N Region Diversity of a Transgenic Substrate in Fetal and Adult Lymphoid Cells

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Summary

The rearrangement of immunoglobulin (Ig) and T cell receptor (TCR) genes requires the activity of an as yet undefined V(D)J recombinase. One component of the recombinase appears to be a terminal transferase which may be involved in the addition of untemplated nucleotides (N regions) to the V(D)J joints. It has been observed that rearranged Ig and TCR genes isolated from fetal liver have few if any N regions, whereas in the adult mouse, these genes have a large number of untemplated nucleotides. The presence of N regions greatly alters the composition of the third hypervariable, complementarity determining region of the respective proteins, thus playing a major role in the conformation of the binding site. It was possible that, for functional reasons, N region-containing Ig and TCR genes were not permissible at the fetal stage of development. We have produced transgenic mice with a rearrangement test gene which, after V-J recombination, does not result in the production of functional Ig or TCR proteins. We report here that the rearrangement products have no N regions in fetal liver, but that the majority of joints in adult lymphoid tissues have N additions. The study is also an interesting demonstration of the randomness of rearrangements and the enormous variability that can be created from a single pair of V and J sequences.

The generation of diversity of antigen receptors proceeds in two phases. First, the genetic elements encoding the V regions of the receptor molecules are assembled in the absence of selective pressures. The final repertoire is then shaped by a variety of selective forces that act upon cells expressing different receptor molecules.

In the first phase, V, (D), and J segments are brought together by a lymphoid-specific recombinase complex whose components and mechanism of action are only beginning to be understood (for review, see reference 1). The joining of these segments is known to be imprecise. Small deletions of coding regions are very common. These deletions are often coupled with addition of nontemplated nucleotides (N regions) particularly in IgH genes. It has been postulated that N region addition is carried out by terminal deoxynucleotidyltransferase (TdT)[1], a possibility strengthened by the finding that N region addition is increased in pre-B cells that have been transfected with a TdT expression construct (2). Although probably not entirely random, this assembly of V, (D), and J segments seems to occur in the absence of selection upon the encoded proteins.

Once a functional rearrangement has occurred, B cells are selected to express a restricted repertoire (3, 4). The selective forces and the mode of their action are complex and not completely understood.

To address the question of how junctional diversity differs during the lifetime of an animal, a variety of Ig and TCR loci have been examined in fetal, newborn, and adult mice (5–8). The general finding is that in fetal and newborn mice there is a lack of N regions and that these sequences only become common in the adult. These results have been interpreted as being compatible with the role of TdT in N region addition, as it is known that TdT only begins to be expressed around day 17 of gestation (9). However, an alternative interpretation is, that either positive or negative selection of cells expressing rearranged genes could account for the observed bias in N region frequency. To avoid this problem of selective pressures acting on the products encoded by the rearranged genes we have examined the sequences of V-J junctions in a transgene that contains only very minimal Ig sequences and so is unable to encode a functional antigen receptor.

The transgene, pHRD, is an artificial rearrangement indicator comprised of multiple elements (10, and see Fig. 1). In addition to the IgH intronic enhancer, the only Ig sequences are 53 nucleotides derived from the 3′ end of the Vκ region used by MOPC167 (20 of these are the most 3′ V-coding sequence) and 122 nucleotides around the 5′ end of Jκ1 (in-

[1] *Abbreviations used in this paper:* gpt, *Escherichia coli* xanthine-guanine phosphoribosyl transferase; TdT, terminal deoxynucleotidyltransferase.

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cluding the nucleotides encoding positions 96–108). The remainder of the sequences are derived from the mouse metallothionein promoter, rat preproinsulin, *Escherichia coli* xanthine-guanine phosphoribosyl transferase (gpt), and viral splicing and polyadenylation signals from SV40. Thus this transgene is incapable of encoding a functional Ig molecule.

We have previously described pHRD transgenic mice and have shown that the methylation of the transgene is controlled by a single dominant gene, *Ssm-1* (strain-specific modifier), that is expressed in certain strains (e.g., C57BL/6), but not in others (e.g., SJL and DBA/2) (11). When the transgene is carried in a methylating strain it is heavily methylated and shows no evidence of rearrangement. However, when bred into a nonmethylating strain, the transgene becomes progressively less methylated and rearrangement can be easily detected by Southern blot analysis in lymphoid tissues (spleen, LNs, and bone marrow), but not in nonlymphoid organs (brain, kidney, and adult liver) (11, 12). To look at the V-J junctions in these animals, we specifically amplified these sequences using a transgene-specific primer pair, cloned the resulting products into plasmids, and determined their DNA sequences. The results indicate that V-J junctions of a nonselected transgene from day 15.5 fetal liver lack N regions, whereas 74% of adult junctions have nontemplated additions.

