CELL STRUCTURE AND FUNCTION 18: 261–266 (1993)

Molecular Characterization of Extrachromosomal Circular DNAs from an Embryonal Carcinoma Cell Line Induced to Differentiate into Neuron-like Cells in vitro

Takuji Iwasato†, Takeyuki Shimizu, Yasuyoshi Kanari and Hideo Yamagishi*

Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606, Japan

Key words: P19 EC cell/RA-induction/neuron-like cell/circular DNA

ABSTRACT. **Extrachromosomal circular DNAs isolated from a P19 embryonal carcinoma cell line were induced to differentiate into neuron-like cells by retinoic acid and cloned into an EcoRI site of a phage vector. Of the 26 DNA inserts (2.1 kb in average length) analyzed, 16 contained repetitive sequences. Out of 10 DNA inserts with unique sequence, 6 carried linear chromosomal sequences and 4 showed chromosomal rearrangements in Southern blots. Two unique fragments with germline configuration were enriched in circular DNA clone libraries. We assigned the breakpoints of 3 circular DNA fragments to positions in the germline sequence. Patchy short inverted repeats were found in the vicinity of breakpoints. An intrastrand loop structure between such inverted short homology region may be required for the circularization of excised DNA.**

The discovery of extrachromosomal circular DNAs found in a number of eukaryotic cells suggests that they are derived from chromosomal DNA (Rush and Misra, 1985: Yamagishi, 1986). Among extrachromosomal circular DNAs isolated from lymphoid cells, chromosomal excision products generated by intrachromosomal recombination mediated by V(D)J or class switch signal sequences have been reported (Fujimoto and Yamagishi, 1987; Okazaki *et al.*, 1987; Toda *et al.*, 1989; von Schwedler *et al.*, 1990; Iwasato *et al.*, 1990; Matsuoka *et al.*, 1990; Harada and Yamagishi, 1991; Iwasato and Yamagishi, 1991; Shimizu and Yamagishi, 1992). The recombination activating genes, RAG-1 and RAG-2, are thought to encode components of the V(D)J site specific recombination machinery (Schatz *et al.*, 1989; Oettinger *et al.*, 1990). The finding of transcripts of RAG-1 in embryonic and postnatal neurons (Chun *et al.*, 1991) encouraged us to investigate the extrachromosomal circular DNAs in an embryonal carcinoma (EC) cell line induced to differentiate to neuron-like cells in vitro.

We chose a murine EC cell line P19 which is capable of being induced to differentiate to neuron and glial cells with retinoic acid (RA) stimulation (McBurney and Rogers, 1982; Jones-Villeneuve *et al.*, 1982; Edwards and McBurney, 1983). We cloned extrachromosomal circular DNAs obtained from P19 cells during in vitro differentiation and examined the nucleotide sequence of several circular DNA clones and the chromosomal regions from which they were derived. This paper reports the relative abundance of particular unique sequences and the nature of recombinational events for circular DNA formation.

* To whom correspondence should be addressed.

† Present address: Howard Hughes Medical Institute, Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

MATERIALS AND METHODS

Cell preparation. P19 cells were grown in α-MEM (GIBCO) supplemented with 10% fetal bovine serum (Flow Laboratories) and 0.1 mg/ml kanamycin. Induction was carried out as described by Rudnicki and McBurney (1987). Briefly, P19 cells were monodispersed and seeded at a density of 1×10^5 cells per ml in medium containing 1 μM RA in 90 mm bacterial-grade plastic dishes. After 2 days they were transferred into dishes with fresh medium containing 1 μM RA. On day 4 after the induction cell aggregates were dispersed and plated on 150 mm tissue culture-grade plastic dishes without RA. Medium was changed every other day. The cells were harvested on day 8, when all the cells had differentiated into neuron-like cells.

Construction of circular DNA clone library. Extrachromosomal circular DNA molecules were prepared from P19 cells induced by RA and purified by use of ATP-dependent exonuclease to remove contaminating linear DNA as described previously (Yamagishi *et al.*, 1983). They were digested by EcoRI, ligated to calf intestinal alkaline phosphatase-treated EcoRI arms of λgt10 phage vector and packaged in vitro. Recombinant phage formed clear plaques on E. coli C600 *hfl*A150 but plaque formation of non-recombinant parent phage was suppressed (Hoyt *et al.*, 1982). Recombinant phage

titers per microgram of vector DNA was 2×10^5.

