were obtained. These transformed cell lines were 1-LTR-CAT DNA and permanently transformed cell lines were maintained in normal culture conditions, but were activated by retreatment with 0.2 mM \( \text{H}_2\text{O}_2 \) for 1 h. On treatment with 0.05 mM \( \text{H}_2\text{O}_2 \) for 1 h, the CAT activity of these cell lines maintained in normal conditions remained latent, whereas cell lines pretreated with 0.01 mM \( \text{H}_2\text{O}_2 \) for 25 days were greatly activated by this treatment. Here, the HIV-1 promoter seemed latent in normal culture conditions, but it could be activated by a comparatively low concentration (0.05 mM) of \( \text{H}_2\text{O}_2 \) after treatment with a dilute \( \text{H}_2\text{O}_2 \) (0.01 mM) for about 1 month. 

Many patients infected with human immunodeficiency virus 1 (HIV-1) show a long latent period before development of AIDS. During this latent period, their infected cells may be subjected to oxidative stress due to metabolism and physical movement. The present results indicate that oxidative stress may cause activation of the HIV-1 promoter in patients with latent HIV-1.

Almost all patients infected with HIV-1 show a latent period of several years before development of acquired immunodeficiency syndrome (AIDS) (1). The production of HIV-1 has been found to be very low before onset of the disease, although the provirus DNA is inserted into the host cell DNA (2, 3). The latent HIV-1 can be activated by superinfection with another DNA virus such as the cytomegalovirus or herpes virus (4), but little is known about this mechanism of activation of latent HIV-1. On the other hand, the nuclear factor \( \kappa \)-B (NF-\( \kappa \)-B) is known to be a strong activator of the HIV-1 promoter (5), and activation of NF-\( \kappa \)-B by hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) has been reported (6). These findings suggest that oxidative stress such as hydrogen peroxide and superoxide may be important in the activation of latent HIV-1.

To examine this possibility, we transfected pCD12 (HIV-1-LTR-CAT) plasmid into human lymphoid cell lines and obtained stable transformants (cf. Refs. 4 and 7). These cell lines showed low CAT activity like latent HIV-1 in normal culture condition. The CAT activity was markedly elevated when these transformant cells were cultured in the presence of 0.2 mM \( \text{H}_2\text{O}_2 \) for 1 h, but on treatment of 0.05 mM \( \text{H}_2\text{O}_2 \) the CAT activity remained at a low level. However, when the transformed cells were pretreated with a dilute \( \text{H}_2\text{O}_2 \) (0.01 mM) for about 25 days, the CAT activity of transformed cell lines could be markedly elevated by retreatment with 0.05 mM \( \text{H}_2\text{O}_2 \) for 1 h. This activation of the HIV-1 promoter by a comparatively low concentration of \( \text{H}_2\text{O}_2 \) seems interesting, because patients latently infected with HIV-1 presumably receive various oxidative stresses as results of physical movements and metabolism during the long latency period (8, 9). In the early period of latency, the patients may have only cells of the "low CAT activity type" but when they undergo oxidative stress for a long time like cells during a long term culture with a dilute \( \text{H}_2\text{O}_2 \), the latent virus may be activated and comparatively low oxidative stress may cause marked activation of HIV-1 gene expression. From this viewpoint, our transformed cell lines may act as a good model of lymphoid cells in patients infected with HIV-1 (cf. Refs. 10 and 11).