Materials and Methods

Mice. Transgenic mice of line 342 with seven copies of the pHRD rearrangement indicator have been described (11). Mouse 342-2-20 (7-mo-old) was produced by mating the female founder with a (C57BL/6J × SJL/J)F_1. This mouse had a partially methylated transgene array and is referred to as II.2 (generation II, mouse 2) in the simplified system of reference 11. Unmethylated transgenic mouse 2967 (13-mo-old) was derived by breeding into DBA/2J for three generations and has been referred to as IX.35. Mouse 3295 (4-mo-old), with an almost completely methylated transgene array, had been bred into DBA/2J for two generations and has been referred to as VIII.66. Mouse 2562 (2.5-mo-old), with a partially methylated array, was derived from the same transgenic parent as 3295 and is labeled VIII.40. Fetal liver was from a single 15.5-d embryo from unmethylated male transgenic mouse XI.2 bred with a C3H/HeSnJ (a nonmethylating strain [11]) female.

Cloning and Sequencing V-J Rearrangements. DNA was prepared from adult spleen, LNs, bone marrow, or fetal liver by overnight digestion with proteinase K (Boehringer Mannheim Corp., Indianapolis, IN), extraction with phenol and chloroform, and ethanol precipitation. Two different methods were used to isolate fragments containing V-J junctions.

DNA from various organs of mouse 342-2-20 was subjected to 30 cycles of PCR using unphosphorylated primers complementary to sequences in the metallothionein promoter (CCTCACTTACTCCGTAGCTCC) and in gpt (CGCTCATGTGAAGTGTCCCAG). The amplified products were cut with BamHI (New England Biolabs, Beverly, MA), which cuts just upstream of the gpt primer, and fragments of ~0.1 kb were isolated from an agarose gel using hydroxyapatite (Bio-Rad Laboratories, Richmond, CA). These fragments were ligated into the polylinker of a modified pUC vector that had been cut with BglII and SmaI (New England Biolabs) and gel purified. No color selection was used to avoid any possibility of selection at this step.

DNAs from the other mice were first cleaved with PstI (New England Biolabs), which cuts in the portion of the transgene deleted during rearrangement, to favor amplification of rearranged copies. Thirty cycles of PCR were carried out using phosphorylated primers and fragments of appropriate size were isolated as above and cloned into EcoRV (New England Biolabs) -cut pBluescript KS+ (Stratagene Inc., La Jolla, CA) which had been treated with phosphatase (Boehringer Mannheim). Again no color selection was used.

Double-stranded plasmid DNA prepared by the alkaline lysis method was used as template for sequence determination using modified T7 polymerase (Sequenase; US Biochemical, Cleveland, OH) and primers complementary to lacZ, T3 promoter, or T7 promoter sequences.

Results

Analysis of pHRD Rearrangement Joints in Fetal Liver. The DNA sequences from 10 joints of pHRD isolated from fetal liver showed that rearrangement has occurred correctly with no or moderate deletions (maximally 10 nucleotides) into the V or J coding regions (Fig. 2 *A*). No N regions were found. This is in sharp contrast to the high frequency of N regions in adult joints (see below). Most of the V and J ends show evidence of a short inverted repeat of CG (nos. 1–3, and 6–10). Two cases are compatible with short direct repeats which may have given rise to homologous recombination (nos. 5, GT; and 6, TC). Sequences 1 and 2 and sequences 8–10, respectively, were identical. Since we are unable to rule out the possibility that these repeated sequences arose from amplification of the same B cell clone, they have been omitted from our statistical analysis. However, these sequences and no. 7 recurred in independent amplifications from different organs of adults, often from different mice. These repeated sequences are marked in Fig. 2 with geometric symbols.

