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Use of D gene segments with irregular spacers in terminal deoxynucleotidyltransferase (TdT)$^{+/+}$ and TdT$^{-/-}$ mice carrying a human Ig heavy chain transgenic minilocus

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ABSTRACT **D gene segments with irregular spacers (DIR) are D gene segments that are specific to higher primates. Their use is controversial because of their G+C-rich long sequences. In the human, it has always been tempting to assume that a complementarity-determining region 3 sequence has been added by terminal deoxynucleotidyltransferase (TdT) activity and is not derived from DIR recombination. Herein, we examine the use of human DIR gene segments by cross-breeding the human Ig heavy chain minilocus pHC1 transgenic mice and TdT-deficient mice. In the absence of TdT and with a defined set of human D gene segments, it is relatively easy to demonstrate that DIR2 is used to form human Ig heavy chains, contributing to 7% of the human heavy chain rearrangements. V_HDJ_H rearrangements (where H is heavy chain) in the minilocus TdT$^{-/-}$ mice use small portions of DIR2 located throughout the coding sequence. These results constitute the strongest evidence to date that DIR gene segments are used to form human antibodies. Additionally, we show that direct and inverted DIR2J_H and V_HDIR2 rearrangements occur in the minilocus transgenic mice. During these rearrangements, D_{M2} 3′ signal sequence and a new DIR2 5′ signal sequence are used. These rearrangements generally follow the 12/23 recombination rule. Our results at the V_HDJ_H, DJ_H, and V_HD levels indicate that DIR2 is used to form human heavy chains in transgenic mice. The rearrangement of this gene segment likely involves, however, other mechanisms in addition to the classical V_HDJ_H recombination.**

The variable region of Ig heavy chains (IgH) and T cell receptor β and δ chains derive from the combination of three separate DNA elements termed the variability (V), diversity (D), and junctional (J) gene segments through a cell-specific process termed V_HDJ_H recombination (where H is heavy chain). Typically, the recombination process is performed in two steps with D to J_H rearrangement preceding V_H to DJ_H rearrangement (1–3). The rearrangement is directed by recombination signal sequences (RSSs) flanking each coding gene segment. RSSs are formed of a consensus palindromic heptamer related to the sequence CACAGTG and a nonamer related to the sequence ACAAAAACC separated by 12 ± 1 or 23 ± 1 nucleotide spacers. D gene segments are flanked on both sides by 12 ± 1 spacer RSSs, whereas V_H and J_H gene segments are flanked by 23 ± 1 spacer RSSs at the 3′ and 5′ sides, respectively. Generally, recombination occurs between segments flanked by RSSs with different spacer lengths (12/23 rule) (4–7). During recombination, coding gene segments are joined in an imprecise manner to form the coding joint, whereas signal sequences are generally brought together without deletions or nucleotide additions to form the signal joint. Exceptions to the precise signal junction rule have, however, been recently described (8). The formation of coding joints is imprecise and includes nucleotide deletions as well as non-germ-line-encoded (N) and germ-line-encoded (P) nucleotide additions. N segments are added by the terminal deoxynucleotidyltransferase (TdT), whereas P (palindromic) nucleotides result from hairpin structures in cleavage intermediates (9–13). Hybrid junctions corresponding to recombination products in which a RSS is joined to a coding element have also been described (14).

To study the mechanisms of Ig V_HDJ_H recombination, we engineered mice transgenic for a human Ig heavy chain minilocus, pHC1 (15–22). One of the D gene segments present in the transgenic minilocus belongs to the *D* gene segments with *ir*regular spacers (DIR) family. These gene segments are specific to higher primates. In the human, DIR gene segments are adjacent to D_M gene segments. Segments from the DIR family are characterized by the presence of RSSs composed of several heptamers and nonamers separated by both 12 ± 1 and 23 ± 1 nucleotide spacers, in both direct and inverted orientation. Additionally, several cryptic heptamers have been described within DIR gene segment coding sequences. DIR gene segments are greater than 180 bp long and are G+C-rich (Fig. 1) (21, 23–25).

The use of DIR gene segments in human IgH remains controversial. All DIR genes segments may not have been identified. Because their sequences are G+C-rich, it is difficult to determine whether a sequence derives from a DIR gene segment or was added by TdT activity. Moreover, it is unclear how only short portions of DIR can be used to form the heavy-chain complementarity-determining region 3 (CDR3), the coding sequence of DIR gene segments being too long to be used in their entirety.

In sequencing the Ig V_H region from more than 200 transcripts and rearranged genes, we previously suggested that DIR2 is used in human IgH produced in the transgenic minilocus mice. The CDR3 of several V_HDJ_H rearrangements studied could be explained only if portions (6–11 bp) of DIR2 were used singly, in either direct or inverted orientation without D–D fusion (18, 19). These portions derive from different locations along the DIR coding sequence. In these mice, involvement of TdT in the formation of the junctions could not be ruled out.

Herein, we further analyze the mechanisms involved in DIR gene segment recombination. (*i*) To rule out the involvement of TdT, we analyzed DIR gene segment use in human Ig heavy chain minilocus transgenic mice on the TdT$^{-/-}$ background.

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Abbreviations: DIR, D gene segments with irregular spacers; TdT, terminal deoxynucleotidyltransferase; H (as subscript), heavy chain; IgH, Ig heavy chain; RSS, recombination signal sequence; CDR3, complementarity-determining region 3.

