Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 6577–6581, July 1995
Genetics

Persistence of immunoglobulin heavy chain/c-*myc* recombination-positive lymphocyte clones in the blood of human immunodeficiency virus-infected homosexual men

JÜRGEN R. MÜLLER*, SIEGFRIED JANZ*, JAMES J. GOEDERT†, MICHAEL POTTER*, AND CHARLES S. RABKIN†

*Laboratory of Genetics and †Viral Epidemiology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

Contributed by Michael Potter, March 6, 1995

ABSTRACT **We studied blood lymphocytes of human immunodeficiency virus (HIV)-seropositive and -negative homosexual men for the presence of T(8;14) translocations that recombine c-*myc* and immunoglobulin heavy-chain (IgH)μ/IgHα switch regions. Clones with T(8;14) translocations were detected in 10.5% (12/114) of the HIV-positive and in 2.0% of the 99 uninfected patients. The majority of recombinations were found at a single time point only. Four patients, however, harbored multiple (up to four) and persistent (up to 9 years) translocation-positive cell clones. No correlation between the presence of these aberrant lymphocytes and a later lymphoma could be established. The exon 1/intron 1 region of the recombined c-*myc* was investigated for the presence of point mutations and these were found in the nonpersistent clones. Additional alterations detected in these clones included duplications and a deletion in the c-*myc* gene. The pattern of base substitutions indicates that they were introduced after the translocation event.**

Individuals infected with the human immunodeficiency virus (HIV) develop non-Hodgkin lymphomas (NHLs) at a frequency 60 times higher than the general population (1). AIDS NHLs are usually derived from B cells and the majority of them carry rearrangements and point mutations in the oncogene c-*myc* (2–4). Several mechanisms by which lymphomagenesis in these immunocompromised patients may be promoted have been proposed (for review, see ref. 5): (*i*) the absence of immunosurveillance, (*ii*) abnormal growth factor expression, and (*iii*) chronic antigenic stimulation.

NHL in AIDS patients, in contrast to NHL in the general population, originates in extranodal locations as frequently as at nodal sites (6, 7). Here we show that c-*myc* rearrangements can be detected in blood lymphocytes of some lymphoma-negative individuals over extended periods of time.

METHODOLOGY

The study included a cohort of 114 HIV-positive and 99 HIV-negative homosexual men (8). All patients were originally recruited in 1982 from three private medical practices in New York, NY, and Washington, DC. Peripheral blood was obtained at intervals of ≈1 year. The lymphocytes from 60 ml of blood were freshly purified by Ficoll/Hypaque density-gradient centrifugation and were stored in liquid nitrogen. For DNA preparation, we further lysed these cells in 50 mM KCl/10 mM Tris·HCl, pH 8.3/2.5 mM $MgCl_2$/0.5% Tween 20/proteinase K at 300 μg/ml, which was followed by ethanol precipitation. The samples were given coded numbers and were then subjected to PCR analysis.

