Unique sequences are interspersed among tandemly repeated elements in the murine $\gamma 1$ switch segment

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ABSTRACT

Early in its differentiative pathway, a given B lymphocyte expresses immunoglobulin of the μ heavy chain class (IgM). Subsequent differentiative processes may involve rearrangement within the immunoglobulin heavy chain chromosomal locus to enable cells of the same lineage to synthesize immunoglobulins of other heavy chain classes (e.g. IgG, IgE or IgA), but with specificity for the same antigen as the original IgM molecule. Switch recombination, the molecular event which facilitates this chromosomal rearrangement, has been shown to occur between segments of DNA consisting of tandemly repeated unit sequences. These DNA segments have been functionally defined as switch regions. We have cloned the $\gamma 1$ switch region from the BALB/c germline, and have demonstrated that significantly divergent sequence elements are interspersed among the tandemly repeated units characteristic of this switch region. We show that these unique elements exist in at least three copies within the switch segment, and discuss the implications of this novel and previously unreported primary structure.

INTRODUCTION

The differentiation of B lymphocytes is marked by two types of independent DNA recombination events specific for the generation of functional immunoglobulin heavy chain genes (1-3). The first recombination involves joining of the physically separated genetic elements V_H , D_H and J_H , to yield a complete heavy chain variable region gene: VDJ_H (1, 3). This variable region is expressed as a single transcription unit in conjunction with the μ constant region gene (C_μ) to produce mRNA which encodes a complete IgM heavy chain. Later in differentiation a second recombination event can occur which involves a switch from the linkage of VDJ and C_μ to the linkage of the same variable region gene and one of the other six C_H genes ($C_{\gamma 3}$, $C_{\gamma 1}$, $C_{\gamma 2b}$, $C_{\gamma 2a}$, C_δ , and C_α). This immunoglobulin class switch, manifested by the expression of different heavy chain isotypes, is mediated by a chromosomal deletion that begins just upstream of the C_μ gene and ends just upstream of the next C_H gene to be expressed (4-7). A schematic representation of switch recombination appears in figure 1.

Figure 1. Schematic diagram of immunoglobulin heavy chain switch recombination. The top bar depicts the configuration of the immunoglobulin heavy chain locus in a B cell which has undergone V-D-J joining and can synthesize a complete μ heavy chain. Switch recombinations which permit expression of the same variable region gene (V) with $\gamma 1$ or α constant regions to produce IgG1 or IgA are depicted in the second and third bars, respectively. Solid boxes denote coding sequences while striped boxes show the various switch regions.

Switch recombinations occur in segments of DNA functionally defined as switch (S) regions. These S regions are located one to five kilobases (kb) upstream of each C_H gene except delta (8), and appear to consist of tandemly repeated unit sequences which vary in length from 5 to 80 base pairs (3, 9-12). Hybridization studies using cloned switch sequences show that: i) switch regions vary in size from approximately one to nine kb (8, 13), and that ii) under conditions of low stringency the μ switch segment (S_μ) cross-hybridizes with $S_{\gamma 3}$, S_ϵ and S_α , but not with $S_{\gamma 1}$, $S_{\gamma 2b}$ or $S_{\gamma 2a}$, although the latter S_γ segments cross-hybridize with each other (8, 13). In addition, direct DNA sequencing of the $\gamma 2b$ switch region suggests that, based on homology considerations of adjacent 49 base repeating units (49mer), this switch region is composed of a larger repeat consisting of six tandemly repeated 49mer segments (11). More extensive sequence analysis suggests also that the prevalent unit sequence of the seven switch regions share the common oligonucleotides (G)AGCT(G) and TGGG(G) (13). To date, no report suggests that any S region contains sequences other than those closely related to its prevalent unit repeat.