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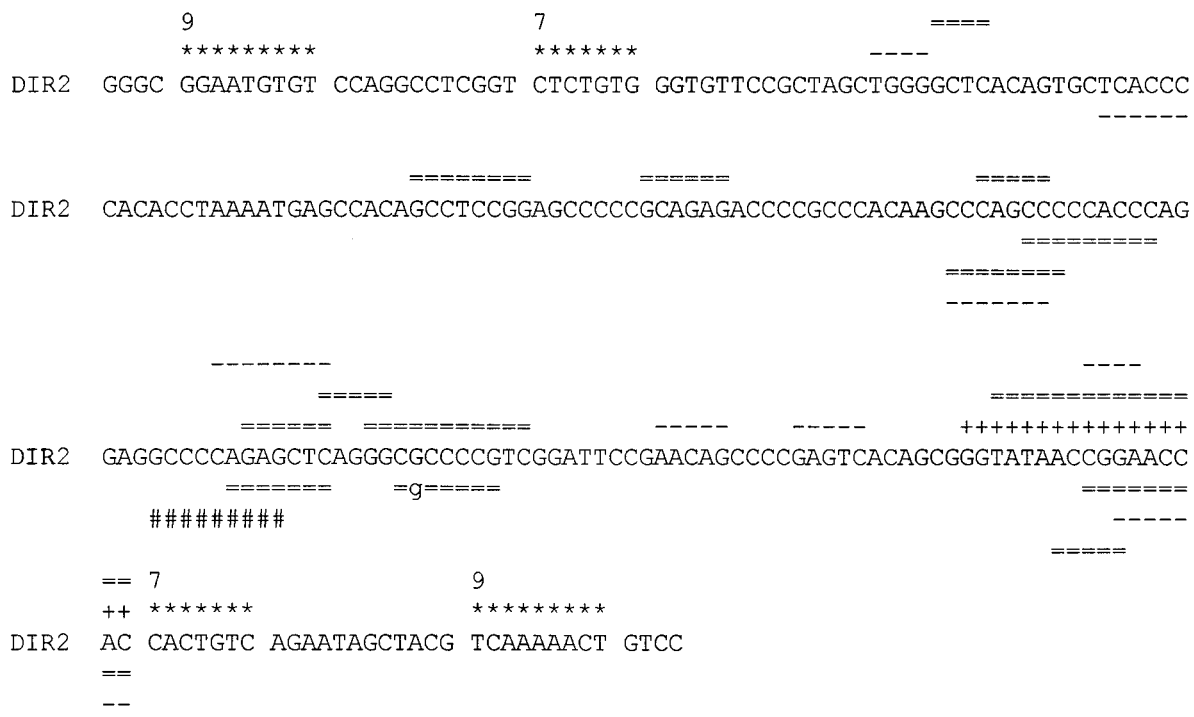

FIG. 1. DIR2 gene segment sequence. DIR2 179-nt coding region is flanked on both sides by two RSSs composed of heptamers and nonamers separated by 12-nt spacers. The location of the two most external RSSs is indicated. D_{M2} coding sequence is indicated by plus signs. Double lines and dashes indicate portions of DIR2 observed in V_HDJ_H rearrangements of pHC1 mice in the $TdT^{+/+}$ and $TdT^{-/-}$ backgrounds, respectively. The portion of DIR2 coding sequence used in the hybrid joint MI2 is indicated by pound signs.

We show that in these mice, human V_HDJ_H rearrangements are diverse and show little evidence of N nucleotides. Additionally, we show that 7% of the rearrangements use the human DIR2 gene segment. This is unequivocal evidence of DIR gene segment use to form human antibodies. (*ii*) We analyzed DIR gene segment use in the first steps of V_HDJ_H recombination, namely, DJ_H and V_HD rearrangements. We show that DIR2 is found in direct V_HD and DJ_H and in inverted DJ_H rearrangements. Our results at the V_HDJ_H, V_HD, and DJ_H levels clearly document that gene segments of the DIR family can be used in human Ig heavy chains. However, the data suggest the existence of additional mechanisms besides the classical V_HDJ_H recombination process. These mechanisms must involve the same recombination machinery as the classical V_HDJ_H recombination because DIR2 is rearranged in transgenic mice despite the absence of a murine DIR counterpart. The existence of DIR gene segments creates an additional level of diversity to the antibody repertoires of higher primates.

MATERIALS AND METHODS

DNA Construct and Mice. pHC1, the construct used to establish the transgenic founder line 119, is 60 kb long and has been described (16–20). $TdT^{-/-}$ mice have been described (11, 26). pHC1-transgenic $TdT^{-/-}$ mice were obtained by crossbreeding and selection by Southern blot filter hybridization. Briefly, tail DNA was cut with the *Bgl*II restriction enzyme and probed with a *Hin*dIII fragment covering the two human V_H gene segments to detect the pHC1 transgene and with an *Eco*RI–*Hin*dIII fragment covering TdT exons IV–VI (a gift from D. Mathis, Université Louis Pasteur, Strasbourg, France) to analyze the TdT locus. Probing with the TdT probe results in two bands of 6.5 and 1 kb in $TdT^{+/+}$ mice but results in two bands of 2.1 and 1.5 kb in $TdT^{-/-}$ mice.

