Detection and cloning of unique integration sites of retrotransposon, intracisternal A-particle element in the genome of acute myeloid leukemia cells in mice

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Received 9 October 1997

Abstract We previously found retrotransposition of the intracisternal A-particle (IAP) element in the genome of acute myeloid leukemia (AML) cells induced by X-irradiation of C3H/He mice (FEBS 16333). To analyze the occurrence of the IAP-mediated retrotransposition in AML cells, we compared integration sites of the IAP element by polymerase chain reaction (PCR) in the genomes of five AML strains derived from different C3H mice. Unique PCR products were found in all of the above independent leukemia cells, whereas no such products were detected in normal cells. Results of cloning, sequencing and Southern analyses showed that the PCR products were derived from novel integration sites of the IAP element in the genome. The data suggest that IAP-mediated retrotransposition occurs frequently in radiation-induced AML cells from C3H/He mice.

Key words: Retrotransposon; Retrotransposition; Intracisternal A-particle; Gene rearrangement; Polymerase chain reaction; Nucleotide sequence

1. Introduction

Radiation-induced leukemia can be experimentally produced in several inbred mice. Acute myeloid leukemia (AML) occurs in 20–30% of C3H/He inbred mice 1–2 years after whole-body exposure to ionizing radiation [1,2]. The murine AML cells from different individuals with a wide variety of histochemical phenotypes can be treated as 'independent' strains. These cells can be used to analyze genetic events which are inscribed in the genome through initial radiation damage and leukemogenesis. We previously found that one of seven independent AML cells tested has a rearrangement in the interleukin-3 gene by integration of the intracisternal A-particle (IAP) element [3].

The IAP is one of the murine retrotransposons and is believed to be a kind of incomplete endogenous retrovirus [4]. Like retroviruses, the reverse transcription product can be introduced into genomic DNA by retrotransposition [5], resulting in an IAP element with two long terminal repeat (LTR) sequences containing enhancer/promoter function [6]. Whereas retroviral infection and subsequent events are rare in normal mice, normal genomes naturally contain large numbers (ca. 1000 copies/haploid) of the IAP element due to accumulation of retrotransposition in the germline cells [4,5]. This means that any normal cell has the possibility of being modified in the genome by IAP retrotransposition, so that the IAP is regarded as an endogenous mutagen in mouse cells [4,5]. Examples of IAP-mediated gene rearrangement that contributes to the specific features of the tumor have been observed in various tumor cell lines in mice [4,5,7–10]. If retrotransposition by the IAP commonly occurs in tumor cells or during tumorigenesis, the integrated IAP element should be observed in the genome from any tumor cell. However, the large copy number of the IAP provirus strongly prevents detection of the tumor-specific integration site.

Our previous finding of gene rearrangement by integration of the IAP element in AML cells from C3H/He mice led us to investigate the possibility that retrotransposition may be a frequent event in AML cells. In this study, we compared the integration sites of the IAP element in genomes of five independent AML cells and normal cells. Genomes of all the AML cells possessed unique integration sites of IAP, whereas no such retrotransposition was observed in normal cells. This suggests that IAP-mediated retrotransposition is a frequent event in radiation-induced AML in C3H/He mice.

2. Materials and methods

2.1. Mice and leukemia cells

Radiation-induced AML was generated 1–2 years after whole-body X-irradiation to C3H/He mice at a dose of 3 Gy [1]. Leukemia cells obtained from the AML mice were maintained by in vivo passage by injection of the cells into 6–8-week-old normal C3H/He mice inbred in our institute [1,3]. The amplified leukemia cells were collected from the spleen 2–4 weeks after injection. High molecular weight DNA samples of five independent leukemia strains derived from different AML mice were prepared by the standard method [11].

2.2. Primers, PCR and inverted PCR

The six PCR primers for detection of AML-specific integration sites are shown in Fig. 1. Four primers (RS-IAP-F, U-IAP-F, RS-IAP-R and U-IAP-R) correspond to IAP-LTR and two primers (SINE-B2-F and SINE-B2-R) correspond to the consensus sequence of the short interspersed element (SINE)-B2. 50 ng of total genomic DNA with a pair of the primers was amplified by PCR with 0.25 units of ExTaq DNA polymerase (Takara Shuzo Co.) (94°C, 1 min; then 32 cycles of 98°C, 20 s; 55°C, 1 min; 72°C, 8 min) using a thermal cycler (Perkin-Elmer model 480).

