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Alteration of a Single Nucleotide Allows Efficient Binding of H2TF1/KBF1 to the Immunoglobulin κ Enhancer B Motif

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NF-κB (a protein present constitutively only in B cells) and H2TF1/KBF1 (a more ubiquitously distributed protein[s]) are two transcription factors that recognize very similar DNA sequences. However, the binding site associated with the κ immunoglobulin gene enhancer (κB) is recognized predominantly by NF-κB. Using synthetically altered recognition sequences, we showed that the B-cell-specific NF-κB-binding site in the κ enhancer can be converted to one that binds both NF-κB and the ubiquitous protein(s) H2TF1/KBF1 by substitution of a single nucleotide. Furthermore, transient transfection experiments suggested that NF-κB and H2TF1/KBF1 are functionally different even though their DNA recognition specificities are very similar.

A somewhat surprising offshoot of the extensive search for transcription factors has been the recurrent observation of families of DNA-binding proteins. For example, NF-κB and H2TF1/KBF1 are two transcription factors which are capable of interacting with related sequence elements. NF-κB was first identified as a factor binding to the κB site in the murine immunoglobulin κ light-chain gene enhancer (17). Although the active protein is found constitutively only in mature B cells and plasma cells, it can be induced in pre-B cells by bacterial lipopolysaccharide (LPS) and in T cells and nonlymphoid cells by phorbol myristate acetate (18). Methylation interference experiments have localized the murine NF-κB-binding site to the sequence GGGACTTTCC (17). This site is necessary for κ enhancer function in B cells (1, 12) and confers phorbol myristate acetate inducibility in T-lymphoid (16) and -nonlymphoid cell types (14). H2TF1/KBF1 was identified as a factor binding to the $H2K^b$ promoter (2, 9). This factor is not restricted to B-lymphoid cells and can be detected in different cell types, e.g., fibroblasts, T cells, and epithelial cells. Israël et al. (9) demonstrated that this factor also binds to a sequence element, GGGACTTTCCC, upstream of the mouse β2 microglobulin (β2m) gene and postulated that it may mediate coordinate regulation of $H2K^b$ and β2m genes. Baldwin and Sharp (3) have further shown that NF-κB can bind the $H2K^b$ sequence as well as the κB sequence in B-cell extracts, whereas the affinity of H2TF1/KBF1 for the κB sequence is approximately 10- to 20-fold less than its affinity for the $H2K^b$ sequence. Because transcription of the κ gene is restricted to B lymphocytes only, it was not surprising that the activity of its tissue-specific enhancer was dependent upon a sequence that binds a B-cell-specific factor. However, the occurrence of a more ubiquitously distributed DNA-binding protein with a very similar sequence specificity raises the intriguing question of what the molecular basis is for the recognition of the κB site by NF-κB and not by H2TF1/KBF1. To determine the sequence requirements of NF-κB and H2TF1/KBF1 interactions, we have performed in vitro binding and competition experiments with synthetic oligonucleotides carrying wild-type and mutant sequences. Our results demonstrated that the B-cell-specific NF-κB-binding site in the κ enhancer can be converted to one that binds both NF-κB and the ubiquitous H2TF1/KBF1 protein by alteration of a single nucleotide. Functional implications of this mutation have been further investigated by transient transfection experiments in different cell types.

As a guide for dissection of the binding sites, we compared the sequences that bind NF-κB predominantly (e.g., mouse κB, human κB, and TβB sequences [Fig. 1]) with sequences that presumably bind both NF-κB and H2TF1/KBF1 (e.g., β2m and $H2K^b$ sequences [Fig. 1]). The comparison revealed two points. First, the κB sequences and the TβB sequence share striking nucleotide conservation (Fig. 1, boxed residues) in the sequences flanking the core-binding site (as identified by methylation interference), whereas $H2K^b$ and β2m share minimal homology outside the binding domain. Secondly, $H2K^b$ and β2m sequences exhibit at least three consecutive C residues at the 3′ end of the core-binding site, whereas the sites which bind NF-κB strongly and H2TF1/KBF1 weakly have only two C residues. Because either or both features could contribute to the characteristic binding patterns observed, we tested each parameter independently.

