Instability of the 12-Nucleotide Repeat in *c-myb* Gene of Bovine T-Lymphoma Cells

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ABSTRACT. Insertion of a 12-nucleotide repeat in *c-myb* gene exon 9 was observed in about 15% of sporadic bovine T-lymphomas. The 12-nucleotide repeat in the T-lymphoma cells showed deletion and insertion of the repeat units during cultivation of the cells. To know whether deficiency in DNA loop repair is involved in the instability of the repeat, abilities to bind and correct the loop structure in nuclear extracts were examined. The nuclear extracts of all examined cells had ability to bind and correct the loop structure. These data suggest that instability of the 12-nucleotide repeat in bovine T-lymphoma cells might be independent of deficiency of DNA loop repair function.

— KEY WORDS: instability, loop repair, repeat.

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The mammalian genome contains many sequences that consist of one- to twelve-nucleotide motifs repeated numerous times [2]. Deletion and addition in such simple repeats are an important source of genomic change and associated with a variety of tumors exhibiting genome instability [1, 7, 9, 14, 17]. For example, tumors developing in hereditary non-polyposis colon cancer (HNPCC) patients display frequent mutations within (CA)_n and other simple repetitive sequences. Germ line mutations in hMSH2, hMLH1, hPMS1, and hPMS2 have been implicated in HNPCC [3, 4, 12]. Since these proteins are involved in the correction of DNA replication errors, deficiencies of function of these proteins appear to be the cause of the genome wide instability in HNPCC.

The *c-myb* is a proto-oncogene encoding a DNA-binding transcriptional activator that is thought to regulate genes critical for hematopoietic cell growth and development [11, 15]. In previous study, we reported a tandemly repeated 12-nucleotide motif (TGATCTGCCCCGT) was inserted into the *c-myb* gene exon 9 of about 15% of sporadic bovine T-lymphomas [16]. The number of repetitive unit ranged from five to 22 copies. The 12-nucleotide motif was comprised of sequence similar to flanking sequences of the insertion. The insertion of a 12-nucleotide repeat have also been reported in *K-ras* gene exon 1 of chemically induced tumors in rats [6] although the sequence of the motif was differ from the 12-nucleotide motif in *c-myb* gene.

Bovine T-lymphomas containing the 12-nucleotide repeat expressed two kinds of *c-myb* mRNA: 1) are that included the repetitive sequence in exon 9, and 2) are that lacked the whole sequence of exon 9. It was demonstrated that the insertion of the 12-nucleotide repeat enhanced exon skipping and mutant *c-Myb* lacking exon 9 had 3- to 8-fold higher transcriptional activating activity than wild-type *c-Myb*. Thus we tried to know features of the 12-nucleotide repeat.

To examine the stability of the 12-nucleotide repeat, we used bovine T-lymphoma cell line BTL26, a clone of BTL-

PC3 cells [8]. Twelve subclones were derived from BTL26 cells and chromosomal DNA was isolated from each subclone at indicated times and subjected to the polymerase chain reaction (PCR) using a primer pair, myb20 (5'-ATCTTTCCTCTAACAGCATC-3', intron 8) and myb17 (5'-AGAGGCACTTTCTTCAGGTAG-3', 1116–1096). Fifty microliter PCR reactions contained genomic DNA equivalent to 100 cells, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100, each 20 pmol of the primer set, 50 μ M deoxynucleoside triphosphates, and 1U of Taq DNA polymerase (Promega, Madison, WI). For radiolabeling of products, 0.2 to 0.4 μ l of [α -³²P] dCTP (deoxycytidine triphosphate) (10 μ Ci/ μ l, 3000 Ci/mmol) were added to the reaction. The genomic DNA templates were denatured at 96°C for 5 min, and amplified for 25 rounds at 96°C for 40 s, 54°C for 30 s, and 74°C for 1.5 min. One tenth of the reaction was analyzed by electrophoresis in 0.5 $\times$ TBE on 10% polyacrylamide nondenaturing gels.

PCR products from each subclone showed variable DNA fragment lengths in addition to wild type. Figure 1 represents examples of the PCR products detected from the subclones of BTL26 cells. The large PCR product detected from BTL26–26 cells at 16 cell divisions contained 17 copies of the 12-nucleotide motif, 9-nucleotide interrupted sequence, and two nucleotides substitution [T(TGATCTGCCCCGT)₈TCTGGGCGT(TGATCTGCCCCGT)₉T] in codon 359 of *c-myb*. At 20 cell divisions, a DNA fragment containing 4 copies of the 12-nucleotide motif [T(TGATCTGCCCCGT)₄T] was detected in addition to the wild-type fragment and the fragment with 17 copies of the motif. The DNA fragment with 4 copies of the motif was disappeared at 30 cell divisions. While, in BTL26–49 cells, the DNA fragment containing 4 copies of the 12-nucleotide motif was predominated at 16 cell division. However, it was not detected thereafter. At 30 to 40 divisions, a DNA fragment containing 11 copies of the 12-nucleotide motif was detected. These results indicate that the 12-nucleotide repeat in subclones of BTL26 cells was highly unstable and deletion and insertion of the repeat occurred frequently.