A nested-type PCR amplification was used to detect recombinations between immunoglobulin heavy-chain (IgH) switch loci and c-*myc*. Primer pairs with defined annealing sites in IgHμ and IgHα switch regions (Sμ/Sα) and in c-*myc* were used. These primers permit the detection of recombinations involving breakpoints in c-*myc* between intron 1 and the 5′ flank ≈2.5 kb upstream of exon 1. Between 2 and 10 recombination structures have to be present in 500 ng of DNA to be detected by PCR (data not shown). The sequences and locations of the oligonucleotides were derived from published sequences (GenBank entries HUMMYCC, HSJHCMU, and HUMIGACHSR) and are as follows: HMY5′8, 5′-CCGCAGCCCCTCCCAACCTTCCCTCTCCAC-3′; HMY5′7, 5′-TTTGCCAGCTTTTCTTCTTTCTCTCG-3′; HMY5′6, 5′-GTTTTCCCTCTGCCTTCTCCTCTCCCATCTTGACA-3′; HMY5′5, 5′-CCCCCACCCCAGCCCCAGTTACCATA-3′; HMY5′4, 5′-GAGGGTGGGGAGGGTGGGGAAGGTGGGGAGGAGAC-3′; HMY5′3, 5′-CGCGCCTACCATTTTCTTTTGCTCCCTCTC-3′; HMY5′2, 5′-GTCCTCTTTCCCCTTTTTATTATTGG-3′; HMY5′1, 5′-CTGATCAAAGAAGAGGAGAGGAGTATT-3′; HSM5′4, 5′-GGGAGGGTGAGTGTGATGGGGAACGCAGTGTAGAC-3′; HSM5′3, 5′-TGAGCTCAGACCACCTAAGACCA-3′; HSM5′2, 5′-CTCGCCTCAGCCCGTTCAGCCAGTT-3′; HSM5′1, 5′-CTCAGCCCAGCCCAGCCCAGCGTAGC-3′; HSA5′2, 5′-GTCCCGCCCAGTCCAGCCCAAGTCATCCTA-3′; HSA5′1, 5′-ATCCCATCCCAGCCCAGGTCAGCCCAGTTA-3′; HMY3′1, 5′-GAAAGAATAACAAGGAGGTGGCTGGAAACT-3′; HMY3′2, 5′-CCCCACCTGACCCCCGCCCTCGTTGACATC-3′; HMY3′3, 5′-CAAATCATGTGTGGGGCTGGGCAACTA-3′; HMY3′4, 5′-CTGAGTCGAAGCGTAAATAAAATGTGAA-3′; HMY3′5, 5′-ATCGTTTTCCTCCTTATGCCTCTATCA-3′; HMY3′6, 5′-CTCCCTATCTACACTAACATCCCACGCTCTGA-3′; HMY3′7, 5′-GCACTGGAACTTACAACACCCGAGCAA-3′; HMY3′8, 5′-CTTTAATGCTGAGATGAGTC-3′; HSM3′1, 5′-TCAAGTAGAGGGAGACAAAAGATGGAAGC-3′; HSM3′2, 5′-AGGAACCCGGCAATGAGATGGCTTTAG-3′; HSM3′3, 5′-GGCTGAGCAGGTTGTATC-3′; HSM3′4, 5′-GGGCCAAGCTCAGTAGA-3′. The PCR mixture contained a final concentration of 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, gelatin at 0.1 mg/ml, each primer at 0.5 μM, each dNTP at 200 μM in a vol of 50 μl. Cycling conditions involved a 5-min initial denaturation at 95°C with the reaction mixture subsequently being held at 80°C for the addition of 1.25 units of *Taq* polymerase. This was followed by 40 cycles of denaturation (15 sec at 95°C), annealing (15 sec at 65°C), and extension (between 30 sec and 2 min at 72°C). For nested PCR, a 1-μl reaction mixture from round one was used as a template for an additional round of 40 cycles. All products were PCR amplified at least twice. PCR products were sequenced directly with *Taq* polymerase (Promega) or they were cloned into pBluescript vector and sequenced with Sequenase (United States Biochemical).

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Abbreviations: HIV, human immunodeficiency virus; NHL, non-Hodgkin lymphoma; IgH, immunoglobulin heavy chain; Sμ/Sα, IgHμ/IgHα switch regions; Chr, chromosome.

For analysis of point mutations in the recombined c-*myc*, we sequenced a 500-bp region spanning the exon 1/intron 1 boundary, which was coamplified with the IgH/c-*myc* junction. In each case, products of several independent PCRs were sequenced either directly or after cloning into pBluescript vector (Table 1, patients 1, 10, and 11). Only identical patterns of base substitutions detected in two or more independent PCR products were considered to be nonartifactual. All base substitutions in c-*myc* were compared to the germ-line c-*myc* sequence, which was amplified in a one-step PCR from each individual patient.

RESULTS

Occurrence of c-*myc* Rearrangements. Five hundred eighty-five samples from HIV-infected patients and 400 samples from uninfected patients were tested for the occurrence of recombinations between c-*myc* and the Sμ or Sα region of IgH. Recombinations were detected in both groups; 10.5% (12/114) of the HIV-infected and 2.0% (2/99) of the uninfected men harbored IgH/c-*myc* recombination-positive clones in their blood lymphocytes. Ten men including the two HIV-negative patients had rearrangements detected at a single time point only (Fig. 1*B*). In the other four patients, a total of 12 multiple clones with different IgH/c-*myc* recombinations were found (Fig. 1*A*). Ten of the 12 multiple clones persisted for periods of at least 1 year; one clone was still present 9 years after the initial detection.

