The D-J<sub>H</sub> complex is an intermediate to the complete immunoglobulin heavy-chain V-region gene

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Received 21 September 1983; Accepted 11 October 1983

ABSTRACT

We have examined the organization of the immunoglobulin J<sub>H</sub> segments in three clones derived from a single Abelson murine leukemia virus-transformed cell. Cloning and nucleotide sequence analyses of the J<sub>H</sub>-containing fragments have revealed the rearrangement from the preformed D-J<sub>H</sub> complex to the complete V<sub>H</sub>-D-J<sub>H</sub> gene, which was accompanied by the expression of the intra-cytoplasmic V chain. In one case a J<sub>H</sub> segment downstream to the preformed D-J<sub>H</sub> was used to create a new V<sub>H</sub>-D-J<sub>H</sub> gene. Upon the D-J<sub>H</sub> and V<sub>H</sub>-D-J<sub>H</sub> rearrangements the intervening D segments were deleted from the chromosome. One of the expressed V<sub>H</sub> genes suffered from a large deletion of the 3' portion (including the 95th cysteine residue) of the V<sub>H</sub> segment. We discuss the possible mechanism of the allelic exclusion.

INTRODUCTION

An immunoglobulin heavy-chain variable region (V<sub>H</sub>) gene is comprised of three segments, namely the V<sub>H</sub>, D and J<sub>H</sub>, which are separated from each other in the germline genome (1,2,3). On the other hand, the light-chain variable-region (V<sub>L</sub>) gene is comprised of two germline segments, V<sub>L</sub> and J<sub>L</sub> (4,5). It is demonstrated by the nucleotide sequence comparison that the heptamer and nonamer sequences are conserved immediately 3' and 5' to the germline V and J segments, respectively, and at both sides of D segments (1). The conserved sequences located 3' to the V<sub>H</sub> and D segments can form stable complementary base pairs with ones placed 5' to the D and J<sub>H</sub> segments, respectively. The spacers between the heptamer and the nonamer are 23+1 bp in the germline V<sub>H</sub> and J<sub>H</sub> segments, and the D segments have the conserved sequences with a 12-bp spacer on each side. It was proposed that a putative recombinase has two functional units, one recognizing the heptamer and nonamer separated by the shorter (12-bp) spacer and the other recognizing the same signal sequences separated by the longer (23-bp) spacer (1,2).

During B lymphocyte differentiation the V<sub>H</sub>-D-J<sub>H</sub> recombination proceeds
the V<sub>L</sub>-J<sub>L</sub> recombination (6,7). Since many T lymphocytes have undergone D-J<sub>H</sub> rearrangements (8), the initiation of the V<sub>H</sub>-D-J<sub>H</sub> recombination is close to the segregation of the T- and B-lymphocytes during ontogeny. Although the triggering of the V<sub>H</sub>-D-J<sub>H</sub> recombination is a key step of B-cell differentiation, little is known about the molecular mechanism of the V<sub>H</sub>-D-J<sub>H</sub> recombination.

One of the most powerful approaches to this goal is the establishment of the pre-B cell line transformed by the Abelson murine leukemia virus (A-MuLV) (7,9,10). The A-MuLV-transformed pre-B cell lines were shown to rearrange the J<sub>K</sub> and J<sub>H</sub> segments while grown in culture. Cloning and characterization of the rearranged J<sub>K</sub> segments revealed new insights into the molecular mechanism of the V<sub>K</sub>-J<sub>K</sub> joining -- the structure of the rearranged J<sub>K</sub> segments was most easily explained by inversion and deletion mechanism (9,11).

Alt et al. (7) have shown that most pre-B cell lines that do not produce any immunoglobulin have rearrangements of both J<sub>H</sub> segments and suggested that such rearrangement might be D-J<sub>H</sub>. In another A-MuLV transformed cell line they have found that D-J<sub>H</sub> is invertedly fused to J<sub>H3</sub> and proposed that D-J<sub>H</sub> joining is an intermediate in the V<sub>H</sub>-D-J<sub>H</sub> assembly process (12). However, the direct evidence to prove the above assumption requires analyses of the cell lineage that rearranges from D-J<sub>H</sub> to V<sub>H</sub>-D-J<sub>H</sub>.

