinking is shown at the top; each of the bold positions was individually substituted by BrdUrd.

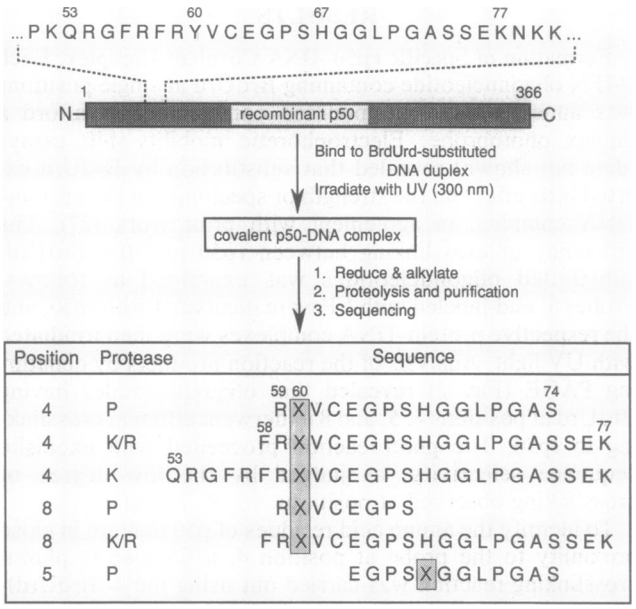

FIG. 3. Scheme for identification of nearby amino acid residues by large-scale photocrosslinking. Position refers to the location in the sequence at which BrdUrd was present in the crosslinking experiment (refer to Fig. 1 or 2). The identities of the proteases are as follows: P, Pronase; K/R, chymotrypsin-free trypsin; K, lysyl endopeptidase.

The amounts (pmol) of identified amino acids and background (in parentheses) at each cycle were 282.4 (0), 56.5 (13.4), 33.0 (1.6), 32.5 (3.6), 23.3 (5.9), 21.8 (7.5), 0 (0), 15.4 (1.5), 0 (0), 11.2 (1.7), 11.3 (3.4), 8.7 (2.2), 4.9 (1.3), 2.2 (0.6), 6.7 (4.6), 7.8 (4.6), 4.5 (1.5), 4.1 (1.7), 5.4 (4.4), 3.3 (1.2), 2.1 (1.2), 2.3 (1.2), 1.2 (0.6), and 0.2 (0.1). Given that three overlapping peptides were obtained in this analysis, all of which possessed modifications at Y60, we conclude that this amino acid residue of p50 is in close proximity to thymine-4 of the β-IFN site. Similar experiments performed on the complex formed between rp50 and the 8-(BrdUrd) probe gave analogous results (Fig. 3). Thus, BrdUrd at position thymine-8 of the β-IFN site also photocrosslinks Y60 of rp50. An oligonucleotide with the photoprobe at position 5 crosslinks rp50 to yield, after digestion with pronase, the peptide EGPSXGGLPGAS, which reveals H67 at the point of attachment (Fig. 3).

Site-Directed Mutagenesis and DNA Binding Analysis. The importance of Y60 for DNA binding was tested by mobility-shift assays on mutant rp50 proteins. At the same time, we examined the effect of changing residues F56 and V61 to methionine and lysine, respectively, since the latter are present in p65. For these studies, the MHC probe was used because it binds p50 more strongly than does the β-IFN site and hence is less likely to show a complete loss of binding to mutant proteins (the MHC site is a consensus sequence for p50; ref. 12). Changing Y60 to phenylalanine, leucine, arginine, glutamine, or asparagine (Y60F, Y60L, Y60R, Y60Q, and Y60N proteins, respectively) led to a substantial loss of DNA-binding affinity (Fig. 4*A*). The F56M/V61K mutant, however, has only slightly lower affinity for wild-type rp50; reciprocal mutations in p65 have been shown to cause a similar effect (28). Some residual binding was observed for the Y60F and Y60R proteins. Hence these proteins were then tested at higher protein concentration for specific binding in competition mobility-shift assays (Fig. 4*B*). Whereas the Y60F and F56M/V61K mutants were observed to bind the MHC site specifically, Y60R exhibited a large increase in nonspecific binding. Quantitative binding assays revealed that the Y60F mutant, which maintains the aromatic ring of