Synthetic oligonucleotides corresponding to the sequences shown in Fig. 1 were cloned into pUC-derived vectors, and the recombinant *Hin*dIII-*Eco*RI restriction fragments were used in electrophoretic mobility shift assays (17). The $H2K^b$ probe bound to H2TF1/KBF1 to generate a complex (labeled H2TF1/KBF1) in extracts derived from the murine T-cell lines RLM11 and BW5147 (Fig. 2A, lanes 2 and 3), from the pre-B-cell line PD31 (lane 6), from PD31 cells treated with bacterial LPS for 20 h (lane 7), and from the B-cell line AJ9 (lane 8). Consistent with a previous report (3), this complex was not readily detectable in extracts derived from 70Z/3 cells before or after treatment with LPS (Fig. 2A, lanes 4 and 5). The $H2K^b$ probe also bound to NF-κB to produce a complex with slower mobility (labeled NF-κB) in extracts from those cell lines in which this protein is present (e.g., 70Z LPS, PD LPS, and AJ9 [Fig. 2A, lanes 5, 7, and 8]). The κB probe showed good complex formation only in stimulated pre-B- or B-cell extracts (Fig. 2B, 70Z LPS [lane 5], PD LPS [lane 7], and AJ9 [lane 8]). It is quite clear that the H2TF1/KBF1 nucleoprotein complex is much weaker or not present at all with the κB probe (compare Fig. 2B, lanes 2, 3, 6, 7, and 8, with Fig. 2A, lanes 2, 3, 6, 7, and 8). Extracts derived from PD31 cells and BW5147 cells

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Mouse κB	C A G A G ▇▇▇▇▇▇▇▇▇▇ G A G A G
Human κB	C A G A G G G G G A T T T C C A A G A G
Mouse TβB	A A G C A G G G A G A T T C C A A G A G
β 2m	A C A A A G G G A C T T T C C C A T T T
H2Kb	G G C T ▇▇▇▇▇▇▇▇▇▇▇ A T C T C
κ B / H2	G G C T G G G G A C T T T C C A T C T C
κ B G--C	C A G A G G G G A C T T T C C _C_ A G A G
SV40	G C C T G G G G A C T T T C C A C A C C

FIG. 1. Comparison of sequences that interact predominantly with NF-κB and those that interact with H2TF1/KBF1 and NF-κB. Mouse κB sequence is from the murine κ enhancer which has been shown to interact with the B-cell-specific protein NF-κB (15, 17). Human κB sequence is derived from the human κ enhancer which also binds a B-cell-specific protein (5, 6). Mouse TβB sequence is derived from the murine T-cell receptor β2 gene intron which shows the same pattern of binding as does the κB fragment (13; unpublished results). H2K^b is the murine major histocompatibility complex class I gene regulatory sequence shown to interact with a ubiquitous factor termed H2TF1 or KBF1 (2, 9, 10). β2m is the murine β2 microglobulin gene sequence which has been shown to bind KBF1 (9, 10). κB/H2 is the sequence of the hybrid mutant corresponding to the NF-κB-binding site derived from the murine κ enhancer with the flanking sequences of the H2K^b promoter. κBG→C is the sequence of the mutant corresponding to the mouse κB site with a single G-to-C substitution; the underlined residue marks the substitution. SV40 is the sequence of the NF-κB site derived from the SV40 enhancer (19). The previously identified core-binding sites of NF-κB on the mouse κB sequence (17) and of H2TF1/KBF1 on the H2K^b sequence (2, 9) are shown indicated (▭). Conserved sequence elements flanking the core NF-κB-binding site are boxed.

showed a weak band corresponding to NF-κB with both probes (Fig. 2A and B, lanes 3 and 6), presumably corresponding to a small number of cells in which NF-κB is already activated.