V_HDJ_H, V_HD, and DJ_H PCR Amplifications. Genomic DNA was isolated from spleen, bone marrow, and liver of 2-month-old mice by phenol/chloroform extraction after proteinase K digestion. It was amplified by PCR as described (18–20). The amplification of 1–6 μg of DNA was performed with 40 cycles of 1-min denaturation (94°C), 2-min annealing (50°C to 54°C depending on the primer combination), and 2-min elongation (72°C) and used 1 unit of *Taq* polymerase. The final cycle was completed by a 7-min elongation at 72°C. V_HDJ_H amplifications were performed by using a V_H oligonucleotide complementary to both V_H5–251 and ψV_H3–105 gene segments (5′-AGGTGCAGCTGGTG(CG)AGTCTG-3′) and a human J_H consensus oligonucleotide (5′-ACCTGAGGAGACGGTGACCAGGGT-3′). Direct and inverted $DIRJ_H$ amplifications were performed by using an oligonucleotide complementary to the DIR2 5′ RSS (DIR2-J_H amplification, 5′-GCTGGGGCTCACAGTGC-3′) or to the DIR2 3′ RSS (inverted DIR2-J_H amplification, 5′-CGCTGTGACTCGGGGC-3′) and the J_H consensus oligonucleotide. Alternatively, D_{M2} 3′ RSS (5′-CAGTTTTTGACGTAGCTATTC-3′) was used to amplify inverted $DIRJ_H$ rearrangements. Direct and inverted V_HDIR amplifications were performed by using an oligonucleotide complementary to the DIR2 3′ RSS (V_HDIR amplification) or to the DIR2 5′ RSS (inverted V_HDIR amplification) and the V_H oligonucleotide. Ten picomoles of each primer was used as appropriate. The PCR products were purified by using Microcon 50 (Amicon) as described by the supplier.

Cloning and Sequencing of PCR Products. The purified PCR products were blunt-end-ligated into *Eco*RV-digested pBluescript KS^+ plasmids (Stratagene) and the ligation mixture was used to transform *Escherichia coli* (XL1-Blue) competent cells. The resulting colonies were screened with ^{32}P-labeled oligonucleotides corresponding to the DIR2 (5′-GCCACAGCCTCCGGAGCCCCCG-3′) or V_H (5′-TGTATTACTGTG(CT)GAGA-3′) gene segment coding sequences. Double-stranded DNA was prepared from positive colonies and sequenced with a Perkin–Elmer ABI Prism 377 automated DNA sequencer.

Southern Blot Filter Hybridization. For Southern blot filter hybridization, direct and inverted V_HDIR rearrangements were PCR-amplified as described above from 1 μg of splenic

or liver DNA or from 10 μg of bone marrow DNA, size-fractionated on 1% agarose gel, and transferred onto Hybond N^+ membrane (Amersham). Membranes were probed with an oligonucleotide specific for the DIR2 coding sequence by using Rapid Hyb (Amersham) as described by the supplier.

RESULTS

The Repertoire of Human V_HDJ_H Rearrangements from Minilocus Transgenic $TdT^{-/-}$ Mice Is Diverse and Shows Little Evidence of N Nucleotides. Eighty and 30 unique human V_HDJ_H rearrangements using the functional V_H5–251 and the ΨV_H3–105 gene segments, respectively, have been sequenced from five pHC1-transgenic $TdT^{-/-}$ mice (data not shown). The repertoire of human heavy chains produced in these mice is extremely diverse. Every human D gene segment present in the IgH minilocus is used with a preferential use of D_{HQ52} (28% of the rearrangements). Every human J_H gene segment is observed in V_HDJ_H rearrangements. J_H4 is preferentially used (34% of the rearrangements). Rearrangements from these mice are characterized by the low frequency of N regions. Only two sequences using conventional D gene segments (2%) show evidence of 1–2 additional nucleotides versus 66% of the rearrangements containing 1–10 additional nucleotides in $TdT^{+/+}$ minilocus transgenic mice. The frequency of P nucleotides is, however, similar in $TdT^{+/+}$ and $TdT^{-/-}$ mice. Fifty rearrangements (45% of the rearrangements containing a D gene segment) occurred at sites of short homology between the V_H and D gene segments. Thirty-nine rearrangements (35% of the rearrangements containing a D gene segment) occurred at sites of short homology between the D and J_H gene segments.

The DIR2 Gene Segment Is Used to Form Human Heavy Chains in Minilocus Transgenic $TdT^{-/-}$ Mice. Eight of the V_HDJ_H rearrangements (7%) obtained from $TdT^{-/-}$ mice cannot be explained solely by the conventional D gene segments present in the human heavy chain minilocus. These rearrangements can, however, be explained by invoking the use of short portions (5–8 nucleotides) of the DIR2 gene segment (Fig. 2). These short portions derive from various internal areas of the DIR2 coding sequence. As for rearrangements using conventional D gene segments, recombination using DIR2 often occurs at sites of short homology between gene segments. Four rearrangements occurred at sites of short homology between the V_H and DIR2 gene segments. Two rearrangements occurred at sites of short homology between the DIR2 and J_H gene segments. The frequency of additional