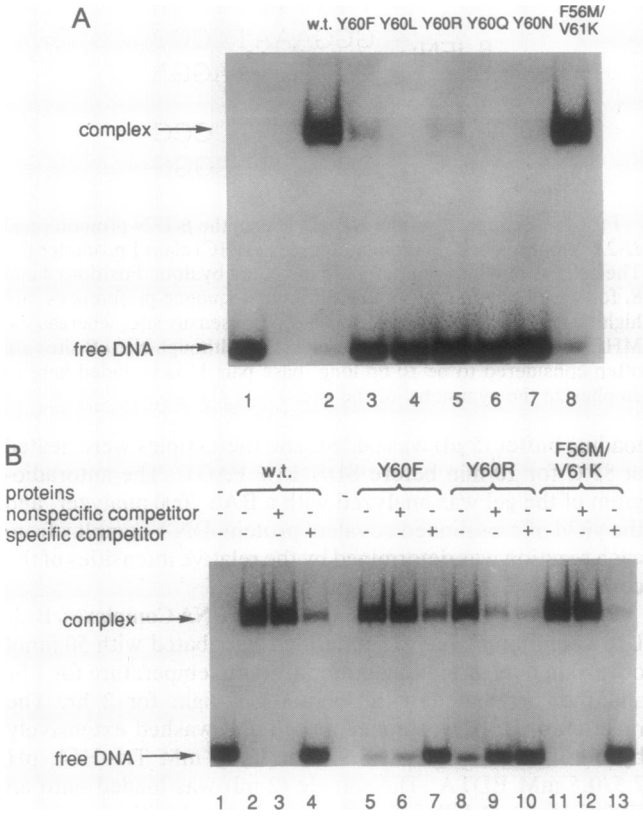

FIG. 4. (*A*) Electrophoretic mobility-shift assay of wild-type (w.t.) and mutant rp50 proteins. Proteins (10 nM) were incubated with a ^{32}P-end-labeled MHC probe, 5′-AGGGCTGGGGATTCCCCATCTCC-3′ (1.5 nM), at room temperature for 30 min in binding buffer. Lane 1 is a control containing no protein. (*B*) Specificity analysis of wild-type rp50 and mutants. Each binding reaction mixture contained 1.5 nM MHC probe, 100 nM protein and, where applicable, 20 μM nonspecific competitor DNA or 15 μM specific competitor DNA. Lane 1 is a control containing no protein. Mutations were generated with the Bio-Rad Muta-Gene kit by site-directed mutagenesis in the single-stranded form of the rp50-overproducing phagemid. The F56M/V61K mutant of rp50, which was constructed to change several residues of p50 to those in p65, was similar in binding affinity to p50.

Y60 but lacks its hydroxyl group, binds the MHC site 36-fold more weakly than the wild-type protein, whereas the Y60L mutant, which maintains a large apolar side chain, bound 120-fold more weakly than wild type (Table 1). A mutant of rp50 with H67 changed to alanine was also made to examine the effect of the histidine residue on DNA binding. Quantitative mobility shift assay showed that this single mutation decreased the specific binding affinity for the MHC site by 50-fold, but resulted in only a 2-fold effect on binding affinity for the β-IFN site. This difference is dramatic in comparison with the Y60F mutation, which decreased the binding affinity of rp50 for MHC and β-IFN sites to a similar extent (Table 1).

DISCUSSION

Y60 Lies Close to the Symmetry-Related Thymine-4 and Thymine-8 and Is Important for DNA Binding. The photocrosslinking results suggest that Y60 lies close to thymine-4 and -8 and probably contacts either these or adjacent bases in the DNA. This is further supported by the following considerations. (*i*) The exceptionally high efficiency with which these crosslinks are formed, especially as compared with other reported cases (17, 29), suggests that Y60 is close to the photoprobe. (*ii*) The 4-(BrdUrd) and 8-(BrdUrd)

Table 1. Relative affinity of wild-type (w.t.) and mutant rp50 proteins for the β-IFN and MHC sites determined by mobility-shift assay

Probe	Relative affinity		
	w.t./H67A	w.t./Y60F	w.t./Y60L
MHC	50	36	127
β-IFN	2.5	31	ND

ND, not determined.

