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Three lymphoid-specific factors account for all junctional diversity characteristic of somatic assembly of T-cell receptor and immunoglobulin genes

[V-(D)-J rearrangement/Rag1–Rag2 recombination/terminal deoxynucleotidyltransferase/N region/P nucleotide]

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ABSTRACT **The somatic diversity immunoglobulin and T-cell receptor diversity is largely provided by the junctional variation created during site-specific rearrangement of separately encoded gene segments. Using a transient transfection assay, we demonstrate that the recombination activating genes Rag1 and Rag2 direct site-specific rearrangement on an artificial substrate in poorly differentiated as well as in differentiated nonlymphoid cell lines. In addition to a high frequency of precise recombination events, coding joints show deletions and more rarely P-nucleotide insertions, reminiscent of immunoglobulin and T-cell receptor junctions found in fetal tissues. N-region insertions, which are characteristic of adult junctional diversity, are obtained at high frequency upon transfection of a terminal deoxynucleotidyltransferase expression vector together with Rag1 and Rag2. These results show that only three lymphoid-specific factors are needed to generate all types of junctional diversity observed during lymphoid development.**

Immunoglobulin (Ig) and T-cell receptor (TCR) gene assembly is achieved through site-specific recombination events, from separately encoded variable (V), in some cases diversity (D), and junction (J) gene segments. Much of the immunoglobulin and TCR diversity is generated by the combinatorial rearrangement of a large number of V, D, and J gene segments (for a review see refs. 1 and 2).

Recombination signal sequences (RSSs) situated adjacent to each gene segment provide the targets for recombination. RSSs are composed of a palindromic heptamer and an (A+T)-rich nonamer separated by a spacer of 12 or 23 base pairs (bp) (3). Rearrangement only occurs between RSSs with spacers of different length. RSSs are sufficient to target rearrangement of artificial substrates containing no other antigen receptor sequences (4–7).

Two main types of joints are formed during the recombination process: coding joints created by the juxtaposition of the gene segments and reciprocal joints by contiguous RSSs (8). Whereas the heptamers in the reciprocal joints are generally joined back to back without nucleotide insertions or deletions, the coding joints are subjected to extensive processing (8). The junctions formed during rearrangement constitute another source of diversity. Several nucleotides can be deleted and two types of insertions can be found. Random nucleotide additions, resulting in N-region insertions, are thought to be introduced by terminal deoxynucleotidyltransferase (TdT) (9–11). P-nucleotide insertions represent the inverted repeat of the adjacent coding sequence. Their addition has been proposed to be a compulsory step of the recombination mechanism (12).

Genomic DNA transfection experiments have allowed the isolation of two recombination activating genes, Rag1 and Rag2 (13–15). Together these genes are able to confer site-specific recombination activity to NIH 3T3 fibroblasts. Although it is not formally excluded that they activate other genes that would be responsible for the recombination activity, Rag1 and Rag2 are thought to encode at least the lymphoid-specific part of the recombination machinery. Other ubiquitous and/or lymphoid-specific factors could be recruited to participate in the recombination process.

The contribution of Rag1 and Rag2 in site-specific rearrangement was studied by analyzing the characteristics of the coding joints formed in a transient transfection assay in fibroblasts. It is shown that cotransfection of Rag1 and Rag2 together with TdT is sufficient to reconstitute all of the junctional diversity found *in vivo*. Finally, the ability of Rag1 and Rag2 to confer recombination activity to various cell lines engaged in separate differentiation pathways was tested.

MATERIALS AND METHODS

Cell Lines. NIH 3T3 mouse embryo fibroblasts (ATCC CRL 6442) and A9, an L-cell derivative (ATCC CRL 6319), were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. BW1J (16) mouse hepatoma cells were cultivated as described. CHO-K1 Chinese hamster ovary cells (ATCC CCL 61) were cultured in RPMI medium with 10% fetal calf serum. BASP-1 pre-B cells (17) were grown in RPMI medium with 10% fetal calf serum and 50 μM 2-mercaptoethanol.