Rearrangement in Adult Lymphoid Tissues. Correctly rearranged joints were found in the spleen of three adults and the spleen, bone marrow, and LNs of an additional adult pHRD mouse (Fig. 2, *B–G*). There was only one sequence (no. 58, 59) that was isolated twice from a single organ. This duplicated sequence was not used in the statistical analysis. Transgene rearrangement is apparently restricted to B cells as we have found no evidence of rearrangement in carefully dissected thymus or in LN T cells that had been expanded in vitro in the presence of anti-CD3 antibodies (12). 50 of

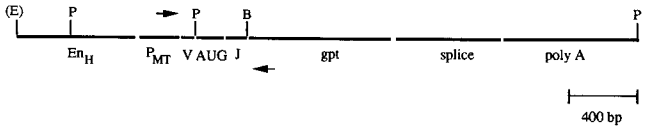

Figure 1. Map of the rearrangement test gene pHRD. The transgene, pHRD (10, 11) contains the mouse Ig H chain intron enhancer (En_H), the mouse metallothionein-1 promoter (P_{MT}), a portion of Vκ including the RSS (*V*), a portion of the rat preproinsulin gene including the translation initiation region (*AUG*), part of Jκ1 including the RSS (*J*), the *E. coli* xanthine-gpt gene, and the SV40 splicing (*splice*) and polyadenylation (*poly A*) signals. (*Arrows*) Primers for PCR (not to scale). Restriction sites are indicated as (*E*) EcoRI (eliminated in the transgene), (*B*) BamHI, and (*P*) PstI. Rearrangement deletes the middle PstI site.