To determine the contribution of the conserved flanking sequences of the murine κB site in preventing the binding of H2TF1/KBF1, a synthesized oligonucleotide, κB/H2 (Fig. 1), corresponding to the NF-κB-binding site sequence derived from the κ enhancer with the flanking sequences of H2K^b, was used in gel mobility shift-binding assays. The hybrid κB/H2 probe showed the same pattern of nucleoprotein complex formation as did the κB probe (Fig. 2C). Thus, a band corresponding to H2TF1/KBF1 was weak or absent in extracts in which it was easily detectable with the H2K^b probe (compare Fig. 2C, lanes 1 to 4, with Fig. 2A, lanes 3 and 6 to 8). The relatively strong complex seen in BW5147 cell extract (Fig. 2C, lane 1), which migrated somewhat faster than the complexes in PD LPS or AJ9 cells (Fig. 2C, lanes 3 and 4), was not reproducibly present in different BW5147 cell nuclear extract preparations, and we have not pursued its origin further. The NF-κB complexes are stronger in Fig. 2C than in Fig. 2B because of longer autoradiography times. Conversely, alteration of the flanking sequences of the H2K^b site did not affect binding of either H2TF1/KBF1 or NF-κB (data not shown). Taken together, these data suggest that the sequences flanking the core-binding sites of each of these factors play only a minor role in determining binding specificity.

To determine whether the presence of three C residues at the 3′ end of the site is crucial to allow binding of H2TF1/KBF1, an oligonucleotide which corresponded to the mouse κB site with a single G-to-C substitution (κBG→C; Fig. 1) was synthesized and utilized in mobility shift-binding assays. The nucleoprotein complex pattern observed with this mutant κB site in various extracts (Fig. 2D) corresponded closely to that seen with the H2K^b probe (Fig. 2A). This single-base change significantly enhanced interaction with H2TF1/KBF1 in extracts from the murine T cells RLM11 and BW5147 (Fig. 2D, lanes 2 and 3), in PD and PD LPS extracts (lanes 6 and 7), and in AJ9 nuclear extract (lane 8). Thus, substitution of a single nucleotide in the κ enhancer sequence was sufficient to considerably enhance binding of the ubiquitous H2TF1/KBF1 factor to this sequence. To verify that the factors were binding at the expected position on the κBG→C probe, we performed methylation interference experiments using LPS-induced PD cell extracts which contained both H2TF1/KBF1 and NF-κB (17). The patterns of interference for the binding of NF-κB and H2TF1/KBF1 on the coding strand were identical; there was a clear effect of the modification of three of the four consecutive G residues, whereas modification of the first G residue did not interfere significantly with NF-κB and H2TF1/KBF1 binding (Fig. 3A, compare lanes 6 and 7 with lane 5 or 8; the interfering G residues are indicated by arrows). On the noncoding strand, the pattern of interference differed slightly