Previous analyses of A-MuLV transformants have shown that the cell clone AT11-2-5-1-5(P) has rearrangements of two J<sub>H</sub> segments though the cell clone does not produce any immunoglobulin (10). Its variant subclones, AT51-1(S) and AT10-1(C) synthesize the intra-cytoplasmic w chain, accompanied by further rearrangements of J<sub>H</sub> segments. In this report, we have cloned all the J<sub>H</sub> segments of the cell clone and subclones, P, S, and C, and determined nucleotide sequences surrounding the J<sub>H</sub> segments. The results have demonstrated unequivocally that the formation of the V<sub>H</sub>-D-J<sub>H</sub> gene proceeds via the D-J<sub>H</sub> complex in the A-MuLV-transformed pre-B cell lines.

MATERIALS AND METHODS

Cells

The cloned A-MuLV transformed pre-B cells, AT11-2-5-1-5, AT51-1 and AT10-1, which we refer to P, S and C, respectively, in this report, were isolated as described (10).
Southern Blotting

High molecular weight genomic DNA of each cell clone was prepared as described (13). Restriction endonuclease digests of genomic DNA were electrophoresed through a 0.6% agarose gel at 250V for 2hr. DNA was transferred to a nitrocellulose filter according to the method of Southern (14), and hybridized to appropriate nick-translated probes (13).

DNA Cloning

The EcoRI fragments containing the rearranged J_H segments of each cell clone were isolated by the preparative agarose gel electrophoresis, and ligated with λgtWES vector with T4 ligase as described (15). The hybrid DNA was packaged in vitro and the recombinant phages were screened by using the J_H4 fragment (16) as probe according to the method of Benton and Davis (17). Cloning experiments were carried out in accordance with the Japanese Guideline for Recombinant DNA experiments.

Nucleotide Sequencing

DNA sequence determination was performed according to the method of Maxam and Gilbert (18).

RESULTS

Cloning of the Rearranged J_H Fragments in the A-MuLV Transformants

The pre-B cell clone P, which does not produce any immunoglobulin protein was established by the injection of A-MuLV into newborn BALB/c mice. The subclones S and C are variants derived from the cell clone P and were previously shown to produce the ι chain in the cytoplasm (10). We ascertained by Southern blotting hybridization with the A-MuLV probe (19) that the integration sites of A-MuLV are identical in the cell clones P, S and C, showing that these clones are derived from a single transfectant (Fig. 1). The clone P has two EcoRI fragments (11 and 5 kb) of the J_H segment while the EcoRI fragment of the germline J_H segment is 6.6 kb as shown previously (10). The subclone S contains a single EcoRI fragment (5 kb) of the J_H segment which seems to be a doublet as assessed by the relative intensity of hybridization and the subclone C contains two EcoRI fragments (6.5 and 2.5 kb).

We fractionated EcoRI digests of DNAs derived from the clone P and subclones S and C by agarose gel electrophoresis and purified the fragments containing the J_H segments. The separated fragments were ligated to λgtWES vector and recombinant phages (2x10^6) of each fragment were screened with the ^32p-labeled J_H probe except that only 5x10^5 phages of the 2.5 kb
Fig. 1 A-MuLV integration sites in the cell clones P, S and C.

Total DNA from the cell clone was digested with BamH1 (lanes 1 through 4) or HindIII (lanes 5 through 8) and electrophoresed in a 0.6% agarose gel. A Southern blot was then hybridized to the nick-translated v-abl-containing BglII fragment (1.5 kb) of pYJ1 (19). Origins of DNA in each lane is as follows; lanes 1 and 5, BALB/c liver; lanes 2 and 6, P; lanes 3 and 7, S; lanes 4 and 8, C. The sizes of the bands are indicated in kb.

A fragment of the subclone C were screened. We isolated four and eight recombinant phages containing 11- and 5.0-kb fragments, respectively, of the clone P. Nine recombinant clones were obtained from the 5.0-kb fraction of the subclone S. Five and one phage clones were isolated from 6.5- and 2.5-kb fractions, respectively, of the subclone C. All the clones derived from

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<td>λgtWES<em>M</em>Ig*J_H-C5 (C5)</td>
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a, Abbreviated names are shown in parentheses.
Fig. 2  Restriction maps of the cloned JH segments.
Horizontal lines show the cloned JH segments. Smaller segments containing structural sequences are enlarged below each clone. Sequenced VH segments are indicated by open rectangles, and broken rectangles show the estimated position of VH segments. Closed and dotted rectangles represent D and rearranged JH segments, respectively. The directions and ranges of the sequences read are shown by horizontal arrows. The horizontal bar A is a probe used for the 5' flanking region to the Dsp2.8 (Fig. 5). Restriction sites are abbreviated as follows: E, EcoRI; X, XbaI; S, SacI; B, BamHI; H, HindIII; Bg, BglII; D, DdeI; P, PstI; Si, SinI; Ha, HapII.

