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Antimitotic and antiviral activities of Kelletinin A in HTLV-1 infected MT2 cells

I. Silvestri[a,c], L. Albonici[a,b], M. Ciotti[a], M. P. Lombardi[a,c], P. Sinibaldi[a], V. Manzari[a], P. Orlando[d,*], F. Carretta[d], G. Strazzullo[e] and P. Grippo[f]

[a]*Dipartimento di Medicina Sperimentale e Scienze Biochimiche, II Università di Roma 'Tor Vergata'*; [b]*Istituto Tecnologie Biomediche C.N.R., v. di Tor Vergata 134, Rome*; [c]*Dipartimento di Medicina Sperimentale, Università 'La Sapienza', Rome*; [d]*Istituto Biochimica delle Proteine ed Enzimologia, Fax +39 81 866 1341; and* [e]*Istituto Chimica Molecole Interesse Biologico, C.N.R., v. Toiano n 6-80072 Arco Felice, Naples; and* [f]*Istituto Biochimica Macromolecole, Fac. Medicina, II Università di Napoli, v. Costantinopoli 16, Naples (Italy)*
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Abstract. Kelletinin A [ribityl-pentakis (p-hydroxybenzoate)] (KA), a natural compound isolated from the marine gastropod *Buccinulum corneum*, showed antiviral activity on the human T-cell leukemia virus type-1 (HTLV-1) and antimitotic activity on HTLV-1-infected MT2 cells. KA inhibited cellular DNA and RNA synthesis, without influencing protein synthesis, and interfered with viral transcription by reducing the levels of high molecular weight transcripts. Finally, the compound inhibited HTLV-1 reverse transcriptase in vitro.
Key words. Kelletinin A; HTLV-1; antiviral-activity; reverse-transcriptase.

Natural compounds with antiviral activity have received much attention in recent years because of their potential pharmacological utilization[1,2].

Previously, we have investigated the biological properties of Kelletinins: natural compounds isolated from the marine gastropod prosobranch *Kelletia kelletii*[3] and from the Mediterranean species *Buccinulum corneum*[4,5]. We have demonstrated that, among kelletinins, Kelletinin A [ribityl-pentakis (p-hydroxybenzoate)] (KA) inhibited cell division and reduced the rate of DNA synthesis in a human lymphoblastoid cell line (Raji cells) by prolonging the G1-S phase[6]. Furthermore, KA exhibited antimitotic properties in phytohemagglutinin-stimulated lymphocytes (unpubl. results) and caused a delay in early development of *Paracentrotus lividus* sea urchin embryos[7]. Although the mechanism of action is unknown, a possible target of the compound might be the DNA-polymerizing enzymes. Indeed, KA inhibited DNA polymerase α[8] and HIV-1, Mo-Mu LV and AMV reverse transcriptases[9,10].

These findings prompted us to test KA as an antimitotic and antiviral agent on HTLV-1-infected CD4$^+$ T-lymphocytes (MT2 cells)[11,12]. HTLV-1, the etiological agent of adult acute T-cell leukemia[13], is a retrovirus which exhibits a complex control mechanism for its expression, including transactivation and splicing regulation. Though the virus does not contain oncogene sequences it shows transforming properties[14].

Here we report data indicating that KA caused a marked decrease in production of HTLV-1 virions and a reduction of two high molecular weight viral transcripts. Furthermore, at nontoxic concentrations the compound inhibited proliferation of MT2 cells.

* To whom correspondence should be addressed.

Materials and methods

Materials. Kelletinin A was purified according to Cimino et al.[4] and dissolved in dimethylsulfoxide at a concentration of 25 mM. Dimethylsulfoxide concentration in cell cultures and in RT assays did not exceed 0.2%; the same concentration was present in control samples.

[^{3}H] thymidine (spec. act. 60 Ci/mmol), [^{3}H] uridine (spec. act. 30 Ci/mmol), [^{3}H] leucine (spec. act. 70 Ci/mmol), [^{3}H] dTTP (spec. act. 30 Ci/mmol) and [^{32}P] dCTP (spec. act. 800 Ci/mmol) were from Amersham (International plc, Buckinghamshire, UK); poly(rA) · oligo(dT)$_{12-18}$ and poly(dA) · oligo(dT)$_{12-18}$ were from Pharmacia (Biotech Inc, Milwaukee, WI, USA). DNA probes pMT2-64[15] and gag region were obtained from LTCB, (NIH Bethesda, USA) and were labelled with [^{32}P] dCTP by random primer system (Amersham), at specific activities of 1 to 5×10^8 cpm/μg.