Molecular Cloning of Mouse TdT. RNA was prepared from the thymus of 5-week-old mice, as described by Auffray and Rougeon (18), except that 4 M guanidine thiocyanate was used instead of 6 M urea. Poly(A)-containing RNA was purified by oligo(dT)-cellulose chromatography and analyzed by Northern blots. First strand synthesis was carried out on 5 μg of poly(A) RNA primed with oligo(dT) using Moloney murine leukemia virus reverse transcriptase (BRL). Second strand synthesis was performed in the presence of DNA polymerase I and RNase H. *Bst*XI double-stranded adapters were ligated to blunt-ended cDNA and cloned in the *Bst*XI restriction site of pCDNA2 (Invitrogen, San Diego). The mouse thymus cDNA library was screened with two mixed oligonucleotides covering position 121–142 and position 1471–1494 on the mouse TdT cDNA sequence (19). cDNA-positive clones estimated to be long enough to contain the entire region of mouse TdT were sequenced on both strands, by the dideoxy termination method (20).

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Abbreviations: TCR, T-cell receptor; RSS, recombination signal sequence; TdT, terminal deoxynucleotidyltransferase; V, variable; D, diversity; J, junction.

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Vectors. pBlueRec was described in ref. 7. Rag1 (M2CD.7 designated here as pRag1) and Rag2 (R2RCD.2 designated as pRag2) expression vectors were provided by M. Oettinger (15). Mouse TdT cDNA was cloned into pCDNA1 expression vector (pTdT). Rag1, Rag2, and TdT are all under control of cytomegalovirus promoter.

Assay for Site-Specific Recombination. Transfections were performed by electroporation following the conditions described by Chu *et al.* (21). Cells (2×10^6) were transfected with 2.5 μg of pBlueRec either with or without 6 μg of pRag1 or 4.8 μg of pRag2 (≈2× molar excess compared to pBlueRec). To assess the effect of TdT on N-region insertion, 4.5 μg of TdT expression vector (≈2× molar excess compared to pBlueRec) was added to the three vectors mentioned above.

Cells were harvested after 40–48 hr of incubation at 37°C and washed with phosphate-buffered saline; plasmid DNA was prepared according to ref. 22. DNA pellets were resuspended in 20 μl of sterile water. Seven microliters of the DNA solution was digested by *Dpn* I, to eliminate nonreplicated plasmids, in the presence of 0.1 ng of a *dam* methylated kanamycin-resistant plasmid. Forty microliters of XL1-blue competent bacteria (Stratagen) were transformed by electroporation (23) and plated on LB agar plates containing 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal; 80 μg/ml), isopropyl β-D-thiogalactoside (IPTG; 150 μM), ampicillin (100 μg/ml), and tetracycline (10 μg/ml). pRag1, pRag2, and pTdT do not give rise to colonies since they do not contain the β-lactamase gene. As a control for digestion, a fraction of the transformation was plated on kanamycin (70 μg/ml) LB agar plates. Rearrangement frequency was calculated as the (amount of blue clones × 3)/total number of clones (7). When rearrangement was scored at different time points after transfection, *Dpn* I digestion was omitted.

Sequencing of the Recombinant Clones. Blue colonies were picked and isolated on LB agar plates containing X-Gal, IPTG, ampicillin, and tetracycline. Small-scale DNA preparations were made according to the boiling method (24), resuspended in 50 μl of 10 mM Tris·HCl, pH 8/1 mM EDTA/10 μg of RNase per ml, and incubated for 2 hr at room temperature prior to double-strand sequencing with reverse primer as recommended by the supplier (United States Biochemical).