### Materials and Methods

**Transformation**—HL60, U937, and MOLT4 cells were maintained in RPMI 1640 with 10% fetal bovine serum. About 5 \( \times \) 10\(^{6} \) cells were fused with the Escherichia coli DH1 containing plasmid pSV2CAT (10\(^{6} \) cells (12)) plus DH1 containing pSV2neo (5 \( \times \) 10\(^{7} \) cells) or mutant pCD12 (5 \( \times \) 10\(^{5} \) cells) plus DH1 containing pSV2neo (5 \( \times \) 10\(^{4} \) cells) by the protoplast fusion method (15). For construction of mutant pCD12, 91-bp Haell fragment containing two NF-\( \kappa \)-B motif (AGGGACCTTCC and GGGGACCTTCC) was replaced with synthetic 91-bp fragment containing two mutant NF-\( \kappa \)-B motif (ACTCTATTCC and GCTCACTTTCC (cf. Ref. 5)). The medium was changed 18 h after protoplast fusion, and the cells were cultured further for 2 days. Then they were selected with G418 in RPMI 1640 medium. After the selection with G418 for about 3 weeks, permanently transformed clones (HL60CD, U937CD, MOLT4CD (transformed by pCD12) and HL60SV, U937SV, MOLT-TSV (transformed by pSV2CAT), respectively (cf. Ref. 7)) were obtained. MOLT4CD* cells were obtained after fusion with DH1 cells containing mutant pCD12 plasmid and MOLT4 cells. The insertions of these DNA were investigated previously by Southern hybridization (7).

**\( \text{H}_2\text{O}_2 \) Treatment and CAT Assay**—A part of the transformed cell lines were maintained in normal RPMI 1640 and treated with a low concentration of \( \text{H}_2\text{O}_2 \) (0.01 mM) for 4 h per day for 25 days and maintained with or without 20 \( \mu \)M of N-acetyl-L-cysteine (NAC) for 24 h. These cell lines were treated with \( \text{H}_2\text{O}_2 \) (0, 0.05, or 0.2 mM) for 1 h and then were cultured in normal RPMI 1640 for 48 h, collected, and washed with phosphate-buffered saline. Samples of 5 \( \times \) 10\(^{5} \) cells were suspended in 0.25 mM Tris-HCl (pH 8.0), and cellular extracts were prepared by five cycles of freezing (\( -80^\circ \text{C} \)) and thawing. Chloramphenicol acetyltransferase (CAT) activity was measured by incubating whole cell extracts with \( ^{14} \text{C} \)-labeled chloramphenicol and 5 mM acetyl coenzyme A at 37 \( ^\circ \text{C} \) for 18 h. Acetylated chloramphenicol was separated from nonacylated chloramphenicol by ascending thin-layer chromatography (16). Chromatograms were examined and quantitated with a Fuji image analyzer BA100.

**Binding of Nuclear Proteins to the NF-\( \kappa \)-B Binding Motif DNA**—Nuclear proteins binding to the NF-\( \kappa \)-B binding motif were detected by
Briefly, 42-mer DNA containing two NF-κB DNA motifs (GGGGACTTTCC) was end-labeled with \[^{32}P\]ATP for binding with nuclear extracts. Nuclear extracts were prepared at intervals by the method of Dignam et al. (17) after treatment of the cells with H2O2. Samples of 5 ng of end-labeled DNA fragments were bound with 3 mg of nuclear proteins in a solution of 20 mM Hepes buffer (pH 7.9), 100 mM KCl, 20% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10 mM MgCl2, 125 mM spermidine, and 3 mg of poly(dI-dC) for 20 min. The preparations were then separated by electrophoresis in 4% polyacrylamide gel in Tris borate-EDTA buffer and autoradiographed. For competition assays, excess amounts of cold 42-mer fragments and a synthetic mutant sequence of the NF-κB motif (TCGACAGAATTCACTTTCCGAGAGGCTCGA) (18) were used for binding assays. For supershift assays, 10-fold diluted rabbit antiserum against NF-κB (p65) (Santa Cruz Biotechnology) was also added to the binding reaction. The complexes of NF-κB motif DNA, nuclear protein, and antibody were identified by electrophoresis as described previously (19).