Cell cultures. MT2 cells infected and transformed with HTLV-1 (LTCB NIH Bethesda) were seeded at a concentration of 1×10^5 to 1×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FCS (Flow Lab.), 50 U/ml penicillin G, 50 mg/ml streptomycin and 2 mM glutamine, and cultured as described[12]. Cell numbers were determined with a Coulter counter; three to five aliquots per sample were counted and the standard percentage error of the medium did not exceed 10%. Cell viability was determined by the Trypan blue dye exclusion test.

Preparation of HTLV-1 virions. HTLV-1 virions from MT2 supernatants were collected by centrifugation at 100,000 × *g* for 1 h. The pellet was lysed in buffer 25 mM Tris-HCl pH 7.5, 4 mM dithiothreitol, 1 mM EDTA, 50 mM KCl, 50% glycerol, 0.25% Triton X-100 (TKE) buffer and used for reverse trousoliptose (RT) assay. Alternatively the pellet was resuspended in buffer 50 mM

Tris pH 7.8, 150 mM NaCl, 5 mM EDTA (TNE buffer), layered on a sucrose gradient 25–60% in TNE, and centrifuged at 38,000 rpm in a SW 41 Spinco rotor for 16 h. Fractions containing the virus particles were pooled, diluted four times in TNE buffer and centrifuged at 100,000 × *g* for 1 h. Virus was disrupted in TKE buffer and used in the RT assay.

Evaluation of DNA, RNA and protein synthesis. Cells were cultured in microtiter wells (200 µl/well) at a concentration of 2×10^5 cells/ml and pulse-labelled with 10 µCi/ml of [^{3}H] thymidine or [^{3}H] uridine or [^{3}H] leucine for 4 h, 15 min and 1 h respectively. Cells were harvested on glass microfibre by a cell harvester (PHD Cambridge Technology Inc.) and counted by liquid scintillation.

Reverse transcriptase assay. Reverse transcriptase was assayed in 100 µl of a stardard reaction mixture containing: 40 mM Tris-HCl pH 7.8, 4 mM dithiothreitol, 40 mM KCl, 10 mM $MgCl_2$, 50 µg/ml of poly(rA) · oligo(dT)$_{12-18}$, 15 µM [^{3}H] dTTP (0.8–1.2 µCi/nmol) and 10–30 µl of virions extract in TKE buffer. The reaction mixture was incubated at 37 °C;