RESULTS

NIH 3T3 Fibroblasts Transfected with Rag1 and Rag2 Have the Same Recombination Frequency as a Pre-B-Cell Line. We analyzed the recombination activity mediated by Rag1 and Rag2 expression in NIH 3T3 fibroblasts using a transient transfection assay. pRag1 and pRag2 were cotransfected into NIH 3T3 fibroblasts together with the recombination substrate pBlueRec. After 48 hr plasmid DNA was recovered and tested in *Escherichia coli* for recombination. The *LacZ* coding sequence of pBlueRec is interrupted by a 280-bp DNA fragment flanked by two RSSs. Site-specific rearrangement will delete this insert and, in one case out of three, will restore the correct reading frame, giving rise to blue clones after transformation of *E. coli*. This rapid test permits examination of a large number of rearrangement events. Transfection experiments with either pRag1 or pRag2 alone did not give rise to any recombinant clone. A high recombination frequency was observed, however, when both plasmids were cotransfected (Table 1). Furthermore, the frequency (geometric mean, $X_g = 1.26$) is comparable to that observed after transfection of the recombination substrate in a pre-B-cell line, BASP-1 ($X_g = 1.46$).

Table 1. Rearrangement in fibroblasts and in B-cell precursors

Cell line	DNA	Amp^r colonies		R
		Total	Blue	
NIH 3T3	pBlueRec	70,000	0	0
	pBlueRec, pRag1	34,860	0	0
	pBlueRec, pRag2	12,140	0	0
	pBlueRec, pRag1, pRag2	144,000	183	0.38
	pBlueRec, pRag1, pRag2	38,000	128	1
	pBlueRec, pRag1, pRag2	60,000	1059	5.3
BASP-1	pBlueRec	14,400	186	1.3
	pBlueRec	14,800	237	1.6
	pBlueRec	12,960	190	1.5

R, recombination frequency [(number of blue clones × 3/total number of clones) × 10^{-2}]; Amp^r, ampicillin-resistant.

Sequence of Coding Joints Obtained by Transfection of Rag1 and Rag2 in NIH 3T3 Fibroblasts. To compare the coding joints formed after Rag1–Rag2-mediated recombination in fibroblasts with those observed in lymphoid cells, the joints on rearranged plasmids recovered after NIH 3T3 transfection were sequenced. All sequences shown in Fig. 1 represent independent recombination events—i.e., identical sequences come from different transfection experiments. Seven joints out of 17 have no deletions or insertions. Four joints have deletions on one side and 4 have deletions on both sides. Only

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CCGCTCTAGAACTAGTGGATCC-CACAGTG-(12)
                        (23)-CACTGTG-GTCGACCTCGAGGGG

CCGCTCTAGAACTAGTGGAT--          ----ACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC          GTCGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC          GTCGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC          GTCGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC          GTCGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC          ---GACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC          GTCGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC          ---GACCTCGAGGGG
CCGCTCTAGAACTAGTGG----          --CGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC          ---GACCTCGAGGGG
CCGCTCTAGAACTAGTG-----          -------TCGAGGGG
CCGCTCTAGAACTAGTGGATCC          GTCGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC          ---GACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC          GTCGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC      GG  --CGACCTCGAGGGG
CCGCTCTAGAACTAGTGG----          --CGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC      C   -TCGACCTCGAGGGG
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FIG. 1. Sequences of the junctions formed on pBlueRec after cotransfection with pRag1 and pRag2 in NIH 3T3 fibroblasts. Sequences of recombined plasmids are aligned with the sequence of native pBlueRec. Dashes indicate nucleotide deletions. Putative P nucleotides are underlined. The heptamers of the RSSs are in bold type.

one joint presented a P-nucleotide insertion of 2 bp associated with a 2-bp deletion on one side of the junction. The last joint shown has a 1-bp deletion and a nucleotide that can be attributed to the heptamer, probably a result of imprecise excision.