Our approach toward understanding the mechanism of switch recombination has been to analyze the unrearranged (germline) switch regions in an attempt to expand the sequence database available for comparison with rearranged switch sequences. To this end we have cloned the germline $\gamma 1$ switch segment from BALB/c mice. Our results demonstrate that the tandemly repeated sequences (49mer) characteristic of this segment are interspersed with significantly divergent sequences. We discuss the possibility that these non-49mer sequences serve as signals in switch recombination.

EXPERIMENTAL PROCEDURES

Southern Hybridization Analysis

Restriction endonucleases purchased from New England Biolabs or Bethesda Research Laboratories were used to digest genomic and cloned DNA samples. Restricted samples were fractionated electrophoretically through agarose gels and transferred to nitrocellulose (14). Prehybridization, hybridization to probes nick-translated (15) to high specific activity ($>10^7$ cpm/microgram), and filter washings were performed as described (9), with two exceptions: i) dextran sulphate was not added to hybridizations, and ii) salt concentrations in hybridization and wash solutions were varied as noted in figure legends.

Bacteriophage Library Construction and Screening

High molecular weight DNA was prepared from kidney and liver tissue of BALB/cJ mice as described (16). DNA (600 μ g) was digested to completion with EcoRI, and enrichment of the 16 kb fraction containing the germline γ 1 switch region was accomplished by sucrose gradient fractionation. Construction of a Charon 4A library was essentially as described (9).

Approximately 100,000 non-amplified plaques were screened by the Benton-Davis procedure (17). Hybridization to the nick-translated plasmid γ 1/IF2P.1.8 (9) was in 6xSSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate). A positive clone was plaque-purified twice and designated γ M33.

Plasmid Subcloning and Screening

The BamHI and PstI fragments of γ M33 were subcloned in pBR322 (18). The high density plasmid screening method of Hanahan and Meselson (19) was accomplished using as a probe M13/73, an M13mp2 subclone of γ 1/IF2P.1.8 which contains Sy1 sequences (W. Dunnick, unpublished). BamHI and PstI subclones were named according to the fragment inserted, as shown in figure 2.

The 10kb EcoRI-HindIII fragment of γ M33 was purified by electroelution (20) from a 0.8% agarose gel and subcloned in pBR325 (21). The plasmid was designated γ 1/EH.10.0.

Verification of recombinant plasmid inserts was accomplished by digestion with the appropriate enzyme(s) and Southern blot comparison with γ M33 using M13/73 as a probe.

M13 Subcloning and Screening

Three strategies were utilized for subcloning γ M33 in the bacteriophage vector M13: i) BamHI fragments of γ M33 were subcloned by the shotgun method in the BamHI site of M13mp7 (22), ii) Sau3A fragments of the γ M33 subclone

py1/B.V1 (pBR322 containing the BamHI V1 fragment shown in figure 2) were shotgun cloned in the BamHI site of M13mp8, iii) random fragments generated by sonication of the plasmid py1/B.V1 were size selected by polyacrylamide gel electrophoresis, rendered blunt-ended by incubation with deoxyribonucleotide triphosphates and the large fragment of E. coli DNA Polymerase I, and ligated into the SmaI site of M13mp8 as described (23). In all three cases, recombinant plaques were screened by the Benton-Davis procedure using either γ M33 or the agarose gel purified BamHI V1 fragment as a probe.

DNA Sequencing

The dideoxy chain termination method of Sanger (24) was used to sequence M13 clones of interest, followed by either standard or buffer gradient 6% polyacrylamide gel electrophoresis as described (25).

DNA Sequence Analysis

DNA sequence analysis programs written for the Apple II microcomputer by Larson and Messing (26) and Fristensky *et al.* (27) were used to overlap and further analyze DNA sequences.

RESULTS

Repeated Elements in the γ 1 Switch Region

This study involved cloning the 16 kilobase EcoRI fragment (figure 2A) containing the γ 1 switch region from the BALB/c germline. Southern hybridization analysis of the clone obtained, designated γ M33, demonstrated a complex pattern of BamHI and PstI recognition sites within Sy1. The sites were mapped by the method of Smith and Birnstiel (28). The results (figure 2B) show that these sites occur frequently throughout this switch region, an observation suggestive of the presence of tandemly repeated sequences and consistent with previously published results (8, 13, 29).