DNA hybridization. Plaque and Southern hybridizations were performed according to the methods of Maniatis *et al.* (1982). The mitochondrial DNA probe was a 0.2 kb and a 2.0 kb EcoRI fragment of BALB/c mouse mitochondrial DNA. DNA probes used for Southern hybridizations were circular DNA fragments shown in Table I. All DNA probes were used as purified inserts. All post-hybridization washes were at high stringency (0.2 × SSC, 0.1% SDS, 68°C). In plaque hybridization, every probe-positive clone was confirmed by a duplicate membrane.

DNA sequence analysis. EcoRI-digested circular DNA and genomic DNA clones were recloned into the pHSG399 plasmid vector. Nucleotide sequences were determined by the dideoxy chain termination method (Sanger, 1981) using the universal M13 primer M4, reverse primer RV, or appropriate specific primers synthesized based on available sequences as shown in Figure 2A. Specific primers were as follows:

TCCTTGATCGCCAGCGTTAG (SPS15),
TTGCAGAGCTGCTGAAGCAC (SPS16),
GAGAAGGCACTTGAGTTAGC (SPS17),
TTATACGCCCAGGTGAGAAG (SPS18),
CTGCTGACTTTGGTTCA (FS1),
TTATCTGGGGCCGTTCT (FS2),
AACAAGATCTACTTGCAG (FS5),
TTGAGATGGATTCCTTG (FS11) and
ATATCTATCACTCACA (FS17).

The sequence data in this paper are available from the EMBL, Gen-Bank and DDBJ Nucleotide sequence Databases under the accession number D17298–17308.

RESULTS

Chromosomal organization of circular DNA clones isolated from RA-treated P19 cells. Extrachromosomal circular DNAs were prepared from P19 cells differentiated into neuron-like cells. A circular DNA library was made by cloning EcoRI-digested circular DNA into λgt10 phage vector (cloning capacity up to 7.6 kb). We selected 352 clear plaque-forming units at random and further characterized them by DNA restriction analysis and hybridization with mitochondrial DNA probes. Of these phage clones, 283 (80%) carried mitochondrial DNA sequences and 24 were found to have 26 DNA inserts of various length (Table 1).

These DNA inserts were used as hybridization probes for Southern blots of undifferentiated P19 DNA that had been digested with EcoRI. Out of 26 DNA inserts of 2.1 kb in average length, 16 contained repetitive sequences mostly showing a hybridization smear. A chromosomal DNA fragment corresponding in size to the probe used was detected for 6 clones (PR10, 17, 14, 15, 11, 4) and discrete bands different from the probe were observed for 4 clones (PR2, 19, 12, 214). Two discrete bands 7.8 and 1.7 kb were observed with the probe PR2 (0.5 kb), 6.0 and 2.2 kb bands for PR19 probe (1.0 kb), 7.5 and 1.8 kb bands for PR12 probe (1.9 kb) and only a single 8.2 kb band for PR214 probe (5.0 kb) (Fig. 1). The partner band of 8.2 kb supposed to be hybridizable to PR214 probe may be too short to be detected. These results strongly suggest that the circularization joint is located within these four fragments derived from circular DNA.

Breakpoints on circular DNA fragments. To determine the joint for circle formation, we prepared a genomic library of P19 DNA which was digested with EcoRI and cloned into the λgt10 phage vector, and screened for germline sequence fragments homologous to the rearranged fragment of circular DNA clones. Among 7 Southern bands shown in Fig. 1, 4 fragments of a relatively small size were recovered (PR8, 1.7 kb; PR23, 2.2 kb; PR24, 6.0 kb; PR21, 1.8 kb) using probes of circle clones PR2, PR19 and PR12, respectively. Three

Table I. CHARACTERIZATION OF CLONED P19 CIRCULAR DNA FRAGMENTS.

Fragments		Genomic Bands[a]
clone	size (kb)	
PR 10	6.0	6.0
120	6.0	R
214	5.0	8.2
17	4.3	4.3
13	3.7	R
201	3.5	R
206	3.3	R
223	2.8	R
235	2.5	R
108	2.1	R
12	1.9	7.5, 1.8
18	1.7	R
14	1.6	1.6
15	1.3	1.3
202	1.2	R
225	1.2	R
227	1.2	R
19	1.0	6.0, 2.2
203	0.9	R
1	0.6	R
2	0.5	7.8, 1.7
11	0.4	0.4
3	0.2	R
4	0.2	0.2
111	0.2	R
106	0.1	R

[a] Every Southern banding pattern of EcoRI-digested genomic DNA was either unique sequences (shown by kb) or repetitive sequences (R).