Cotransfection of Rag1, Rag2, and TdT in NIH 3T3 Fibroblasts. Although deletions and a P-nucleotide insertion were observed after Rag1 and Rag2 transfection in fibroblasts, no N regions were found. In an attempt to reconstitute all the junctional diversity observed in pre-B or pre-T cells, NIH 3T3 fibroblasts were transfected with the TdT expression vector pTdT together with pRag1, pRag2, and pBlueRec. Recombined plasmids were sequenced. Control transfections with the pCDNA1 vector without the TdT cDNA yielded no N-region insertions (results included in Fig. 1). As shown in Fig. 2, 88% of the joints show non-template-encoded nucleotide insertions or N regions. The insertions are superimposed onto the pattern of junctional diversity observed after pRag1–pRag2 transfection. Most of the N regions were 1–4 nucleotides long, with an average of 3 nucleotides per junction. One exceptionally long insertion of 18 nucleotides was also obtained. TdT has been shown to incorporate G residues more efficiently than any other nucleotide (11, 25). A slight GC preference in the N-region insertions (64%) was observed. The frequency of P-nucleotide insertion (underlined in Fig. 1) seems to be increased in this experiment. It should be noted, however, that it is not possible to distinguish them from N regions.

Rag1 and Rag2 Are Sufficient to Confer Recombinase Activity to Various Types of Differentiated Cell Lines. Rag1 and Rag2 are able to confer recombinase activity to rather undifferentiated NIH 3T3 fibroblasts (ref. 15; this paper). It was of interest to test if the activity of these two genes is affected by the differentiation state of the host cell line. It is plausible that some of the accessory factors participating in the recombination process together with Rag1 and Rag2 might be missing in these cell lines, leading to the absence of rearrangement. Alternatively, the coding joints might be structurally different from those observed in pre-B cells. pRag1, pRag2, and pBlueRec were cotransfected in BW1J fetal mouse hepatoma, a Chinese hamster ovary cell line (CHO-K1), and A9 cells (L-cell derivative). The results obtained in each transfection experiment are listed in Table 2. There is some variation between transfections in the same cell line, but, surprisingly, the rearrangement frequency obtained in hepatoma (X_g = 4.82) as well as in Chinese hamster ovary cells (X_g = 10.45) was conspicuously higher than in 3T3 fibroblasts. Rearrangement could be detected as early as 13 hr after cotransfection of pBlueRec, pRag1, and pRag2 in NIH 3T3 and CHO-K1 cells. Sequencing of the recombined plasmids revealed that deletions at the coding joints occur in all cell lines tested (Fig. 3). One P-nucleotide insertion was found in the junction of a recombinant clone recovered after A9 transfection.

DISCUSSION

Rag1 and Rag2 Are Likely to Encode the Tissue-Specific Components of the Recombinase Complex. It has previously been reported that together Rag1 and Rag2 were able to confer recombinase activity to rather undifferentiated NIH

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CCGCTCTAGAACTAGTGGATCC-CACAGTG-(12)
                       (23)-CACTGTG-GTCGACCTCGAGGGG

CCGCTCTAGAACTAGTGG----        GTTC          GTCGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC        GG            -----CCTCGAGGGG
CCGCTCTAGAACTAGTGG----        GGCC          GTCGACCTCGAGGGG
CCGCTC----------------        TTTC          GTCGACCTCGAGGGG
CCGCTCTAGAACTAGT------        TT            -----CCTCGAGGGG
CCGCTCTAGAACTAGTGG----        G             GTCGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC        CCA           ---GACCTCGAGGGG
CCGCTCTAGAACT---------        T             -TCGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC        GAC           GTCGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC        ATC           --CGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC        A             ----ACCTCGAGGGG
CCGCTCTAGAACTAGTG-----                      ----ACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC        TCC           GTCGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC                      GTCGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC        CTC           GTCGACCTCGAGGGG
CCGCTCTAGAACTAGTGG----        GTCC          GTCGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATC-        GGG           -----CCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC        GAG           GTCGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATC-        G             ---GACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC                      GTCGACCTCGAGGGG
CCGCTCTAGAACTAGTGGAT--  ACCATACCCCTTTACCAA  -TCGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC        CCCCCCGCC     GTCGACCTCGAGGGG
CCGCTCTAGAACTAGTGG----        TCCT          -----CCTCGAGGGG
CCGCTCTAGAACTAGTGGATC-        AC            ----ACCTCGAGGGG
CCGCTCTAGAACTAGTGG----        CC            ---GACCTCGAGGGG
CCGCTCTAGAACTAG-------        CCCTAC        --CGACCTCGAGGGG
CCGCTCTAGAACTAGTGG----        CCC           --CGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC                      GTCGACCTCGAGGGG
CCGCTCTAGAACTAGTGG----        TCC           --CGACCTCGAGGGG
CCGCTCTAGAACTAGTGGAT--        TC            GTCGACCTCGAGGGG
CCGCTCTAGAACTAGTGG----        TC            -TCGACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC        A             ----ACCTCGAGGGG
CCGCTCTAGAACTAGTGGATCC        CCC           GTCGACCTCGAGGGG
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FIG. 2. Sequence of the junctions formed on pBlueRec after cotransfection with pRag1, pRag2, and pTdT in NIH 3T3 fibroblasts. Sequences are displayed as described in the legend to Fig. 1.