The cause of deletion and insertion in simple repeats has

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Fig. 1. Instability of the 12-nucleotide repeat in subclones of BTL26 cells. DNA from subclones of BTL26 (BTL26-26 and BTL26-49) was used as template for PCR with primers specific for the *c-myc* gene. The PCR products were subjected to electrophoresis, and representative autoradiograph of the gel is shown.

been thought to be due to loss of function to correct DNA loop structure arisen by slippage during the replication of simple repeats [17, 19]. The first step of the repair has been considered to be recognition of the loop structure. Thus, we examined T-lymphoma nuclear extracts for an ability to bind DNA loop structure. Electrophoretic mobility shift assay (EMSA) was performed as previously reported by Hernandez-Munain and Krangel [5], with some modifications. Briefly, nuclear extracts (1–9 μ g of proteins) were mixed with 0.5 μ g of poly(dI-dC) in 10 μ l of reaction mixture containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA 1 mM dithiothreitol, and 2% glycerol. A radio labeled oligonucleotide ON1 (5'-ACTCCATCTCTGCCCCGCGGATCCCGGCTCCCTAC-3') was annealed to an oligonucleotide ON8 (5'-G T A G G G A G C C G G G A T C A A C G G G C A GATCAACGGGCAGAGATGGAGT-3') containing the 12-nucleotide motif (14 unpaired bases) at its center. The radio labeled heteroduplex probe ON1-8 was added to the reaction mixtures and incubated for 15 min at 37°C. As competitors, unlabeled ON1-8 and ON1-11 (ON11; 5'-

G T A G G G A G C C G G G A T C A G T G A G C G AACACACGGGCAGAGATGGAGT-3'), a oligonucleotide with the 14 unpaired nucleotides of ON1-8 realigned randomly, were added to the reaction mixtures before incubation with the radiolabeled probe. Samples were electrophoresed through 7.5% polyacrylamide gel in 0.5 $\times$ TBE and exposed to X-ray film.

When 3 μ g of nuclear extracts were used, one abundant complex (indicated by arrowhead) and an array of minor complexes with lower and higher electrophoretic mobility were observed in nuclear extracts of BTL26 (parent) and Pr2181, a bovine T-lymphoma cell line without the insertion of the 12-nucleotide repeat in *c-myc* gene (Fig. 2). By addition of 5-fold molar excess of non-radioactive competitor oligonucleotide ON1-8, the signal of the band indicated by the arrowhead was weakened in nuclear extracts of Pr2181, YS1 (a bovine T-lymphoma cell line), and BTL26 cells. The band was hardly visible in the presence of 40-fold molar excess of the unlabeled ON1-8. On the other hand, the band was not weakened until addition of 40-fold molar excess of the unlabeled ON 1-11. These results suggest that nuclear factor(s) bound specifically to the unpaired bases of oligonucleotide ON1-8 and that binding activities of nuclear factor(s) in all cells examined were almost the same.

To investigate whether the BTL26 cells were defect in the DNA loop repair function, an ability to correct the loop structure was compared between whole cell extract of the BTL26 cells (parent) with 17 copies of the 12-nucleotide motif and whole cell extracts of Pr2181 and YS1 cells, bovine T-lymphoma cell lines without the insertion of the 12-nucleotide repeat. The circular heteroduplex substrates (WT:D and D:WT, Fig. 3a) for *in vitro* loop correction were prepared using pKS+WT and pKS+D plasmids [16] as described by O'Regan *et al.* [13]. The 11-nucleotide loop (TTGGGCCCCGTT) is positioned in a heteroduplex cassette sequence such that correction to WT strand without the inserted sequence creates a *Bam*HI site, whereas correction to D strand with the insertion generates an *Apal* site (Fig. 3a). These *Bam*HI and *Apal* sites are cut at position 0 by appropriate restriction enzymes. The heteroduplexes contain a site-specific nick within an *Not*I site. The loop repair

Fig. 2. Binding of T-lymphoma nuclear extracts to the loop structure. Radio labeled ON1-8 oligonucleotide was incubated with increasing amounts of nuclear extracts (1, 3, and 9 μ g of proteins) in the absence of a competitor or with 3 μ g of nuclear extract in the absence or presence of increasing amounts of competitors (5-, 10-, 20-, and 40-fold molar excess). ON1-8 complexes were resolved by electrophoresis. Arrowhead denotes the DNA-protein complex.