The initial aim of our study was to assess the organization of repeated elements which characterize the γ 1 switch region. As a starting point for sequence analysis of Sy1, we chose the BamHI V1 fragment (figure 2, b and c).

The sequence of the V1 fragment is presented in figure 3. It is 773 bp in length and includes nearly 14 tandemly repeated copies of 49mer, not one of which diverges from the consensus sequence by more than 17%. In order to better understand the distribution and homology relationships among the 49mer elements repeated within Sy1, we used the 3' Sau3A fragment of V1 (figure 2c) as a probe of the cloned γ 1 S region in Southern hybridization

Figure 2. Restriction map of the germline $\gamma 1$ switch region clone $\gamma M33$. A. Selected restriction sites flanking the germline BALB/c $\gamma 1$ switch region; X, XbaI; H, HindIII; E, EcoRI (7,8). The hyphenated bold bar spans the DNA segment that hybridizes to the plasmid $\gamma 1$ /IF2P.1.8 (9), which contains approximately ten tandem copies of the $\gamma 1$ 49 base repeat unit. $\gamma 1$ coding sequences begin approximately 3 kb to the right of the 3' EcoRI site shown here. B. Restriction map of the germline $\gamma 1$ switch region clone $\gamma M33$. A deletion of greater than 2 kb was suffered by $\gamma M33$ in the course of large scale preparation, and is reflected by the open box. The top bar shows the EcoRI, XbaI and HindIII sites aligned with the known germline sites, as well as KpnI (K) and BglII (Bgl) sites. The PstI and BamHI maps appear on the bottom bar with respective fragments denoted by letter. C. Strategy for sequencing the germline BamHI V1 fragment. The three cloning strategies are described in Experimental Procedures. The arrows represent V1 subclones in $\gamma M33$. Asterisks denote Sau3A clones while the open circle marks a BamHI clone. All other clones were generated by the sonication procedure.

analysis. This probe, denoted mp9.MAS74, contains five copies of 49mer which are homologous to the consensus by an average of 97% (figure 3). The results of this experiment prove that 49mer sequences extend at least 2 kb 5' (BamHI X; PstI F and G) and at least 6 kb 3' (BamHI U, T and Y; PstI D1, C and a) to V1 (figure 4, lanes 3 and 4). However, high stringency wash conditions abrogate detection of the BamHI X and PstI F, G and a fragments (figure 4, lanes 1 and 2). In addition, mp9.MAS74 hybridizes poorly to the BamHI V2 fragment (between V1 and U in figure 4, lane 3), while the PstI A fragment (from the 3' end of Sy1; see figure 2b) hybridizes well to both the BamHI V2 and PstI F fragments (not shown). Taken together, these data imply: i) that 49mers in the central portion of Sy1 are more closely related to each other than to those at the 5' and 3' ends, and ii) that

Figure 3. DNA sequence of the germline Syl BamHI V1 fragment. The nucleotide sequence of the BamHI V1 fragment is aligned in a best fit manner relative to the consensus $\gamma 1$ 49 base repeat unit (9). Lines represent identity with the consensus; differences are replaced with the appropriate nucleotide, while deletions are reflected as blank spaces. Arrows indicate the Sau3A sites which delimit the insert in mp9.MAS74 (see figure 4 and text). The average homology of 49mers in this fragment were calculated, the standard deviation of which appears in parentheses.

these terminal sequences bear homology to each other. In fact, comparison of the repeated elements in the central part of Syl (figure 3) with those at the 3' end (30, 34) do show some notable differences (see Discussion).

