Recognition of Angiotensin II: Antibodies at Different Levels of an Idiotypic Network Are Superimposable

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Genetic and sequence information are reported for an angiotensin II–reactive antibody (Ab1, MAb 110) and an anti–anti-idiotypic antibody (Ab3, MAb 131) that have identical antigen binding properties and that are related by an anti-idiotypic antibody (Ab2-β) that satisfies accepted biochemical criteria for an internal image–bearing antibody. The sequences of the variable regions of the Ab3 and of the Ab1 are nearly identical, even though the Ab1 is an antibody to a peptide and the Ab3 is an antibody to a globular protein. Significantly, amino acid residues that make critical contacts with antigen in the crystal structure of the Ab3-antigen complex are highly conserved in Ab1, suggesting that the epitopes of the Ab2-β recognized by the Ab3 do indeed resemble the bound structure of the antigen.

The immunologic determinants specific to a particular antibody molecule (Ab1) define its idiotype. It has been proposed that some anti-idiotypic (Ab2) antibodies (designated Ab2-β) can express a surface substructure—the internal image—that is a molecular mimic of the original immunizing antigen (*1*). An Ab2-β could therefore elicit anti–anti-idiotypic antibodies (Ab3) with antigen-binding characteristics similar to those of Ab1. Yet, in the three-dimensional (3-D) structure of an Ab2 in complex with an antibody to a globular protein Ab1, the Ab2 did not structurally resemble the original antigen, lysozyme (*2*). A short, flexible peptide, however, has physical-chemical properties that render it more likely to elicit an idiotypic series in which molecular mimicry could exist. Peptide hormones represent a class of molecules with the desired characteristics.

The use of anti-idiotypic antibodies to identify and purify receptors has emerged as one practical strategy to isolate an unknown binding protein of a known ligand. Specific binding and biochemical modulation of physiological membrane receptors by anti-idiotypic antibodies have now been described in several systems. Two explanations have been proposed to rationalize this phenomenon: (i) the anti-idiotypic antibody (Ab2) acts as a "mimic" or "internal image" of the ligand recognized by the physiological receptor and (ii) the Ab2s recognize structural homologies shared by the antibody to ligand (Ab1) and the receptor. Both mechanisms could result in the isolation of the desired receptor but, if one is relying strictly on molecular mimicry of a ligand to isolate a receptor, the anti-idiotypic approach may be limited to ligands with particular structural characteristics.

Monoclonal antibodies (Ab1s) have been produced against the octapeptide hormone angiotensin II (AII) that binds the hormone with high affinity (association constant $K_a > 10^9\ M^{-1}$) (*3*). In addition, monoclonal Ab3s have been produced against the Ab2-β that was affinity-purified by using the Ab1 to AII, MAb 110 (*4*). With respect to its high affinity for AII and its binding specificity, this Ab3 (MAb 131) is almost indistinguishable from its Ab1 counterpart (MAb 110). We determined the nucleotide sequences encoding the variable regions of MAb 131 (Ab3) and MAb 110 (Ab1) and found that they are remarkably similar (Fig. 1).

The immunoglobulin κ chain genes are assembled from two gene segments, V_κ and J_κ (*5*). The light chain genes of MAb 131 and MAb 110 exhibit nearly identical V_κ sequences (95% nucleotide sequence identity), and both contain the $J_\kappa 2$ gene segment (*6*, *7*) (Fig. 1A). Although V_κ 131 and V_κ 110 show 94 to 97% identity to a series of V_κ sequences from antibodies to DNA (DNA5, DNA6, and DNA7) that have been attributed to the $V_\kappa 8$ family (*8*), they appear to differ somewhat from the $V_\kappa 8$ consensus (*9*). Thus V_κ 131 and V_κ 110 are apparently derived from the same V_κ family, but the identity of this family is not certain.