CLONE	VH5-251	PNP	DIR2	PNP	JH
	TGCGAGACA		TGTGGGTGT		
D82 (7)	-------	T	-----		TGGTA (JH2)
			CCGAGTCACA		
D28 (-)	---		===-=		TGGTA (JH2)
			CGAACAGCC		
D336(-)	--------		=----		CTGGT (JH5)
			inverted DIR2		
			GTGGGGTGAG		
D52 (4)	---------		---===		CTACT (JH4)
D360(-)	---------	C	-------	G	TACTT (JH4)
	ΨVH3-105		DIR2		
	TGTGAGAGG		CCAGAGCTCA		
D171(8)	-----		--------		TACTG (JH2)
			inverted DIR2		
			GGGCTGGGCT		
D200(-)	-------		=======	A	TGGTA (JH2)
			GTGGGCGGGG		
D342(-)	-------		====---	A	TTTGA (JH4)

FIG. 2. CDR3 of eight V_HDJ_H rearrangements using the DIR2 gene segment obtained from minilocus transgenic $TdT^{-/-}$ mice. The V_H gene segment 3′ end and part of DIR2 coding sequence are indicated. For each clone, homologies with V_H and DIR coding sequences are indicated by dashes. Double lines indicate nucleotides that can derive from either gene segment, suggesting recombination at sites of short homologies between gene segments. The J_H gene segment is indicated at the right of the figure. P and N nucleotides are indicated. The CDR3 length is indicated in parenthesis when the rearrangement is productive, whereas nonproductive rearrangements are indicated by a dash.

nucleotides at the gene segment junctions is higher for DIR2 rearrangement than for conventional D gene segment rearrangement. Two DIR2 rearrangements contain one additional nucleotide at the V_HD junction and three DIR2 rearrangements contain one additional nucleotide at the DJ_H junction.

The DIR2 Gene Segment Is Used in DJ_H Rearrangements from Human Ig Heavy Chain Transgenic Mice. Twenty-five $DIR2J_H$ rearrangements have been sequenced from human Ig heavy chain minilocus transgenic $TdT^{+/+}$ mouse spleens (Fig. 3). Most of the rearrangements (92%) use the J_H4 gene segment.

Ten of the $DIR2J_H$ rearrangements result from deletional recombination (Fig. 3*A*). Most of these rearrangements occur at the J_H RSS and at the D_{M2} 3′ RSS. No evidence of rearrangements within the DIR2 coding sequence was found. Because DIR2 and D_{M2} gene segments are adjacent in the genomic DNA, 9 of the $DIR2J_H$ rearrangements cannot be differentiated from $D_{M2}J_H$ rearrangements. During these rearrangements, 0–3 nucleotides of the D_{M2} coding end were removed by exonuclease activity and 0–9 nucleotides were added at the coding joint. Only 1 rearrangement (D13) does not include the D_{M2} coding sequence. This rearrangement occurred either through D_{M2} 3′ RSS with 19 nucleotides removed by exonuclease activity or through D_{M2} 5′ RSS. If D_{M2} 5′ RSS was used, the D13 rearrangement corresponds to a hybrid joint of D_{M2} 5′ RSS and J_H4 coding sequence. In this hybrid joint, two nucleotides were deleted from the signal sequence by exonuclease activity.

Fifteen rearrangements result from inversional recombination (Fig. 3*B*). Rearrangements by inversion occur at the J_H RSS and at the previously described 5′ RSS or at a new DIR2 5′ RSS, located more upstream. Twelve rearrangements occur at the J_H RSS and at the new DIR2 5′ RSS. During these rearrangements, 0–16 nucleotides were removed at DIR2 coding end by exonuclease activity and 0–12 nucleotides were added at the coding joint. Two rearrangements (DI12 and DI10) occurred either through the new 5′ RSS with 20 or 21 nucleotides deleted by exonuclease activity or more likely through the previously described 5′ RSS. This RSS is in inverted orientation relative to the DIR2 coding sequence. Therefore, if this RSS was used, the two rearrangements correspond to hybrid joints in which DIR2 5′ inverted RSS is rearranged to J_H4 coding sequence. Additionally, one inverted $DIR2J_H$ rearrangement occurred within the DIR2 coding sequence (sequence DI3). In rearrangement DI3, 73, 52, or 45 nucleotides were removed if the new 5′ RSS, the previously described inverted 5′ RSS, or the previously described direct 5′ RSS was used, respectively. Alternatively, this rearrangement may have occurred directly within the coding sequence or may have been mediated by DIR2 internal cryptic heptamers in a nonamer independent fashion. However, no clear evidence of a cryptic heptamer near this region can be found. Cryptic heptamers may have, however, been involved in the formation of the rearrangement MI2 (Fig. 3*C*). This DJ_H rearrangement was obtained by PCR amplification using an oligonucleotide specific for D_{M2} 3′ RSS. It contains D_{M2} 3′ RSS and J_H4 coding sequence separated by 9 nucleotides derived from an internal portion of DIR2. It is noteworthy that several cryptic heptamers can be detected at the 5′ side of the DIR2 portion used. These cryptic heptamers may have mediated the formation of the hybrid joint between the DIR2 coding sequence and the D_{M2} 3′ RSS. In this rearrangement, the DIR2 portion used is located 107 nucleotides 3′ of the DIR2 new 5′ RSS and 61 nucleotides 5′ of D_{M2} 3′ RSS.