Non-49mer Sequences in the $\gamma 1$ Switch Region

Interestingly, while hybridization results suggest that $\gamma 1$ 49mer unit sequences span the length of Syl, our sequence of the germline V1 fragment clearly demonstrates that unique sequences, bearing poor homology to the Syl 49mer, exist among these tandemly repeated elements. To assess the existence of multiple copies of the V1 non-49mer sequence within Syl, we undertook the construction of a probe consisting of this sequence but

Figure 4. Hybridization of V1 49mer and unique sequences to other segments of the γ 1 switch region. One microgram of p γ 1/EH.10.0 DNA was digested with either BamHI (odd numbered lanes) or PstI (even numbered lanes). Lanes 1 through 4 were hybridized with mp9.MAS74, a M13mp9 subclone of p γ 1/B.V1 (see text), while lanes 5 through 8 were hybridized with mp8.V13.2 (see figure 5). Both single stranded M13 probes were labeled to specific activities of greater than 10^7 cpm/microgram by the method of Hu and Messing (35). After hybridization at 65°C for 16 hours, the filters were washed in either 0.2xSSC, 0.5% SDS (sodium dodecyl sulfate) (lanes 1, 2, 5 and 6) or 6xSSC, 0.5% SDS (lanes 3, 4, 7 and 8) at 65°C for 3 x 30 minutes. Autoradiography was performed for 6 hours (lanes 1 through 4) and 7 days (lanes 5 through 8). The letter designations on the left side of the figure denote BamHI fragments while those on the right denote PstI fragments (see figure 2). The upper case T denotes a doublet of the T1 and T2 BamHI fragment. The lower case a denotes the S $_{\gamma$ 1-pBR325 PstI fusion fragment of p γ 1/EH.10.0 which corresponds to the PstI A fragment of γ M33. The bands marked by stars reflect hybridization of pBR325 with pBR322 sequences contained in mp9.

suitably free of 49mer sequences. The V1 subclone M13mp8.V13, generated by the sonication procedure, was utilized as the starting molecule to create a probe containing only non-49mer sequence from V1. The sequence of mp8.V13 and relevant restriction sites, as well as the details of the construction are shown in figure 5. We took advantage of the existence of SacI and BstEII sites, which cut within the non-49mer sequence, to generate a probe which included nucleotides 33 through 90 of mp8.V13 (figure 5a, underlined sequence). Note that the probe mp8.V13.2 is completely devoid of both pBR322 sequences and sequences closely related to the consensus 49mer.

Figure 5. Generation of a V1 non-49mer sequence hybridization probe. A. The top numbered lines show the nucleotide sequence of the V1 subclone mp8.V13 as well as relevant restriction sites. This clone contains greater than 250 bp of pBR322 (from p_{yl}/B.V1) and includes the 5' BamHI site of the V1 fragment. Nucleotide 153 represents the SmaI recognition site junction between the sonicated fragment and mp8. The underlined sequence denotes the insert in the derivative clone mp8.V13.2 (see B below). Nucleotide sequences derived from the BamHI X fragment are aligned below the mp8.V13 sequences in a best fit manner (see figures 2, 4 and text for discussion). B. Strategy for generating a hybridization probe devoid of 49mer sequences. The two-step procedure was performed as follows: 1. The replicative form of mp8.V13 was first digested with BstEII (Bs) and HincII (H). The linear molecule was rendered blunt-ended by incubation with the large fragment of *E. coli* DNA polymerase I, and circularized with T4 DNA ligase. The resulting clone was verified by sequence, and designated mp8.V13.1. 2. The replicative form of mp8.V13.1 was digested with EcoRI (E) and SacI (S), and rendered blunt-ended with mung bean nuclease. The resulting molecule was circularized by ligation, its sequence verified, and the clone designated mp8.V13.2. Enzymes were purchased from New England Biolabs and PL Biochemicals, with incubations performed as suggested.