The Ig heavy chain genes are assembled from three separate germ line gene segments (*5*), V_H, D, and J_H. The V_H 131 and V_H 110 segments share 94% nucleotide sequence identity and are 92 to 96% identical to the known members of the V_H 110 family (*10*), indicating that they were derived from that set. The observed nucleotide sequence differences may reflect the effects of somatic mutation, the use of different members of the $V_H 10$ family, or both. The MAb 131 and MAb 110 heavy chain genes both use the $J_H 4$ gene segment (*11*) (Fig. 1B). They differ most in the region encoding the complementarity determining region (CDR) H3 (codons 96 through 105), which spans the V_H-D-J_H junction (Fig. 2A). In both instances, codon 96 is contributed by the V_H segment and codons 103 through 105 by J_H (*4*). The two genes differ, however, with respect to the probable origins of codons 97 through 102. In this region of the MAb 131 heavy chain gene, the most extensive homology to any known D segment is a 5-bp (GGGAC) alignment to a portion of D_{Q52} (*12*). Within the same region, the MAb 110 heavy chain gene has an interval (8 of 9 bp) homologous to the sequences of several D_{SP2} segments (*13*). In both genes, nucleotides not accounted for by known V_H, D, and J_H segments are likely the result of de novo nucleotide insertion, as is commonly observed at V_H-D-J_H junctions (*14*). Despite these differences, the third CDRs encoded by the 131 and 110 heavy chain genes are highly conserved with respect to length and amino acid sequence. In the corresponding light chain genes, codons 89 through 95 specify CDR L3. This region spans the V_κ-$J_\kappa 2$ crossover point, which lies at the same position for both genes (Fig. 2B).

A second Ab1, MAb 133, also binds AII with high affinity ($K_a > 10^9\ M^{-1}$) but shows a specificity pattern distinct from that of MAbs 110 and 131 (*3*). The variable (V) regions of MAb 133 differed markedly from the V regions of MAb 131 and MAb 110 (49 and 59% amino acid sequence identity in the heavy and light chains, respectively), and significant differences were found in the sequences and lengths of the CDRs (Fig. 1, A and B). A monoclonal Ab1 to AII that was produced by another group (*15*) has a V region sequence and binding specificity similar to MAb 133, and therefore also differs markedly from MAbs 110 and 131. Thus the similarity between Ab1 MAb 110 and Ab3 MAb 131 does not simply result from a lack of alternative genetic strategies for AII binding.

Crystals of Fab fragments of MAb 131, in complex with AII, have been grown (*16*), and the atomic structure of the complex has been solved and refined at 3 Å resolution (*17*). Crystals of the Fab fragment of MAb 110 in complex with AII have also been grown, and these crystals are of the same space group ($P4_1$ or $P4_3$) and have two unit cell dimensions [$a = b = 110$ Å (*18*)] in common with the Fab 131–

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complex crystals. Given the sequence similarity between these antibodies, the congruence of the crystal parameters strongly suggests that they have similar structures. Chothia *et al.* have shown that CDRs fall into distinct structural categories that can often be predicted accurately based on their sequences and sizes (*19*). The CDRs of MAb 110 and MAb 131 are exactly the same length, and all have the same canonical structures. We therefore expected that a model of the MAb 110 combining site could be constructed using the 3-D structure of the Fab 131–AII complex.

In the structure of MAb 131, a deep pocket is formed by the unusually long CDRs L1 and H2. Five of the six CDRs contact the hormone, and the heavy chain dominates the interaction. When the sequence substitutions of MAb 110 were made in the structure of Fab 131, only a few of the key residues in contact with the hormone were changed. Van der Waals contact residues in the Fab 131–AII complex are indicated by asterisks in Fig. 1, A and B. The CDR H3 is most intimately in contact with the hormone in the Fab 131–AII complex, and only two of ten residues in this CDR are changed in MAb 110. The most significant difference is the replacement of Arg^{H99} by Tyr in Fab 110. It has been shown recently, however, that Arg and Tyr are often used for the same chemical purposes in antibody combining sites, and that Arg often assumes a "pseudoaromatic" role (*20*). The CDR H2 is also strongly involved in contact with the hormone, especially a hydrophobic cluster Phe-Asn-Phe, which is preserved in MAb 110 as Tyr-Thr-Tyr. Of the 13 residues of Fab 131 involved in van der Waals contact with AII, only four are different in MAb 110 (Fig. 3).