The DIR2 Gene Segment Is Used in V_HD Rearrangements from Human Ig Heavy Chain Transgenic Mice. Eleven V_H-DIR rearrangements have been sequenced from human Ig heavy chain minilocus transgenic mouse spleens (Fig. 4). All of the rearrangements use the functional V_H5–251 gene segment and result from deletional recombination. These rearrange-

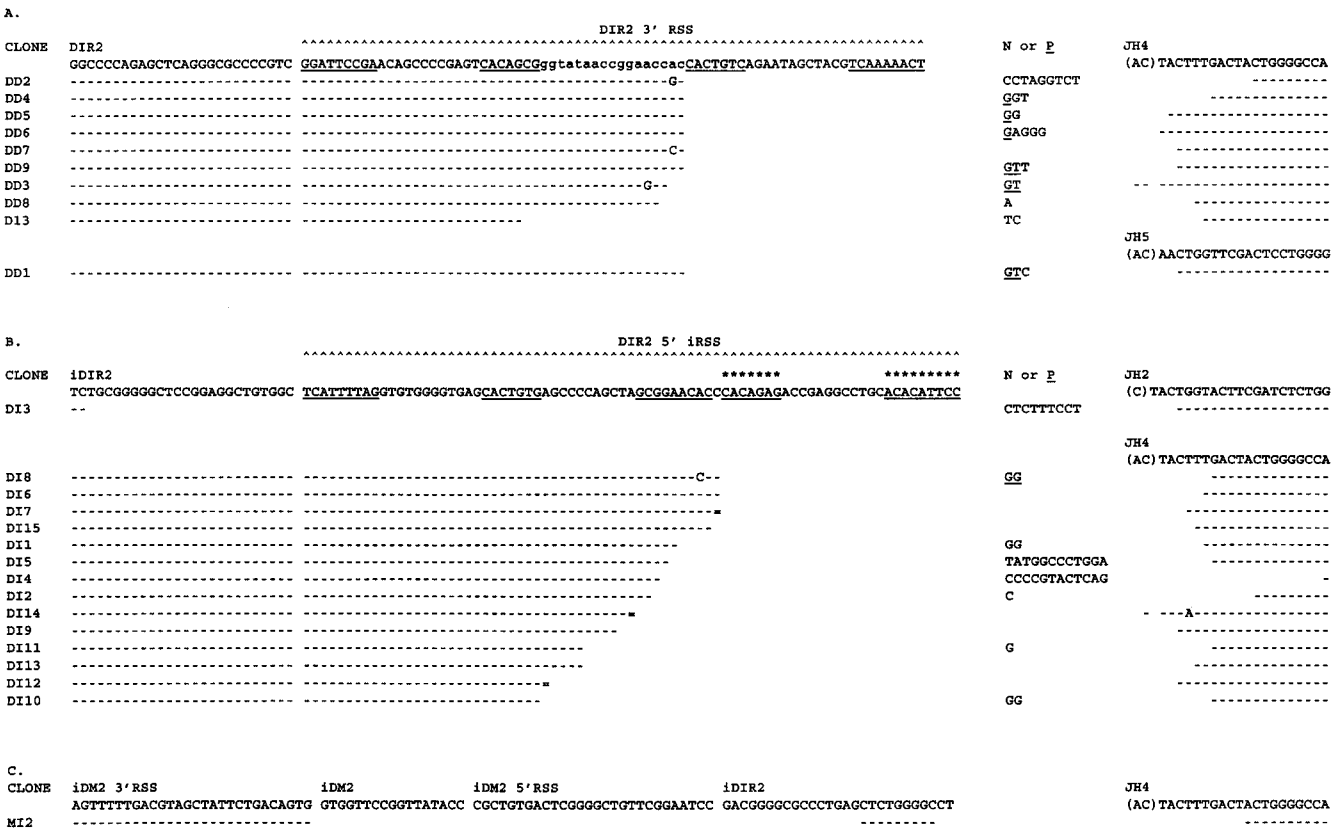

FIG. 3. Junctional sequence of direct and inverted DIR2J_H rearrangements obtained from minilocus transgenic mice. Germ-line DIR2 RSS, DIR, and J_H coding sequences are indicated in uppercase type at the top. D_{M2} coding sequence is indicated in lowercase type. Heptamers and nonamers as described (23) are underlined. The heptamer and nonamer of the new 5′ RSS determined from this study are indicated by stars. iDIR, iDM2, and iRSS indicate inverted DIR, D_{M2}, and RSS sequences. For each clone, homologies with V_H and DIR coding sequences are indicated by dashes. Double lines indicate nucleotides that can derive from either gene segment. Mutations are indicated in uppercase type in each rearrangement. N and P nucleotides are indicated. (*A*) Nucleotide sequence of 10 DIRJ_H rearrangements. (*B*) Nucleotide sequence of 15 inverted DIRJ_H rearrangements. (*C*) Nucleotide sequence of one hybrid joint involving D_{M2} 3′ RSS, DIR2 coding sequence, and J_H4.

ments occur at the V_H5–251 RSS and at the DIR2 new 5′ RSS. None of the rearrangements occur within the DIR2 coding sequence. No inversional rearrangements were obtained by sequencing. Inverted V_HDIR rearrangements are also not detected by PCR followed by Southern blot filter hybridization, whereas deletional V_HDIR rearrangements were easily detected in the murine spleen and bone marrow by this method (Fig. 5).