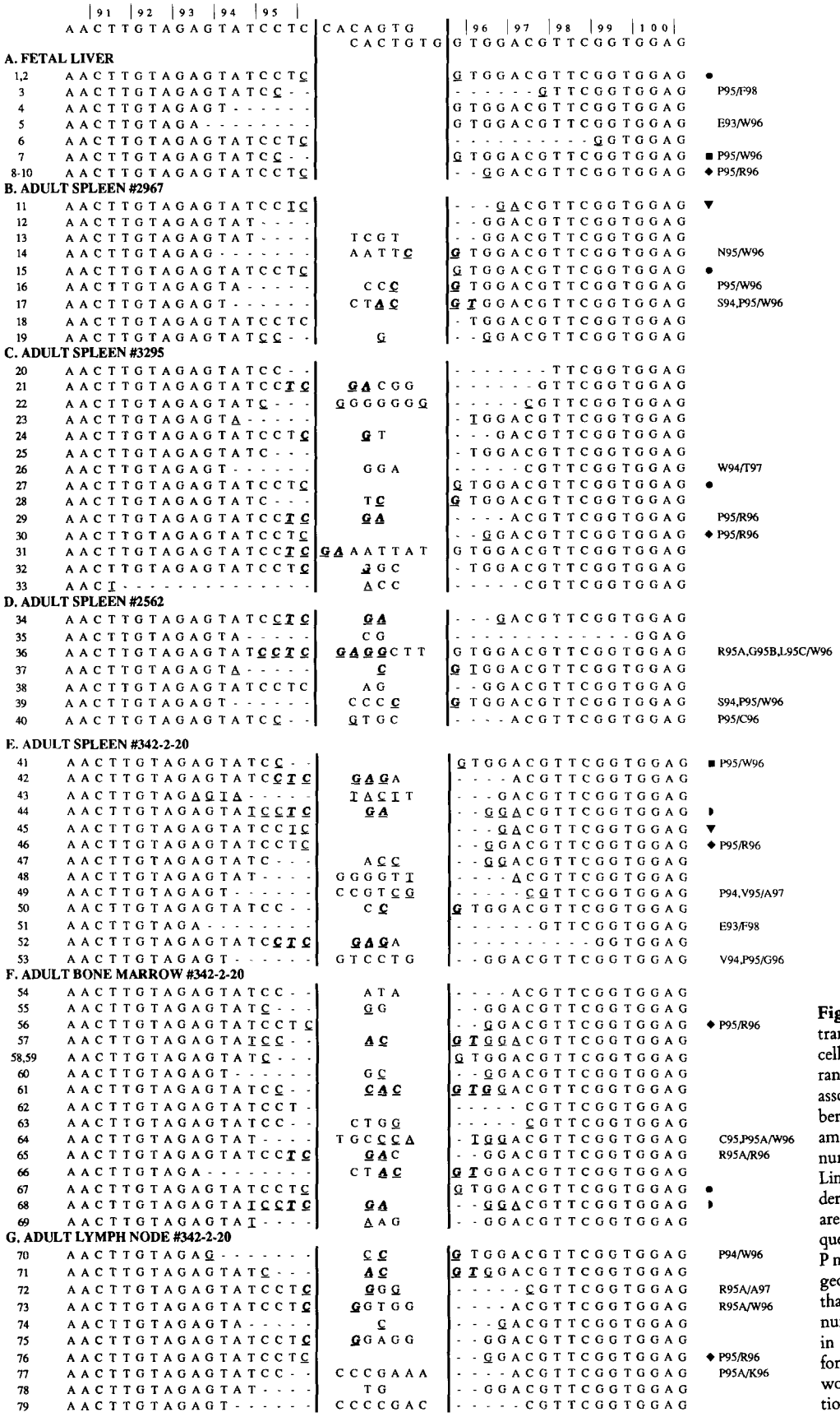

Figure 2. VJ joints of the pHRD transgene in fetal and adult lymphoid cells. The top lines show the unrearranged sequence of V and J with their associated heptamers (*center*). The numbers above these sequences refer to the amino acid positions according to the numbering system of Kabat et al. (13). Lines 1–79 are independent DNA clones derived by PCR. Noncoded nucleotides are shown in the center of each sequence. Palindromes are underlined and P nucleotides are in bold italic type. The geometric symbols indicate sequences that appear more than once. Letters and numbers (*right*) show the amino acids in one letter code and their positions for those sequences in which the joint would have created a correct translational reading frame.

the 68 (74%) definitely independent joints sequenced had N regions added, comprising from one to eight nucleotides. The presence of N regions in the adult compared with the absence in fetal joints is a highly significant difference ($p = 2.4 \times 10^{-4}$ by Fisher's Exact test). Small deletions into the V and/or J coding region were found, on average 5.2 nucleotides and maximally, 19 nucleotides (no. 33). There was no correlation between the length of the deletions and the existence or length of the N regions.

Most of the joints showed inverted repeats, often of considerable length (Fig. 2). The longest repeats of 2 × 5 nucleotides in nos. 44, 57, and 68 were accompanied by only two nucleotides of N region. However, most sequences without any N regions had inverted repeats of only 2 × 1 nucleotides, except for nos. 11 and 45 with 2 × 2. Thus, no consistent correlation between the existence and length of N regions and the existence and length of inverted repeats was observed.

Discussion

The analysis of VJ joints of a transgenic rearrangement test gene has allowed sampling of the coding joints arising from a single precursor gene. The 79 sequences presented in Fig. 2 are almost all different except for five joints that occur two, four, or five times in different organs (three of which also recur in the same organ). This exemplifies the enormous variety of potential antibody genes that can be produced even from a single VJ combination. About one third (28 of 79) of the joints are in-frame with respect to the translational codons for the V and the J region, as expected from random associations. In none of them has a translational stop codon been created. However, perhaps not all the in-frame joints would result in functional Igs, because 10 of the 28 (36%) have deletions of more than one terminal amino acid. Deletion of more than one amino acid is rare in known antibody molecules (13, and Table 1). Of 691 mouse L chain amino acid sequences reported, 611 (88.4%) have joined amino acid 95 encoded by the V region to amino acid 96 encoded by J. Most of the remaining known κ L chains have a single amino acid deleted (in Table 1, nos. *2* and *3*). Deletion of two amino acids is rare and deletion of three amino acids occurs in only 0.2% of the known κ chains (Table 1, nos. *5* and *6*). Deletion of four amino acids, as in no. 51 of the pHRD sequences (Fig. 2) has not been observed among the mouse κ chains, nor in the known rat and rabbit κ chains (13). It is, however, found in one human κ chain of subgroup III where amino acid 92 is joined with 97 (no. 56 on pg. 120 of reference 13, and [14]). In that case it is not clear whether the deletion of four amino acids resulted from the joining event or, whether an unusually short Vκ gene is involved. However, this appears to be a functional κ chain, because it can associate with γ H chains into IgG (14), although, whether such a large deletion in a L chain can be functional may depend on the H chain present in the particular B cell. Overall, the results from this unselected pool of VJ joints suggest that probably fewer than one third of VJ joints, i.e., of possible κ or λ rearrangements, would normally result in a functional B cell.