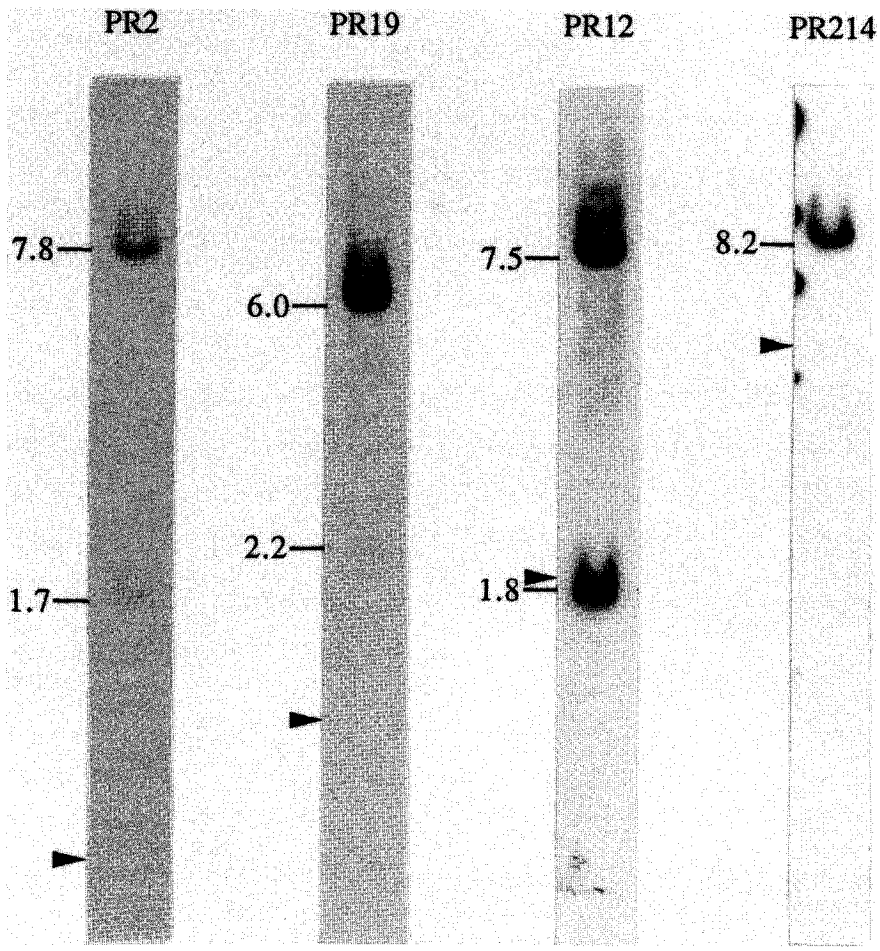


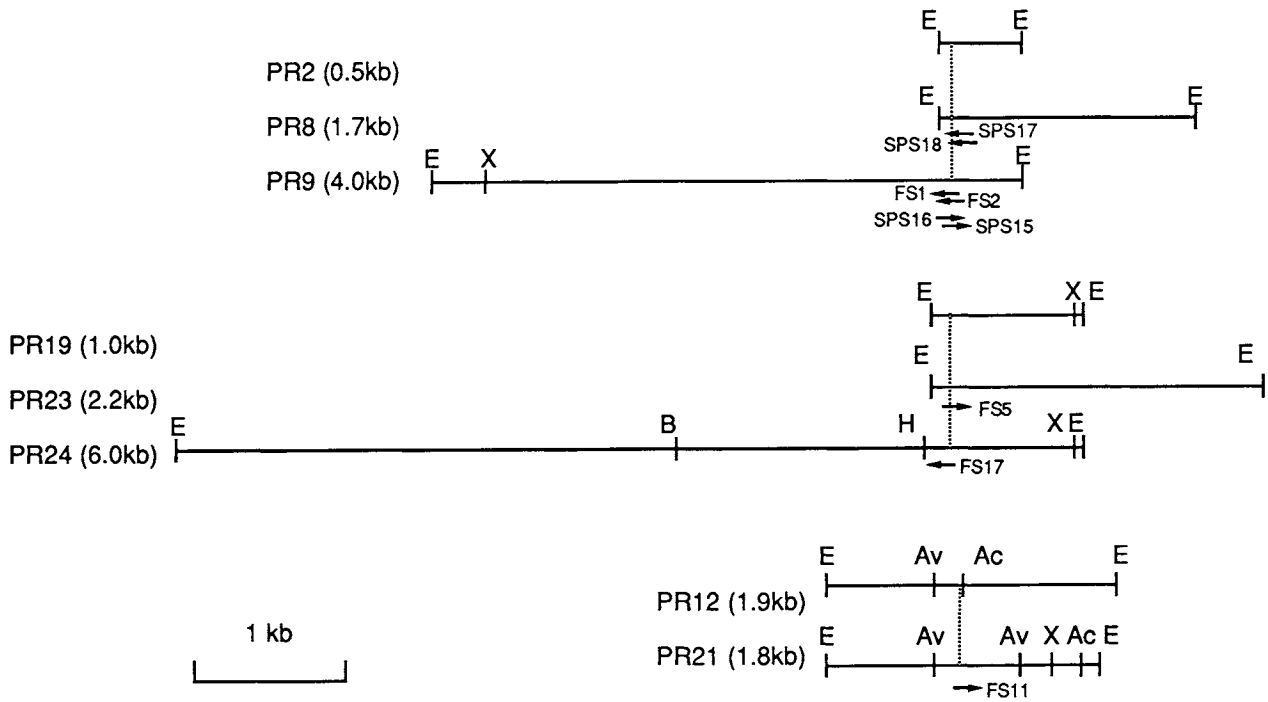
Fig. 1. Genomic Southern blots of EcoRI-digested P19 DNA probed with circular DNA fragments, PR2, PR19, PR12 and PR214. Molecular sizes, estimated with marker DNA (λ-HindIII fragments and ϕX174-HaeIII fragments), are indicated in kb on the left of each lane. Arrowheads indicate the estimated positions of DNA probes.

larger fragments (7.8 kb; 7.5 kb; 8.2 kb) were not found in this library possibly due to the upper limit of cloning capacity. However, we isolated a germline 4.0 kb fragment (PR9) homologous to the rearranged circular DNA fragments of 0.5 kb (PR2) which may have been derived from the expected 7.8 kb fragment by deletional events during DNA cloning. We compared the restriction fragments of circular DNA with the corresponding germline restriction maps (Fig. 2A) and sequenced the expected breakpoints with sequence primers (Fig. 2B). We determined the nucleotide sequences surrounding the breakpoints of 3 fragments (PR2, 19, 12) as shown in Fig. 2B. The homology between recombined germline sequences at the breakpoints was one or two bases. Patchy or very limited inverted repeats were found in close proximity to recombinant junctions.

Homology among circular DNA clones. We tested for the enrichment of unique sequences of circular DNA fragments in circular DNA libraries. Out of 3.7×10^5 phage containing 80% mitochondrial DNA fractions, we obtained 3 PR4-positive clones and 2 PR17-positive clones (data not shown). However, there were no clones hybridizable with other unique sequences including the rearranged fragments, PR2, PR19, PR12 and PR214. PR4 contained a sequence 65% homologous to the 3′ half of the B1 consensus sequence (Kayev

Fig. 2. Breakpoints on circular DNA clones. A) Restriction maps of circular DNA fragments (PR2, 19, 12) are compared with those of their germline counterparts. Dotted vertical lines indicate the breakpoints. Orientations of sequencing primers are shown by arrows starting from the primer sequence. E, EcoRI; X, XbaI; Av, AvaI; Ac, AccI; B, BamHI; H, HindIII. B) Nucleotide sequences surrounding breakpoints of circular DNA fragments. The recombinant structure is compared with the corresponding germline sequences. Nucleotides which are homologous to circular DNA fragments are indicated by a dash. Inverted repeats of interest are marked by a pair of divergent superscript arrows and cross-over junctions by dotted lines.