Fig. 3. *In vitro* correction of loop structure. a. The substrates for the reaction were form II duplex molecules constructed by annealing viral (v) and unmatched complementary (c) strands. Each substrate contained the loop shown within a heteroduplex cassette sequence. The position of the loop is designated 0. The numbers in parentheses indicate the distance (kb) measured in a clockwise direction from the loop site. Correction of the loop structure in the WT strand generates a *Apal* site and in the D strand generates a *Bam*HI site. b. Correction assay. Substrates (10 ng) were incubated with whole cell extracts (70 μ g) for 1 hr at 37°C. The DNA was recovered and digested with *Apal* or *Bam*HI plus *Eco*RV. Digestion products were separated on agarose gels, transferred by Southern blotting, and probed with radiolabeled pKS+ plasmid.

assay mixtures (20 μ l) contained 20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM glutathione, 50 μ g/ml bovine serum albumin, 0.1 mM each dNTP, 1.5 mM ATP, 70 mM KCl, and 10 ng of heteroduplex DNA. The reactions were started by addition of extract (70 μ g), incubated for 60 min at 37°C, and the reactions were terminated by the addition of 30 μ l of 25 mM EDTA, 0.5% SDS and proteinase K (50 μ g/ml). After further incubation for 15 min at 37°C, the mixtures were extracted with phenol: chloroform and DNA was recovered by ethanol precipitation. Precipitated DNA was dissolved in the buffer for digestion with restriction enzymes (Boehringer Mannheim GmbH, Mannheim, Germany). Digestion products were separated on 0.7% agarose gels, denatured, and transferred to Hybond N filters (Amersham Life Science, Buckinghamshire, UK). The

Table 1. *In vitro* correction of the loop structure

Cell	WT:D		D:WT	
	<i>Apal</i>	<i>Bam</i> HI- <i>Eco</i> RI	<i>Apal</i>	<i>Bam</i> HI- <i>Eco</i> RI
Pr2181	56.4*	24.7	12.6	74.4
YS1	55.3	19.1	10.9	73.9
BTL26	54.1	18.5	10.0	72.9

*: A percentage of 4.1 kb fragment shown in Fig. 3.

filters were probed with radiolabeled random-primed pKS+ plasmid linearized with *Eco*RI. Radioactive DNA was localized by autoradiography.

When the WT:D heteroduplex treated with whole cell extracts of Pr2181 and YS1 was digested with *Apal*, 56.4 and 55.3% of WT:D were detected as a fragment of 4.1 kilobases (kb) (Fig. 3b and Table 1). Similarly, the fragment of 4.1 kb were detected from the WT:D heteroduplex treated with the cell extract of BTL26 on a percentage of 54.1. These results indicate that the cell extract of BTL26 carried out repair of the loop structure as well as those of Pr2181 and YS1. By digestion with *Apal*, 54.1–56.5% of the WT:D heteroduplex treated with these cell extracts were detected as the 4.1 kb fragment, while by digestion with *Bam*HI and *Eco*RV, 18.5–24.7% of them were detected as the 4.1 kb fragment. These data suggest that the WT strand with the nick was repaired more efficiently than the D strand by these cell extracts. When D:WT was treated with extracts of BTL26 cells, Pr2181 cells, or YS1 cells, digestion with *Apal* or *Bam*HI plus *Eco*RV yielded fragments of 4.1 and 5.1 kb. Contrary to the WT:D heteroduplex, the 4.1 kb fragment of D:WT heteroduplex produced by digestion with *Bam*HI and *Eco*RV was more abundant than the 4.1 kb fragment yielded by digestion with *Apal*. The observations suggest that the nicked strand was repaired more efficiently than the strand without the nick. These data are consistent with loop repair by human cell extracts that correction is directed to the nicked strand [19]. Our observations suggest that instability of the 12-nucleotide repeat might be independent of DNA loop repair function.