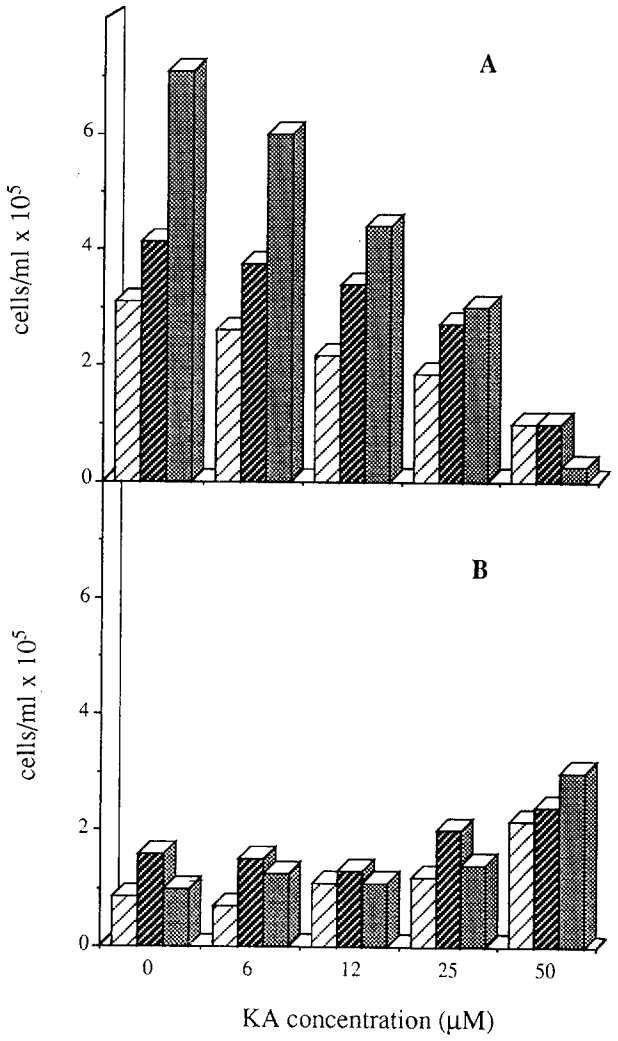

Figure 1. Effect of KA on MT2 cells in culture. MT2 cells were seeded at initial concentration of 2×10^5 cells/ml and cultured for 24 h ▨ 48 h ▨ and 72 h ▩ in presence of the indicated concentrations of KA. Live (*A*) and dead (*B*) cells were evaluated as described in 'Materials and methods'.

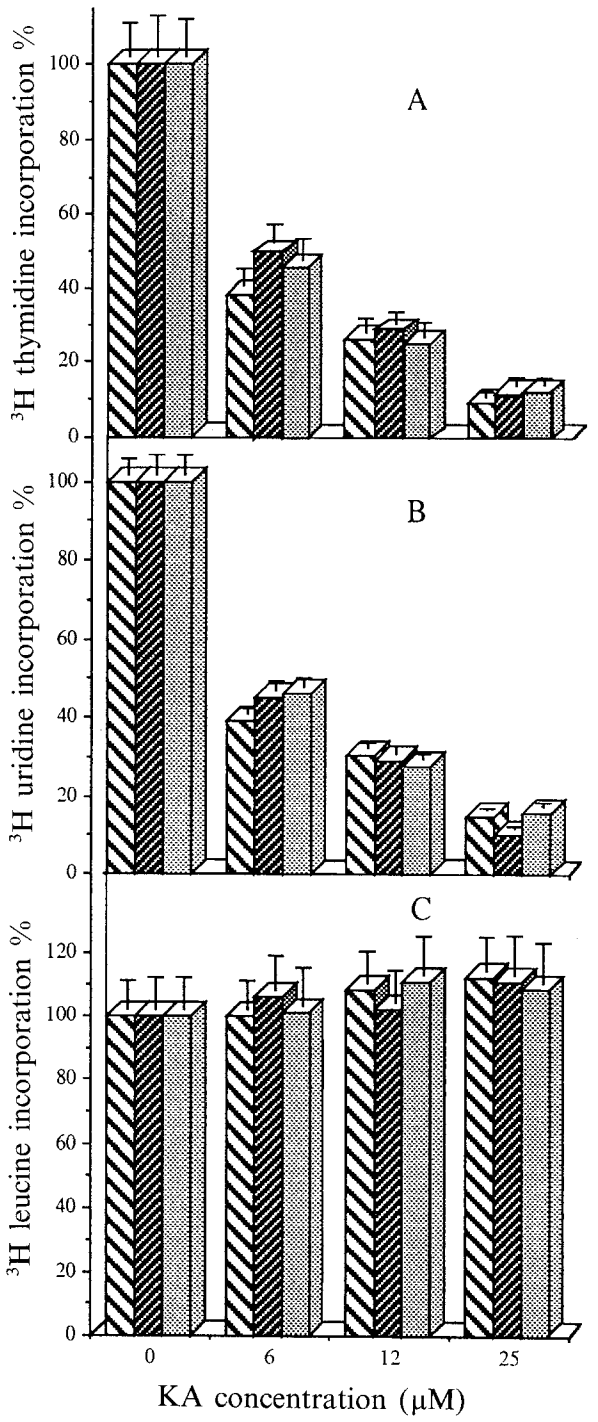

Figure 2. DNA, RNA and protein synthesis in KA-treated MT2 cells. MT2 cells were seeded at initial concentration of 5×10^5 cells/ml and cultured in presence of the indicated concentration of KA for 24 h ▧ 48 h ▨ and 72 h ▩; cells were then harvested, resuspended at 2×10^5 cells/ml and pulse-labelled with [^{3}H] thymidine (*A*), [^{3}H] uridine (*B*) and [^{3}H] leucine (*C*) as described in 'Materials and methods'. The standard deviation did not exceed 10%.