Each fraction contain the same EcoRI fragment except for the 5.0-kb fraction of the subclone S, from which 2 kinds of clones were obtained. We refer to the phage clones derived from the 11- and 5.0-kb fragments of the cell clone P as λgtWES'M·Ig'JH-P2 (P2) and λgtWES'M·Ig'JH-P6 (P6), respectively. Two kinds of the recombinant phages containing the 5.0-kb fragment of the subclone S were designated as λgtWES'M·Ig'JH-S4 (S4), and λgtWES'M·Ig'JH-S9 (S9). The phage clones λgtWES'M·Ig'JH-C2 (C2) and λgtWES'M·Ig'JH-C5 (C5) were obtained from the 6.5- and 2.5-kb fragments, respectively, of subclone S.
C. Cloned phages were summarized in Table 1.

The restriction maps of all the phage clones were constructed by digestion with various combinations of the restriction enzymes as shown in Fig. 2. The restriction maps and Southern blot hybridization analysis have revealed that the clone S4 is the same as the clone P6. The recombination sites in each clone were estimated by comparison with the restriction map of the germline JH segment, and nucleotide sequences surrounding the recombination regions were determined according to the sequence strategy shown (Fig. 2).

The JH Segments of the Clone P

The nucleotide sequence of the phage clone P2 not only has the JH3 segment rearranged immediately after the heptameric signal, but also contains new recombination signals (heptamer and nonamer) separated by the 12-bp spacer 15 bp upstream of the rearrangement site (Fig. 3). There are two termination codons in frame with the JH3 segment within the 150-bp region 5' to the recombination site. The results suggest that the clone P2 contains the D-JH3 form. A very similar structure was found in the clone P6. When compared with the germline JH3 sequence the recombination site is in the JH3 segment which is also flanked by new heptamer and nonamer signals separated by the 12-bp spacer, suggesting that the clone P6 also contains the D-JH form. There are two termination codons in the 50-bp region 5' to the rearrangement site (Fig. 4A).

Comparison of the restriction map of the 5' flanking region of the clone P2 with that of the Dsp2 family (8) indicates that the rearranged D of the clone P2 seems to be Dsp2.8. The nucleotide sequence of the clone P2 matches with that of the Dsp2.8 except for one base change in the coding region and one base insertion previously designated as N region (12, Fig. 3). The D segment of the clone P6 is derived from DFL16,1, judging from the homology of the nucleotide sequences in the 5' flanking and coding regions.

Fig. 3 Nucleotide sequences of the rearranged JH regions of the clones P2 and S9.

The nucleotide sequences of the clones P2 and S9 are aligned with germline VH101, D and JH segments (2, 8). Amino acid sequence deduced from the nucleotide sequence is indicated at the bottom line of each row by one-letter code. Vertical lines represent the borders of the homology between the sequences. The conserved nonamer and heptamer of the putative recognition signal are underlined. The dotted region is not sequenced. The arrow heads show nucleotides of S9 different from those of the germline VH101. The heptamer like sequence at the 3' portion of the germline VH101, and termination codons in frame with the JH segment are marked with the broken and overhead lines, respectively.
A)  

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**100**

**AGA6AGAGACTACGCTGAGGCTTTCTGGATACCTACGAGGCTACCCAGACGATAGCTGCTTATACACGAGCTACCCAGACGATAGCTG**

**200**

**CEGFRWFYRDISQSLYLMWNAELRAEDNYEC**

**70 80 90**

**ARITTVDYAMDYWGQGTGSVTYSS**

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**100 110**

**OERMLINE JHH**

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(Fig. 4A). P6 has three base long N region. DFL16.1 and Dsp2.8 are the most 5' and 3' member, respectively, of the D cluster located 19.5 kb 5' to the JH1 (8,20).

The JH Segments of the Subclone S

The clone S4 is identical to the clone P6 as described above. The nucleotide sequence of the clone S9 is homologous to that of the clone P2 in the D-JH3 region, but different upstream of the D segment. The comparison between the nucleotide sequence of the clone S9 and published sequences of VH genes has revealed that the 5' portion of the clone S9 is most similar to the germline VH101 (3) although there are scattered four base replacements in the VH segment (Fig. 3). The restriction sites of EcoRI and HindIII in the 5' flanking region of the clone S9 agree with those of the germline VH101. It is, therefore, clear that the complete VH gene of the clone S9 was created by the recombination of the germline VH101 to the preformed Dsp2.8-JH3 segment of the clone P2. In addition, the results indicate that somatic mutation takes place at the pre-B cell stage.