The inverted PCR [12] was used to clone the normal allele of the AML-specific rearranged DNA. After sequencing of the first PCR product specific for AML cells, a primer set suitable for the inverted PCR was prepared. Germline DNA was digested by appropriate restriction enzymes, end-filled by Klenow fragment of DNA polymerase I, and recircularized by T4 DNA ligase at low DNA concentration (2 ng/ml). The recircularized DNA was used for the inverted PCR with a

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The sequences in this paper will appear in DDBJ, EMBL and GenBank nucleotide sequence databases under accession numbers D85906 and D85907.

PII S0014-5793(97)01383-5

set of primers and ExTaq DNA polymerase (35 cycles of 94°C, 1 min; 52°C, 1 min; 72°C, 4 min). By comparing the sequences between an AML-specific PCR product and the corresponding product of the inverted PCR from germline DNA, AML-specific insertion sites of the IAP element were determined. Since the first PCR product is one side of the AML-specific junction of the IAP element, the sequence corresponding to the other side of the junction in the AML genome can be predicted by a comparison. A primer matched to the other side was prepared, together with an IAP-LTR primer and AML genomic DNA, and was used for PCR (35 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 2 min) by Taq DNA polymerase (Perkin Elmer). The 6 primers (Q11-F, Q11-R, Q11-B, Q14-F, Q14-R and Q14-B) used in the subsequent PCR are shown in Fig. 3.

2.3. Nucleotide sequence analysis and Southern blot hybridization

The PCR products were electroeluted, cloned using the T-vector pMOSblue (Amersham), and sequenced using Sequenase-2 (USB). Probes for genomic Southern analysis were prepared from PCR products corresponding to the AML-specific insertion site in the normal allele which does not contain IAP- or SINE-derived sequences by appropriate primers (Q11-R plus Q11-B or Q14-R plus Q14-B) and germline DNA. Using the standard method [11], the probes were labeled with ^{32}P using a random primer labeling kit (BRL), hybridized with blots and analyzed with the BAS2000 system (Fuji Photo Film Co.).

3. Results

We have mainly studied whether IAP-mediated retrotransposition is a frequent event in the AML cells from C3H/He mice. If so, AML-specific integration sites of the IAP element should be detectable in the genomes of the AML cells from any type of independent AML. However, it is difficult to detect the specific integration sites due to the 1000 copies of the IAP provirus in the normal genome. Even though 2-dimensional genomic Southern blot analysis was used to visualize the whole provirus, non-IAP-derived genes and RFLP produced large numbers of background spots and interfered with the analysis.

3.1. Detection of AML-specific PCR product using IAP-specific primers

We attempted to amplify the integration sites by PCR using a primer corresponding IAP-LTR as one of a pair. To construct another primer, a sequence for the SINE-B2, a repetitive sequence with a high copy number (80 000–120 000) dispersed throughout the mouse genome, was used. Since both SINE-B2 and IAP have directions, four combinations can be analyzed as follows: (a) the region from the 5′-end of the IAP-LTR to the 3′-end of the SINE; (b) from IAP-LTR-5′ to SINE-5′; (c) from IAP-LTR-3′ to SINE-3′; (d) from IAP-LTR-3′ to SINE-5′.

To construct IAP-LTR-specific primers, we focused on the central area of the U3 region of the IAP-LTR. Examination of this area among IAP-LTR sequences registered in the GenBank database showed that they can be classified into two subtypes. One subtype is named the RS group which is similar to the IAP element isolated from C3H/He-derived AML cells [3], and the other is named the U group (Fig. 1a). Therefore, we constructed four primers for IAP-LTR (RS-IAP-F, U-IAP-F, RS-IAP-R and U-IAP-R). On the other hand, two primers in both directions corresponding to consensus sequences for SINE-B2 were prepared (SINE-B2-F and SINE-B2-R, Fig. 1b).