Table 2. Rearrangement frequency in various cell lines

Cell line	DNA	Amp^r colonies		
		Total	Blue	R
A9	pBlueRec	16,800	0	0
	pBlueRec, pRag1, pRag2	5,620	71	3.8
BW1J	pBlueRec	12,000	0	0
	pBlueRec, pRag1, pRag2	5,600	83	4.4
	pBlueRec, pRag1, pRag2	1,800	8	1.3
	pBlueRec, pRag1, pRag2	16,000	1050	19.6
CHO-K1	pBlueRec	3,434	0	0
	pBlueRec, pRag1, pRag2	2,300	38	4.9
	pBlueRec, pRag1, pRag2	1,996	149	22.3

See Table 1 for abbreviations.

3T3 fibroblasts (15). Two alternative models for the mechanism of action of Rag1 and Rag2 have been proposed: these genes might encode the lymphoid-specific components of the recombinase complex and thus directly participate in the enzymatic steps of site-specific recombination. Alternatively, they might encode factors that induce genes or activate proteins required for recombination activity.

Transient expression of Rag1 and Rag2 in highly differentiated and rather undifferentiated cell lines leads to a high level of recombinase activity, comparable to that found in pre-B-cell lines (refs. 7 and 26; this paper). The rapid appearance of recombination activity and its detection in all cell lines tested constitute arguments in favor of the hypothesis of Oettinger *et al.* (15), that Rag1 and Rag2 are the tissue-specific components of the recombinase complex.

Coding Joints Generated in Rag1–Rag2 Transfections Present Deletions and P-Nucleotide Insertions. Transient transfection of pRag1 and pRag2 in NIH 3T3 fibroblasts and the other cell lines allows recombination of an artificial episomic substrate creating coding joints similar to those found in fetal immunoglobulin and TCR gene rearrangements (27–31). It is interesting to note that a large proportion of the rearranged coding joints do not present deletions or insertions. This would suggest that V(D)J rearrangement is basically a conservative reaction in which diversity is secondarily generated by processing of the junction.

Coding joints featuring nucleotide deletions are obtained after pRag1 and pRag2 transfection in all cell lines tested. These results suggest that the enzymatic activity responsible for nucleotide deletion could be due to a ubiquitous exonuclease. It is also interesting to note that the loss of nucleotides from coding sequences during immunoglobulin heavy chain gene rearrangement is roughly equivalent in newborn and adult mice and is not differentially regulated during B-cell ontogeny (28). Another kind of junctional diversity that was found on recombined plasmids after Rag1 and Rag2 transfection in fibroblasts was P-nucleotide insertions. P-nucleotide insertions as defined by Lafaille *et al.* (12) represent the inverted repeat of the adjacent coding sequence. It has been proposed that two P-nucleotides are systematically added to the coding joint during the recombination process, before the extremities become accessible to exonuclease activity, which, most of the time leads to their loss. The frequency of P-nucleotide insertions observed in our experiments is very low, especially given the large number of junction extremities that were not subjected to nucleotide deletion. Furthermore, the junctions formed on the same recombination substrate after transfection in a pre-B-cell line showed roughly the same low frequency of P-nucleotide insertions (data not shown). This suggests that P-nucleotide insertions arise only rarely during the recombination process, since it seems unlikely that these two nucleotides would be uniquely sensitive to the exonuclease activity and thus specifically lost in the assay. We proposed that P-nucleotide insertion may occur occasionally during the recombination process but this is not a compulsory step. This interpretation is further supported by the very low frequency of this type of insertion in immunoglobulin κ gene rearrangements (32). Moreover, abnormally long P-nucleotide additions, which cannot be explained by the model of Lafaille *et al.*, have been described for TCR γ coding joints found in severe combined immunodeficient T-cell lymphoma (33).

