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Identification of an Octamer-Binding Site in the Human Kappa Light-Chain Enhancer

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Octamer motifs contribute to the function and tissue specificity of immunoglobulin heavy- and light-chain gene promoters and the heavy-chain enhancer. A variant octamer-binding site within a conserved region of the human kappa light-chain gene enhancer which contributes to the function of this enhancer has been identified.

The expression of immunoglobulin genes is a paradigm for tissue-specific and developmental control of eucaryotic gene transcription through *cis*-acting DNA sequences (3). Different sequence motifs within the promoters and enhancers of heavy- and light-chain immunoglobulin genes have been shown to interact specifically with nuclear proteins which modulate transcription (15, 23, 25–27). While some of these motifs interact with ubiquitous nuclear factors, others interact with factors which are developmentally regulated and restricted to cells of the B-lymphoid lineage (12, 24, 27). The octamer motif (ATTTGCAT) interacts with both ubiquitous (OTF-1, also called Oct-1) and B-cell-specific (OTF-2, also called Oct-2) factors and has been shown to be important for the function of heavy- and light-chain promoters and the heavy-chain enhancer (5, 7, 14, 16, 19, 23, 27–29). It has been proposed that OTF-1 and OTF-2 binding to the heavy-chain promoter and enhancer may allow promoter-enhancer interactions which result in the synergism observed with these control elements (8, 13).

Recently, a variant octamer motif was identified in the mouse kappa immunoglobulin light-chain gene enhancer (4). This motif was found in a region upstream of other protein-binding sites within the enhancer which has previously been shown to be important for maximal enhancer function (20). We have also identified a novel protein-binding site within this region of the mouse kappa enhancer approximately 50 base pairs (bp) upstream of the NF-κB binding site (18). This site appears to be important for maximal function of the kappa enhancer as an inducible *cis*-acting element in murine pre-B cells, and it interacts with a protein only after induction of kappa gene transcription in pre-B cells. Sequence homology between this site in the mouse enhancer and the corresponding region in the human kappa enhancer led us to investigate nuclear-factor interaction with this region in the human enhancer.

To facilitate binding studies, oligonucleotides which contain human kappa enhancer sequences (hKE-OCT) found approximately 50 bp upstream of the NF-κB binding site were synthesized (Fig. 1). The double-stranded oligonucleotide hKE-OCT contains a stretch of eight nucleotides which differs from the promoter octamer-binding site at two positions (TTTTACAT). This oligonucleotide was used as a probe in mobility shift assays, as previously described (18). Nuclear extracts were prepared from mouse pre-B (7OZ/3 and 3-1) and human nonlymphoid cells (HeLa), which do not express the kappa gene and contain only the ubiquitous factor OTF-1. Extracts were also prepared from pre-B cells stimulated with bacterial lipopolysaccharide (LPS) and human (Namalwa) or mouse (S107 and S194) mature B-cell lines, which express the kappa gene and contain both OTF-1 and OTF-2. hKE-OCT interacted with a factor in each extract which comigrated with OTF-1 bound to an oligonucleotide probe (hPr-OCT) (Fig. 2A) containing the kappa promoter octamer (Fig. 1B). Similarly, a factor found only in cells expressing the kappa gene bound hKE-OCT and comigrated with OTF-2 bound to hPr-OCT (Fig. 2A). The specificity of binding to the hKE-OCT probe was tested by including a 25-fold molar excess of unlabeled oligonucleotide competitor in mobility shift reactions. The hKE-OCT competed for binding of the two complexes comigrating with OTF-1 and OTF-2, as did the oligonucleotide containing the promoter octamer motif (hPr-OCT) (Fig. 2B). An oligonucleotide containing the NF-κB sequence of the mouse kappa