The results of hybridization experiments using mp8.V13.2 as a probe of p_{yl}/EH.10.0 are shown in figure 4. Under conditions of moderate stringency (lanes 7 and 8), mp8.V13.2 detects both the BamHI V1 and PstI D2 fragments, as expected. In addition, however, it is apparent that related sequences exist in the BamHI X and corresponding PstI G fragments. Furthermore,

extended exposures in similar experiments reveal hybridization with Y and C as well. When high stringency wash conditions are employed (lanes 5 and 6), V1 and D2 hybridization persists, although X and G hybridization is drastically reduced. In conjunction with the 49mer hybridization data discussed above, these data indicate that non-49mer sequences related to those in the V1 fragment are interspersed among the tandemly repeated elements of Sy1. Indeed, sequence analysis of the 1.8 kb KpnI fragment containing the 5' portion of Sy1 has permitted us to identify the non-49mer sequences found in the BamHI X (and PstI G) fragment (figure 5a). With the exception of a 7 bp deletion and a single nucleotide insertion, the sequence found in X is 100% homologous to the 58 bp insert in mp8.V13.2, and greater than 80% homologous to the 100 bp stretch of non-49mer sequence from V1.

DISCUSSION

Homology Relationships among Sy1 49mers

We have determined the sequence of fourteen 49mer repeat elements from the central portion of the $\gamma 1$ switch region. Our results (figure 3) demonstrate that these 49mers are highly homologous to each other and to the consensus sequence derived by Dunnick *et al.* (9). In addition, hybridization experiments indicate that 49mers bearing similar homology to this consensus characterize the bulk of the $\gamma 1$ switch region (figure 4, lanes 1-4).

Kataoka *et al.* (11) described 500 bp of sequence from the 3' end of Sy1, and suggested that these sequences are arranged as tandemly repeated 49 bp elements. By comparing these 49mers pairwise the authors calculated an average homology of 46.4 ± 11.3 % for the set of twelve repeated units. In contrast to these data, our 49mers from the middle of Sy1 show a mean homology of 88.8 ± 5.5 % when compared in all pairwise combinations. Furthermore, a comparison of 49mer consensus sequences generated from the two data sets reveals differences at 8 positions (83.7% homology).

Our conclusion from this analysis of Sy1 repeat elements is that the average homology of a group of tandemly repeated 49mers reflects the location of this group within the switch region itself. Hence, the repeat elements we sequenced (from the middle of the Sy1 region) are very homologous to each other and yield a consensus sequence which may represent the majority of 49mers in the Sy1 region. On the other hand, the sequences derived by Kataoka *et al.* (from the 3' end of Sy1) are not closely related to each other and yield a different (though related) consensus sequence.

Figure 6. Germline DNA sequences flanking a $\gamma 1$ switch recombination site and comparison with S $\gamma 1$ non-49mer sequence from the BamHI V1 fragment. a. Rearranged S $\gamma 1$ sequences from the IgG1 myeloma MC101 (30, 34). Lower case letters denote sequence derived from S α ; the point of recombination is indicated by the asterisk. b. Germline S $\gamma 1$ sequences flanking the switch recombination site in MC101 (30, 34). c. Non-49mer sequence derived from the BamHI V1 fragment (residues 33-90 from figure 5a).

Interestingly, published data indicate a similar organization of repeated elements for Sy2b (11), Sy2a (13) and for Sy3 (11, 13, and unpublished data from this laboratory).

Novel Organization of Non-49mer Elements in S $\gamma 1$

Previous studies of cloned switch regions have suggested that they are composed solely of tandemly repeated unit sequences which vary in length from 5 to 80 bp (3, 9-13). In contrast to these findings, our results clearly demonstrate that the $\gamma 1$ switch region is characterized by an interspersion of significantly divergent sequences among its tandemly repeated 49mer elements. At the gross level, therefore, it appears that S $\gamma 1$ is organized in a different manner than the other switch regions.

In a recent report, Wu *et al.* (31) described a novel long identical repeat element (LIR) present in two copies in the $\gamma 2b$ switch region. Computer assisted analysis of all available switch region sequences demonstrated that this organization scheme was unique to Sy2b. Each LIR consists of approximately ten 49mers which bear a mean homology to the consensus Sy2b 49mer of $88.3 \pm 3.7\%$. In this light, the organization schemes of Sy2b and S $\gamma 1$ must be considered distinct since sequences unrelated to the 49mer form the very basis of S $\gamma 1$'s novel organization.

