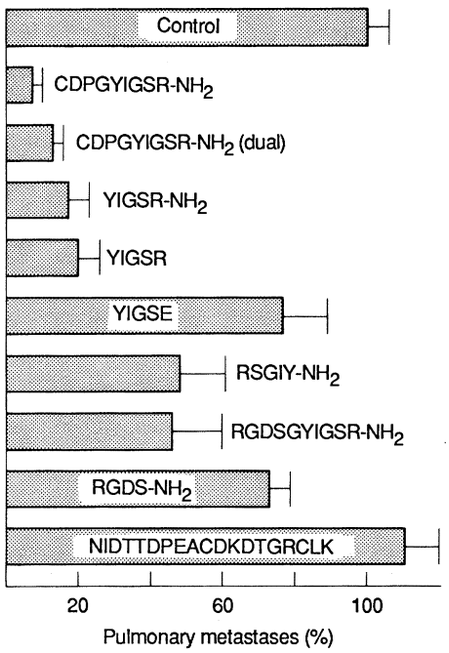

Fig. 5. Inhibitory effect of synthetic peptides on the formation of lung tumors. Peptides were solubilized at either 2 mg/ml (YIGSR) or 10 mg/ml (other peptides) in PBS and injected into mice as described (Fig. 3). Control mice received the same amount of cells and PBS without the peptide. In this group given dual injections of cells plus CDPGYIGSR-NH_2, mice were injected sequentially with melanoma cells via one tail vein and the peptide via the other. Each treatment and control group consisted of eight mice. Two weeks after the injection, mice were killed, lungs were removed, and the number of lung tumors was counted visually. At the time of autopsy, no extrapulmonary tumors were found. In the control mice (lacking the peptide), the average number of tumors was 60. Bar represents standard error of the mean.

laminin caused a fourfold increase in collagenase production (*18*). Taken together, we speculate that YIGSR may inhibit lung tumor colony formation by competing with laminin for the laminin receptor on tumor cells, thus blocking the binding of the cells to basement membranes. The fibronectin/vitronectin cell attachment peptide GRGDS also shows inhibitory activity in tumor cell colonization (*15*). The YIGSR sequence is specific to laminin whereas the RGDS sequence is present in over a hundred proteins (*20*). Since cells interact with both of these proteins via separate and specific receptors, it is likely that these peptides block tumor cell colonization by different mechanisms.

REFERENCES AND NOTES

1. R. H. Timpl *et al.*, *J. Biol. Chem.* **254**, 9933 (1979); J. E. Engel *et al.*, *J. Mol. Biol.* **150**, 97 (1981); H. K. Kleinman *et al.*, *J. Cell. Biochem.* **27**, 317 (1985).
2. H. L. Malinoff, J. P. McCoy, J. Varani, M. S. Wicha, *Int. J. Cancer* **33**, 651 (1984); P. M. Hand, A. Thor, J. Schlom, C. N. Rao, L. A. Liotta; *Cancer Res.* **45**, 2713 (1985); L. A. Liotta, *Am. J. Pathol.* **117**, 339 (1984); J. P. McCoy, R. V. Lloyd, M. S. Wicha, J. Varani, *J. Cell Sci.* **65**, 139 (1984).
3. V. P. Terranova, L. A. Liotta, R. G. Russo, *Cancer Res.* **42**, 2265 (1982).
4. V. P. Terranova, J. E. Williams, L. A. Liotta, G. R. Martin, *Science* **226**, 982 (1984).
5. T. Turpeeniemi-Hujanen, U. P. Thorgeirsson, C. N. Rao, L. A. Liotta, *J. Biol. Chem.* **261**, 1883 (1986).
6. V. P. Terranova, C. N. Rao, T. Kalebic, I. M. Margulies, L. A. Liotta, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 444 (1983); M. L. Malinoff and M. S. Wicha, *J. Cell Biol.* **96**, 1475 (1983).
7. S. H. Barsky, C. N. Rao, J. E. Williams, L. A. Liotta, *J. Clin. Invest.* **74**, 843 (1984).
8. C. C. Howe and D. Dietzschold, *Dev. Biol.* **98**, 385 (1983); D. P. Barlow, N. M. Green, M. Kurkinen, B. L. M. Hogan, *EMBO J.* **3**, 2355 (1984); M. R. Paulsson *et al.*, *ibid.* **4**, 309 (1985).
9. M. Sasaki, S. Kato, K. Kohno, G. R. Martin, Y. Yamada, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 935 (1987).
10. The following abbreviations are used for amino acids: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.
11. J. Graf *et al.*, *Cell* **48**, 989 (1987).
12. A. Albini *et al.*, *Cancer Res.* **47**, 3239 (1987); H. K. Kleinman *et al.*, *Biochemistry* **25**, 312 (1986).
13. I. J. Fidler, *Nature (London) (New Biol.)* **242**, 148 (1973).
14. J. Graf *et al.*, *Biochemistry*, in press.
15. M. J. Humphries, K. Olden, K. M. Yamada, *Science* **233**, 467 (1986).
16. S. K. Akiyama and K. M. Yamada, *J. Biol. Chem.* **260**, 4492 (1985); S. S. Brown, H. L. Malinoff, M. S. Wicha, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5927 (1983); S. M. Barsky, C. N. Rao, L. A. Liotta, *Breast Cancer Res. and Treat.* **4**, 181 (1984).
17. Y. Iwamoto, unpublished data.
18. H. K. Kleinman, unpublished data.
19. R. Reich, unpublished data.
20. E. Ruoslahti and M. D. Pierschbacher, *Cell* **40**, 517 (1986).