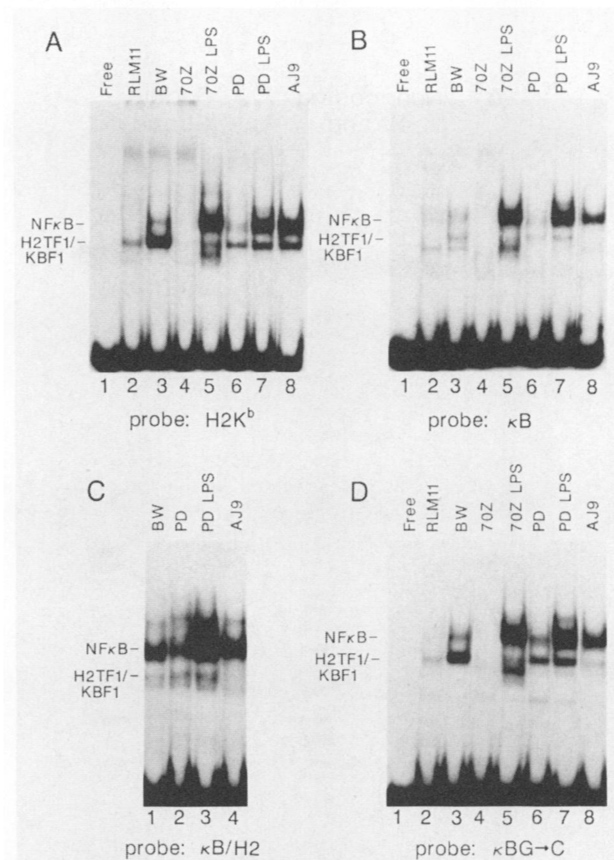

FIG. 2. Electrophoretic mobility shift analysis of nucleoprotein complex formation on κB- and H2K^b-related sequences in extracts derived from murine lymphoid cell lines. Probes used were H2K^b (A), κB (B), κB/H2 (C), and κBG→C (D). Nuclear extracts (4) are indicated above each lane. Positions of complexes characteristic of NF-κB and H2TF1/KBF1 proteins are indicated. (Autoradiogram presented in panel C is from a longer exposure than those used for the other panels.)

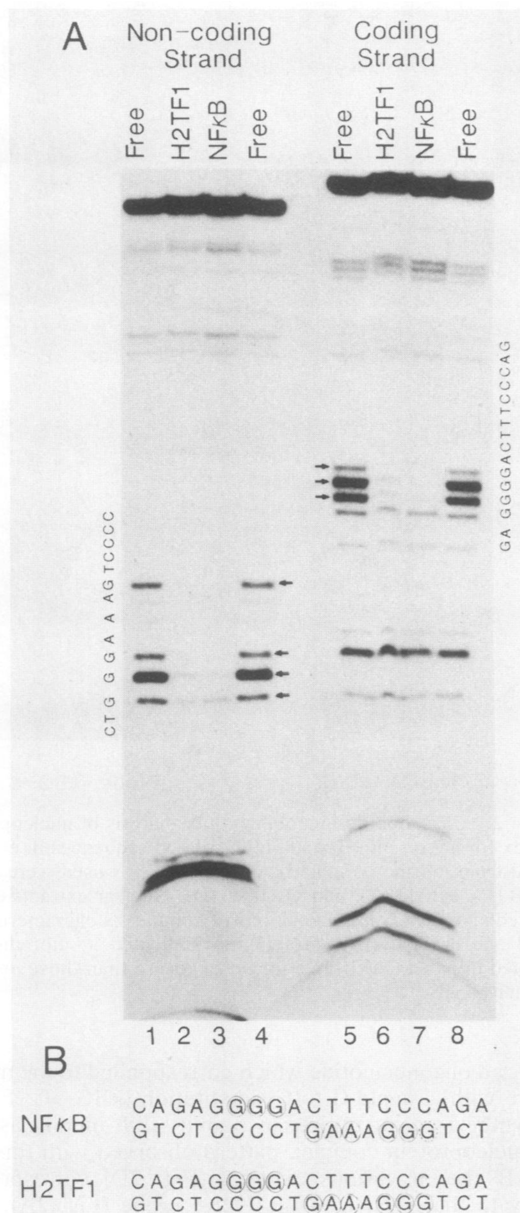

FIG. 3. Methylation interference analysis of proteins binding to the κBG→C sequence. (A) Localization of contact residues on the coding and noncoding DNA strands of κBG→C. Reactions were carried out as described previously (17), using LPS-treated PD31 nuclear extracts. Lanes: 1, 4, 5, and 8, DNA corresponding to the free fragment; 2, noncoding strand DNA from H2TF1/KBF1 complex; 3, noncoding strand DNA from NF-κB complex; 6, coding strand DNA from H2TF1/KBF1 complex; 7, coding strand DNA from NF-κB complex. G residues whose methylation interferes with DNA-protein interactions are indicated by arrows, and the sequence of the appropriate region is shown next to the lanes. (B) Summary of interference analysis. The coding (top) and noncoding (bottom) strands of the κBG→C oligonucleotide are shown. G and A residues whose modification significantly interferes with binding are circled, and those that interfere partially are circled with a dotted circle. NF-κB sequence summarizes interference observed with NF-κB complexes, and H2TF1 sequence summarizes interference obtained with H2TF1/KBF1 complexes.