DISCUSSION

The use of DIR gene segments in human IgH has long been a subject of controversy. The first point of debate relates to whether or not DIR gene segments are used at all. All D gene segments may not be known. Additionally, because DIR sequences are G+C-rich, it is difficult to determine whether a transcript sequence derives from a DIR gene segment or was added by TdT activity. The second point of contention relates to the mechanisms by which DIR gene segments are used. Indeed, it is unclear how only short portions of DIR are used to form the heavy chain CDR3 because the coding sequence of DIR gene segments is too long to be used in its entirety.

A few human V_HDJ_H sequences have previously been reported that could derive from a DIR gene segment. These rearrangements use up to nine nucleotides of one of the six DIR gene segments previously reported (24, 25, 27–31). Additionally, one sequence was reported that contained 35 nucleotides from DIR (27). It has been suggested that DIR gene segments may contribute to up to 12% of the human IgH repertoire (24). It is, however, intriguing that in the human, CDR3s show large discrepancies compared with DIR germline sequences. Sequence alignment often requires the introduction of mismatches and gaps, even in situations where somatic mutation is minimal. These disparities are probably

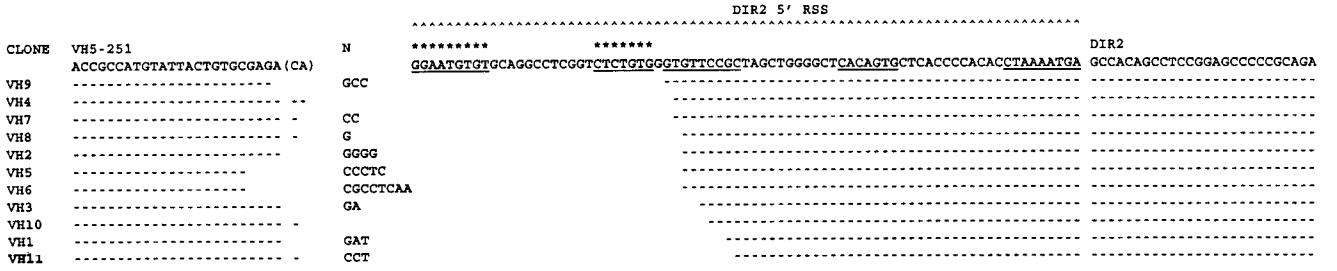

FIG. 4. Junctional sequence of 11 V_HDIR2 rearrangements obtained from minilocus transgenic mice. Sequences are indicated as in Fig. 3.

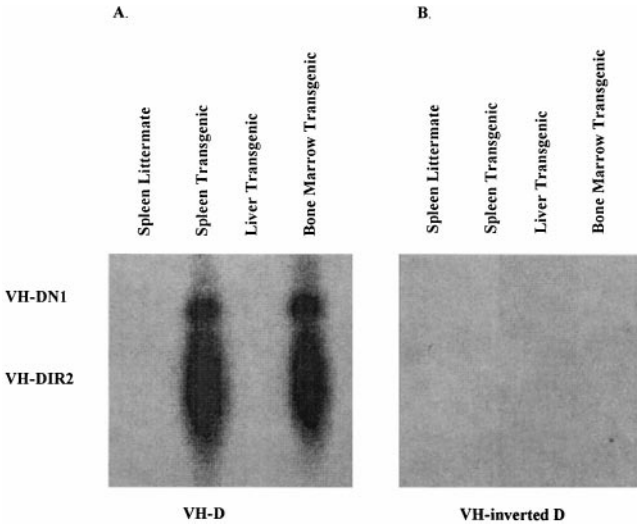

FIG. 5. PCR amplification and Southern blot filter hybridization of direct and inverted V_HDIR2 rearrangements. (*A*) Direct V_HDIR rearrangements were PCR-amplified from 1 μg of splenic or liver DNA or from 10 μg of bone marrow DNA of transgenic or littermate $TdT^{+/+}$ mice and probed with a DIR2-coding-sequence-specific oligonucleotide. The positions of V_HDIR2 and V_HDN1 rearrangements are indicated. (*B*) Inverted V_HDIR rearrangements were PCR-amplified from 1 μg of splenic or liver DNA or from 10 μg of bone marrow DNA of transgenic or littermate, $TdT^{+/+}$ mice and probed with a DIR2-coding-sequence-specific oligonucleotide.

not caused by DIR polymorphism (24). An alternative explanation for these sequences involves TdT activity. An additional possibility is that rearrangements use unknown conventional D gene segments containing DIR-like coding sequences.

Human IgH minilocus transgenic mice constitute an ideal model to study DIR gene segment use in human antibodies. All D gene segments present in the minilocus are well characterized. In the absence of immunization, the human heavy chain repertoire is extremely diverse and contains virtually no evidence of somatic mutation. In the minilocus transgenic mice, endogenous heavy chain gene segment recombination occurs as well. Additionally, in founder line 119, a line we have studied extensively, xenotypic exclusion does not occur. Under these circumstances, selection is more likely to occur on the endogenous murine genes. The nonselected repertoire can also be analyzed by studying the repertoire associated to the ΨV_H3–105 gene segment (18–22).