PCR amplification from SINE-B2 to IAP gave a limited number of DNA species which could be analyzed by agarose gel electrophoresis (Fig. 2). When the DNA area from the 5′-end of RS-IAP-LTR to the 3′-end of SINE-B2 was amplified using the reverse primer for RS-IAP-LTR and the forward primer for SINE-B2, the genomes of all leukemia cell strains, except L-8028, produced evident novel bands of different sizes (Fig. 2a, lanes 4–8) as compared with the normal genome (Fig. 2a, lanes 2 and 3). On the other hand, the U-IAP-LTR-specific primer gave different sizes and numbers of amplified bands (Fig. 2a, lane 1), though an AML genome-specific band was not detected (data not shown). Similarly, AML-specific bands were observed in the genomes of strains L-8028 and L-8065 at the area from RS-IAP-LTR-5′ to SINE-B2-5′ (Fig. 2b, lanes 5 and 7), in the L-8032 genome at the area from RS-IAP-LTR-3′ to SINE-B2-3′ (Fig. 2c, lane 6) and in the L-8002 and L-8032 genomes at the area from RS-IAP-LTR-3′ to SINE-B2-5′ (Fig. 2d, lanes 4 and 6). No RFLPs were observed among genomic DNA of spleen and liver from 15 different individuals of C3H/He mice containing different production lots (details not shown, see Fig. 2a–d, lanes 2 and 3). These results show that all five independent AML genomes have a novel structure lying between the IAP element and SINE-B2.

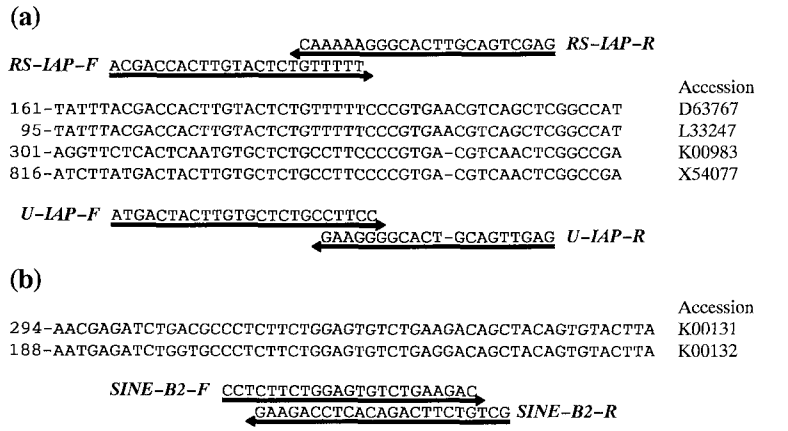

Fig. 1. Nucleotide sequences of parts of IAP-LTR and SINE-B2. a: Sequences at the center of the U3 region of IAP-LTR were compared with those from the GenBank database. Accession number D63767 is the sequence of IAP elements previously isolated from AML cells. PCR primers used in this study are mentioned at the top and bottom of the figure, with arrows indicating the direction (5′ to 3′) of the primers. b: The consensus sequence of SINE-B2 and corresponding primers are shown.