It is often found that the 3' terminal few bases of the germline VH segment (1, 2, 3) is missing from the complete VH gene. Although the VH gene of the clone S9 has an open reading frame from the leader sequence through the JH segment, the last 20 bp of the germline VH101 segment are deleted. Since the S9 VH gene does not have cysteine at residue 95, it can not form the intra-domain disulfide bond. We will discuss later about this deletion.

The JH Segments of the Subclone C

The nucleotide sequence of the clone C5 is homologous to that of the clone P6 in the D-JH3 region but different upstream of the D segment. The clone C5 has an open reading frame, the amino acid sequence of which is homologous to that of the VH region of the myeloma TEP601 between residues 68-98 except for four amino acid residues dispersed in the region (21). Note that six bases are deleted at the 5' end of the D segment. It is

Fig. 4 Nucleotide sequences of the rearranged JH regions of the clones P6, C5, and C2.

A) Nucleotide sequences of the JH and D segments of P6 and C5, and B) nucleotide sequence of the JH segment of C2. The germline JH and D sequences are shown for comparison (2,8). Conserved nonamers and heptamers were underlined. Recombination sites are indicated by vertical lines. Nucleotide sequence of the VH segment of C2 was translated into amino acid sequence by the omission of G base indicated by an arrow head. Termination codons in frame with the JH segment are lined above.

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likely that the $V_H$ gene of the clone C5 was formed by the fusion of a germline $V_H$ segment ($V_{HC5}$) and the preformed $D_{FL16.1-JH3}$ of the clone P6, accompanied by the deletion of the 5' six bases of the $D_{FL16}$ segment. The clone C5 must be expressed as a $\mu$ chain as the clone C2 is abortively rearranged as described below.

The nucleotide sequence of the clone C2 contains the recombined $V_H-D-J_{H4}$ gene which does not have the common sequence with the D-J segment of either P2 or P6 (Fig. 4B). The D segment used in C2 seems to belong to the $D_{FL16}$ family as assessed by the nucleotide sequence comparison (8).

There are two termination codons in the $V_H$ segment in phase with the $J_{H4}$ sequence. The omission of a G base at the junction of the $V_H$ and D segments of the clone C2 would yield an open reading frame of the $V_{JJ}$-like sequence, the sequence of which resembles that of the clone C5 with 63% and 66% homology in terms of amino acid and nucleotide sequences, respectively.

Since C5 is derived from P6, C2 is most likely to be the descendant of P2 in the subclone C. If so, the germline $V_H$ ($V_{HC2}$) and a D segment were recombined to the $J_{H4}$ segment, skipping over the preformed D-J$H3$ complex.

Deletion of the D Segment Cluster in the $\mu$-positive Subclones

To determine whether the $V_H-D$ rearrangement involves the deletion of the intervening DNA segment, DNA from each cell line was digested with EcoRI and analyzed by Southern blot hybridization. The $^{32}$P-labeled SacI/PstI fragment (600bp) of the clone P2 (probe A in Fig. 1) seems to hybridize to
all the members of the Dsp2 family due to the mutual homology because the hybridized bands in the BALB/c liver DNA represent all the EcoRI fragments of the germline Dsp2 segments (Fig. 5). The flanking sequences (40 bp) of the D segments in the Dsp2 family are highly conserved at both ends (8). The 11-kb fragment corresponding to the clone P2 is present in DNA of the cell clone P instead of the 17.5-kb fragment containing the germline Dsp2.8. By contrast, no bands are found in DNAs of the subclones S and C. If the deletion model is correct, the joining of Dp116.1 and JH should remove all the Dsp2 segments from the chromosome because the Dp116.1 is 5′ to the Dsp2 family (8). Naturally, the deletion model expects the disappearance of unused members of the Dsp family which is located between the VH family and the JH segments from the chromosome that undertook the VH-D-JH recombination. The results obtained are consistent with the deletion mechanism for the VH-D-JH rearrangement. The mechanism of VH-D-JH rearrangement might be different from that of VL-JL rearrangement since in the latter case the reciprocal recombination products remain in the myelomas (22, 23).