To estimate the load of lymphocytes with IgH/c-*myc* recombinations, all samples with recombinations were subjected to repeat PCR analysis. PCR amplifications were done between 5 and 40 times with samples of 500 ng of DNA from the same blood cell preparations. This amount of DNA is representative of $\approx 10^5$ lymphocytes. The entire blood circulation contains $\approx 10^{10}$ lymphocytes. Identical recombinations were detected in 5–35% of PCR amplifications (data not shown). This indicates the presence of at least several thousand IgH/c-*myc* recombination-positive lymphocytes in the bloodstream of these individuals.

Currently, 12 of the 114 HIV-infected men have been diagnosed with NHL. The tumors were histologically classified as five large cell diffuse lymphomas, five immunoblastic lymphomas, and one T-cell lymphoma. One central nervous system tumor was not biopsied and could not be classified. In 3 of these 12 cases, tumor tissue (two large cell diffuse and one immunoblastic lymphoma) was available from paraffin blocks for PCR analysis. None of these three tumors had a c-*myc* recombination detected by PCR. Only 1 patient (Fig. 1*B*, patient 11) had aberrant lymphocytes carrying IgH/c-*myc* illegitimate recombinations before the diagnosis of NHL and this patient's tumor was one of the three analyzed. Thus, no correlation between IgH/c-*myc* recombination-positive clones in the blood and the later diagnosis of a lymphoma could be established.

Point Mutations in c-*myc* Exon 1/Intron 1. Base substitutions in the exon 1/intron 1 region are frequently detected in Burkitt lymphomas (9, 10). We analyzed the c-*myc* sequence in the vicinity of the junction site with the immunoglobulin locus for the occurrence of point mutations. None of the four patients who harbored multiple and persistent recombination-positive cells had point mutations in their sequences (Fig. 1*A*) as determined by direct sequencing. However, the presence of smaller subclones with base substitutions could be masked by the predominance of cells without point mutations. In addition, the presence of multiple clones with different sets of base substitutions in the DNA sample would be detected as germ-line sequences. IgH/c-*myc* recombination-positive tumor DNA (BL67) was diluted into recombination-negative DNA. In these experiments, we consistently detected as few as two recombination structures in 500 ng of DNA (data not shown). In repetitive experiments with identical lymphocyte samples, between 5% and 35% of the reactions generated the same PCR product. Therefore, the number of recombination structures in any sample containing 500 ng of DNA was unlikely to prevent the detection of base substitutions. Rearranged c-*myc* DNA

Table 1. Flanking sequences around chromosomal breakpoints on Chr 14q+ and on the reciprocal Chr 8q− in blood cells