Reconstitution of All Lymphoid Junctional Diversity by Coexpression of Rag1, Rag2, and TdT in NIH 3T3 Fibroblasts. N-region insertion is highly regulated during B- and T-cell ontogeny (27–31). Whereas N-region insertions are found in immunoglobulin heavy chain and TCR gene rearrangements of adult mice, none or very little is observed during fetal life or in immunoglobulin light chain gene rearrangements. TdT expression correlates very well with the N-region insertion pattern (9). Moreover, transfection of a TdT expression vector in pre-B cells increases the frequency of N-region insertions (10).

We have shown that two lymphoid-specific factors, Rag1 and Rag2, are sufficient to direct site-specific recombination in non-lymphoid cell lines reproducing fetal junctional diversity pattern, devoid of N-region insertions. Moreover, transfection of TdT together with Rag1 and Rag2 generates joints with N-region insertions, which are typical of adult junctional diversity. N-region addition thus seems to be the only lym-

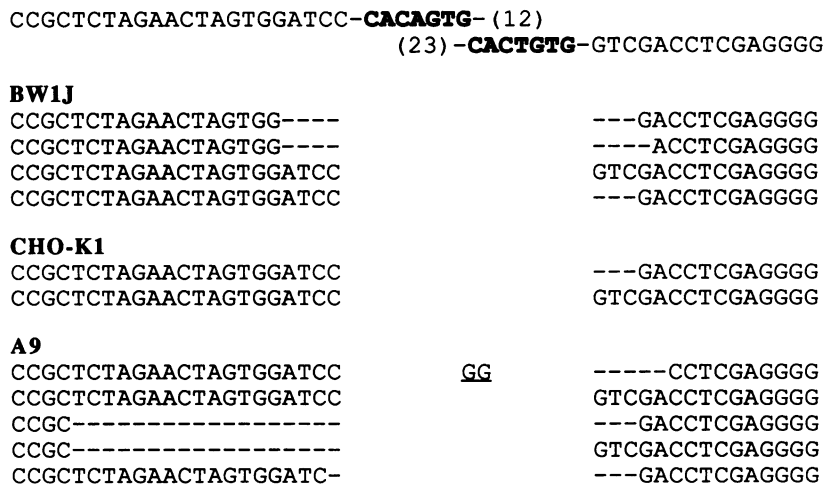

FIG. 3. Sequence of the junctions formed on pBlueRec after cotransfection with pRag1 and pRag2 in various cell lines. Sequences are displayed as described in the legend to Fig. 1.

phoid-specific junctional diversification mechanism not mediated by Rag1 and Rag2. Although the ratio of TdT/α DNA polymerase activities in thymus and transformed T cells is surprisingly high (34), the only known function of TdT, which is conclusively confirmed by this work, is the addition of a few deoxynucleotidylphosphate residues on a rearrangement intermediate.

In this paper we have conclusively demonstrated that just three lymphoid-specific factors are needed to reproduce all types of junctional diversity found in immunoglobulin and TCR rearrangements. These conclusions are in agreement with the results showing that a very limited number of nuclear proteins mark the stages competent for immunoglobulin gene rearrangement in B-cell development (35).

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