DISCUSSION

Sequential Rearrangement from D-JH to VH-D-JH

We have clearly demonstrated that a Dsp2.8-JH3 complex (P2) of the clone P is converted to VH101-Dsp2.8-JH3 (S9) of the subclone S as summarized in Fig. 6. Similarly, the other allele DpL16.1-JH3 (P6) of the clone P is rearranged to VHCS-DpL16.1-JH3 (C5) in a different subclone C. Both V-D-J genes are expressed as µ chain (10). The results unequivocally demonstrate that the D-JH complex can serve as an intermediate for the VH-D-JH formation although we do not know whether or not an alternative pathway which includes the VH-D complex as an intermediate coexists.

We have also shown that the preformed Dsp2.8-JH3 complex was skipped over by another VH and D to form a novel VH-D-JH4 gene, resulting in the deletion of the preformed D-JH3. The results indicate that the D-JH fusion does not provide any stop signal for further D-JH rearrangement. The transition from P2 to C2 requires at least two recombinational events. Since we have not detected the rearrangement of the VHCS segment in the cell clone P (data not shown), the preexisting VHCS-D in the clone P is unlikely.

Deletion of Intervening DNA Segments upon V-D-J Recombination

We have demonstrated that the intervening D segments were deleted upon both D-JH and VH-(D′JH) joinings. Unlike VK-JK recombination in which a
significant portion of the intervening segment is retained (22, 23) the intervening segment of \( V_H - D - J_H \) recombination is not retained although an example of inverted \( D - J_H \) joining was demonstrated (12). Our present analyses are consistent with the deletion model for both \( V_H - D \) and \( D - J_H \) recombination and suggest that different enzymic systems operate for \( V_L - J_L \) and \( V_H - D - J_H \) recombination.

The large deletion of the \( V_{H101} \) gene in the subclone S9

The deletion of the several base-pairs at the joining region is generally observed in the mouse \( \lambda \) chain (24) and heavy chains (1, 2, 3). But the deletion of the 20 bp of the 3' portion in the germline \( V \) segment is not known. It is worth noting that this deletion in S9 removed cysteine at residue 95 which is conserved in every immunoglobulin \( V_H \) region and necessary to form the domain structure of the \( V_H \) region. It is not known whether or not the deletion is due to an error of the recombinational machinery. We would like to point out that the \( V_{H101} \) sequence (TACTGTG) at positions 455-460 is similar to the conserved heptameric signals. The deletion might be due to the misrecognition of the above sequence by recombinational machinery. We consider it unlikely that S9 used another germline \( V \) segment similar to the \( V_{H101} \) segment which had deleted the 3' portion of the \( V_{H101} \) because of the homology of the DNA sequence and restriction map.
between the V gene of S9 and the germline V_{H101} gene. We cannot exclude the possibility of the cloning artifact completely but this is also unlikely because we do not find any repetitive sequences, or direct or inverted repeat surrounding the deleted region in the germline V_{H101} sequence.

**Mechanism of Allelic Exclusion**

Several models were proposed to account for the mechanism that allows expression of a single H chain protein in a lymphocyte. There are at least four biochemical steps that are essential to the differentiation of the stem cell to the pre-B cell. First of all, the recombinational machinery is activated to form the D-J_{H} rearrangement. Secondly, the complete V_{H} gene is created. Then, the V_{H}-C_{H} gene is transcribed and the ϖ transcripts are processed into the mature mRNA. Finally, the mRNA is translated into the ϖ chain. If there were no negative feed-back to the "recombinase" activity, every germline D segment would disappear to form V_{H}-D-J_{H} complexes on both chromosomes. This is not the case. Most pre-B, B and myeloma cells have J_{H} rearrangements in both chromosomes, one of which is expressed as heavy-chain protein (7,10). The J_{H} segment of the inactive chromosome is in the form of D-J_{H} (25) or abortively rearranged V_{H}-D-J_{H} (Fig. 4B). The results suggest that the production of the ϖ chain protein provides a signal to terminate further rearrangement of the V_{H} locus. The subclone S which produces the ϖ chain has a stable D-J_{H} form in the other chromosome in agreement with the above assumption that the ϖ chain production gives the termination signal for the V_{H} rearrangement. The ϖ chain produced by the subclone S has a large deletion in the V_{H} segment, resulting in a loss of intra-V domain disulfide bond. This results may suggest that the C_{H} portion of the ϖ chain may play an important role for the termination signal of V_{H}-D-J_{H} recombination. Since the clone P has undergone further rearrangements, there is no doubt about that the D-J_{H} rearrangement per se is unable to terminate the V_{H} rearrangement.

**ACKNOWLEDGEMENT**

We thank Dr. D. Baltimore for pYJ1 and Miss K. Kawabata for preparation of the manuscript. This work was supported by Grant-in-aid-for Special Distinguished Research.

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