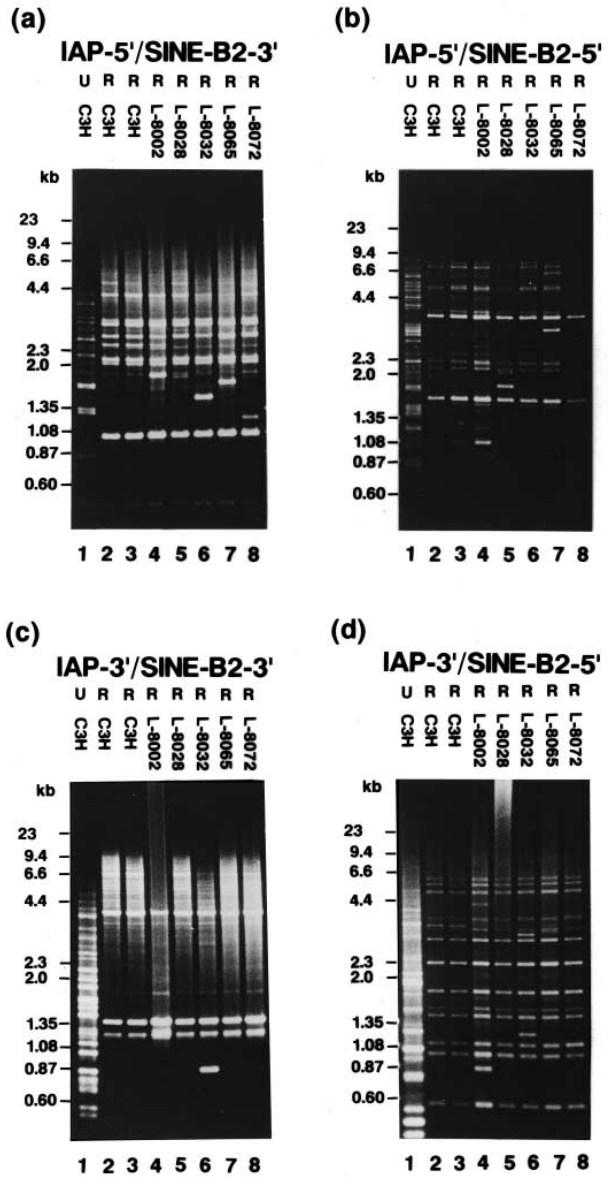

Fig. 2. PCR amplification of DNAs from germline and AML cells. DNAs from normal livers of C3H/He mice (lanes 2 and 3) and from AML cells of different origins (lanes 4–8) were PCR-amplified using RS-IAP-LTR primer (R) and SINE-B2 primer. Results of PCR following replacement of the RS-IAP-LTR primer with primer U (U) using germline DNA are shown in lane 1. a: Backward IAP-LTR plus forward SINE-B2. b: Backward IAP-LTR plus backward SINE-B2. c: Forward IAP-LTR plus forward SINE-B2. d: Forward IAP-LTR plus backward SINE-B2.

3.2. *Cloning and analysis of the AML-specific integration site of IAP*

The AML-specific PCR product was cloned and sequenced. Fig. 3a shows the nucleotide sequence at the junction of the IAP element isolated from the first PCR product of 1.7 kbp DNA derived from the L-8002 AML cell strain (Fig. 2a, lane 4). A sequence which was not found in the database was linked to the 5′-end of the IAP element.

To isolate the normal allele corresponding to the junction, inverted PCR was used. The nucleotide sequence of the first PCR product which was not related to any multicopy sequence recorded in the database was used to construct a pair of oligonucleotide primers (Q11-F and Q11-R, Fig. 3a) for the inverted PCR. The normal allele was amplified by inverted PCR when germline DNA was used. By comparing the sequences of the inverted PCR product and the first PCR product in Fig. 3a, the original structure before integration of the IAP element in L-8002 AML was estimated as shown in Fig. 3c. If the sequence is compatible with a normal allele, a part of the sequence which is not found in Fig. 3a must be linked to the 3′-end of the IAP element in the L-8002 genome. To confirm this, a PCR primer (Q11-B) was prepared based on the germline sequence shown in Fig. 3c. By priming Q11-B plus RS-IAP-F for PCR, amplification of a single band was detected using DNA from L-8002 AML cells but not using other genomic DNAs. The product was sequenced as shown in Fig. 3b and contained the junction of the 3′-end of the IAP element and the genomic sequence. When these three sequences were compared, a target duplication of 6 bp long which was generated by retrotransposition was found. Therefore, it is suggested that the construction of the AML-specific structure (Fig. 3a,b) occurred by retrotransposition of IAP at the normal site (Fig. 3c) during or after leukemogenesis of L-8002 AML cells.

Similarly, subsequent PCR and sequencing analyses were performed using the first PCR product of 1.5 kbp DNA derived from L-8065 AML (Fig. 2a, lane 7). The sequences obtained were compared as described above. Fig. 3d shows the target duplication site and surrounding germline sequence where the arrangement occurred in the L-8065 genome.