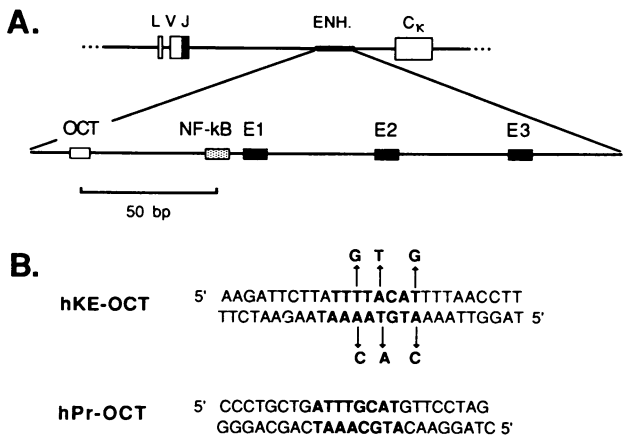

FIG. 1. (A) Diagram of a rearranged kappa gene and the human kappa enhancer. Protein-binding sites within the human kappa enhancer (ENH.) are indicated by boxes. A variant octamer motif (OCT) found approximately 50 bp upstream to the NF-κB-binding site (25) is indicated. E1 through E3 are E motifs homologous to sequences in the immunoglobulin heavy-chain enhancer (6). L, V, J, and $C_κ$ indicate leader, variable, joining, and constant gene segments, respectively. (B) Oligonucleotide probes used in mobility shift assays. The hKE-OCT probe contains a sequence from the human kappa enhancer encom-passing a variant octamer motif (boldface type). Specific mutations made within the variant octamer are indicated by arrows. Analogous mutations in the mouse immunoglobulin heavy-chain enhancer octamer have been shown to eliminate octamer binding in vitro. (9). The hPr-OCT probe contains a sequence from a human kappa gene promoter and includes the octamer motif. Oligonucleotides were end-labeled with [γ-^{32}P]ATP and annealed for use in binding assays, as described previously (18).

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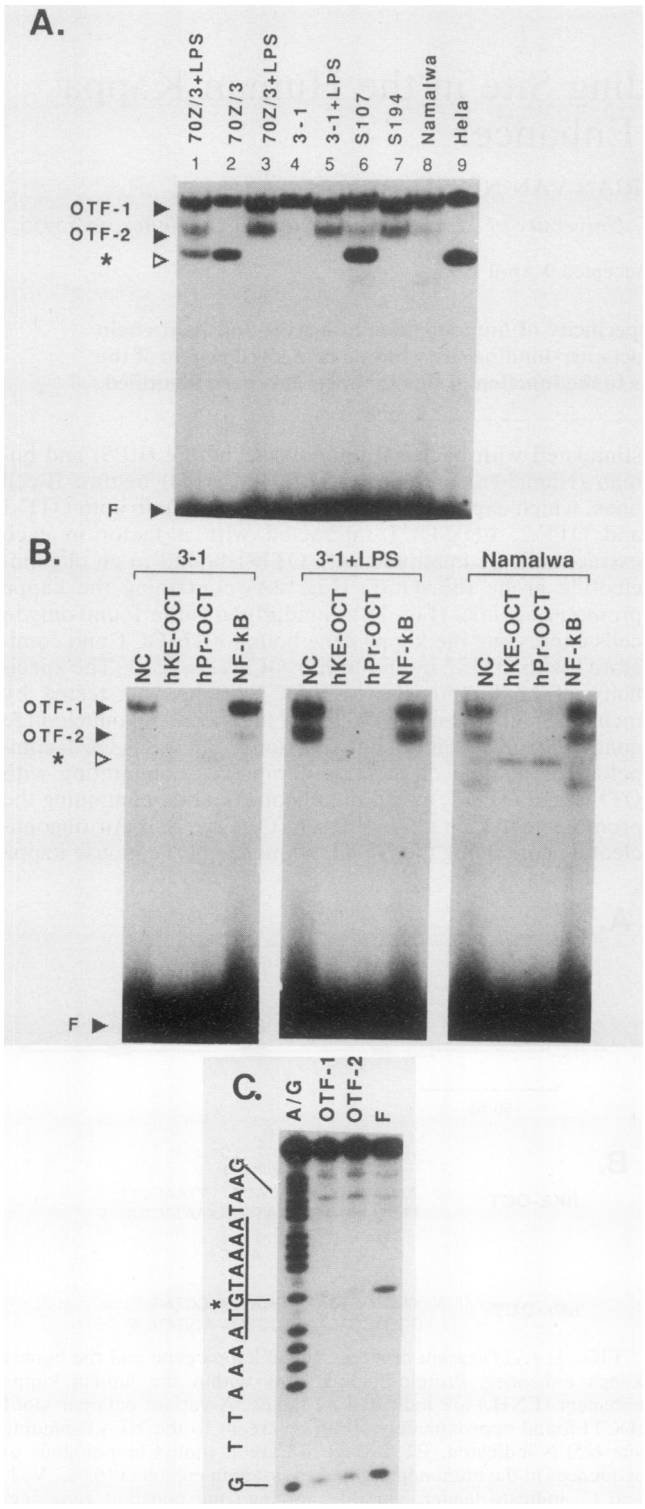