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Two Pairs of Recombination Signals Are Sufficient to Cause Immunoglobulin V-(D)-J Joining

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The minimum sequence requirements for antigen receptor V-(D)-J joining were studied by constructing recombination-substrates containing synthetic recombination signals and introducing them into a recombination-competent pre-B cell line. Two sets of heptamer (CACTGTG) and nonamer (GGTTTTTGT) sequences were shown to be sufficient to cause the V-(D)-J joining, if the 12– and 23–base pair spacer rule is satisfied. A point mutation in the heptamer sequence, or a change in the combination of the two spacer lengths, drastically reduced the recombination.

COMPLETE IMMUNOGLOBULIN (IG) and T cell receptor (TCR) variable region genes are generated by a somatic DNA rearrangement process, which assembles variable (V), diversity (D), and joining (J) gene segments during the differentiation of lymphocytes (*1–5*). Two blocks of sequences, a heptamer CACTGTG and a nonamer GGTTTTTGT are highly conserved adjacent to the germline V, D, and J segments (*6, 7*). The joining takes place between two pairs of recombination signal sequences (RSS's); one pair is separated by a 12–base pair (bp) spacer and the other by a 23-bp spacer (*8, 9*). Three approaches have been taken to study the molecular mechanism of V-(D)-J joining. One approach is the sequence analysis of the recombination region in the V, D, and J segments. Both Ig and TCR genes were shown to contain the heptamer and nonamer sequences separated by a spacer of constant length (Table 1). The second approach is the biochemical characterization of the enzymatic machinery responsible for V-(D)-J joining. It is assumed that at least three activities are needed in the V-(D)-J joining reaction: a DNA binding activity, an endonucleolytic activity, and ligase activity. Candidates for the endonucleolytic activity (*10–13*) were identified in pre-B cells. The third approach is the introduction of artificial recombination substrates with appropriate selectable markers into recombination-competent pre-B cells. Alt and Baltimore and their colleagues (*14, 15*) demonstrated that V-(D)-J joining could take place on exogenous genes. Lewis *et al.* (*14, 16*) identified V-J joining by DNA inversion between the exogenous V_κ and J_κ genes with a retroviral vector system (*17*). Blackwell and Alt (*15*) demonstrated that Ig heavy chain D-J joining occurred by DNA deletion on a plasmid vector introduced by DNA transformation. Yancopoulos *et al.* (*18*) found that even transfected TCR genes could rearrange in the pre-B line 38B9 (*15, 19*).

To define the minimum sequence requirements for the V-J type of recombination, we synthesized two oligonucleotides containing a pair of recombination signals, a heptamer and a nonamer; one pair was separated by a

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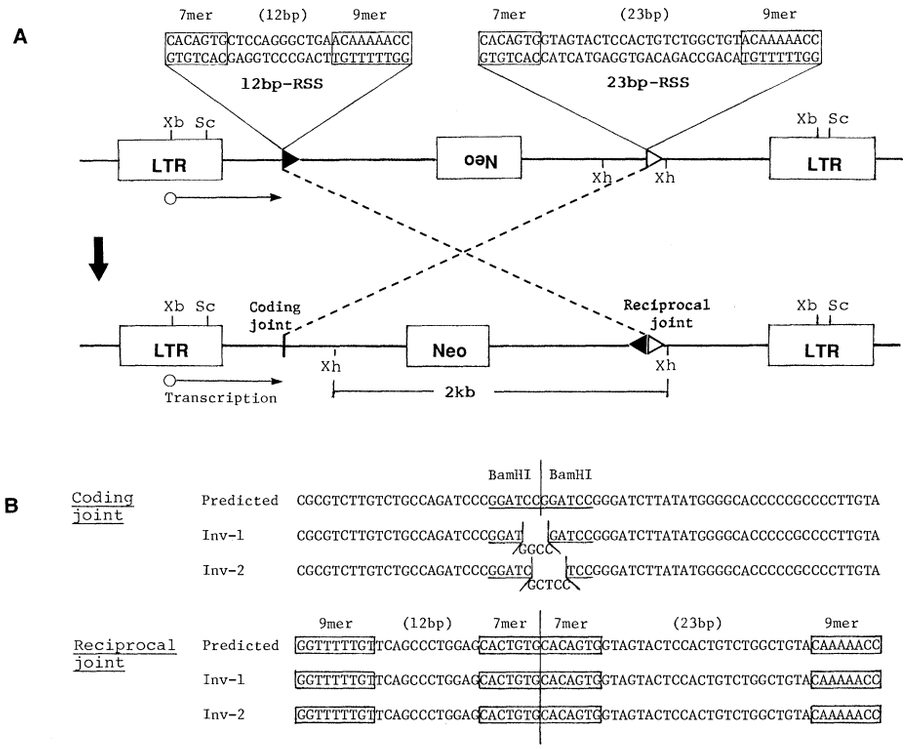