Taken together, the data pertaining to the structures of S $\gamma 1$ and Sy2b raise the point that structural differences among S regions may reflect differences at the level of function. Indeed, Wu *et al.* suggest that structural differences among S regions may confer isotype specificity to the mechanism of switch recombination. However, until each switch region is completely characterized at the nucleotide level and the mechanism of the switch is elucidated, differences in structural organization and their relation to function will remain difficult to assess.

What Role do Non-49mer Sequences Play in Switch Recombination?

The demonstration of non-49mer sequences in the $\gamma 1$ switch segment is particularly interesting in the respect that a heretofore unrecognized factor may be involved in generating the switch recombination. Our working hypothesis has been that while tandemly repeated elements may facilitate switch region pairing, unique sequences might contribute the actual site of recognition and/or action for the switch recombinase(s). This hypothesis is well supported in the case of switches involving S_{μ} , since switch recombinations in the S_{μ} segment usually occur in the random 5' flanking sequences, and not in the tandemly repeated elements (32). Of the recombination sites in S_{γ} sequences reported to date, one is in completely random sequences (MOPC141, ref. 3) and four are in regions with very poor homology to 49mer consensus sequence (MPC11 nonexpressed gene, MPC 11 expressed gene, and MC101 expressed gene, refs. 8, 33 and 34). These recombination sites, in $S_{\gamma 3}$, $S_{\gamma 2b}$ and $S_{\gamma 1}$ segments, occur in regions with 75% or poorer homology to the consensus sequence of the repeat element, even though the average homology of unit repeats is nearer to 90% (this paper, refs. 8-13).

As a means of evaluating our hypothesis, we examined the sequences flanking the site of $\gamma 1$ switch recombination in the IgG1 myeloma MC101, as reported by Obata *et al.* (34). In figure 6 the germline and corresponding rearranged $S_{\gamma 1}$ sequences from MC101 are aligned relative to the V1 non-49mer sequences. The underlined and overlined sequences represent stretches of homology between germline $S_{\gamma 1}$ sequences upstream of the recombination site and a core portion of the V1 non-49mer sequence. Although a precise homology calculation is complicated by the insertion of 8 bp, the overall match between the sequences upstream of the MC101 switch recombination site and the core sequence remains impressive. While this information regarding the functional significance of the $S_{\gamma 1}$ non-49mer sequence, in particular its 20 bp core, is obviously limited, other bits of circumstantial evidence weigh in its favor. First, even though it is unrelated to the $S_{\gamma 1}$ 49mer repeat, the core sequence contains the GAGCTG oligonucleotide implicated in switch recombination (13). In addition, if the core sequence is important for switch recombination, one would anticipate its conservation among the non-49mer sequence segments interspersed among 49mers in $S_{\gamma 1}$. In fact, the sequence obtained from the BamHI X fragment (figure 5) demonstrates complete conservation of this core, despite a 7 bp deletion upstream and a single nucleotide insertion downstream. Finally, one might argue that this

recombination in MC101 is unusual in that S_{α} and not S_{μ} has been joined to $S_{\gamma 1}$. However, until a μ to $\gamma 1$ switch recombination site is completely characterized, the significance of the MC101 α - $\gamma 1$ switch will remain unclear.

The hypothesis that our $S_{\gamma 1}$ non-49mer core sequence plays a role in switch recombination, at least of the $\gamma 1$ class, affords several testable predictions: i) the core sequence should be conserved among other $S_{\gamma 1}$ non-49mer segments, if these can serve as functional switch loci, and ii) switch recombination sites will be found in other copies of the non-49mer segment within $S_{\gamma 1}$. Completion of the germline $S_{\gamma 1}$ sequence and analysis of switch recombination sites from more IgG1 myelomas and hybridomas should serve to clarify these issues.

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