FIG. 2. Characterization of nuclear-factor interaction with a region of the human kappa enhancer containing a variant octamer motif. (A) Mobility shift assays were performed as described previously (18), with nuclear extracts and with the oligonucleotides hPr-OCT (lane 1) and hKE-OCT (lanes 2 through 9) as probes (Fig. 1B). Nuclear extracts were prepared from the mouse pre-B cell lines 3-1 and 70Z/3 before or after a 24-h induction of the kappa light-chain gene with 10 μg of LPS per ml (lanes 1 through 5). Extracts from mouse (S107 and S194) and human (Namalwa) mature B-cell lines and a nonlymphoid (HeLa) cell line were also used in binding assays (lanes 6 through 9). Reaction mixtures typically contained 10 μg of nuclear extract, 1 μg of poly(dI-dC) · (dI-dC), and 0.1 ng of end-labeled probe. Specific complexes formed by the octamer-binding factors OTF-1 and OTF-2 are indicated. Nonspecific complexes (*) and free probe (F) are also identified. (B) Competition analysis of nuclear factors binding to the hKE-OCT probe. No competitor (NC) or a 25-fold molar excess of double-stranded oligonucleotide was included in mobility shift reaction mixtures containing nuclear extract from uninduced 3-1, LPS-induced 3-1, or Namalwa cells. An oligonucleotide containing the NF-κB binding site from the mouse kappa light-chain enhancer was used as a nonspecific competitor (18). (C) Methylation interference analysis of OTF-1 and OTF-2 binding to the hKE-OCT probe. Partially methylated hKE-OCT probe was used in mobility shift reaction mixtures containing LPS-induced 3-1 nuclear extract, as described previously (18). The methylated guanine on the noncoding strand of hKE-OCT, which interfered with OTF-1 and OTF-2 binding, is indicated by an asterisk. The variant octamer motif is underlined. A/G lane shows A+G cleavage pattern of free probe.

enhancer (18) did not compete for binding to hKE-OCT (Fig. 2B). Thus, the variant octamer (TTTTACAT) found within the human kappa enhancer sequence appears to interact specifically with OTF-1 and OTF-2 in vitro.

To verify that the sequence within hKE-OCT which interacted with OTF-1 and OTF-2 included the variant octamer motif, methylation interference assays were performed (18) with the hKE-OCT oligonucleotide as a probe. Methylation of a guanine residue on the noncoding strand interfered with OTF-1 and OTF-2 complex formation (Fig. 2C). This residue is within the variant octamer, showing that it is this motif within hKE-OCT which interacts with OTF-1 and OTF-2.