Table 1. RT levels in culture supernatants of KA-treated MT2 cells.

Drug concentration (μM)	Live cells/ml × 10^5	Enzymatic activity	
		rA-$dT_{(12-18)}$ (pmoles incorp./h.)	dA-$dT_{(12-18)}$ (pmoles incorp./h.)
no drug	9.12 (100%)	15.10 (100%)	0.57
6	8.90 (98%)	12.75 (84%)	0.53
12	8.43 (92%)	11.25 (74%)	0.48
25	6.40 (70%)	4.80 (31%)	0.50
50	2.00 (22%)	0.58 (3%)	0.75

MT2 cells (5×10^5 cells/ml) were grown for 24 h in the presence or absence of KA. 5 ml of culture supernatants were used for HTLV-1 virion preparation. Enzymatic assays were performed as described in 'Materials and methods', and the values represent the mean of two independent determinations in a duplicate experiment; the standard deviation did not exceed 5%. rA-$dT_{(12-18)}$: poly rA-oligo$dT_{(12-18)}$; dA-$dT_{(12-18)}$: poly dA-oligo $dT_{(12-18)}$.

under these conditions the reaction rate was linear with time and extract concentration. The reaction was stopped by adding 1 ml of cold 10% trichloroacetic acid (TCA), 100 μl of 0.5 M K_2HPO_4 and 10 μl of 10 mg/ml tRNA (Boehringer Mannheim GmbH, Mannheim, Germany). The acid precipitate was collected on 0.45 μm filters BA 85 (Schleicher & Schuell); the filters were washed with 5% cold TCA and then with 70% ethanol, dried, and the radioactivity measured. Cell culture supernatants were also assayed for cellular DNA polymerases, replacing poly(rA) · oligo$(dT)_{12-18}$ by poly(dA) · oligo$(dT)_{12-18}$ in the standard reaction mixture.

Northern blot analysis. Total RNA was purified from MT2 cells by the RNA-zol method (Cinna Biotecx Lab., Houston, TX, USA). RNA samples were electrophoresed on a 1% denaturing agarose gel, transferred to a Hybond N membrane (Amersham) by electroblotting, prehybridized in 5 × SSPE, 10 × Denhardt's, 0.5% SDS at 65 °C for 2–4 h and hybridized at 65 °C for 16 h with [^{32}P] dCTP-labelled pMT2-64 and gag region probes at a concentration of 3×10^6 cpm/ml. Filters were washed with 0.1 × SSPE, 0.5% SDS at 65 °C and autoradiographed overnight at −80 °C using an X-AR film (Kodak).

Results

Inhibition of MT2 cell proliferation. Figure 1 shows a typical experiment in which HTLV-1-infected MT2 cells were exposed to KA for 24, 48 and 72 h. The number of live cells (panel A) decreased with increasing concentrations of KA and time of treatment. On the other hand, cell mortality (panel B) did not vary significantly with concentrations of KA up to 25 μM but did increase at 50 μM. Comparable effects were obtained when the cells were seeded at higher densities.

Effects on DNA, RNA and protein synthesis. The rate of DNA and RNA synthesis (fig. 2A, 2B) was reduced by increasing the concentration of KA and did not change when varying the time of treatment with the compound. Since pulse-labeling is very short (15 min), the reduced RNA synthesis rate could be mainly ascribed to the inhibition of mRNA synthesis. Finally, the rate of protein synthesis, measured by pulse-labeling the cultures with [^{3}H] leucine, was not affected by the compound at the concentrations and time tested (fig. 2C). These results were also confirmed when [^{3}H] methionine was used instead of [^{3}H] leucine and the radioactivity measured in the acid precipitable fraction (data not shown). Furthermore, the analysis of de novo-synthesized proteins by SDS-PAGE and autoradiography did not show qualitative or quantitative differences in the synthetic patterns of treated or normal cells (data not shown).