Although the Ab2-β in this system satisfies the classical biochemical criteria (*21*) for an internal image–bearing antibody, two possibilities still exist to explain the near identity of MAb 131 and 110: (i) Ab2-β is exhibiting a perfect structural mimic of AII bound to MAb 110; or (ii) Ab2-β has simply elicited a clone similar to MAb 110 (which we call MAb 131 at the Ab3 level) because it is specific for an idiotope on MAb 110 that may not necessarily represent the AII binding site. Which of these mechanisms is a priori more probable depends on the physical-chemical nature of the antigen. The second mechanism would be more likely in cases where the original immunizing antigen is a large globular protein or a small hapten, molecules which would be physically or chemically intractable to duplicate with the CDRs of an antibody combining site. If molecular mimicry by an Ab2-β could occur, it would

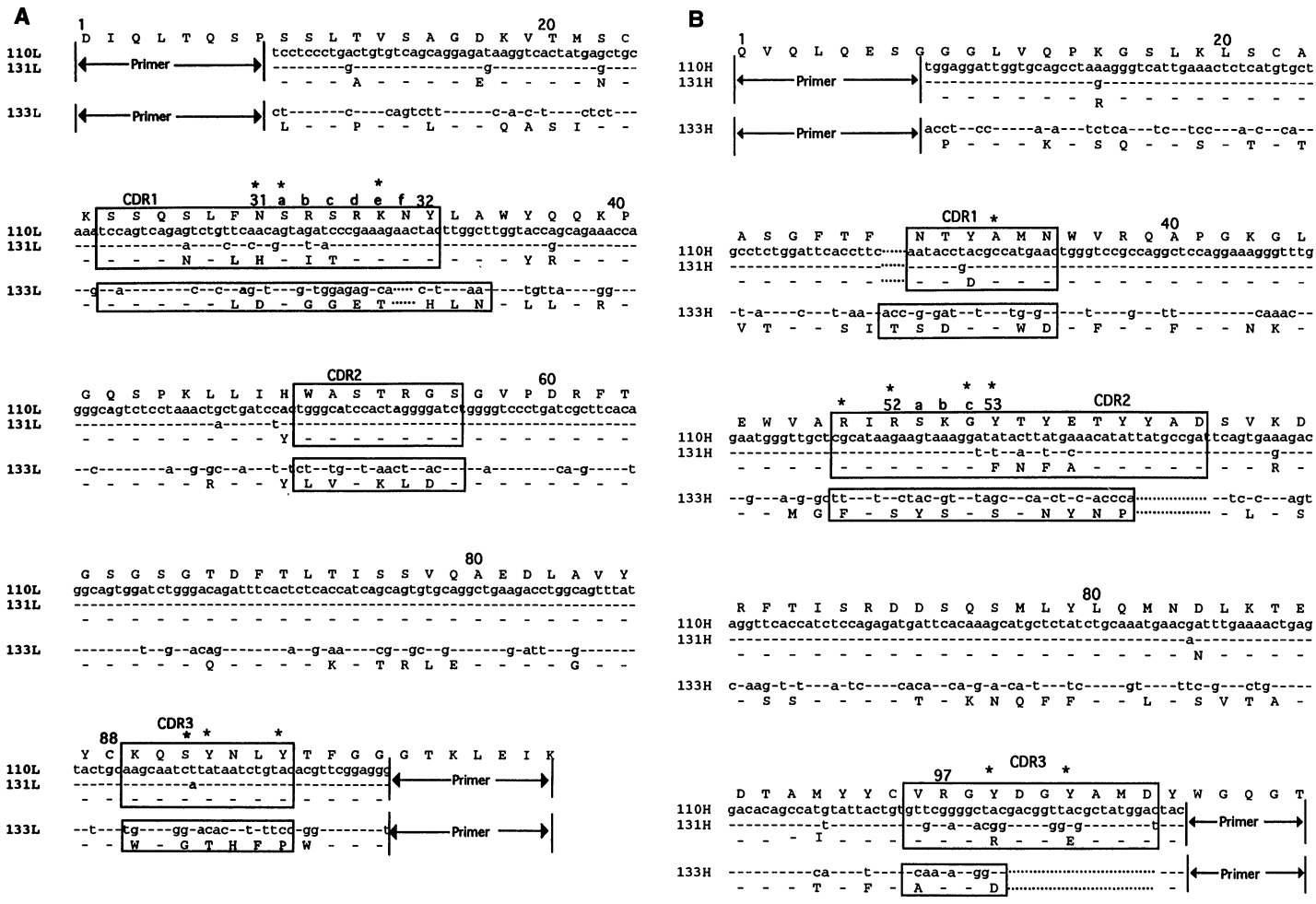

Fig. 1. Variable regions of MAbs to AII (*26–32*). Nucleotide sequences are shown in small letters and were aligned by inspection to maximize homology. Sequence identities are indicated by hyphens and gaps by ellipses. Key contact residues between Fab 131 and AII are indicated by asterisks. Amino acid translations (single-letter code) are shown in capital letters (*33*). Codons are numbered above, in Kabat nomenclature (*34*). Complementarity-determining regions (CDRs) are boxed. Sites corresponding to primers used in polymerase chain reaction amplification are indicated by arrows. (**A**) Variable regions of κ (light) chain genes. The nucleotide sequences of κ chain genes from MAbs 110 (110L), 131 (131L), and 133 (133L) were determined in the region encoding amino acid residues 9 through 99 (Kabat nomenclature). (**B**) Variable regions of heavy chain genes. The nucleotide sequences of heavy chain genes from MAbs 110 (110H), 131 (131H), and 133 (133H) were determined in the region encoding amino acid residues 8 through 106 (Kabat nomenclature).

A

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Jel318    ...TAT TAC TGT GTG aGA
H220-3    ...TAT TAC TGT GTG aGA
MRL-RF24  ...TAT TAC TGT GTG
MRL-DNA4  ...TAT TAC TGT GTG aGA
131H      ...TAT TAC TGT GTG CGA GGA CGG GAC GGG GAG GCT ATG GAT TAC...
DQ52                        c aac tGG GAC
JH4                                    At tac tAt GCT ATG GAC TAC...

110H      ...TAT TAC TGT GTT CGG GGC TAC GAC GGT TAC GCT ATG GAC TAC...
DSP2.3                  tc tac tat GGt TAC GAC
DSP2.4                  tc tac tat GGt TAC GAC
DSP2.6                  cc tac tat GGt TAC GAC
JH4                                    At tac TAt GCT ATG GAC TAC...

131H a.a....Tyr Tyr Cys Val Arg Gly Arg Asp Gly Glu Ala Met Asp Tyr...
110H a.a.... -   -   -   -   -   -   Tyr -   -   Tyr -   -   -   - ...
             93  94  95  96  97  98  99 100 101 102 103 104 105
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B

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DNA5      ...TAC TGC AAG CAA TCT TAT AAT CTt tcg
DNA6      ...TAC TGC AAG CAA TCT TAT AAT CTt cgg
6A4       ...TAC TGC AAG CAA TCT TAT AAT CTt cgg
DNA7      ...TAC TGC AAG CAA TCT TAT AAT CTt cgg
131L      ...TAC TGC AAG CAA TCA TAT AAT CTG TAC ACG TTC GGA GGG GGG...
Jk2                                  TG TAC ACG TTC GGA GGG GGG...