Fig. 1. Inversion recombination with two pairs of synthetic V-J joining signals. (**A**) Structure of inversion substrate. A 2.1-kb Bam HI–Eco RI fragment containing the *neo*^{r} gene (*20*) flanked by 12-bp (▶) and 23-bp (▷) RSS's was inserted into the retroviral vector pZIPneoSV(X)1 (*21*), using Bam HI and Xho I sites. In the substrate, sequences for the 12-bp and 23-bp RSS's were taken from the mouse Ig V_κ21C and J_κ1 genes, respectively (*6*). The 12-bp RSS and the promoter-deleted *neo*^{r} gene were placed in an inverse orientation relative to the LTR transcription. Restriction sites for Xba I (Xb), Xho I (Xh), and Sac I (Sc) are indicated. (**B**) Detection of inversion recombination. The substrate plasmid and hygromycin B phosphotransferase gene (*32*) containing plasmid, pY3, were cotransfected into a mouse fibroblast cell line, ψ2, by a calcium-phosphate DNA transformation method (*33*). DNA-transformed ψ2 cells were selected with hygromycin B (Calbiochem) (150 μg/ml) for 2 weeks. After the selective media were removed, a recombination-competent pre-B cell line, 38B9 (*15*), was cocultured with the DNA-transformed ψ2 cells for 2 days. In coculture, pre-B cells were infected with the substrate containing M-MuLV particles released from ψ2 cells. The culture was distributed to 24-well culture plates and incubated for 2 weeks in the presence of G418 (Gibco) (*22*) at a concentration of 2 mg/ml. Cells with the Neo^R phenotype were studied by Southern hybridization for DNA rearrangement, with the *neo*^{r} gene used as a probe. All the DNA samples tested gave a 2.0-kb Xho I band, which was generated by DNA inversion. Nucleotide sequences at both coding joint and reciprocal joint were analyzed for two separate samples, Inv-1 and Inv-2. Reciprocal joints have a head-to-head structure of two RSS oligomers, as predicted, whereas nucleotide deletions and additions occur at the coding joints. It is of interest to know whether the particular 38B9 cells used in the study have the terminal deoxynucleotidyl transferase (TdT) activity, because the TdT is thought to be responsible for the nucleotide addition at the coding joint (*34*).

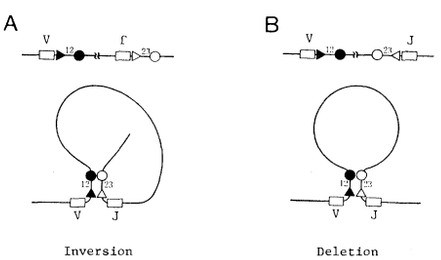


Fig. 2. Schematic diagram of V-J joining by DNA inversion (**A**) or deletion (**B**). Coding sequences, V and J, are in boxes. Heptamers (▲ and △), nonamers (● and ○), and spacer lengths (12 and 23) are also indicated. When the two RSS's are arranged in the same orientation, recombination occurs by DNA inversion (A). Recombination takes place by DNA deletion (B) when the two RSS's are arranged in a head-to-head fashion.

12-bp spacer and the other by a 23-bp spacer (the 12-bp and 23-bp RSS's) (Fig. 1A). The synthetic RSS's carried on a retroviral vector were introduced into pre-B cell line 38B9, kindly provided by Alt (*5*), to test whether these sequences are sufficient to mediate the V-J type of recombination. Figure 1A shows a schematic diagram of the substrate structure in the retroviral vector.

We first examined the site-specific recombination with an inversion assay (Fig. 2A). This assay with the retroviral vector system is particularly useful because a single copy of the substrate can be introduced into the host chromosome. Furthermore, two joints, both coding and reciprocal, generated from one recombination event can be characterized. The coding joint in V-(D)-J joining is a recombination site where the V-(D)-J structure is formed. The reciprocal joint contains two RSS's joined in a head-to-head fashion. In the inversion assay, a selectable gene that was placed in an inverse orientation between the two RSS's is activated by DNA inversion followed by a readthrough transcription from the viral long terminal repeat (LTR) promoter. We used the neomycin phosphotransferase gene (*neo*^{r}) (*20*) as a selectable marker. As shown in Fig. 1A, the *neo*^{r} gene, flanked by two synthetic RSS oligomers that are located in the same orientation, was inserted into a Moloney virus genome on the plasmid vector pZIPneo(X)1 (*21*). As detailed in the legend to Fig. 1B, the recombination-competent pre-B cell line 38B9 was infected with the virus particles containing the recombination substrate. The virus-infected pre-B cells were transferred to a 24-well culture plate and cultured with a neomycin derivative G418 (*22*) in order to enrich for cells undergoing the *neo*^{r} gene inversion.

G418-resistant 38B9 cells with the Neo^R phenotype were obtained from all wells. The G418-resistant cells from five separate wells were studied by Southern blotting with the *neo*^{r} gene as a hybridization probe. As shown schematically in Fig. 1A, the inversion of the *neo*^{r} gene could yield the 2.0-kb *neo*^{r} gene–containing fragment, whereas no 2.0-kb Xho I fragment is expected from the unrearranged substrate. The 2.0-kb Xho I fragment was in fact detected in all the G418-resistant samples. The Sac I or Xba I digestion did not reveal the DNA rearrangement, suggesting that the *neo*^{r} gene rearrangement did not affect the distance between the two viral LTR's (Fig. 1A). These observations indicate that the rearrangement of *neo*^{r} was caused by DNA inversion, most likely with the 12-bp and 23-bp RSS's.