A



B

```
PR2    AAAGATAAAG ATGCAGGCAA CTTCAGCATC CACAGCAGTA GCCAGCACTG TGGAGAGAGT
PR8    ---------- ---------- ---------- ---------- ---------- ----------
PR9    AGTCTGCTTG AAGTTATGAA AGCAGAGCAG GCTGATCCAA TTACATAAGA AAAGTGCTCA

PR2    TCCACACAGG ACCATCTGGG CTGTGAATGC TGGGCTCTCT GAACCAAAGT CAGCAGCTTC
PR8    ---CCCGCAC ATTTCCTGAT GCTGAGCTTA GCAGTCTCTG AAGCTCACGT GGGGGGAAAG
PR9    GT-------- ---------- ---------- ---------- ---------- ----------

PR19   GCAGTATTAT TTGGTTTAAA CTAGATTAGC TTAATTATAAG AGGGAGTTGG GTTTGGTGAG
PR23   ---------- ---------- ----------  -GCCATTTCT GCTAAAAAAA AACTAAAGCA
PR24   GAGACAAAAG AGATAAAAGT AACATCAAA- ----------- ---------- ----------

PR12   ATGAGAGAGA CACGGTTGAG ATGGATTCCT TGTTTTCAGC AGGAATGACC GTGAGACTGG
PR21   ---------- ---------- ---------- ---------- ---------- ----------

PR12   ACTGTGGGGT TTTGCTGAGG TAGACCTGTG GGAGGAAATG TGATGTTTGG AGAGAGTATC
PR21   ---------C AGAAACGAGG AACTAGGAAA GGGAAGTGTT TTTGTGGGGG AGAGGGCAAG
```

Fig. 2.

```
PR4              GAATTCTGGG CTAGCCTTGG CTACTTATCA AGTTCAAGGC TAGCCCAGAT TA CCTGA  GA  C
B1 CONSENSUS     --G---GA-- -C-----G-T ----AG-GTG -----C---A C----AG-GC --A--A--AA--AA-

PR4              CCTGT:AGGGT TTGCTCACCA GGTCTGTCTT CACCATGTTA AACCCAAGTC TGGCAGGCTC
B1 CONSENSUS     -----:CT
                      :

PR4              CCTTCCCCAG CCCTCTGTGG GCCTACCTCA AAGAGCCAGA CGAGAAGTGT GACGACGCCC

PR4              ATGGAATTC
```

Fig. 3. Nucleotide sequence of PR4 compared with B1 consensus sequence (Krayev *et al.*, 1980). Sequences are aligned to give maximal homology by introducing gaps. Dash indicates the homologous base.

et al., 1980) (Fig. 3) but several hundred base stretches sequenced from both ends of PR17 had no homologue in the EMBL DNA database.

DISCUSSION

The presence of extrachromosomal circular DNAs composed of chromosomal sequences seems to reflect the plasticity of eukaryotic genomes. However, their presence does not necessarily mean the reciprocal recombination product of any rearranging chromosomal DNA as verified in V(D)J recombination and class switch recombination in immune system (Fujimoto and Yamagishi, 1987; Okazaki *et al.*, 1987; Toda *et al.*, 1989; von Schwedler *et al.*, 1990; Iwasato *et al.*, 1990; Matsuoka *et al.*, 1990; Harada and Yamagishi, 1991; Iwasato and Yamagishi, 1991; Shimizu and Yamagishi, 1992). Extrachromosomal fragments may be also generated by other mechanisms, such as retrotranscription and replicon-misfiring as reviewed previously (Rush and Misra, 1985; Yamagishi, 1986).

Extrachromosomal circular DNA was purified in the presence of ATP-dependent exonuclease and hence free from linear DNA as assessed by electron microscopy (Yamagishi *et al.*, 1983). EcoRI DNA fragments from the circular DNAs purified from differentiated P19 EC cells were cloned into λgt10 phage vector. We selected 26 non-mitochondrial DNA fragments at random. Of these fragments, 16 contained repetitive sequences and 10 contained unique sequences. Their size distribution ranged from 0.1 kb to 6.0 kb, with a mean length of 2.1 kb. We identified 4 chromosomal breakpoints in 10 circular DNA fragments covering a total 22 kb unique chromosomal sequence. The high incidence of breakpoints suggests that the average size of intact circular DNAs must be no longer than 17 kb for an extrachromosomal circular DNA from mouse thymocytes (Fujimoto *et al.*, 1985) that has been characterized as an excision product of T-cell receptor V(D)J gene rearrangements (Fujimoto and Yamagishi, 1987).