between NF-κB and H2TF1/KBF1. Specifically, modification of the outermost guanine residue at the 3′ end of the site interfered more with H2TF1/KBF1 binding than with NF-κB binding, whereas modification of the central guanine residue (of the three consecutive G residues) had the opposite effect (Fig. 3A; compare lanes 2 and 3 with lane 1 or 4). Two of the three A residues on the noncoding strand were also visible in this ladder, and modification of these also interfered with binding of either NF-κB or H2TF1/KBF1, as shown by the absence of these bands in the lanes corresponding to the DNA-protein complexes. Thus, on the noncoding strand, modification of six of seven contiguous G and A residues interfered with protein binding. Two other methylation interference experiments with the κBG→C probe in BW5147 cell extracts that contained predominantly H2TF1/KBF1 and in LPS-induced 70Z/3 cell extracts that contained predominantly NF-κB showed the same patterns of interference (data not shown). These results are summarized in Fig. 3B. The H2TF1/KBF1 contacts observed with the κBG→C probe were consistent with those reported on the $H2K^b$ sequence (2, 9, 21), since they identified a core-binding domain (defined as the sequence between the first and last G residues scored in the interference assay) of 11 base pairs. Unexpectedly, mapping the sites of NF-κB interaction with the κBG→C oligonucleotide showed an additional contact with the DNA that had not been identified previously (17). This alteration extended the core-binding site of NF-κB to 11 base pairs, making it identical to that of H2TF1/KBF1, and highlighted the similarity of DNA recognition by the two factors. These observations suggest an interesting inverse correlation between regulation of expression of a factor and its DNA-binding specificity. Thus, the more stringently regulated factor, NF-κB, is sufficiently flexible in its specificity that it binds efficiently even if the last residue of its core-binding site is changed, whereas the affinity of the ubiquitously distributed factor, H2TF1/KBF1, falls considerably when this contact is altered.

To further compare the ability of the related sequences κB, κBG→C, κB/H2, $H2K^b$, β2m, and simian virus 40 (SV40) (Fig. 1) to bind either NF-κB or H2TF1/KBF1 (or both), in vitro competition experiments were performed. Briefly, various amounts of nonradioactive competitor DNA fragments were included in binding reactions with either radioactive κB or $H2K^b$ sequences and with extracts that were particularly rich in either NF-κB or H2TF1/KBF1, and the binding reactions were followed by electrophoresis and autoradiography (data not shown). Quantification of the autoradiograms showed that the various sequences could be ordered in their ability to compete for H2TF1/KBF1 binding as follows: $H2K^b$ > β2m, κBG→C >> κB, κB/H2, and SV40. There was less than a twofold difference between the amounts of $H2K^b$, β2m, and κBG→C sequences that were required to compete with H2TF1/KBF1 binding, whereas 6- to 10-fold-greater amounts of the κB, κB/H2, and SV40 sequences were needed to achieve the same extent of competition. In contrast, all sequences competed equivalently for the binding of NF-κB to the κB sequence, except for κBG→C, which was somewhat better (approximately 1.5- to 2-fold). Thus, the originally identified κB site within the κ enhancer was a suboptimal one for NF-κB binding. We concluded that the single-nucleotide substitution in the κB sequence allowed binding of H2TF1/KBF1 with affinity comparable to that of the wild-type $H2K^b$ or β2m sequences. Furthermore, there is a good correlation between efficient binding of H2TF1/KBF1 and the presence of three consecutive C residues at the 3′ end of the core-binding site.

TABLE 1. Expression of plasmids dependent on κB and related sequences in S194 (plasma) and RLM11 (T) cells

Plasmid[a]	Expression[b] in:	
	S194	RLM11
pSPCAT	1	1
p(κB)$_2$ CAT−	4.3	1.1
p(κBG→C)$_2$ CAT−	10.3	1.8
p(κBG→C)$_2$ CAT+	8.4	1.9
p(H2K^b)$_2$ CAT+	2.0	0.9

[a] +, Binding sites were inserted in the orientation corresponding to the sequences shown in Fig. 1 5′ to the CAT gene; −, binding sites were inserted in the opposite orientation.