In the present study we showed that the 12-nucleotide repeat was highly unstable in the T-lymphoma cell line BTL26. Because several specific mutations in the same cell are necessary in order to develop a tumor, such a high mutation rate, probably based on a disturbance in the stability of the genome, might be contribute a tumor development. It has been thought that deficiency in repair of loop structure risen by slippage during the replication causes the insertion and deletion of repetitive sequences [17]. Thus we had expected the BTL26 cells was deficient in DNA loop repair. However, nuclear factor(s) in extracts of BTL26, Pr2181, and YS1 cells had almost the same binding activities to oligonucleotide probe with loop. Besides, the BTL26 cell extract had ability to correct the loop structure as the other cell extracts. Namely, activity of DNA loop repair in the BTL26 cell extract seemed to be normal. These results suggest that high mutation rate seen

in the cells containing 12-nucleotide repeat might be independent of loop repair function. Recently, dodecamer repeat expansion was found in human cystatin B gene in progressive myoclonus epilepsy [10], whereas any correlation with deficiency of loop repair mechanisms have not been reported. Perhaps, some causes for the repeat instability other than deficiency of the loop repair system might be exist.

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REFERENCES

1. Aaltonen, L. A., Peltomäki, P., Leach, F. S., Sistonen, P., Pylkkänen, L., Mecklin, J.-P., Järvinen, H., Powell, S. M., Jen, J., Hamilton, S. R., Petersen, G. M., Kinzler, K. W., Vogelstein, B., and de la Chapelle, A. 1993. *Science* 260: 812–816.
2. Beckman, J. S. and Weber, J. L. 1992. *Genomics* 12: 627–631.
3. Bronner, C. E., Baker, S. M., Morrison, P. T., Warren, G., Smith, L. G., Lescoe, M. K., Kane, M., Earabino, C., Lipford, J., Lindblom, A., Tannergård, P., Bollag, R. J., Godwin, A. R., Ward, D. C., Nordenskjöld, M., Fishel, R., Kolodner, R., and Liskay, R. M. 1994. *Nature (Lond.)* 368: 258–261.
4. Fishel, R., Lescoe, M. K., Rao, M. R., Copeland, N. G., Jenkins, N. A., Garber, J., Kane, M., and Kolodner, R. 1993. *Cell* 75: 1027–1038.
5. Hernandez-Munain, C. and Krangel, M. S. 1994. *Mol. Cell. Biol.* 14: 473–483.
6. Higinbotham, K. G., Rice, J. M., Buzard, G. S., and Perantoni, A. O. 1994. *Oncogene* 9: 2455–2459.
7. Ionov, Y., Peinado, M. A., Malkhosyan, S., Shibata, D., and Perucho, M. 1993. *Nature (Lond.)* 363: 558–561.
8. Ishiguro, N., Ohzono, T., Shinagawa, T., Horiuchi, M., and Shinagawa, M. 1994. *J. Biol. Chem.* 269: 26822–26829.
9. Kolodner, R. D., Hall, N. R., Lipford, J., Kane, M. F., Rao, M. R., Morrison, P., Wirth, L., Finan, P. J., Burn, J., Chapman, P., Earabino, C., Merchant, E., and Bishop, D. T. 1994. *Genomics* 24: 516–526.
10. Lalioti, M. D., Scott, H. S., Buresi, C., Rossier, C., Bottani, A., Morris, M. A., Malafosse, A., and Antonarakis, S. E. 1997. *Nature (Lond.)* 386: 847–851.
11. Mucenski, M. L., McLain, K., Kier, A. B., Swerdlow, S. H., Schreiner, C. M., Miller, T. A., Pietryga, D. W., Scott, W. J., and Potter, S. S. 1991. *Cell* 65: 677–689.
12. Nicolaidis, N. C., Papadopoulos, N., Liu, B., Wei, Y.-F., Carter, K. C., Ruben, S. M., Rosen, C. A., Haseltine, W. A., Fleischmann, R. D., Fraser, C. M., Adams, M. D., Venter, J. C., Dunlop, M. G., Hamilton, S. R., Peterson, G. M., de la Chapelle, A., Vogelstein, B., and Kinzler, K. W. 1994. *Nature (Lond.)* 371: 75–80.
13. O'Regan, N. E., Branch, P., Macpherson, P., and Karran, P. 1996. *J. Biol. Chem.* 271: 1789–96.
14. Richards, R. I. and Sutherland, G. R. 1994. *Nature Genet.* 6: 114–116.
15. Sheiness, D. and Gardinier, M. 1984. *Mol. Cell. Biol.* 4: 1206–1212.
16. Shinagawa, T., Ishiguro, N., Horiuchi, M., Matsui, T., Okada, K., and Shinagawa, T. 1997. *Oncogene* 14: 2775–2784.
17. Strand, M., Prolla, T. A., Liskay, R. M., and Petes, T. D. 1993. *Nature (Lond.)* 365: 274–276.
18. Thibodeau, S. N., Bren, G., and Schaid, D. 1993. *Science* 260: 816–819.
19. Umar, A., Boyer, J. C., and Kunkel, T. A. 1994. *Science* 266: 814–816.