3.3. *Genomic Southern blot analysis*

Evidence for IAP-derived rearrangement in AML cells was further supported by Southern analysis of genomic DNA from normal and AML cells. It is expected that both PCR products of the normal allele using Q11-R plus Q11-B (Fig. 3c) or Q14-R plus Q14-B (Fig. 3d) primers can be used as probes for the analysis, since there are no repetitive or repeated sequences. Actually, a single size of DNA was definitely detected by PCR using either of these primer pairs (data not shown). These PCR products were thus used as probes. They hybridized to the corresponding allele in the genomic DNA without any artificial bands (Fig. 4). Rearrangement in half of the allele of the genome was observed only in L-8002 DNA with the Q11 probe (Fig. 4a, lane 2). When the Q14 probe was used, it appeared that both alleles in the L-8065 genome contained rearrangement (Fig. 4b, lane 5). No RFLP was detected among germline DNAs from 15 C3H/He mice in this study (data not shown).

4. Discussion

It is well known that the genomes of most tumor cells contain a variety of abnormal structures, and at least part of them contribute to properties of the tumor. Since rearranged genes activated by IAP-mediated retrotransposition were found in several murine tumor cell lines, the possibility that IAP-mediated retrotransposition is one of the mechanisms responsible for tumorigenic changes has been put forward [4,5]. Activation of the retrotransposition can increase the frequency of gene rearrangement and some of these events may affect the function of genes close to the integration site due to the insertion of a functional LTR sequence [6] or disruption of the target gene structure. Indeed, most of such