Table 1. *VJ Joints of Mouse κ Chains*

Joint	Deletions/Insertions	Number	%*
1. V95-J96‡	0§	611‖	88.4
2. V94-J96	deletion 1	37	5.4
3. V95-J97	deletion 1	16	2.3
4. V94-J97	deletion 2	3	0.4
5. V94-J98	deletion 3	1	0.1
6. V95-J99	deletion 3	1	0.1
7. V95A-J96	insertion 1	16	2.3
8. V95B-J96	insertion 2	6	0.9

VJ joints of mouse κ chains compiled from sequences published in Kabat et al. (13).
* % of the sequenced VJ joints reported in reference 13.
‡ Numbering of amino acid positions as in Kabat et al. (13).
§ Number of amino acids deleted or inserted assuming that all Vκ genes end by a heptamer located within 0–2 bp 3′ of the V95 codon.
‖ Number of κ chains reported in which the region around the joint has been completely sequenced.

Insertion of one or more amino acids occurs only in 3.2% of the recorded mouse κ chains (Table 1), but is seen in 5 of the 28 (18%) in-frame pHRD joints. It is generally assumed, that insertions derived from N regions are only added to $V(D)J_H$ joints and not to L chain genes, because the terminal transferase, implicated in the addition of N regions, is only found during H gene rearrangement (15, 16). In the case of the pHRD transgene, the presence of H chain enhancer would presumably permit rearrangement already during the early pre-B cell stage. The rare additions of amino acids, presumably due to N regions, in normal κ chains (Table 1) may indicate that some κ chain genes are rearranged early or that sometimes the N region addition persists in late pre-B cells.

The data presented here show that the addition of N regions to the pHRD transgene is mainly a property of adult B cells and that only minimal Ig sequences are required for N region addition. The difference between the absence of N regions in the fetal VJ joints and their presence in the adult is statistically highly significant ($p = 2.4 \times 10^{-4}$ by Fisher's Exact test). Since selection for particular joints in the pHRD gene cannot occur, apparently, there is a mechanistic difference between the rearrangement process in fetal liver pre-B cells and in pre-B cells in the adult. Others have shown that Ig H chain and TCR-β genes of fetal or newborn mice have very few N regions (5–8). In these cases it was possible that the cells were selected for survival based on the particular gene products. Conceivably, different Ig and TCR repertoires could be selected in early versus adult life. Previous studies (17) have shown that different V_H families are preferentially used in fetal pre-B cells, thus the difference in N region addition might be explained by some intrinsic feature of these sequences. In this study, we have examined the rearrangement of exactly the same V and J sequences in fetal and adult pre-B cells, thus eliminating this potential source of variability. The rearranged test gene pHRD results in mRNA which can be trans-

lated into gpt protein regardless of the VJ joint, since the initiation codon of the gpt gene lies about 69 nucleotides downstream of the VJ joint (Fig. 1). Thus no cell selection can occur for particular joints in the pHRD transgene.

Although none of the B cells could have been selected on the basis of pHRD rearrangement, it is possible that they were selected because of the endogenous Igs produced by the cells. If such a selection took place, the pHRD transgene could reflect the joints of the endogenous Ig genes simply as a bystander. One could postulate that at all ages, two different types of pre-B cells exist. Those, in which the recombination machinery is prone to N regions and those in which it is not. Presumably, in the 15.5-d fetal liver or the bone marrow pre-B cells, selection could have occurred based on expression of a μ chain in combination with surrogate L chains (18).

Our data combined with those of others (5–8) do not support the hypothesis that selection of pre-B cells takes place in the fetal liver based on H genes without N regions in cells which do and can add N regions (to transgenes). The results are, however, compatible with at least two possibilities. One is that N regions are not added during Ig gene rearrangement in fetal cells. The other is that the pre-B cells in the fetal liver were selected based on H genes without N regions in cells that did not add N regions to either endogenous genes or transgenes. This latter possibly would not exclude that there are pre-B cells in the fetus which can add N regions, but which do not survive the conditions in the fetal liver. They would have to be eliminated very soon after rearrangement, otherwise N regions would have been seen in the randomly amplified samples of Ig genes performed by us and others (5–8). Taken together, the data seem to favor the hypothesis that the overall state of the recombination machinery is different in the fetal liver compared with adult bone marrow. This may be a reflection of the different levels of TdT at the two stages (9). On the other hand, at least in tissue culture cells, the levels of TdT vary and there is no strict correlation with N region addition (2). Also, fibroblasts transfected with RAG-1 and RAG-2 genes which seem not to express TdT can display N regions in test substrates (19). The final molecular analysis of V(D)J recombinase composition and function at the two developmental stages will have to await a cell free system of Ig gene rearrangement. In bone marrow pre-B cells N additions are mainly seen at the early H gene rearrangement stage. Presumably, the immature pre-B cell's recombinase differs from that of the mature pre-B cell, and the latter resembles the fetal recombinase.