probes, in which the photoactive nucleoside is located at symmetry-related positions in different half-sites (Fig. 1), crosslink the same amino acid residue. (*iii*) The Y60 mutant proteins other than Y60R are decreased in their affinity for specific DNA but similar in their affinity for nonspecific DNA (J.L. and G.L.V., unpublished results). We note that whereas operator positions 4 and 8 are occupied by thymines in the β-IFN site (Fig. 1), they are occupied by cytosines in the MHC site, to which p50 binds more strongly. Mutation of Y60 affects the binding affinity for the MHC and β-IFN sites to a similar extent (Table 1), suggesting that this residue is not important for sequence discrimination. This is consistent with the observation that the residue corresponding to Y60 is conserved in all known Rel proteins (Fig. 5), despite their preferences for different pyrimidines at positions 4 and 8 (2, 12, 22). A comparison of the binding affinity of Y60F and Y60L with rp50 (Table 1) indicates that both the aromatic ring and hydroxyl functionality of Y60 are required for tight, specific binding of p50 to DNA.

H67 Is a Key Residue for the Discriminative Binding of p50 and p65 to MHC and β-IFN Sites. The large difference in binding affinity for MHC and β-IFN sites demonstrated by the H67A mutant suggests that amino acid 67 contributes to sequence discrimination at positions 4 and 8. Even though H67 was identified by virtue of its crosslinking to BrdUrd at position 5 of the β-IFN site, it is possible that H67 contacts adenine-4 either alone or in addition to thymine-5. RHR-containing proteins such as p65 and Rel, which prefer an A·T base pair at position 4 and T·A at position 8 (as in the β-IFN site), all possess an alanine at position 67, whereas Rel proteins such as p50 and Lyt-10, which prefer G·C at position 4 and a C·G at position 8 (as in the MHC site), all possess a histidine at position 67 (Fig. 5). Our findings are thus consistent with reports suggesting that position 67 is involved in determining p50-like versus p65-like sequence specificity in Rel proteins (11, 28).

Residues 51–72 Are Important for DNA Binding of p50. Y60 lies just two residues before C62 (Fig. 5), which has been shown to regulate the DNA binding activity of p50 and v-Rel in a redox-dependent manner, presumably by participating in disulfide bond formation (10, 19). Indirect evidence suggests C62 may lie close to DNA or even contact it directly: (*i*) DNA protects C62 from alkylation by iodoacetate (19) and (*ii*) mutation of C62 to serine increases the affinity of the protein for DNA (10). Mutagenesis and domain-swap experiments have also implicated the stretch of residues surrounding C62, including Y60 and H67, as being involved in DNA recognition by Rel proteins (10, 11, 28). Our results furnish direct evidence that this region of the RHR makes sequence-specific contacts to DNA; further, this analysis provides information regarding specific amino acid–DNA base contact pairs, which form a basis for model-building exercises. Although evidence for sequence-specific contacts involving a cysteine and a phenyl-containing amino acid were documented in the case of the papillomavirus E2 protein (39), this protein shares no apparent sequence similarity with Rel proteins. Sequence-specific contacts involving histidine and alanine have been widely documented (13, 14).

Peptide G51–G72 of p50 May Possess a DNA-Recognition Motif Consisting of a β-Turn–β Structure. With a few notable exceptions (15, 16), DNA-binding modules of transcription factors share the common feature of presenting an α-helix, residues of which make most of the sequence-specific contacts to DNA (13, 14). Secondary structure analysis of the RHR in the region around Y60 and H67 suggests that Rel proteins use an altogether unrelated architectural motif. The segment containing Y60 and H67 is not only bracketed by potent helix-destabilizing residues (P51, G55, G68, G69, P71, and G72 in p50) but also contains within it a strong helix-breaking sequence (G64-P65). Based on the locations of these helix-destabilizing residues, it appears that only the stretch between residues 56 and 63 has the potential to form an α-helix. However, several features of the 56–63 stretch argue strongly against it being α-helical. It consists of alternating hydrophobic and hydrophilic residues, a pattern that is more consistent with an extended structure such as a β-sheet or loop than with an α-helix, and the amino and carboxyl termini of the 56–63 stretch possess a net positive and negative charge, respectively, which would destabilize an α-helix by