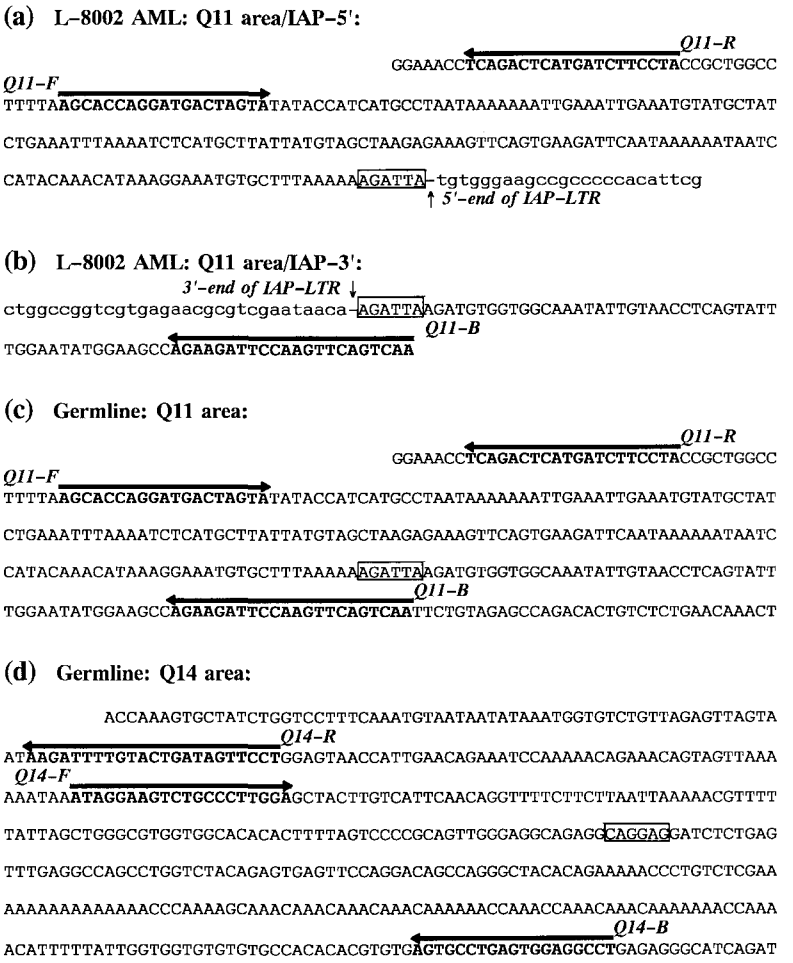

Fig. 3. Nucleotide sequences of target sites for IAP elements specific to AML strains. Small letters indicate the IAP-element-derived sequence. Bold letters with arrows showing the directions correspond to primers used in subsequent PCR analyses (Q11-F, Q11-R, Q11-B, Q14-F, Q14-R and Q14-B). The target duplication site for IAP-mediated retrotransposition site is boxed. a: Sequence of the junction of the IAP element (5'-side) from L-8002 AML cells of a 1.5 kb fragment (Fig. 2, lane 4). b: Sequence of another junction of the IAP element (3'-side) from an abnormal allele in L-8002 AML cells. c: Sequence of a normal allele corresponding to (a) and (b) estimated by comparison of (a) and the inverted PCR product from germline DNA. d: Sequence of a normal allele where the retrotransposition of IAP occurs in the L-8065 genome estimated by sequence comparison in subsequent analyses of a 1.5 kb PCR product (Fig. 2, lane 7) derived from L-8065 AML cells.

integrated genes are attributed to changes in gene function as discussed elsewhere [3]. If the retrotransposition occurs frequently in a tumor cell line, integrated IAP elements must be detectable with or without functional changes in the cell line. However, the tumor-specific insertion of an IAP element as a result of retrotransposition is hard to detect since the normal murine genome naturally possesses 1000 copies of the IAP element.

The IAP elements have been divided into subtypes based on their physical structure [5] and on the sequence heterogeneity of LTR [13,14]. Using 34 LTR sequences of different IAPs in the GenBank database, we compared the central area of the U3 region which has a low level of heterogeneity. Seven of these 34 sequences were similar to the IAP that was isolated from one of the AML cells reported previously [3] and were named as the RS subtype. The total amount of the RS subtype can be estimated at ca. 200 copies per mouse genome. We designated a PCR primer corresponding to the RS-LTR to analyze members of this group selectively (Fig. 1).

In this study, we amplified part of the integration site of the IAP element by PCR using a primer set of the RS-IAP and the SINE-B2, a retroposon which is a widely dispersed element in the mouse genome (Fig. 1). Therefore, sequences between the IAP element and SINE-B2 less than 4 kbp in size were analyzed. Whereas the PCR products of all genomic DNA from normal C3H/He mice were identical, unique bands were observed only when the genomes of AML cells were used (Fig. 2). The unique IAP integration sites were confirmed by subsequent analyses using two examples of the PCR product (Figs. 3 and 4). One allele in the L-8002 genome was rearranged at the Q11-related locus (Fig. 4a) showing the typical structure of retrotransposition-mediated rearrangement. However, both alleles in the L-8065 genome were rearranged at the Q14-related locus (Fig. 4b), and this was probably due to loss of the normal allele after retrotransposition by chromosomal heterogeneity. The most important point in this study was that unique integration sites were commonly observed among different AML cells generated independently (Fig. 2). This fact strongly suggests the universality of this event at least in radiation-induced AML from C3H/He mice.

In the PCR analyses, a total number of 30 sites (Fig. 2) were detected out of ca. 400 junctions of RS-type IAP in the

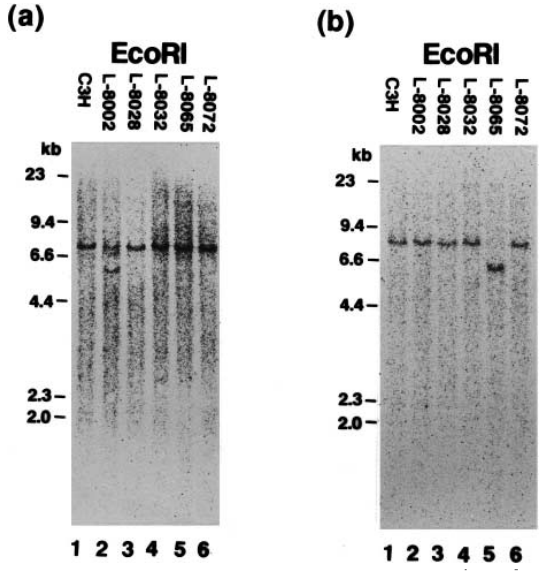

Fig. 4. Genomic Southern analysis. Each lane contained 10 mg of total DNAs from germline and AML cells digested with *Eco*RI. Probes from PCR products primed with Q11-R plus Q11-B (a) and with Q14-R plus Q14-B (b) were used. Lane 1, germline DNA; lane 2, L-8002 AML; lane 3, L-8028 AML; lane 4, L-8032 AML; lane 5, L-8065 AML; lane 6, L-8072 AML.