In order to analyze nucleotide sequences at the rearrangement sites, we prepared DNA libraries from G418-resistant cells and screened recombinant phages with the *neo*^{r} probe. Two joining sites, a coding joint and a reciprocal joint, were identified outside of the inverted *neo*^{r} gene (Fig. 1A). Recombination sites in two different clones, Inv-1 and Inv-2, were characterized by DNA sequencing (Fig. 1B). In both cases, loss and addition of a few nucleotides were detected at the coding joint. In contrast, the joining sites were flush at the reciprocal recombination site; that is, two heptamers were joined in a head-to-head fashion without any loss or addition of nucleotides (Fig. 1B). These results are in all respects consistent with the

features seen in normal V-J joining processes (*15, 16, 23–25*).

In order to confirm that the V-J type recombination also occurs via deletion with the two synthetic RSS's, we constructed another recombination substrate (Fig. 3A) to assay DNA deletion (Fig. 2B). The substrate contains the *neo*^{r} gene flanked by two RSS's and a thymidine kinase gene (*tk*) (*26*) further downstream. Expression of these genes is mediated by a promoter consisting of the SV40 and *tk* gene promoters. To test the site-specific recombination with the two RSS oligomers, we introduced the plasmid into a pre-B cell line lacking *tk*, 38B9 (*15*), by electroporation (*27*). DNA-transformed cells were first selected for the Neo^R phenotype with the neomycin-derivative G418 (*22*). Cells that had undergone the rearrangement were then selected for the Tk$^+$ phenotype in hypoxanthine-aminopterin-thymidine (HAT) medium (*28*). With this system, the joining of two RSS sequences would remove the *neo*^{r} gene by DNA dele-

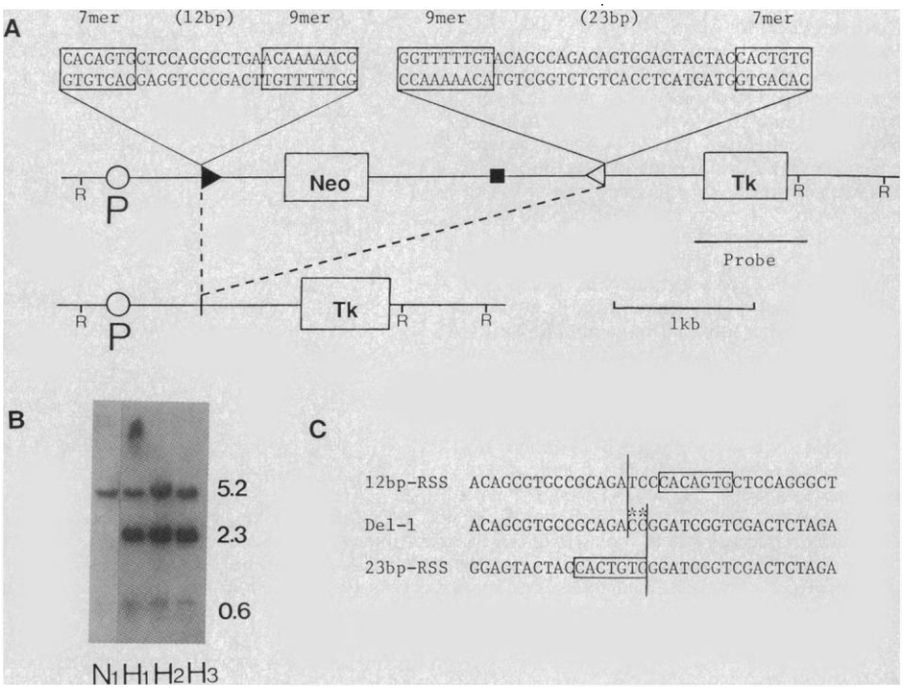


Fig. 3. Deletion recombination with two pairs of synthetic V-J joining signals. (**A**) Structure of the deletion substrate. Immunoglobulin RSS's of the mouse V_κ21C (▶) and J_κ1 (◁) were synthesized and introduced into a plasmid vector pIBI76 (IBI) containing *neo*^{r} gene and *tk* gene (the 3.5-kb Bgl II–Bam HI fragment) (*26*) as selectable markers. The plasmid DNA was transfected into the 38B9 pre-B cells by electroporation, and the transformants were selected with G418. Transcription of the promoter-deleted *neo*^{r} gene is driven by the SV40 early region promoter (*22*) and HSV *tk* gene promoter (*26*). The SV40 polyadenylate site (■) was placed downstream from the *neo*^{r} gene. Joining between the two recombination signals removes the *neo*^{r} gene as well as the polyadenylate site by DNA deletion and places the *tk* gene immediately downstream from the promoter region. Eco RI sites are indicated (RI). (**B**) Southern analysis of DNA-transformed 38B9 cells. A 38B9 derivative carrying the substrate plasmid (N1) and three Tk$^+$ subpopulations of N1 (H1, H2, and H3) were analyzed. The 5.2- and 0.6-kb probe-positive Eco RI fragments were derived from the unrearranged sequences. A 2.3-kb Eco RI fragment (rearranged) was seen only in the Tk$^+$ subpopulations. (**C**) Nucleotide sequences at the deletion site. The 2.3-kb rearranged Eco RI fragment (Del-1) was cloned from the Tk$^+$ 38B9 (H1). A nucleotide sequence at the joint was determined and compared with the substrate sequences (12-bp and 23-bp RSS's). As in the inversion system in Fig. 1, nucleotide loss and addition are seen at the joint. Inserted nucleotides are indicated by asterisks. In this system, a reciprocal joint cannot be recovered from the chromosomal DNA because it is excised into a circular DNA molecule (*24, 25*).