[b] Transient transfection experiments and CAT assays were carried out as described previously (7, 8, 20), and each experiment was repeated at least twice with duplicate samples. Numbers are mean values and represent relative CAT activity normalized to the pSPCAT level.

To assess the functional significance of the increased NF-κB and H2TF1/KBF1 binding to the κ enhancer mutant sequence (κBG→C), we carried out transient transfection analysis in a variety of lymphoid and nonlymphoid cell lines. The κB, H2K^b, and κBG→C sequence elements were excised as *Sma*I-*Hin*cII fragments from the cloned oligonucleotides in pUC vectors and inserted as dimers into the vector pSPCAT, which carries the bacterial chloramphenicol acetyltransferase (CAT) gene downstream from an enhancerless SV40 early promoter (14). Introduction of these plasmids into the murine plasma cell line S194 showed that the κB dimer can efficiently direct CAT enzyme expression (Table 1), as expected (16). The H2K^b dimer also activated transcription, although less efficiently (Table 1). Interestingly, the κBG→C dimer in either orientation activated transcription more efficiently than did the wild-type κB and H2K^b sequences (Table 1). These results showed that the greater affinity of NF-κB for the κBG→C sequence is reflected in its ability to activate transcription. To check whether the ubiquitous distribution of H2TF1/KBF1 permits transcriptional activity of p(κBG→C)$_2$ CAT and p(H2K^b)$_2$ CAT plasmids in non-B cells, we performed transient transfections into murine NIH 3T3 fibroblasts, human HeLa cells, and the murine T-cell line RLM11. No CAT expression was evident in either of the two nonlymphoid cell lines (NIH 3T3 and HeLa), except when the cells were treated with phorbol myristate acetate (data not shown). This suggests that the H2TF1/KBF1 factor by itself is apparently a much weaker transcriptional activator than NF-κB and that a dimer of its binding site is insufficient for activity in these nonlymphoid cell lines. Perhaps H2TF1/KBF1 needs to interact with other factors in order to efficiently activate transcription, similar to the proposed role for the ubiquitous octamer-binding protein NF-A1 in activating histone H2B and U small nuclear RNA gene transcription (11). In the murine T-cell line RLM11, plasmids p(κB)$_2$ CAT and p(H2K^b)$_2$ CAT showed activity similar to that of the enhancerless control pSPCAT (Table 1). However, CAT enzyme expression directed by the κBG→C dimers was reproducibly twofold higher than that of the control (Table 1). We favor the hypothesis that this transcriptional activity resulted from the presence of low levels of NF-κB and not from H2TF1/KBF1 for two reasons: first, because the plasmids were inactive when transfected into nonlymphoid cells which contained H2TF1/KBF1 (as discussed above), and second, because the levels of activity in RLM11 cells correlated well with the affinities of NF-κB for the various sequences and not with those of H2TF1/KBF1. This analysis is suggestive that the increased affinity of NF-κB for the κBG→C sequence allows detectable transcriptional activation under conditions in which the wild-type κB would be inactive. We speculate that a reason for the suboptimal κB site in the κ immunoglobulin gene enhancer might be to accentuate the B-cell specificity of this element.

The κB sequence element of the κ gene enhancer is preferentially bound by one (NF-κB) of the two factors (NF-κB and H2TF1/KBF1) that have very similar DNA recognition specificities (3). The initial goal of the experiments presented here was to determine the basis for this selectivity. Our results demonstrated that alteration of a single nucleotide of the κB site was sufficient to create a H2TF1/KBF1-binding site with an affinity comparable to that of the sites present in the major histocompatibility complex class I and β2m gene promoters. Although the in vitro-binding and competition experiments served to enhance the similarities between H2TF1/KBF1 and NF-κB, transient transfection experiments to functionally characterize the various related sequences suggested that the mechanisms of transcriptional activation by the two factors (NF-κB and H2TF1/KBF1) are different.

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