genome. From the fact that all independent AML cells tested gave 2–3 unique junctions, it is expected that there are 400/30 times the observed number present in the genome in AML cells. If the integration occurred randomly throughout the genome, this number seems too small to cause drastic changes in leukemia cells. However, IAP-mediated gene rearrangements have been observed around a locus of tens of kilobases containing the interleukin-3 gene in distinct cell lines [3,7–9,15,16]. Additionally, IAP-mediated retrotransposition adjacent to the *agouti* locus in germline cells was also reported by different authors [17,18]. Both of them suggest the presence of loci which are susceptible to incorporation of IAP elements in the mouse genome. If so, the 20–30 novel integration events may be sufficient to induce marked changes in the properties of AML cells. In addition, Lepage et al. reported that amplification of a multidrug resistance gene in a doxorubicin-resistant derivative of the P388 cell line is the consequence of insertion of an IAP element [19]. If insertion of the IAP element stimulates gene amplification at the target site, a limited number of IAP-mediated retrotransposition event gives greater cytological effects.

At present, there is no answer to the question whether the retrotransposition event is limited in C3H/He mouse-derived tumors, AML-related tumors or radiation-induced tumors. Investigation of a wide variety of types of cells including those from tumors and transformed cells by our method should answer the above question.

References

[1] Seki, M., Yoshida, K., Nishimura, M. and Nemoto, K. (1991) Radiat. Res. 127, 146–149.
[2] Hayata, I., Seki, M., Yoshida, K., Hirashima, K., Sado, T., Yamagiwa, J. and Ishihara, T. (1983) Cancer Res. 43, 367–373.
[3] Tanaka, I. and Ishihara, H. (1995) FEBS Lett. 376, 146–150.
[4] Keshet, E., Schiff, R. and Itin, A. (1991) Adv. Cancer Res. 56, 215–251.
[5] Kuff, E.L. and Lueders, K.K. (1988) Adv. Cancer Res. 51, 183–276.
[6] Lamb, B.T., Satyamoorthy, K., Solter, D., Basu, A., Xu, M.Q., Weinmann, R. and Howe, C.C. (1992) Mol. Cell. Biol. 12, 4824–4833.
[7] Ymer, S., Tucker, W.Q.J., Sanderson, C.J., Hapel, A.J., Campbell, H.D. and Young, I.G. (1985) Nature 317, 255–258.
[8] Hirsh, H.H., Nair, A.P.K. and Moroni, C. (1993) J. Exp. Med. 178, 403–411.
[9] Algate, P.A. and McCubrey, J.A. (1993) Oncogene 8, 1221–1232.
[10] Lepage, P., Devault, A. and Gros, P. (1993) Mol. Cell. Biol. 13, 7380–7391.
[11] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[12] Triglia, T., Peterson, M.G. and Kemp, D.J. (1989) Nucleic Acids Res. 16, 8186.
[13] Christy, R.J., Brown, A.R., Gourlie, B.B. and Huang, R.C.C. (1985) Nucleic Acids Res. 13, 289–302.
[14] Mietz, J.A. and Kuff, E.L. (1992) Mamm. Genome 3, 447–451.
[15] Duhrsen, U., Stahl, J. and Gough, N.M. (1990) EMBO J. 9, 1087–1096.
[16] Leslie, K.B., Lee, F. and Schrader, J. (1991) Mol. Cell. Biol. 11, 5562–5570.
[17] Michaud, E.J., vanVugt, M.J., Bultman, S.J., Sweet, H.O., Davisson, M.T. and Woychik, R.P. (1994) Genes Dev. 8, 1463–1472.
[18] Duhl, D.M.J., Vrieling, H., Miller, K.A., Wolff, G.L. and Barsh, G.S. (1994) Nature Genet. 8, 59–64.
[19] Stocking, C., Loliger, C., Kawai, M., Suciu, S., Gough, N. and Ostertag, W. (1988) Cell 53, 869–879.