In a comparison of fetal and adult TCR-γ/δ joints, not only a low number of N regions, but also a decreased number of deleted nucleotides were found in the fetuses (20). Decreased deletions were not found in fetal TCR-β joints (7). Also, in our sample, the fetal joints had an average of 5.1 nucleotides deleted per joint and the adult joints an average of 5.2 nucleotides. Thus, it is not a general rule that exonuclease is low in fetal lymphoid cells (20).

One unexpected observation in the pHRD gene joints from adult mice was the presence of many examples of long inverted repeats (Fig. 2). In the fetal pHRD joints an inversion of maximally one nucleotide is seen, but in the adult joints palindromes of two or more nucleotides are frequent. The palindromes are indicated by underlining of the respective nucleotides in Fig. 2. Many of them (Fig. 2, bold italic type) are partly compatible with the originally described P nucleotides, namely two untemplated nucleotides contiguous to a full-length coding end of a VJ joint and representing its inverted repeat (20), except that we have several examples with larger than dinucleotide inversions (nos. 36, 42, 52, and 61). The original definition assumed that the palindromes are derived by flipping over the terminal complementary dinucleotides of the full-length coding end (20). However, inspection of our sequences (and other published sequences) show many palindromes that could not have arisen in this way (Fig. 2, underlined roman type). In one group, one complementary sequence represents the full coding end, the other extends into the other, generally partially deleted, coding end (nos. 11, 34, 44, 57, etc.). In yet others, neither coding end is full-length (nos. 43, 49, 64, etc.). Such palindromes may be apparent in the pHRD transgenes, because of the natural inverted repeats existing in the coding ends. The significance of the "classic" P nucleotides, as well as of the additional types of palindromes described here, remains to be elucidated. Because it is not clear that inverted repeats arise by a unique mechanism, we have counted all nucleotides not encoded by the transgene as N regions.

Long palindromes have not been observed in fetal or adult conventional or Ly1 B or T cells from normal mice (5, 7, 21). Especially long palindromes were, however, seen in nearly correct TCR gene rearrangements in mice with *scid* (22–24). In these mice, coding joints are generally defective, but signal joints are mostly correct. Because of the palindromic nature of the hepatamer sequence, signal joints are also characterized by inverted repeats of up to nine nucleotides (25). It could be postulated that inverted repeats aid the joining of severed ends in *scid* as well as in normal cells, and that the integrity of the signal joints in both *scid* and wild type cells is a reflection of this stabilizing effect. Alternatively, the inverted repeats seen increased in *scid* cells may be an indication that the particular joint was formed under conditions which disfavor rearrangement. We do not know if and how in the pHRD transgenic mice conditions for rearrangement may have been unfavorable. We have considered that partial methylation of the transgene may have an untoward effect. However, there is no consistent increase of palindromes in more methylated compared with unmethylated transgenes. The transgenes were increasingly methylated in the order no. 2967 (Fig. 2 *B*, unmethylated); nos. 2562, and 342-2-20 (partially methylated, Fig. 2, *D–G*); and no. 3295 (Fig. 2 *C*, most methylated). Whereas the partially methylated transgenes (shown in Fig. 2, *E–G*) have many long palindromes and the unmethylated transgenes (shown in Fig. 2 *B*) have no long palindromes, the most highly methylated mouse (shown in Fig. 2 *C*) also has none and thus does not support the pattern. It has been shown, however, that hypermethylation inhibits Ig gene rearrangement in transgenic mice (11) and in transfected cells (26, and M. Glymour, P. Engler, and U. Storb, unpublished results). It is possible that other differences be-

tween the transgenic mice (strain, age, sex, parental imprinting, etc.) influenced the effect and it will be interesting to further investigate if situations that decrease rearrangement efficiency are accompanied by long inverted repeats.

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