Nascent fragments derived from chromosome may be linear or circularized with the two ends occasionally modified by base deletion or addition. Therefore, the breakpoints identified on circular DNA fragments merely represent the junction of circle formation. To determine whether chromosomal DNA was actually rearranging, we amplified the DNA sequences surrounding the breakpoint of circular DNA fragments by the polymerase chain reaction (PCR) method (Saiki *et al.*, 1988) with the use of appropriate primers pairs from PR8/PR9 and PR23/PR24 as shown in Fig. 2A. However, we have obtained no evidence so far for deletional events (data not shown). Since extrachromosomal circular DNAs are only a small fraction of the total cellular DNA and the least abundant in the neonatal brain so far examined by electron microscopy (Yamagishi, 1986), chromosomal deletion may be rare, accidental events and hard to be proven even by the PCR method.

Patchy short inverted repeats as we saw in the circularization junctions (Fig. 2B) were often found in novel joints as non-homologous deletions in the human β-globin gene cluster (Orkin and Kazazaian, 1984) and inversions of a myelin basic protein (MBP) fragment (Okano *et al.*, 1988), and truncations in retroposon sequences (Fujimoto *et al.*, 1985; Schindler and Rush, 1985; Jones and Potter, 1985). The canonical signal sequences of V(D)J recombination can be classified as one of the patchy short inverted repeats. Intrastrand loop structure between such inverted short homology regions may be maintained, presumably by DNA-binding proteins such as DNA topoisomerase binding to the loop and then excised. Yet we have obtained no evidence of circular DNA fragments indicating V(D)J-like recombination in neuron-like EC cells as reported in transgenic mouse brain cells (Matsuoka *et al.*, 1991). The relative abundance of two circular DNA fragments PR4 and PR17 in circular DNA libraries may reflect the illegitimate recombination occurring in the unstabilized chromosomal region such as fragile sites.