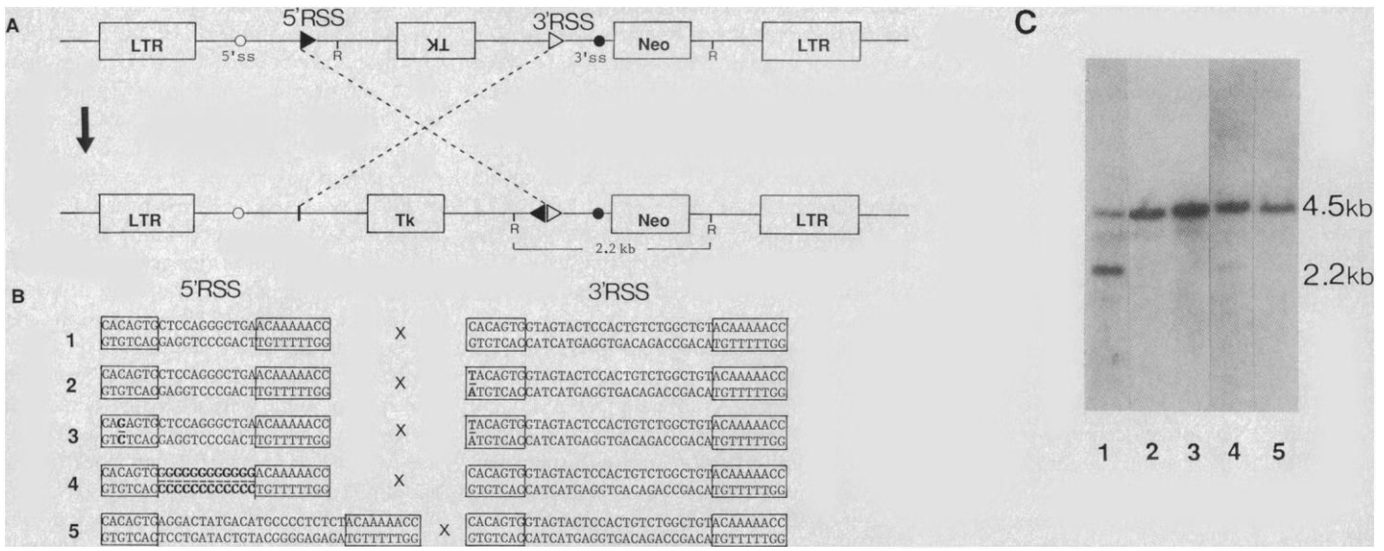


Fig. 4. Comparison of recombinations with various synthetic substrates. (**A**) The inversion substrate in a retroviral vector, pZIPneoSV(X)1 (*21*). The inverted *tk* gene (Bgl II–Eco RI fragment) (*26*) is flanked by two RSS's, a 5′ RSS (▶) and a 3′ RSS (▷). The *neo*^{r} gene, located downstream from the *tk* gene, can be expressed via spliced messenger RNA by utilizing the 5′ (○) and 3′ (●) splice sites (ss). Eco RI sites are indicated (R). (**B** and **C**) Inversion recombinations with variant RSS's. Five different pairs of RSS's were examined. A pre-B cell line, 38B9 (*15*), was infected with the recombinant virus and selected with G418 for the presence of the substrate. Regardless of the Tk phenotype, DNA samples of Neo^R cells were examined for the DNA rearrangement. The DNA was digested with Eco RI and analyzed by Southern blotting with the *neo*^{r} gene as a hybridization probe. The 4.5-kb band represents an unrearranged substrate. The 2.2-kb band represents DNA inversion mediated by two RSS's.

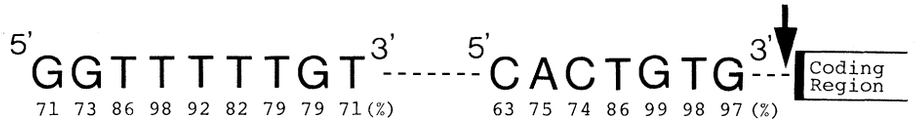
tion. This rearrangement would place the *tk* gene next to the promoter, thus activating the *tk* gene. Three Tk^+ subpopulations (H1, H2, and H3) were analyzed for the DNA rearrangement. The DNA samples were digested with Eco RI, and analyzed by Southern blotting, with the *tk* gene as a hybridization probe. A 5.2-kb band in Fig. 3B represents the intact *tk* gene without any DNA rearrangement, whereas the 2.3-kb band contains the deletion generated by the site-specific recombination. Each Tk^+ sample appears to contain multiple copies of the recombination substrates, two-thirds of which have undergone the rearrangement. Nucleotide sequence analysis showed that the recombination had occurred in the vicinity of the heptamers, with a few base-pair deletions and insertions at the joining sites (Fig. 3C). Thus, the deletion experiment also demonstrated that two RSS oligomers are sufficient substrates for the V-J type of joining.