In minilocus transgenic mice, 90% of the human V_HDJ_H rearrangements can be explained by invoking the use of one of the nine conventional D gene segments present in the minilocus D region. The remaining 10% can be explained by the DIR2 gene segment. In minilocus transgenic $TdT^{-/-}$ mice, 93% of the human V_HDJ_H rearrangements can be explained by conventional D gene segments. The remaining 7% can be explained by the DIR2 gene segment. In transgenic $TdT^{-/-}$ mice, N nucleotides are rare in rearrangements using conventional D gene segments, similar to what was shown for murine junctions, as well as in cell lines lacking TdT activity (11, 12, 32, 33). The finding that additional nucleotides are more frequently found in rearrangements using the DIR2 gene segment is in agreement with data indicating that in the human, CDR3 shows large discrepancies compared with DIR germ-line sequences even in situations where somatic mutation is minimal. Our data indicate that these nucleotides are independent of TdT activity and are probably the result of unconventional mechanisms of rearrangement associated with DIR gene segment use. A recent paper suggests that DIR gene segments are not required to explain human IgH rearrangements (34). Herein, we find that 100% of the human heavy chain rearrangements from pHC1 mice can be explained only if the DIR2 gene segment is invoked. The portions of DIR used are located throughout the coding sequence but several hot spots can be observed (Fig. 1). One hot spot corresponds to the previously described D_{M2} sequence. It is noteworthy that except for the D_{M2} region flanked by two RSSs, no cryptic heptamer can be detected directly flanking the hot spots.

The portions of the DIR2 coding sequence used in V_HDJ_H rearrangements are located up to 100 nucleotides away from the described RSS. This relates to the second controversial point concerning DIR gene segments: What are the mechanisms involved in DIR gene segment rearrangement? Several nonmutually exclusive hypotheses can be invoked to explain DIR gene segment rearrangement: (*i*) the TdT is responsible for the sequences, (*ii*) the sequences derive from unknown gene segments containing DIR-like coding region, (*iii*) DIR recombination is accompanied by extensive exonuclease activity, (*iv*) DIR rearrangement occurs through internal cryptic heptamers, (*v*) DIR gene segment rearrangement occurs at the RSS and is followed by secondary events resulting in the deletion of internal portions of the DIR coding sequence, and (*vi*) DIR gene segments are used through gene conversion resulting in internal portions of DIR coding sequences under the control of conventional D RSS. Alternatively, this could be the result of the formation of hybrid joints involving the DIR coding sequence and conventional D gene segment RSS. Our results in $TdT^{-/-}$ mice rule out possibility *i*. Additionally, every gene segment present in the minilocus is known and every D region that does not derive from a conventional D gene segment can be found in the germ-line DIR2 coding sequence, ruling out possibility *ii*. Exonuclease activity appears to be normal in the $DIRJ_H$ and V_HDIR rearrangements, suggesting that hypothesis *iii* may not be the only explanation.

Rearrangements at the $DIRJ_H$ and V_HDIR levels occur usually at two main RSSs: D_{M2} 3′ RSS and a new 5′ RSS. It is noteworthy that we have been unable to demonstrate the use of the previously described DIR2 RSS by looking for the signal joints involving these RSSs (data not shown). The use of the two most external RSSs suggests that hypothesis *iv* in which only internal cryptic heptamers would be used is not the sole mechanism involved in DIR recombination. Only sequences DI3 and MI2 may result from this mechanism. It is noteworthy that $DIRJ_H$ and V_HDIR recombination follows the 12/23 rule. The two RSSs define a 179-nt coding region. This coding region covers the previously described D_{M2} gene segment. The incorporation of the D_{M2} gene segment within DIR2 gene segment is also suggested by the fact that we did not find evidence of D_{M2} 3′ RSS use to form inverted D_{M2} rearrangement. Our results indicate that DIR gene segment recombination requires additional mechanisms besides the classical V_HDJ_H recombination mechanisms. DIR gene segment recombination may be a two-step process in which the first step corresponds to a classical rearrangement through the 5′ or 3′ RSS. This is followed by secondary recombination resulting in the deletion of internal portions of the DIR coding sequence. These secondary recombinations apparently do not require special recombinase machinery because mice rearrange DIR despite the absence of such gene segments in normal mice. Secondary recombination may be directed by the cryptic heptamers located throughout DIR coding sequences. Sequences DI3 and MI2 may be the result of such secondary recombinations. Alternatively, DIR gene segment rearrangement may involve gene conversion mechanisms in which the DIR coding sequence is put under the control of conventional RSSs. However, to date we have not been able to detect evidence for gene conversion in pHC1-transgenic mice. We are currently testing the two possibilities (secondary recombination and gene conversion) with new transgenic miniloci. DIR gene segment recombination may also involve the formation of hybrid joints. Several V_HD and DJ_H rearrangements

and more particularly the MI2 rearrangement observed in the minilocus transgenic mice suggest that DIR2 may be involved in such hybrid joints more frequently than conventional D gene segments.

DIR gene segments are specific to higher primates, thus appearing relatively recently in evolution. This study unequivocally shows that these gene segments can be used to form Ig heavy chains. The DIR2 gene segment must be invoked to explain all of the human rearrangements in the transgenic mice even in the absence of TdT. Because of the coding region length, some form of secondary recombination is likely required to explain the use of these gene segments. Several nonmutually exclusive mechanisms that do not require new recombinase machinery can be invoked. The use of DIR gene segments results in proline-, alanine-, and glycine-rich CDR3. Antibodies containing such IgH may have advantages in responses against certain antigens. DIR gene segments add an important level of diversity to the Ig heavy chain repertoires of higher primates.