	T_{14q+}*			T_{8q-}†		
Patient	IgH_{14q+}		c-myc_{14q+}	c-myc_{8q-}		IgH_{8q-}
1	CAGCCTAGCTCAGCCCAGCT		GGGGGCTTCGCCTCTGGCCC	n.a.		
2a	CTGCTAGACTTGGCCCAGCT		TGGCCCAGCCCTCCCGCTGA	n.a.		
2b	CCCAGCCCAGCCTCGGCTTA		ATAGCAGCGGGCGGGCACTT	GCATCCACGAAACTTTGCCC		CTCAGCCCAGCCCAGGTTAG
2c	n.a.			GACTTGTCAAGATGCGAGAG	C‡	TGGCTCAGCCCAGCACAGCA
3	CCCAGCTCAGCTCACCCCAG		TGGCTTCTTAAAAAGCAATA	n.a.		
4	CAGCCTTGCCCAGGTTAGCT		CCGGCTGAGTCTCCTCCCCA	n.a.		
5	CAGCCCAGCCCAGCTCAGTC		GCTTAGACGCTGGATTTTTT	n.a.		
6	GCTCAGCTGAGCCCAG	GCAGGCTAT‡	GGAGGAGCAGCAGAGA	TGCGGTTTGTCAAACAGTAC		AGCTCAGCCCAGTCCAGGTT
7a	AGCGCAGCCCAGCCCAGCTC		AGCACCGAAGTCCACTTGCC	AGCGCAGCCCAGCCCAGCTC	AGC‡	ACCGAAGTCCACTTGCCTTT
7b	CCGACACAGCCTCCTCAACC		CGGGCACTTTGCACTGGAAC	n.a.		
7c	n.a.			CCCATTAATACCCTTCTTTC		AGCCCCGCTCAGACCCGCTC
7d	GCCCAGTTCAGCTCAGCTCA	A‡	CGACGCGGGAGGCTATTCTG	n.a.		
8a	n.a.			CCGGTTTTCGGGGCTTTATC		CCAGCTCAGCTCAGCTCAGC
8b	CAGCTCAGCCTTGCCCAGGT		TGAAAGGCTCTCCTTGCAGC	TTCCCCGCCGCTGCCAGGAC		TGGCTCAGCTCAGCCTAGCT
8c	GCTAAGCTCAGCTCGGCTCG		GATGCTCTCTCGCTAATCTC	n.a.		
9	GCCCAGTTCAGCTCAGCTCA		CTCCTTGCAGCTGCTTAGAC	n.a.		
10	TAGCTCAGCTCAGCCCAGCT		CATAAGCGCCCCTCCCGGGT	n.a.		
11	TCAGCCCAGTTCAGCTCGGC		GGGCTGGGGTGGGGGGTAAT	CTTTTCTCAGAGTAGTTATG		CCCAGCCCAGCCCAGCCTAG
12a	AGCCCAGCCTCAGCTCAGCC		CCCGAGCAAGGACGCGACTC	n.a.		
12b	CTTGCCCAGGTTAGCTCAGC		GCGGGCACTTTGCACTGGAA	CGAAACTTTGCCCATAGCAG	T‡	CCAGGTTAGCTCAGCCCAGC
13§	GCTCTGCCCAGGTTAGCTCA		GCCCCGGCCGTCCCTGGCTC	CGGTTTGTCAAACAGTACTG		AAAGCTGGATGGAGTTGTCA
14§	AAACTGGCTGAACGGGCTGA		GGCGAGAGAAAGAAGAAAAG	CTTGTCCCCGTCTCCGGGAG		AGGTGGCGGCAGGACTACCA

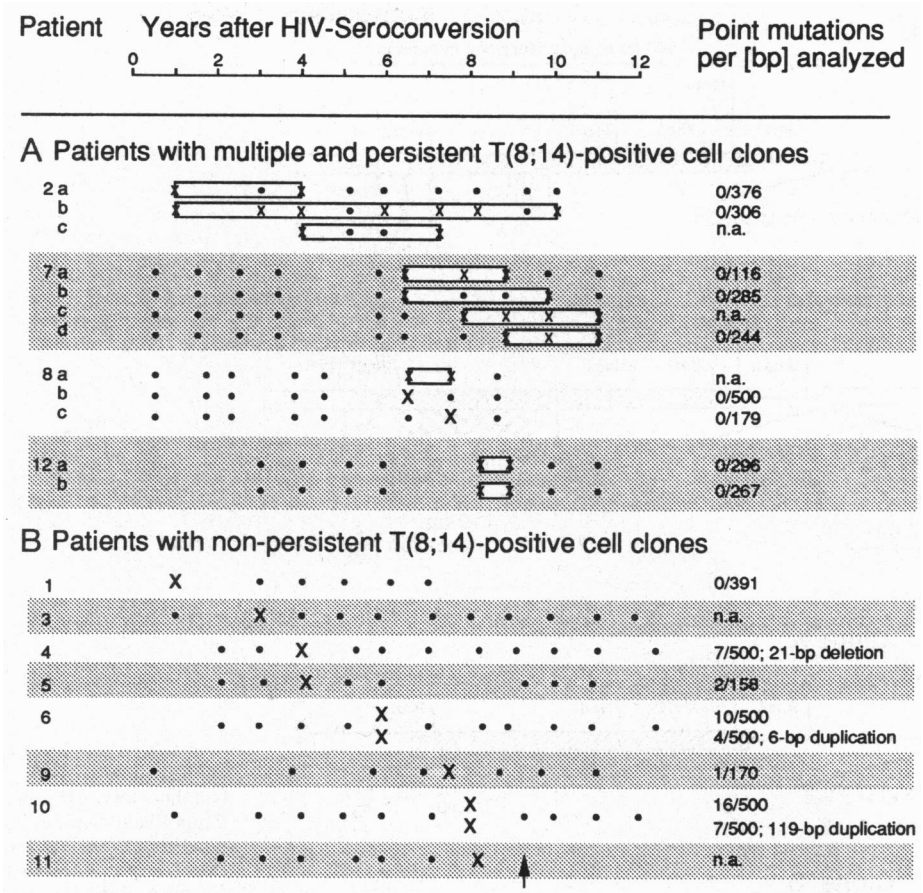
n.a., Not amplified by PCR. Differences of sequences flanking the IgH/c-*myc* junction with germ-line sequences are given in boldface italics; homologies between IgH and c-*myc* at the recombination site are underlined and are arbitrarily assigned to c-*myc*.