It is well established that V-(D)-J joining occurs only between two pairs of signal sequences with different spacer lengths (*8, 9*); that is, one pair is separated by a 12-bp spacer and the other by a 23-bp spacer. No joining has ever been reported between two RSS's with the same spacer length. Table 1 is a comparison of 91 5′ RSS's reported for J and D segments in both Ig and TCR genes. Examination of these sequences reveals almost 100% conservation of the last three nucleotides, GTG (Table 1). In the 3′ RSS's of germline V and D segments, the first three nucleotides of the heptamer, CAC, are always conserved. It appears that the three nucleotides adjacent to the recombination sites are particularly important for V-(D)-J joining. To test whether these restrictions are reproduced with synthetic RSS oligomers, we constructed another recombination substrate with which the relative recombination frequency can be measured. As shown in Fig. 4A, the substrate contains two selectable markers, *tk* and *neo*^{r}, which are under the control of the LTR promoters. In this system the *neo*^{r} gene selects cells containing the recombination substrate regardless of the DNA rearrangement. Although the *neo*^{r} gene is located downstream from the *tk* gene, its expression is ensured by the 5′ and 3′ RNA splice sites (ss) (Fig. 4A). The other selectable marker, *tk*, placed in an inverse orientation is flanked by 5′ and 3′ RSS's and detects the DNA inversion (Fig. 4A). Surprisingly, DNA rearrangement was detected by Southern blotting in Neo^R cells even without HAT selection for the *tk* gene inversion. With wild-type RSS's, more than two-thirds of Neo^R cells underwent recombination (lane 1 in Fig. 4C).

In the assay described in Fig. 4C, multiple recombination events are scored in the G418 culture. It is possible that we may be looking at amplified recombinants derived from a limited number of recombination events which occurred at an early stage of the culture. However, the possibility is unlikely, because the results by Southern blotting do not fluctuate from one experiment to the other. Four pairs of variant RSS's were also examined for their abilities to cause DNA recombination (Fig. 4, B and C). As expected, mutations in the heptamer region adjacent to the joining site reduced the recombination drastically (lanes 2 and 3 in Fig. 4C). Although Southern blotting did not detect the rearrangement with the mutant RSS's, HAT selection for the Tk^+ phenotype still identified cells undergoing the rearrangement. With the combination of two 23-bp RSS's, no recombination was detected by Southern blotting (lane 5 in Fig. 4C) or by Tk^+ selection with HAT medium (*28*). It appears that there is a tighter restriction in the matching of two spacer lengths than in the sequence conservation in the heptamer. It is interesting that the replace-

Table 1. Comparison of heptamer and nonamer sequences in the 5′ RSS's of Ig and TCR genes. Ninety-one published sequences were compared both in the human and mouse [see (*31*) for references]. Three nucleotides, GTG, adjacent to the recombination sites (arrows) are always conserved except in pseudogenes.