110L      ...TAC TGC AAG CAA TCT TAT AAT CTG TAC ACG TTC GGA GGG GGG...

131L a.a....Tyr Cys Lys Gln Ser Tyr Asn Leu Tyr Thr Phe Gly Gly Gly...
110L a.a.... -   -   -   -   -   -   -   -   -   -   -   -   -   ...
             87  88  89  90  91  92  93  94  95  96  97  98  99 100
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Fig. 2. Assembly of heavy and light chain genes encoding MAbs 131 and 110. (**A**) Sequences at V_H-D-J_H junctions. Nucleotide sequences of 131H and 110H are shown in boldface type. Above the 131H sequence are aligned the 3′ ends of V_H10-type segments used in the heavy chain genes of Jel318 (*35*), H220-3 (*36*), MRL-RF24 (*10*), and MRL-DNA4 (*10*). Aligned below the 131H sequence are the coding sequence of D_{Q52} (*12*) and the 5′ end of J_H4. Aligned below the 110H sequence are the coding sequences of $D_{SP2.3}$, $D_{SP2.4}$, $D_{SP2.6}$ (*13*), and the 5′ end of J_H4 (*11*). Nucleotide sequence differences are indicated by small letters. The corresponding amino acid sequences (*33*) (131H a.a. and 110H a.a.) are numbered as in Fig. 1; identities are marked by hyphens. (**B**) Sequences at V_κ-J_κ junctions. Nucleotide sequences of 131L and 110L are shown in boldface type. The 131L sequence is aligned with the 3′ ends of V_κ segments DNA5, DNA6, DNA7 (*8*), and 6A4 (*37*) (above) and with the 5′ end of J_κ2 (below); sequence differences are shown in small letters. The corresponding amino acid sequences (131L a.a. and 110L a.a.) are shown as in (A).

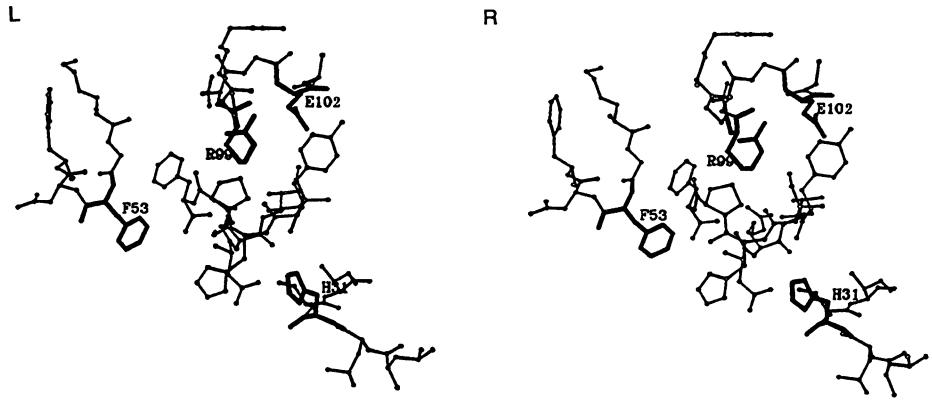

Fig. 3. Stereo drawing of the Fab 131–AII complex. The four CDR residues of Fab 131 that make van der Waals contact with the peptide and that undergo sequence changes in MAb 110 are drawn in thick, bold lines. The peptide is in the middle of the figure in thinner lines; F53 is Phe^{53} of CDR-H2, E102 is Glu^{102} of CDR-H3, H31 is His^{31} of CDR-L1, and R99 is Arg^{99} of CDR-H3. Drawing was plotted by the program PLUTO.

most likely happen with an antigen that is chemically identical to a protein and yet is small and deformable—for example, a short, flexible peptide. Angiotensin II is such a peptide. The AII system presented here has an additional characteristic that suggests that recognition of an internal image was involved in the generation of the Ab3. Sequence comparison shows that the differences in the CDRs of the Ab1 and Ab3 are mostly conservative. In particular, key contact residues in CDR H3 are conserved between Ab1 and Ab3 even though the genes were constructed with different D segments. This result suggests that Ab2-β did function as a molecular mimic of AII.