Inhibition of HTLV-1 production. The production of HTLV-1 virions in MT2 cells was evaluated by measuring the levels of poly(rA) · oligo$(dT)_{12-18}$-directed RT activity in culture supernatants. As shown in table 1, the production of HTLV-1 virions was inhibited by the compound with a dose-dependent effect. In fact, RT activity decreased to 84%, 74% and 31% at 6 μM, 12 μM and 25 μM KA respectively. No RT activity was found using a concentration of 50 μM. Furthermore, at the same concentration of KA, the cytostatic effect on MT2 cells was significantly lower than the antiviral activity, and poly(dA) · oligo$(dT)_{12-18}$-directed polymerase activity, that measures the level of cellular DNA polymerases, was undetectable. Finally, the inhibition of HTLV-1 production persisted in cells treated for 24 h with KA, washed and then cultivated for 24 h in the absence of the compound (table 2).

Table 2. RT levels in culture supernatants of KA-treated MT2 cells after 24 h culture in absence of KA

Drug concentration (μM)	Live cells/ml × 10^5	RT activity %
no drug	11.00 (100%)	100
6	9.75 (89%)	93
12	9.25 (84%)	87
25	8.40 (76%)	45

After 24 h culture in presence of KA (see table 1) MT2 cells were harvested and washed extensively to remove the drug. 5×10^5 cells/ml were cultured for further 24 h. 5 ml of culture supernatant were used for HTLV-1 pellet preparation, and RT assays were performed as described in 'Materials and methods'. RT activity values represent the mean of two independent assays in duplicate experiments; the standard deviation did not exceed 5%.

Table 3. Effect of KA on RT activity from HTLV-1 virions.

Drug concentration (μM)	Residual activity %
no drug	100
3	98
6	80
12	73
25	50

HTLV-1 virions from MT2 cells cultured in absence of KA were purified and homogenized as described in 'Materials and methods'; RT activity of the extract was assayed in the absence or in the presence of the drug. The values represent the mean of four independent assays in a duplicate experiment. The standard deviation did not exceed 5%.

Inhibition of HTLV-1 RT. RT activity in extracts of purified preparations of HTLV-1 virions was inhibited in vitro by KA. Table 3 shows that 6 μM, 12 μM and 25 μM KA caused 20%, 30% and 50% of inhibition, respectively.

Inhibition of viral mRNA synthesis. KA interfered with the synthesis of viral mRNAs. In fact, Northern blot analysis of total RNA, utilizing the full-length RNA probe pMT2-64[15], showed that the two high molecular weight viral RNA species disappeared in KA-treated culture (fig. 3A) while the majority of transcribed RNA species were not affected by the compound. These data were confirmed when a probe for gag gene, which is present only in the high molecular weight unspliced viral RNA, was used (fig. 3B).

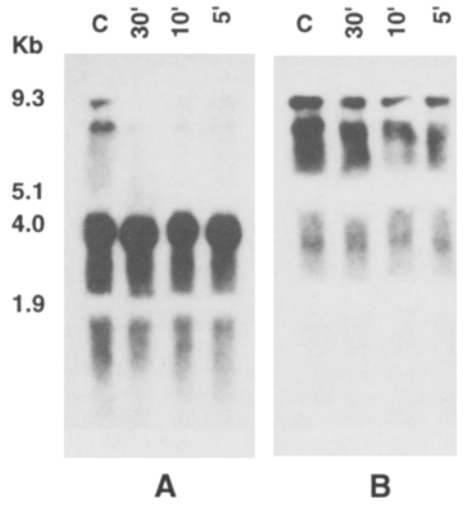

Figure 3. Northern blot analysis of viral RNA species. MT2 cells were cultured in the presence of 25 μM KA for the indicated time. Northern blot analysis of total RNA (15 μg/lane) was performed as described in 'Materials and methods' using pMT2-64 (*A*) or gag region (*B*) as probes.

Discussion

In the present study we report three different effects of KA on HTLV-1-infected and transformed MT2 cells: inhibition of cell growth, inhibition of viral production and interference with viral transcription.

KA caused an inhibition of cell growth which was dependent on the concentration used and duration of treatment. These findings can not be due to a cytotoxic effect because: 1) cell mortality did not change with variations in the time of treatment and KA concentration; 2) DNA and mRNA synthesis rates decreased with increasing KA concentration but did not change when duration of treatment increased. Consequently, the effect of KA on cell growth can be explained by hypothesizing either a decrease in cell division rate in the whole cell population, or an arrest of mitosis in a particular cell fraction. We favor the first hypothesis since in Raji cells KA induced a prolonged G1-S phase[6]. Surprisingly KA did not affect protein synthesis. This result might be explained by either the presence of a stable mRNA population or a more efficient translation process.