**Fig. 1. CAT assay of transformed cell lines.** A, transcriptional activation of HIV-LTR promoter measured by CAT activity after treatment with H2O2. Part a, 5 × 10⁵ cells of each cell line were treated with 0, 0.05, and 0.2 mM H2O2 for 1 h and extracts were separated by thin-layer chromatography, and the CAT activity of each transformed cell line was measured; part b, the percentage conversion of the acetylated form of \[^{14}C\]chloramphenicol. B, Time course of CAT activation in cells transformed with pCD12 on long term dilute H2O2 (LDH) treatment. Part a, time course of CAT activity of cells treated with LDH measured by thin-layer chromatography; part b, the percentage conversion of the acetylated form of chloramphenicol. C, reduction of CAT activity by NAC (20 mM) after long term (25 days) dilute H2O2 (LDH) treatment. Part a, CAT activity of cells treated with NAC after LDH treatment; part b, the percentage conversion of the acetylated form of chloramphenicol. The percentage conversion of acetylated form of \[^{14}C\]chloramphenicol was determined as follows: Conversion (%) = counts/min of acetylated form of chloramphenicol/total counts/min × 100.
Weak Oxidative Stress Can Sensitize HIV-1

**RESULTS AND DISCUSSION**

**Effects of Short Term Treatment with H$_2$O$_2$ on CAT Activity**—Transformed cell lines were obtained by the protoplast fusion method as described previously (7). Three cell lines (HL60, U937, and MOLT4) transformed by pCD12 (HIV-LTR-CAT) plasmid were named HL60CD, U937CD, and MOLT4CD, respectively, and the same cells transformed by pSV2CAT plasmid were named HL60SV, U937SV, and MOLT4SV, respectively (cf. Ref. 7), and MOLT4 cells transformed by mutant pCD12 were named MOLT4CD*. For short term H$_2$O$_2$ treatment, these cell lines were treated with 0, 0.05, or 0.2 mM H$_2$O$_2$ for 1 h, then washed with normal medium (RPMI 1640, 10% fetal bovine serum), and cultured for 48 h. Samples of 5 × 10$^5$ of these cells were then used for measurement of CAT activity by thin-layer chromatography (Fig. 1A, part a). The CAT activities were measured as percentage conversion of the acetylated form of chloramphenicol from extracts derived of transformed cell lines. The CAT activities of HL60CD cell line treated with 0, 0.05, and 0.2 mM H$_2$O$_2$ were 0, 5.2, and 65.3%, respectively, and almost the same results were obtained in U937CD and MOLT4CD cell lines (Fig. 1A, part b). It was found that on short term (1 h) H$_2$O$_2$ treatment with a moderate concentration of H$_2$O$_2$ (0.05 mM), the CAT activity of pCD12-transformed cell lines were only weak; however, a high concentration of H$_2$O$_2$ (0.2 mM) treatment resulted in a marked activation of CAT activity (cf. Ref. 6). The CAT activity of pSV2CAT-transformed cells were extremely high and did not change on treatment with H$_2$O$_2$, but the CAT activity of MOLT4CD* was low and did not change on treatment with H$_2$O$_2$ (Fig. 1A).