*Recombination breakpoint with adjoining sequences on the c-*myc* activating chromosome 14q+. T_{14q+} indicates the translocation breakpoint. IgH sequences are in a transcriptional orientation that is reversed with respect to that of c-*myc* because of the head-to-head recombination.

†Recombination breakpoint with adjoining sequences on the reciprocal chromosome 8q−. IgH sequences are reversed with respect to transcriptional orientation because of the tail-to-tail recombination.

‡Five junctions contain additional nucleotides of unknown origin between c-*myc* and immunoglobulin sequences.

§IgH/c-*myc* recombinations that were detected in HIV-negative men.



C Nature of base substitutions

From \ To	A	G	C	T	Number of mutations	Corrected proportions
A	-	0.67	0.08	0.25	12	0.30
G	0.40	-	0.47	0.13	15	0.28
C	0.00	0.25	-	0.75	12	0.24
T	0.33	0.17	0.50	-	6	0.17

FIG. 1. Occurrence of IgH/c-*myc* recombinations in blood lymphocytes of HIV-positive homosexual men and point mutations in recombined c-*myc* in a subset of these clones. For point mutations, we analyzed a 500-bp region spanning the exon 1/intron 1 boundary (GenBank entry HUMMYCC, 2500–3000). (*A*) Subjects 2, 7, 8, and 12 had IgH/c-*myc* identical cell clones detected repeatedly over 1–9 years (shown by ×s inside boxes). These clones were free of point mutations as determined by direct sequencing. (*B*) Nonpersistent clones (depicted by ×s) with a variety of point mutations, deletions, and duplications were detected in eight of the subjects. Negative samples are shown by dots. Because of the location of the breakpoints in c-*myc* or the successful PCR amplification of only the reciprocal IgH/c-*myc* junction, some recombinations were not analyzed (n.a.). NHL was diagnosed after detection of an IgH/c-*myc* rearrangement in the blood in only one patient (arrow). (*C*) Distribution of the base substitutions is given as proportion of the total. Because of the head-to-head Chr 14q+ recombination, the distribution was calculated by using the noncoding strand of c-*myc*. Corrected proportions show the mutations occurring in each of the 4 nucleotides after correction has been made for base composition.

from individuals with nonpersistent clones had a number of base substitutions. The mutations showed a bias toward transitions and a low frequency of thymidine substitutions (Fig. 1*C*). This resembles the pattern of base substitutions found in nonselected V(D)J region sequences after somatic hypermutation in B lymphocytes (11).