The fact that MAb 131 binds AII tightly yields considerable structural insight into the recognition characteristics of this idiotypic series. First, the conformation of AII bound to both antibodies is most likely "proteinlike"; that is, bound AII forms a compact structure (as opposed to an extended or open one) similar to the epitopes present on protein antigens. Such a structure is more amenable to mimicry by an Ab2 and would be more efficient in eliciting peptide-reactive Ab3s. Also, only a subset of peptide conformations can be represented in the framework of a large protein such as an Ab2-β. That MAb 131 is able to bind AII with high affinity reflects a selection—imposed at the level of the Ab2-β—for an Ab1 (MAb 110) that binds a member of this conformational subset. Second, the epitope on Ab2-β serving as the structural mimic of AII is probably a continuous polypeptide chain segment. This follows from the observation that antibodies to globular proteins are almost never able to bind smaller peptide fragments (*22*), primarily because antigenic determinants on large proteins are almost always discontinuous topographical surfaces (*23*) and therefore not easily represented by a short, continuous polypeptide chain. Yet MAb 131, in spite of being raised against a large globular protein, does bind a short peptide with high affinity. A structural motif of an antibody that could act as the molecular mimic of a bound peptide and that is both proteinlike and continuous might be a single CDR loop protruding from the Ab2-β combining site. In fact, when the conformation of the peptide bound in the Fab 131–AII complex is searched against a database of solved protein structures, the highest similarity was found to be with a loop of an antibody (0.8 Å root-mean-square distance between residues 90 to 96 of the REI light chain dimer and residues 2 to 8 of AII).

Remarkably, it was observed that Ab2-β was more efficient than AII (coupled to carrier) at eliciting high-affinity MAbs to AII (*24*), implying that an immunodominant conformation of the flexible peptide was "trapped" within the static framework of the Ab2-β. This drastic reduction in the flexibility of the peptide could be responsible for better binding to B cell receptors, resulting in enhanced immunogenicity. The study of anti-idiotypic mimicry of flexible peptide hormones may suggest ways to rigidify or scaffold analogs of their bioactive conformations to minimize conformational entropy and enhance binding.

Although raised in different individual mice and elicited against different antigens, MAbs 110 and 131 can be considered members of the same idiotypic network. In principle, these two antibodies, which have similar sequences and peptide-recognition properties, could have been produced as the result of the same immune response. Thus, during a response, if anti-idiotypic antibodies are made (*25*), some of the antibodies that recognize the antigen could actually have been produced against idiotypes of the anti-idiotypic antibodies and not against the antigen itself.

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26. The 131 hybridoma (*4*) secretes an All-reactive, Ab3 MAb (γ1, κ); the Ab1 hybridomas 110 and 133 (*3*) (both γ1,κ) were generated against keyhole limpet hemocyanin (KLH)–coupled All. Both were obtained by fusion of immune BALB/c spleen cells to the nonsecreting myeloma line NS-1. Immunoglobulin class and subclass were determined by Ouchterlony analysis of supernatants with commercial antibodies (Bionetics, Kensington, MD). Total RNA was prepared from 5 × 10^8 hybridoma cells by the guanidinum isothiocyanate–CsCl method (*27*) or by organic extraction (*28*). Polyadenylated RNA was purified by oligo(dT) cellulose chromatography (*29*). Minus-strand cDNA synthesis was carried out with Mo-MLV reverse transcriptase as described (*30*); primers were random hexanucleotides or the oligonucleotides V_H1FOR and V_K1FOR (*31*), modified to include Not I restriction sites. Amplification of V region sequences was carried out in a 20-μl reaction mixture containing 50 ng of the cDNA-RNA hybrid, 25 pmol of 3′ (V_H1FOR or V_K1FOR) and 5′ (V_H1BACK or V_K1BACK) primers, 200 μM each deoxynucleotide-5′-triphosphate, 67 mM tris-Cl (pH 8.8), 10 mM $MgCl_2$, and 2 units of Taq polymerase (Cetus). Light chain sequences were amplified in 30 cycles of 1 min at 95°C, 1 min at 30°C, and 2 min at 72°C. Heavy chain sequences were amplified in 30 cycles of 1 min at 95°C, 1 min at 30°C, and 2 min at 55°C. Amplified DNA was ligated into pBluescript (Stratagene) and nucleotide sequences were determined by the dideoxynucleotide chain termination method (*32*) with Sequenase (USB). The products of three independent amplification reactions were sequenced for each chain.
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33. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val, W, Trp; and Y, Tyr.
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Fusion of the Leucine Zipper Gene *HLF* to the *E2A* Gene in Human Acute B-Lineage Leukemia