		5′			3′
Consensus		**GGTTTTTGT**	(bp)	**CACTGTG**	
Mouse Ig κ	J1	---------	(23)	-------↓	
	J2	A--------	(23)	--G----	
	J3ψ	--G------	(21)	------A	
	J4	---------	(24)	-------	
	J5	---------	(23)	-------	
Human Ig κ	J1	-----C---	(23)	-------	
	J2	A--------	(23)	--T----	
	J3	---------	(23)	-------	
	J4	---------	(23)	-------	
	J5	---------	(23)	-------	
Mouse Ig λ	J1	-T------C	(12)	---A---	
	J2	------G-G	(12)	--T----	
	J3	-----AG-G	(12)	-------	
	J4ψ	-T------C	(12)	--G---T	
Human Ig λ	J1	------G--	(12)	-------	
	J2	---------	(12)	---A---	
	J3	---------	(12)	---A---	
	J4ψ	-----CAA-	(12)	--G--AC	
	J5ψ	-----CAA-	(12)	--G--AA	
Mouse Ig H	J1	A-----A--	(22)	G------	
	J2	---------	(23)	T-G----	
	J3	AT--A----	(23)	--A----	
	J4	---------	(22)	T-T----	
Human Ig H	J1	-----C---	(21)	---C---	
	J2	T--------	(23)	G------	
	J3	-----G---	(23)	-C-----	
	J4	---------	(23)	--A----	
	J5	----C--TG	(22)	--A----	
	J6	---------	(22)	--T----	
Mouse Ig H	DQ52	------GAC	(12)	---A---	
	DSP22	-A-------	(12)	---A---	
	DSP23	-A-------	(12)	T------	
	DSP24	-A-------	(12)	T------	
	DSP25	-A-------	(12)	T------	
	DSP26	-A-------	(12)	T------	
	DSP27	-A-------	(12)	T------	
	DSP28	-A-------	(12)	T------	
	DFL1	-C-------	(12)	T------	
	DFL2	-C-------	(12)	T------	
Human Ig H	D1	--A------	(12)	-------	
	D2	--A------	(12)	-------	
	D3	--A------	(12)	-------	
	D4	--A------	(12)	-------	
	DQ52	--------G	(13)	-------	
Mouse TCR β	J1.1	TA-----C-	(13)	-------↓	
	J1.2	CCA-A--CG	(12)	TGA----	
	J1.3	------GAA	(12)	GG-----	
	J1.4	A-----ACC	(12)	TGT----	
	J1.5	--AG-----	(13)	T------	
	J1.6	------ACC	(12)	AG-----	
	J1.7ψ	-C-CCA-T-	(12)	GG-----	
	J2.1	-AA--C-TG	(12)	TG-----	
	J2.2	-----G--C	(12)	G------	
	J2.3	A--------	(12)	GG-----	
	J2.4	A--------	(12)	GG-----	
	J2.5	A--------	(12)	GG-----	
	J2.6ψ	-----C-C-	(12)	GGT----	
	J2.7	-----G---	(12)	-T-----	
Human TCR β	J1.1	-A----CAC	(12)	-------	
	J1.2	CC----A-A	(12)	TTA----	
	J1.3	------GAA	(12)	GG-----	
	J1.4	------CC-	(12)	TGT----	
	J1.5	--G---GCC	(12)	-------	
	J1.6	--G---TA-	(12)	AG-----	
	J2.1	-AA--C--G	(12)	-------	
	J2.2	-----GC-C	(12)	GG-----	
	J2.3	---------	(12)	GG-----	
	J2.4	A----C---	(12)	GG-----	
	J2.5	---------	(11)	GG-C---	
	J2.6	--------C	(12)	GG-----	
	J2.7ψ	-----GCA-	(12)	-T-C---	
Mouse TCR α	JPHDS	A--------	(12)	-------	
	JTT11	A---A----	(12)	GG-----	
	J80	-----A---	(12)	--GG---	
	J84	A--------	(12)	-------	
	J19	----A----	(12)	-------	
	J65	---------	(12)	---A---	
	J45	A-------C	(12)	--G----	
Human TCR α	JA1	----A-CTC	(12)	---A---	
	JB	T-------A	(12)	-------	
	JC	T-G------	(12)	--TA---	
	JD	CCA------	(12)	---A---	
	JEψ?	--------A	(12)	----A--	
Mouse TCR γ	J10.3	-A-------	(12)	-------	
	J13.4	-A-------	(12)	-------	
Human TCR γ	J1	A-------A	(12)	-------	
	J2	A-------A	(12)	-------	
Mouse TCR β	D1	CT-------	(12)	--T----	
	D2	CT-------	(12)	--T----	
Human TCR β	D1	T--------	(12)	--T----	
	D2	CA-------	(12)	--T----	



ment of the spacer with a stretch of GC pairs did not entirely diminish the joining (lane 4 in Fig. 4C). This observation is consistent with the notion that the length is more important than the sequence for the spacer (*8, 9*).

We analyzed V-(D)-J joining with synthetic RSS oligomers in a recombination-competent pre-B line. It was clearly demonstrated that 12-bp and 23-bp RSS's were sufficient substrates to cause DNA recombination in a manner analogous to the natural V-(D)-J joining. In the endogenous Ig and TCR genes, V-(D)-J joining takes place in a time-ordered fashion during lymphocyte development (*29, 30*) although all the V-(D)-J joining events follow the same 12- and 23-bp spacer rule (Table 1). It remains to be determined what regulates DNA rearrangement. Transcriptional activation of a gene is thought to be prerequisite for DNA rearrangement (*18*). A putative recombinational enhancer may also play an important role in making the chromatin structure open for DNA rearrangement (*30*).