The c-*myc* locus in two patients each had two independent patterns of point mutations adjacent to identical recombination junctions (Fig. 2), suggesting that these mutations were introduced after the illegitimate rearrangement. Two tandemly repeated duplications, one of 119 bp in subject 10 and one of 6 bp in patient 6, and a 21-bp deletion (subject 4) in c-*myc* were detected in the IgH/c-*myc* rearranged DNA. The identical pattern of base substitutions in one of the duplications (Fig. 2, patient 10, clone B) suggests that duplications and deletions may be late events and that they occur after the generation of point mutations in a rearranged c-*myc* locus.

Molecular Characteristics of IgH/c-*myc* Recombinations. The PCR assay used to amplify T(8;14) rearrangements permits the detection of recombination breakpoints as far upstream as 2.5 kbp 5′ of exon 1 in c-*myc*. All breaksites in the T(8;14) recombinations in subjects who have not developed NHL were detected within intron 1, exon 1, and the near 5′ flank of the *myc* gene (Fig. 3*B*). The breakpoint cluster in c-*myc* coincides with a high concentration of switch region motifs in the *myc* gene (Fig. 3 *B* and *C*).

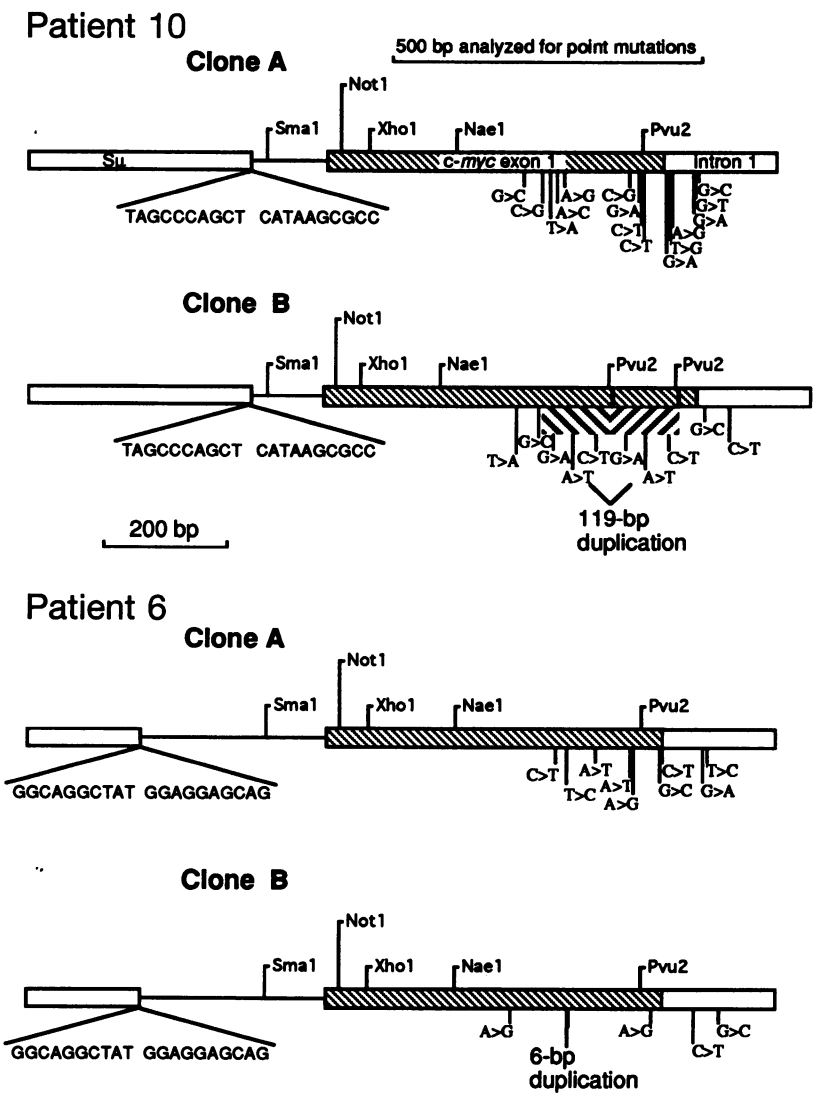


FIG. 2. Partial restriction maps of Chr 14q+ structures as detected in the blood lymphocytes of HIV-positive patients (patients 10 and 6 in Fig. 1). Identical recombination sequences, which characterize two clones in each patient, are depicted. Clones acquired an unrelated set of point mutations in c-*myc* exon 1/intron 1. In patient 10, clone B harbors a tandemly repeated 119-bp stretch of c-*myc* with 3 identical base substitutions in both parts. Structures indicate that the sequence of mutational events was translocation → point mutations → duplication in this single case.