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A t(17;19) chromosomal translocation in early B-lineage acute leukemia was shown to result in chimeric transcripts that contain sequences from the *E2A* basic helix-loop-helix transcription factor gene on chromosome 19, fused to sequences from a previously unidentified gene (*HLF*) on chromosome 17 that encodes a hepatic leukemia factor. The chimeric protein consisted of the amino-terminal transactivation domain of E2A linked to the carboxyl-terminal basic region-leucine zipper domain of HLF. HLF was normally expressed in liver and kidney, but not in lymphoid cells, and was found to be closely related to the leucine zipper–containing transcription factors DBP (albumin D-box binding protein) and TEF (thyrotroph embryonic factor), which regulate developmental stage–specific gene expression.

Molecular analysis of chromosomal translocations in human leukemic cells has led to the identification of new cellular proto-oncogenes that contribute to leukemogenesis (*1*). Genes encoding transcription factors that regulate cell growth and differentiation are frequent targets for such rearrangements (*2*). For example, the chromosomal translocation t(1;19)(q23;p13) in human pre-B cell acute lymphoblastic leukemia (ALL) fuses a basic helix-loop-helix (bHLH) gene (*E2A* or *E12/E47*) on chromosome 19 with a homeobox-containing gene (*PBX1*) on chromosome 1 (*3*). The protein product of the rearranged locus retains the NH_2-terminal activator domain of E2A, but its DNA-binding region is replaced by the homeobox domain of PBX1. Hybrid E2A-PBX1 proteins are expressed in the nucleus and induce malignant transformation when introduced into murine fibroblasts (*4*).

Other nonrandom chromosomal translocations occurring in leukemic cells at band p13 of chromosome 19 might also involve the *E2A* gene. Using an *E2A* cDNA probe, we analyzed the DNAs of t(17;19)(q22;p13)-positive leukemic lymphoblasts from two patients with early B cell precursor ALL and disseminated intravascular coagulation, which is rare in ALL cases lacking this translocation (*5*). Southern blot analysis revealed altered *E2A* restriction fragments in each of these patients, indicating rearrangement within the gene (*5*).

We prepared a genomic library with leukemic cell DNA from one of the patients (patient 1) and isolated two recombinant phages (LT1 and LT2) that contained altered restriction fragments surrounding the breakpoint within overlapping inserts that spanned 17 kb of genomic DNA (Fig. 1A). Probe A hybridized to *E2A* restriction fragments from chromosome 19, while probe B hybridized to human sequences in a mouse-human hybrid cell line that contained only human chromosome 17. Nucleotide sequencing revealed that *E2A* exons were oriented 5′ to 3′ relative to the breakpoint on chromosome 19, which occurred in the same intron of the *E2A* gene that contains the breakpoints of the 1;19 chromosomal translocation (*6*). Two cosmid clones, cosmids 17 and 19, isolated from a library prepared from unrearranged human DNA, were mapped by in situ hybridization with a fluorescent marker to chromosome bands 17q22 and 19p13, respectively (*7*).

We analyzed RNAs from a cell line

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