Besides the effects on cell growth, KA affected HTLV-1 replication. The decrease in HTLV-1 production was significantly higher than the inhibition of cell growth, suggesting a direct antiviral effect, and was not due to reduced cell viability, because cellular DNA polymerase activity in the culture supernatant was negligible. Furthermore, the effects of KA persisted in the cells treated with KA and then cultivated in a medium deprived of the compound, thus indicating a long-lasting mechanism.

RNA-dependent DNA polymerase activity of HTLV-1 RT was inhibited in vitro by KA. Nevertheless, these data do not allow us to conclude that the decrease in viral production was a consequence of reverse transcriptase inhibition. It is interesting to note that the low degree of RT inhibition observed in our experiments could be due to the presence of interfering substances in the viral extract; in fact, purified RT activities of HIV-1 and Mo-MuLV were inhibited to higher extent by the drug[9,10].

The inhibition of viral replication was confirmed by Northern blot analysis of viral transcripts that showed a drastic decrease in two high molecular weight mRNAs. This phenomenon could be explained either by a premature arrest of mRNA synthesis or by an enhanced mRNA-processing, such as takes place in the absence of REX protein, a viral protein which is essential for splicing regulation and transport of genomic viral RNA to the cytoplasm[16,17]. The reduction in viral genomic RNA available for packaging could explain the decrease in viral production.

The anti HTLV-1 properties of KA coupled with its low cytotoxicity in cell culture warrant further characterization of the antiviral activity of the compound and its synthetic derivatives.

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1 Tan, G. T., Pezzuto, J. M., and Kinghorn, A. D., J. nat. Prod. *54* (1991) 143.
2 De Clercq, E., Adv. Virus Res. *42* (1993) 1.
3 Tymiak, A. A., and Rinehart, K. L., J. Am. chem. Soc. *105* (1983) 7396.
4 Cimino, G., De Stefano, S., and Strazzullo, G., J. nat. Prod. *50* (1987) 1171.
5 Ciminiello, P., Fattorusso, E., and Magno, S., Gazz. chim. ital. *118* (1988) 105.
6 Orlando, P., Peluso, G., Carretta, F., and Grippo, P., VIII Congr. naz. Associazione di Biologia Cellulare e del Differenziamento, Salsomaggiore Terme, Italy (1989) p. 29.
7 Mangoni di S. Stefano, G. S., Orlando, P., Peluso, G., and Grippo, P., Rif. med. *108* (1993) 93.
8 Orlando, P., Carretta, F., Grippo, P., Cimino, G., De Stefano, S., and Strazzullo, G., Experientia *47* (1991) 64.
9 Orlando, P., Carretta, F., De Petrocellis, L., Minei, R., and Grippo, P., 5th Int. Congr. Cell Biol., Madrid (1992) abs. P-8.2 n° 152, p. 247.
10 Orlando, P., Strazzullo, G., Carretta, F., De Falco, M., and Grippo, P., submitted.
11 Poiesz, B. J., Ruscetti, F. W., Gardar, A. F., Bunn, P. A., Minna, J .D., and Gallo, R. C., Proc. natl Acad. Sci. USA *77* (1980) 7415.
12 Myoshi, I., Kubonishi, I., Yoshimoto, S., Akagi, T., Ontusuki, Y., and Shiraishi, Y., Nature *294* (1981) 770.
13 Yoshida, M., Miyoshi, I., and Hinuma, Y., Proc. natl Acad. Sci. USA *79* (1979) 2031.
14 Popovic, M., Lange-Wantzin, G., Sarin, P. S., Mann, D. G., and Gallo, R. C., Proc. natl Acad. Sci. USA *80* (1983) 5402.
15 Clarke, M. F., Gelmann, E. P., and Reitz, M. S. J., Nature *305* (1983) 60.
16 Inque, J., Yoshida, M., and Seiki, M., Proc. natl Acad. Sci. USA *84* (1987) 3653.
17 Kinoshita, T., Shimoyama, M., Tobinai, K., Ito, M., Ito, S. I., Ikeda, S., Tajima, K., Shimotohno, K., and Sugimura, T., Proc. natl Acad. Sci. USA *86* (1989) 5620.