Fifteen breakpoints in the immunoglobulin locus were located in Sμ and two were located in Sα (Fig. 3*A*). Recombination sites in Sμ were clustered within the first half of the repetitive region. Both characteristics of these recombinations in blood lymphocytes of HIV-positive patients are unusual. Our own study of Burkitt lymphomas showed a higher frequency of recombinations in Sα (5/15) and in the 3′ half of Sμ (3/15; unpublished data).

Short homologies of IgH and c-*myc* sequences up to 8 bp appear to be the preferential site of recombination on the c-*myc* activating chromosome (Chr) 14q+ (Table 1). Most junctions showed at least a 1-bp identity (10/17). The maximum length of homology was 8 bp in one sample. In contrast, homologies at the illegitimate junction on the reciprocal chromosome were detected less frequently (4/10 junctions with a 1-bp homology), suggesting that different mechanisms may be involved in establishing the two reciprocal IgH/c-*myc* junctions.

DISCUSSION

Immunocompromised HIV-infected patients have a high incidence of NHL. Rearrangements of the c-*myc* oncogene are characteristic for the majority of these tumors (2, 3). The frequency of this mutational change supports a role for deregulated c-*myc* expression in the process of lymphoma development. The data presented in this study show that HIV-positive individuals may harbor expanded cell clones with illegitimate recombinations between c-*myc* and Sμ or Sα in their blood, often over long periods of time. The finding that clonotypic IgH/c-*myc* recombinations are repeatedly found in the DNA samples indicates the presence of at least several thousand of these aberrant lymphocytes in the blood circulation of the patients. The expansion of the translocation-positive cell may be mediated by deregulated c-*myc* expression. In addition, chronic antigenic stimulation may support the expansion of lymphocyte clones. In support of this, we showed evidence in two cases (patients 6 and 10) of two coexisting clonotypes that carried different sets of point mutations. Deregulated c-*myc* is toxic to cells that are not protected by the presence of certain cytokines (12, 13). Increased circulating levels of interleukin 6, which accompany HIV infection (14), may reflect the presence of tissues producing large amounts of this growth factor. In these tissues, interleukin 6 may help in rescuing the clones from apoptosis (15, 16).

Several molecular characteristics of the IgH/c-*myc* recombinations found in blood lymphocytes from HIV-positive individuals are shared by recombinations found in Burkitt lymphoma samples (unpublished data). Short homologies between switch sequences and c-*myc* were detected in the majority of junctions on the c-*myc* activating Chr 14q+. Such homologies were not found on the reciprocal recombinations (Chr 8q−). Furthermore, breakpoints in the *myc* gene coincided with the location of switch motifs. These characteristics