The sequence homologous to the hKE-OCT probe in the mouse kappa enhancer is important for its maximal functioning as an inducible control element in pre-B cells (18). The importance of this variant octamer motif within the human kappa enhancer was examined by mutating the motif and examining the effects of the mutation on enhancer function in transient transfection assays. The polymerase chain reaction (21) was used to amplify a 249-bp fragment containing either wild-type or mutated enhancer octamer motifs and all other previously characterized sites of DNA-protein interaction (6, 10, 15, 17, 25). A wild-type or mutated hKE-OCT oligonucleotide (Fig. 1B) was used as the 5′ primer in polymerase chain reactions, and an oligonucleotide complementary to sequences 60 bp downstream of the KE3 site of the human enhancer was used as the 3′ primer (Fig. 3A). The mutations made in the human kappa enhancer octamer sequence (Fig. 1B) are analogous to mutations which have been shown to eliminate binding of OTF-1 and OTF-2 to the murine heavy-chain enhancer octamer in vitro (9). The polymerase chain reaction-generated 249-bp enhancer fragments were cloned into the *Hin*dIII site of the vector KpCAT, which contains a chloramphenicol acetyltransferase (CAT) gene under the control of a natural mouse $V_\kappa 21E$ light-chain gene promoter (Fig. 3A) (2). This promoter has previously been shown to direct accurate transcription initiation in transfected expression vectors (1). The resulting vectors, which contained wild-type (KpCAT-OCT$^+$) or mutated (KpCAT-OCT$^-$) kappa enhancer octamer motifs, were sequenced to verify that only the specific mutations were made (22). The vectors were then transfected into cell lines by the DEAE-dextran protocol (2). Cell extracts were prepared and assayed for CAT activity (11).

Mutation of the kappa enhancer octamer motif had a significant deleterious effect on the ability of the 249-bp