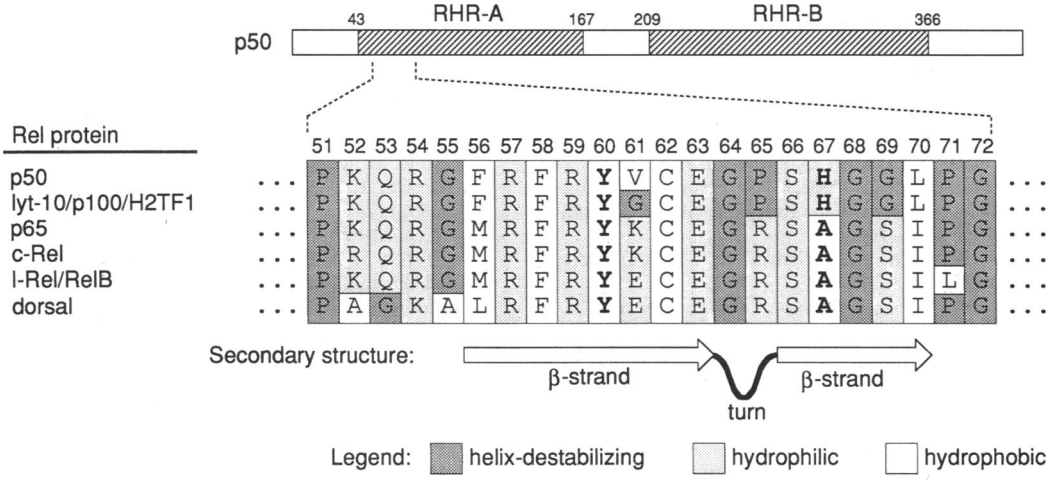

FIG. 5. Schematic representation of the p50 protein, with the two parts of its hyphenated RHR designated as RHR-A and RHR-B. The expansion below is a sequence comparison of the segment of the RHR surrounding Y60 from various human proteins of the Rel family [p50 (6, 7, 30), lyt-10/p100/H2TF1 (31–33), p65 (20, 34), mouse c-Rel (35), I-Rel (36, 37), *Drosophila* dorsal (38)]. Numbering here and throughout the text for all Rel proteins refers to that of human NF-κB p50. The proposed secondary structure of the DNA-binding element is shown below the corresponding amino acid sequence.

increasing its intrinsic dipole moment (40). For these reasons, we propose that residues 56–63 are in a β-type conformation, either a β-strand or a loop. Since Y60 and H67 crosslink adjacent residues in the β-IFN site (thymine-4 and -5, respectively), the peptide chain must undergo a reversal in order to bring these residues close together. We propose that residues 64 and 65, which in p50 are glycine and proline, form a turn, followed by another segment of β-strand or loop (Fig. 5). This putative DNA-binding element can thus be described as a β–turn–β motif. The contacts made between Y60 and H67 with bases in the center of the DNA site would seem to orient R57 and R59 favorably to interact with the guanines at the 5′ end of the site; such arginine–guanine interactions are the single most frequently observed contact pair in protein–DNA complexes (13, 14). Toledano *et al.* (11) have pointed out a modest sequence similarity between the β structural elements of the TFIIIA-like zinc finger and this region of the RHR, thereby suggesting that the two possess a common structure. Although it is possible that the two elements possess local similarities in structure, they must also possess significant differences. For example, the residue corresponding to C62 in the TFIIIA-type zinc finger points into the core of the structure (41), in order to ligate a zinc ion, and is therefore inaccessible.

Although the majority of structurally characterized transcription factors use α-helices as DNA-recognition elements (13, 14, 39, 42), extended secondary structures are also known to participate in sequence-specific recognition. For example, the restriction endonucleases *Eco*RI and *Eco*RV possess nonhelical segments that contact DNA (43, 44). The class of bacterial proteins typified by the *E. coli* Met repressor contact DNA by using residues located on an antiparallel β-ribbon formed by homodimerization of an amino-terminal peptide extension (15). The observation that Rel proteins form heterodimers with discrete half-site specificities (9, 20, 45) rules out their use of such a β-ribbon motif for DNA binding, since in this motif each protein subunit binds both half-sites. Finally, the eukaryotic TATA-box-binding protein, which unlike the RHR binds DNA in the minor groove, has been shown to interact via residues in a saddle-like all-β structural element (46, 47). In summary, it is likely that the Rel proteins embody a unique structural solution to the problem of DNA recognition, a solution that involves the use of non-α-helical secondary structure.

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