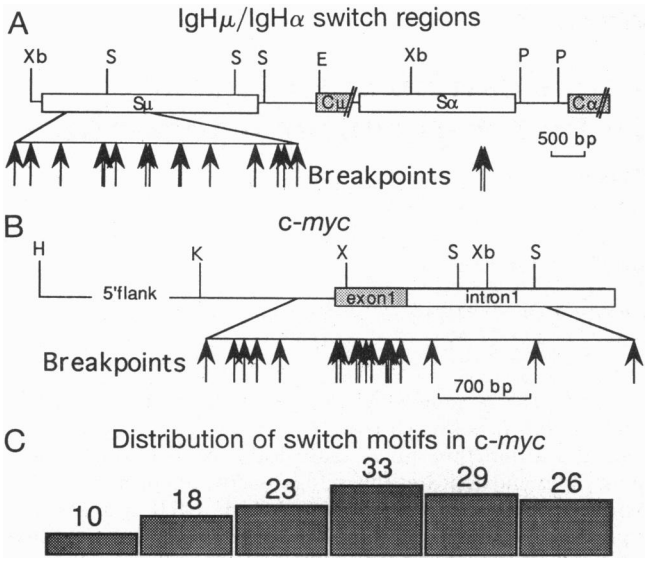


FIG. 3. Distribution of recombination breakpoints in IgH/c-*myc* rearrangements detected in blood lymphocytes of HIV-infected and uninfected homosexual men. (*A*) In the immunoglobulin locus, the majority of breakpoints were detected in the 5′ half of Sμ. (*B*) Recombination sites in c-*myc* were found in intron 1, exon 1, or the near 5′ flank. They coincided with switch motifs in c-*myc*. We consider switch motifs to be any consecutive pentamer of the sequence GCTG(A/G)GCTG that covers ≈50% of the human Sμ and Sα regions. The distribution of these pentamers in the c-*myc* gene is shown for 700-bp segments in *C*. Bars reflect the correct topological position of the c-*myc* gene as shown in *B*. Approximately 14 switch motifs are expected in a random 700-bp DNA sequence; the number of pentamers that were actually found in c-*myc* is given above the bars.

suggest a two-step model of generating IgH/c-*myc* recombinations. The initial event joins immunoglobulin and c-*myc* sequences by an immunoglobulin isotype switch-related mechanism. The two resulting free DNA ends are then repaired by nonhomologous recombination processes (17). Typically, the ubiquitous activity of nonhomologous recombination in mammalian cells joins DNA double-strand breaks preferentially at sites of short homologies (17). The majority of homologies at IgH/c-*myc* junctions were detected on Chr 14q+ in our study. This suggests that the initial recombination event preferentially involved Chr 8q− junctions.

Point mutations in the c-*myc* exon 1/intron 1 region that have the potential to alter regulation sites are frequently found in the c-*myc* oncogene of Burkitt lymphoma tissue (9, 10). The recombination between c-*myc* and Sμ interrupts the immunoglobulin locus and replaces the variable region of the heavy-chain gene by c-*myc* sequences. Because the variable region is the target of somatic hypermutation during B-cell development, it has been hypothesized that the base substitutions detected in the recombined c-*myc* are caused by this mechanism. Several findings in our study support this concept. First, the majority of cells within persisting translocation-positive clones remained free of base substitutions over years, suggesting that point mutations do not gradually accumulate. Second, patients 6 and 10 each had two sequences with identical IgH/c-*myc* junctions that showed no common base substitutions. Because specific mutations are frequently detected in Burkitt lymphomas, these substitutions may well confer a growth advantage. If point mutations did accumulate over an extended period of time, then these specific mutations should be preserved. Third, the process of somatic hypermutation in B cells shows a specific pattern of base substitutions, characterized by a high rate of transitions and a low frequency of thymidine changes (11). These patterns were also present in the translocated c-*myc* of HIV-positive individuals.

Interestingly, somatic hypermutation was found only in the transient nonpersisting lymphocyte clones with c-*myc* rearrangements. It is possible that some subclones harboring point mutations might have escaped detection because of the predominance of nonmutated DNA. We suggest that the persistence of mutation-negative clones in four cases may be due to the fact that the majority of these cells do not enter the developmental stage in which hypermutation occurs. Alternatively, IgH/c-*myc* recombinations could originate in cells after the V(D)J hypermutation mechanism has been silenced. The persistence of these lymphocytes over several years would indicate that c-*myc* deregulation is tolerated well by these cells. In contrast, cells with a mutation-positive c-*myc* gene are detected only sporadically, perhaps due to a more vigorous c-*myc* expression. Differences in the deregulation of the c-*myc* oncogene could be explained by effects of the immunoglobulin 3′ α enhancer, which is placed in the vicinity of the oncogene by the T(8;14) translocation event. The activity of this enhancer is suppressed by the presence of a B-lymphocyte-specific activator protein that is down-regulated in differentiated plasma cells (18).

We are grateful to Dr. Dave Waters for preparing the DNAs and to Ms. Joy Williams for continuous editorial help. We are indebted to Drs. Judith E. Karp and M. Raffeld for reading the manuscript and for making valuable suggestions.

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