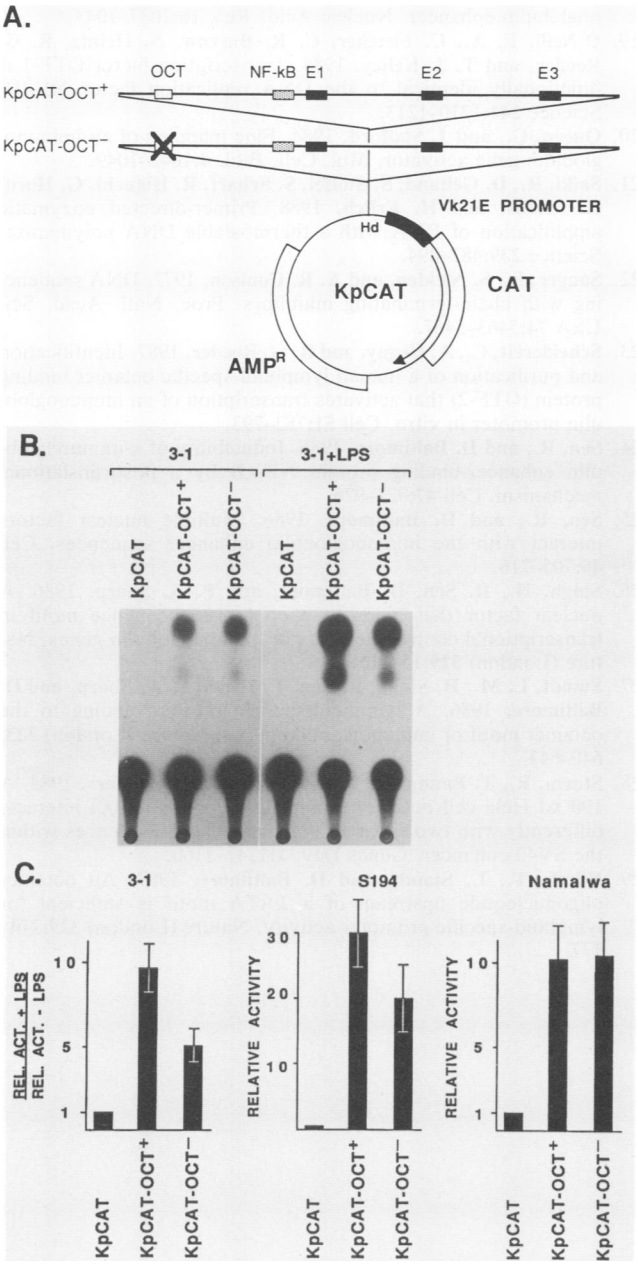

FIG. 3. Functional analysis of the human kappa enhancer octamer motif. (A) Vector constructs used in transient transfection assays. Enhancer fragments containing wild-type (KpCAT-OCT$^+$) or mutated (KpCAT-OCT$^-$) octamer motifs and all other characterized sites of enhancer-protein interaction were generated by polymerase chain reaction and cloned into the *Hin*dIII site (Hd) of the vector KpCAT (described as ECAT$^-$ in reference 2). (B) The pre-B-cell line 3-1 was transfected with KpCAT, KpCAT-OCT$^+$, or KpCAT-OCT$^-$ vector, split into media with and without 10 μg of LPS per ml, and cultured for 24 h. Cell extracts were then prepared and assayed for CAT activity as described previously (18). Acetylated forms of [^{14}C]chloramphenicol were separated by thin-layer chromatography (11). (C) Summary of transient transfection assays in a pre-B-cell line (3-1) as well as in mouse (S194) and human (Namalwa) mature B-cell lines. Mature cell lines were harvested 48 h posttransfection and assayed for CAT activity. Enzyme activity was calculated as the percentage of chloramphenicol converted to acetylated forms. Relative activity is the CAT activity in each extract normalized to the CAT activity in KpCAT-transfected cells (1.0 relative CAT activity). To correct for any differences in transfection efficiency, the values for CAT activities in 3-1 cells are expressed as ratios of the relative activity in LPS-induced cells (REL. ACT. + LPS) to the relative activity in uninduced cells (REL. ACT. − LPS). The induction ratios of KpCAT-OCT$^+$ and KpCAT-OCT$^-$ in 3-1 cells were significantly different ($P < 0.035$, $n = 6$). Values shown for each cell line are averages of at least four independent experiments (± standard error of the mean) done with at least two different preparations of each plasmid.

kappa enhancer fragment to induce CAT expression in response to LPS stimulation in 3-1 pre-B cells, even though it did not affect CAT expression significantly in unstimulated 3-1 cells (Fig. 3B). The ratio of CAT activities in LPS-stimulated 3-1 cells to CAT activities in unstimulated cells, or the induction ratio, was twofold higher with the wild-type enhancer construct (KpCAT-OCT$^+$) than with the mutated construct (KpCAT-OCT$^-$) (Fig. 3C). This result was reproducible in six independent experiments performed with three preparations of each vector. A similar decrease in enhancer function has been observed when the octamer motif was mutated in the context of the full mouse immunoglobulin heavy-chain enhancer (15). In these experiments, 3-1 cells were transfected with each vector and then split into media with and without 10 μg of LPS per ml, ensuring that the transfection efficiency was equivalent for each vector in LPS-induced and uninduced cells. The induction ratios, therefore, indicate the ability of the enhancer fragment in each vector to respond to LPS, independent of transfection efficiency. This result correlates with previous observations which indicate that the homologous region of the mouse kappa enhancer is important for maximal induction in LPS-stimulated pre-B cells (18). The effect of mutating the octamer motif was less dramatic in murine plasmacytoma S194 and human Burkitt's lymphoma Namalwa cells (Fig. 3C). These results suggest that this variant octamer motif in the human kappa enhancer may be more important in pre-B cells than in mature B cells for its function during induction of kappa transcription.

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