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1. Max, E. E. (1993) in *Fundamental Immunology*, ed. Paul, W. E. (Raven, New York), 3rd Ed., pp. 315–382.
2. Hedrick, S. M. & Eidelman, F. J. (1993) in *Fundamental Immunology*, ed. Paul, W. E. (Raven, New York), 3rd Ed., pp. 383–420.
3. Alt, F. W., Yancopoulos, G. D., Blackwell, T. K., Wood, C., Thomas, E., Coffman, R., Tonegawa, S. & Baltimore, D. (1984) *EMBO J.* **3,** 1209–1219.
4. Early, P., Huang, H., Davis, M., Calame, K. & Hood, L. (1980) *Cell* **19,** 981–992.
5. Sakano, H., Maki, R., Kurosawa, Y., Roeder, W. & Tonegawa, S. (1980) *Nature (London)* **286,** 676–683.
6. Hesse, J. E., Lieber, M. R., Mizuuchi, K. & Gellert, M. (1989) *Genes Dev.* **3,** 1053–1061.
7. Eastman, Q. M., Leu, T. M. J. & Schatz, D. G. (1996) *Nature (London)* **380,** 85–88.
8. Candeias, S., Muegge, K. & Durum, S. K. (1996) *J. Exp. Med.* **184,** 1919–1926.
9. Landau, R., Schatz, D. G., Rosa, M. & Baltimore, D. (1987) *Mol. Cell. Biol.* **7,** 3237–3243.
10. Lafaille, J. J., DeCloux, A., Bonneville, M., Takagaki, Y. & Tonegawa, S. (1989) *Cell* **59,** 859–870.
11. Gilfillan, S., Dierich, A., Lemeur, M., Benoist, C. & Mathis, D. (1993) *Science* **261,** 1175–1178.
12. Komori, T., Okada, A., Stewart, V. & Alt, F. W. (1993) *Science* **261,** 1171–1174.
13. Meier, J. T. & Lewis, S. M. (1993) *Mol. Cell. Biol.* **13,** 1078–1092.
14. Lewis, S., Hesse, J. E., Mizuuchi, K. & Gellert, M. (1988) *Cell* **55,** 1099–1107.
15. Lonberg, N., Taylor, L. D., Harding, F. A., Troutsine, M., Higgins, K. M. *et al.* (1994) *Nature (London)* **268,** 856–859.
16. Taylor, L. D., Carmack, C. E., Schramm, S. R., Mashayekh, R., Higgins, K. M., Kuo, C. C., Woodhouse, C., Kay, R. M. & Lonberg, N. (1992) *Nucleic Acids Res.* **20,** 6287–6295.
17. Taylor, L. D., Carmack, C. E., Huszar, D., Higgins, K. M., Mashayekh, R. *et al.* (1994) *Int. Immunol.* **6,** 579–591.
18. Tuaillon, N., Taylor, L. D., Lonberg, N., Tucker, P. W. & Capra, J. D. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3720–3724.
19. Tuaillon, N., Miller, A. B., Lonberg, N., Tucker, P. W. & Capra, J. D. (1994) *J. Immunol.* **152,** 2912–2920.
20. Tuaillon, N., Miller, A. M., Tucker, P. W. & Capra, J. D. (1995) *J. Immunol.* **154,** 6453–6465.
21. Tuaillon, N., Tucker, P. W. & Capra, J. D. (1995) *The Immunologist* **3,** 269–274.
22. Tuaillon, N. & Capra, J. D. (1995) in *The Antibodies*, eds. Capra, J. D. & Zanetti, M. (Gordon & Breach, New York), pp. 1–39.
23. Ichihara, Y., Matsuoka, H. & Kurosawa, Y. (1988) *EMBO J.* **7,** 4141–4150.
24. Sanz, I., Wang, S. S., Meneses, G. & Fishbach, M. (1994) *J. Immunol.* **152,** 3958–3969.
25. Moore, B. B. & Meek, K. (1995) *J. Immunol.* **154,** 2175–2187.
26. Gilfillan, S., Bachmann, M., Trembleau, S., Adorini, L., Kalinke, U., Zinkernagel, R., Benoist, C. & Mathis, D. (1995) *Eur. J. Immunol.* **25,** 3115–3122.
27. Sanz, I. (1991) *J. Immunol.* **147,** 1720–1729.
28. Yamada, M., Wasserman, R., Reichard, B. A., Shane, S., Caton, A. J. & Rovera, G. (1991) *J. Exp. Med.* **173,** 395–407.
29. Raaphorst, F. M., Timmers, E., Kenter, M. J. H., Van Tol, M. J. D., Vossen, J. M. & Schuurman, R. K. B. (1992) *Eur. J. Immunol.* **22,** 247–251.
30. Shin, E. K., Matsuda, F., Fujikura, J., Akamizu, T., Sugawa, H., Mori, T. & Honjo, T. (1993) *Eur. J. Immunol.* **23,** 2365–2367.
31. Minegishi, Y., Akagi, K., Nishikawa, K., Okawa, H. & Yata, J. (1996) *J. Immunol.* **156,** 4666–4671.
32. Komori, T., Pricop, L., Hatakeyama, A., Bona, C. A. & Alt, F. W. (1996) *Int. Rev. Immunol.* **13,** 317–325.
33. Gauss, H. G. & Lieber, M. R. (1996) *Mol. Cell. Biol.* **16,** 258–269.
34. Corbett, S. J., Tomlinson, I. M., Sonnhammer, E., Buck, D. & Winter, G. (1997) *J. Mol. Biol.* **270,** 587–597.