REFERENCES AND NOTES

1. S. Tonegawa, *Nature (London)* **302**, 578 (1983).
2. G. D. Yancopoulos and F. Alt, *Annu. Rev. Immunol.* **4**, 339 (1986).
3. M. Kronenberg *et al.*, *ibid.*, p. 529.
4. B. Toyonaga and T. Mak, *ibid.* **5**, 585 (1987).
5. M. M. Davis, *ibid.* **3**, 537 (1985).
6. H. Sakano *et al.*, *Nature (London)* **280**, 288 (1979).
7. E. E. Max, J. G. Seidman, P. Leder, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3450 (1979).
8. H. Sakano *et al.*, *Nature (London)* **286**, 676 (1980).
9. P. Early *et al.*, *Cell* **19**, 981 (1980).
10. R. J. Aguilera, T. J. Hope, H. Sakano, *EMBO J.* **4**, 3689 (1985).
11. T. J. Hope, R. J. Aguilera, M. Minie, H. Sakano, *Science* **231**, 1141 (1986).
12. S. Desiderio and D. Baltimore, *Nature (London)* **308**, 860 (1984).
13. T. Kataoka *et al.*, *Nucleic Acids Res.* **12**, 5995 (1984).
14. S. Lewis, A. Gifford, D. Baltimore, *Nature (London)* **308**, 425 (1984).
15. T. K. Blackwell and F. W. Alt, *Cell* **37**, 105 (1984).
16. S. Lewis, A. Gifford, D. Baltimore, *Science* **228**, 677 (1985).
17. R. Mann, R. C. Mulligan, D. Baltimore, *Cell* **33**, 153 (1983).
18. G. D. Yancopoulos *et al.*, *ibid.* **44**, 251 (1986).
19. F. Alt *et al.*, *ibid.* **27**, 381 (1981).
20. E. Beck *et al.*, *Gene* **19**, 327 (1982).
21. C. L. Cepko, B. E. Roberts, and R. C. Mulligan, *Cell* **37**, 1053 (1984).
22. P. J. Southern and P. Berg, *J. Mol. Appl. Genet.* **1**, 327 (1982).
23. F. Alt and D. Baltimore, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4118 (1982).
24. K. Okazaki, D. D. Davis, H. Sakano, *Cell* **49**, 477 (1987).
25. S. Fujimoto and H. Yamagishi, *Nature (London)* **327**, 242 (1987).
26. S. L. McKnight, *Nucleic Acids Res.* **8**, 5949 (1980).
27. H. Potter, L. Weir, P. Leder, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7161 (1984).
28. M. Wigler *et al.*, *Cell* **11**, 223 (1977).
29. F. Alt *et al.*, *EMBO J.* **3**, 1209 (1984).
30. M. Hagiya *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 145 (1986).
31. E. A. Kabat *et al.*, in *Sequences of Proteins of Immunological Interest* (NIH, Bethesda, MD, ed. 4, 1987).
32. L. Gritz and J. Davies, *Gene* **25**, 179 (1983).
33. F. L. Graham and A. J. van der Eb, *Virology* **52**, 456 (1973).
34. S. V. Desiderio *et al.*, *Nature (London)* **311**, 752 (1984).

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Deep-Sea Hydrocarbon Seep Communities: Evidence for Energy and Nutritional Carbon Sources

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Mussels, clams, and tube worms collected in the vicinity of hydrocarbon seeps on the Louisiana slope contain mostly "dead" carbon, indicating that dietary carbon is largely derived from seeping oil and gas. Enzyme assays, elemental sulfur analysis, and carbon dioxide fixation studies demonstrate that vestimentiferan tube worms and three clam species contain intracellular, autotrophic sulfur bacterial symbionts. Carbon isotopic ratios of 246 individual animal tissues were used to differentiate heterotrophic ($\delta^{13}C = -14$ to -20 per mil), sulfur-based ($\delta^{13}C = -30$ to -42 per mil), and methane-based ($\delta^{13}C = <-40$ per mil) energy sources. Mussels with symbiotic methanotrophic bacteria reflect the carbon isotopic composition of the methane source. Isotopically light nitrogen and sulfur confirm the chemoautotrophic nature of the seep animals. Sulfur-based chemosynthetic animals contain isotopically light sulfur, whereas methane-based symbiotic mussels more closely reflect the heavier oceanic sulfate pool. The nitrogen requirement of some seep animals may be supported by nitrogen-fixing bacteria. Some grazing neogastropods have isotopic values characteristic of chemosynthetic animals, suggesting the transfer of carbon into the background deep-sea fauna.

WE REPORT HERE A STUDY OF THE energy and nutritional carbon sources of mussels, clams, and tube worms from hydrocarbon seep communities on the Louisiana continental slope (*1*). The organisms were collected in trawls near hydrocarbon seep sites in water depths between 400 and 920 m on R.V. *Gyre* cruises 86-G-1/2. The northern Gulf of Mexico slope is extensively faulted and fractured by salt tectonics, thus providing conduits for the upward migration of oil and gas (*2*). The taxa at these sites are similar to those of the hydrothermal vent sites of the Pacific (*3*), the cold seep sites of the Florida Escarpment (*4*), and the Oregon Subduction Zone (*5*). The Louisiana sites are distinct in that the vent taxa are living in a high hydrocarbon environment derived from deeper reservoired petroleum. These vent-type taxa use organic matter produced in situ by chemoautotrophic, sulfide-oxidizing bacteria and endosymbiotic chemoautotrophs (*6, 7*). Methane use has been demonstrated for the mussels from the Louisiana site (*8*) and from the Florida Escarpment (*4, 9*) and has been suggested for the animals at the Oregon Subduction Zone (*5*).

A variety of tests were used to determine the nature (and presence) of endosymbionts in these seep fauna. The mussel is the only animal with confirmed methanotrophic symbionts (*8*) and is the only one of these seep species that possesses methanol dehydrogenase, an enzyme characteristic of methylotrophy (Table 1). The mussel is also the only animal tested whose bacterial symbionts contain stacked internal membranes (typical of type I methanotrophs). Mussel gills lack the enzymes characteristic of sulfur oxidation [adenosine triphosphate (ATP) sulfurylase and adenosine-5′-phosphosulfate reductase], lack elemental sulfur, and have only trace activities of ribulose-bisphosphate carboxylase (an enzyme characteristic of autotrophic carbon fixation); these factors indicate that symbionts of mussel gills are not sulfur-oxidizing chemoautotrophs.

The other three bivalves and the two

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