**Effects of Long Term Dilute H$_2$O$_2$ Treatment on CAT Activity**—For long term dilute H$_2$O$_2$ treatment, transformed cell lines were treated with 0.01 mM H$_2$O$_2$ for 25 days. At 5-day intervals 0.01 mM H$_2$O$_2$-treated transformed cell lines were collected. The cells were retreated with 0, 0.05, or 0.2 mM of H$_2$O$_2$ for 1 h, washed with normal medium, and cultured for an additional 48 h. The CAT activities of the HL60CD cell line measured as a percentage conversion of the acetylated form of chloramphenicol after short term H$_2$O$_2$ treatment (0, 0.05, 0.2 mM for 1 h) were about 0, 5.2, and 65.3%, respectively (cf. Fig. 1A), but in long term treatment (25 days) the CAT activities were about 7.0, 68.4, and 71.0%, respectively (Fig. 1B). It was found that long term dilute H$_2$O$_2$ treatment (4 h/day for 20 days or more) slightly elevated the CAT activity. And when these cells were retreated with 0.05 mM H$_2$O$_2$ for 1 h, marked elevations of CAT activity were observed. Almost the same results were obtained in U937CD and MOLT4CD cell lines. This sensitization of CAT activity by dilute H$_2$O$_2$ was abolished by the antioxidant (NAC, 20 mM) treatment (Fig. 1C). Marked elevation of CAT activity induced by a moderate concentration (0.05 mM) of H$_2$O$_2$ after long term dilute H$_2$O$_2$ treatment may indicate that the latent HIV-1 promoter in infected cells became sensitive on long term treatment with a low concentration (0.01 mM) of H$_2$O$_2$. In contrast CAT activity of MOLT4CD* cell remained at a low level after long term dilute H$_2$O$_2$ treatment (Fig. 1B; cf. Fig. 1A).
Many patients infected with HIV-1 show a long latency period. If this latency of infected patients corresponds with the "latency" of expression from the HIV promoter in transformed cells, sensitization of the HIV-1 promoter in transformed cells in the presence of a low concentration of H2O2 may represent one of the activation processes of latent HIV-1 in infected patients. Patients presumably suffer oxidative stress during the long latency period, so long term treatment of H2O2 of transformed cells may be a useful model for the onset of AIDS by oxidative stress.

Induction of the NF-κB DNA Motif-binding Protein in Transformed Cells—Binding proteins of the NF-κB DNA motif were detected with a Life Technologies, Inc. kit. A 42-mer DNA containing two GGGGACTTTCC motifs was end-labeled and used for band shift and supershift assays. When the above treated cells were also pretreated with NAC (20 mM), the shifted bands could bind to the HIV-LTR region on long term dilute H2O2 treatment, corresponding with the CAT activity.

Binding of the NF-κB-like Factor to HIV-LTR DNA in Transformed Cells—To examine the binding activity of the NF-κB-like factor with the transcription-activating motif in HIV-1 LTR, a SacI, PvuII fragment (120 bp) containing the NF-κB binding DNA motif of HIV-1-LTR (20) was isolated, end-labeled, and incubated with nuclear proteins of transformed cell lines. The time courses of mobility shift on long term dilute H2O2 treatment (Fig. 3A) and NAC treatment (Fig. 3B) were also examined. Essentially the same results were obtained as with the 42-mer NF-κB DNA binding motif (Fig. 3, cf. Fig. 2). After band shift assay, shifted bands (except supershifted bands) were eluted from the gel and then DNA-protein complexes were immunoprecipitated with anti-NF-κB p65 rabbit serum. The recoveries of radioactivity from shifted bands of transformed cells were about 75% or more. These results also strongly suggest that pCD12 transformed cell lines potentially produce an NF-κB-like transcription activating factor by long term dilute H2O2 treatment corresponding with the CAT activity.

The transformed cells showed a latency for production of CAT activity, but when these cells were maintained in the medium with a low concentration of H2O2, they became potent producers of an NF-κB-like factor, and the HIV-1 promoter was
activated by even a moderate concentration of short term $H_2O_2$ treatment. Patients infected with HIV-1 show a latency for a long time. The latency for the production of HIV-1 in transformed cell seems to correspond well with the latency of the cells of infected patients (8, 9). It is interesting that the activation of transformed cells by treatment with a low concentration of $H_2O_2$ for a long time seems to correspond with the activation of the latent HIV-1 virus in patients. Physical movement and normal metabolism should cause various types of oxygen stress in the body (9). As in transformed cells, this oxygen stress over a long period may result in activation of the latent virus by NF-$\kappa$B. Therefore, the present cell system may be a useful model of HIV-1 virus activation and production of AIDS.

Acknowledgments—We thank Drs. F. Wong-Staal and T. Okamoto for gifts of plasmid pCD12.

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