Acknowledgments. We are grateful to Dr. Ursula Storb for critical reading of the manuscript and Dr. Hiroshi Hamada for the gift of P19 cell lines. This work was supported by Grants-in-Aid for Science Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. Chun, J.J.M., Schatz, D.J., Oettinger, M.A., Jaenisch, R., and Baltimore, D. 1991. The recombination activating gene-1 (RAG-1) transcript is present in the murine central nervous system. *Cell,* **64**: 189–200.
2. Edwards, M.K.S. and McBurney, M.W. 1983. The concentration of retinoic acid determines the differentiated cell types formed by a teratocarcinoma cell line. *Dev. Biol.,* **98**: 187–191.
3. Fujimoto, S., Tsuda, T., Toda, M., and Yamagishi, H. 1985. Transposon-like sequences in extrachromosomal circular DNA from mouse thymocytes. *Proc. Natl. Acad. Sci. USA,* **82**: 2072–2076.
4. Fujimoto, S. and Yamagishi, H. 1987. Isolation of an excision product of T-cell receptor α-chain gene rearrangements. *Nature,* **327**: 242–243.
5. Harada, K. and Yamagishi, H. 1991. Lack of feedback inhibition of Vκ gene rearrangement by productively rearranged alleles. *J. Exp. Med.,* **173**: 409–415.
6. Hoyt, M.A., Knight, D.M., Das, A., Miller, H.I., and Echols, H. 1982. Control of phage λ development by stability and synthesis of cII protein: Role of the viral cIII and host hflA, himA and himD genes. *Cell,* **31**: 565–573.
7. Iwasato, T., Shimizu, A., Honjo, T., and Yamagishi, H. 1990. Circular DNA is excised by immunoglobulin class switch recombination. *Cell,* **62**: 143–149.
8. Iwasato, T. and Yamagishi, H. 1991. Novel excision products of T cell receptor γ gene rearrangements and developmental stage specificity implied by the frequency of nucleotide insertions at signal joints. *Eur. J. Immunol.,* **22**: 101–106.
9. Jones-Villeneuve, E.M.V., McBurney, M.W., Rogers, K.A., and Kalnins, V.I. 1982. Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. *J. Cell Biol.,* **94**: 253–261.
10. Jones, R.S. and Potter, S.S. 1985. L1 sequences in HeLa extrachromosomal circular DNA: Evidence for circularization by homologous recombination. *Proc. Natl. Acad. Sci. USA,* **82**: 1989–1993.
11. Krayev, A.S., Kramerov, D.A., Skryabin, K.G., Ryskov, A.P., Bayev, A.A., and Georgiev, G.P. 1980. The nucleotide sequence of the ubiquitous repetitive DNA sequence B1 complementary to the most abundant class of mouse fold-back RNA. *Nucleic Acids Res.,* **8**: 1201–1215.
12. Maniatis, T., Fritsch, E.F., and Sambrook, J. 1982. *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
13. Matsuoka, M., Yoshida, K., Maeda, T., Usuda, S., and Sakano, H. 1990. Formation of switch circular DNA in cytokine treated mouse splenocytes: evidence for intramolecular DNA deletion in immunoglobulin class switch. *Cell,* **62**: 135–142.
14. Matsuoka, M., Nagawa, F., Okazaki, K., Kingsbury, L., Yoshida, K., Muller, U., Larue, D.T., Winer, J.A., and Sakano, H. 1991. Detection of somatic DNA recombination in the transgenic mouse brain. *Science,* **254**: 81–86.
15. McBurney, M.W. and Rogers, B.J. 1982. Isolation of male embryonal carcinoma cells and their chromosomal replication patterns. *Dev. Biol.,* **89**: 503–508.
16. Oettinger, M.A., Schatz, D.G., Gorka, C., and Baltimore, D. 1990. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science,* **248**: 1517–1523.
17. Okano, H., Ikenaka, K., and Mikoshiba, K. 1988. Recombination within the upstream gene of duplicated myelin basic protein genes of myelin deficient shi^{mld} mouse results in the production of antisense RNA. *EMBO J.,* **7**: 3407–3412.
18. Okazaki, K., Davis, D.D., and Sakano, H. 1987. T cell receptor β gene sequences in the circular DNA of thymocyte nuclei: direct evidence for intramolecular DNA deletion in V-D-J joining. *Cell,* **49**: 477–485.
19. Orkin, S.H. and Kazazaian, H.H. 1984. The mutation and polymorphism of the human β-grobin gene and its surrounding DNA. *Annu, Rev. Genet.,* **18**: 131–171.
20. Rudnicki, M.A. and McBurney, M.W. 1987. Cell culture method and induction of differentiation of embryonal carcinoma cell lines. In *Teratocarcinoma and Embryonic Stem Cells: A Practical Approach,* (E.J. Robertson, ed.). IRL Press, Oxford, pp 19–50.
21. Rush, M.G. and Misra, R. 1985. Extrachromosomal DNA in eucaryotes. *Plasmid,* **14**: 177–191.
22. Saiki, R.K., Gelfand, D.H., Stoffel, S.J., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Ehrlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science,* **239**: 487–491.
23. Sanger, F. 1981. Determination of nucleotide sequences in DNA. *Science,* **214**: 1205–1210.
24. Schatz, D.G., Oettinger, M.A., and Baltimore, D. 1989. The V(D)J recombination activating gene (RAG-1). *Cell,* **59**: 1035–1048.
25. Schindler, C.W. and Rush, M.G. 1985. The KpnI family of long interspersed nucleotide sequences is present on discrete sizes of circular DNA in monkey (BSC-1) cells. *J. Mol. Biol.,* **181**: 161–173.
26. Shimizu, T. and Yamagishi, H. 1992. Biased reading frames of pre-existing D_H-J_H coding joints and preferential nucleotide insertions at V_H-DJ_H signal joints of excision products of immunoglobulin heavy chain gene rearrengements. *EMBO J.,* **11**: 4869–4875.
27. Toda, M., Hirama, T., Takeshita, S., and Yamagishi, H. 1989. Excision products of immunoglobulin gene rearrangements. *Immunol. Lett.,* **21**: 311–316.
28. von Schwedler, U., Jack, H.-M., and Wabl, M. 1990. Circular DNA is a product of the immunoglobulin class switch rearrangement. *Nature,* **345**: 452–456.
29. Yamagishi, H., Tsuda, T., Fujimoto, S., Toda, M., Kato, K., Maekawa, A., Umeno, M., and Anai, M. 1983. Purification of small polydisperse circular DNA of eukaryotic cells by use of ATP-dependent deoxyribonuclease. *Gene,* **26**: 317–321.
30. Yamagishi, H. 1986. Role of mammalian circular DNA in cellular differentiation. *Bio Essays,* **4**: 218–221.

(Received for publication, September 10, 1993 and accepted, September 17, 1993)