

Oxidoreductive Regulation of Nuclear Factor κ B

INVOLVEMENT OF A CELLULAR REDUCING CATALYST THIOREDOXIN*

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We have investigated an oxidoreductive regulatory pathway for the DNA binding activity of a pleiotropic cellular transcription factor, nuclear factor κ B (NF κ B), has been investigated by using NF κ B prepared from the nucleus and the cytosol of the primary human T lymphocytes. We show that a cellular reducing catalyst thioredoxin (Trx) plays a major role in activation of the DNA binding of NF κ B *in vitro* and stimulation of transcription from the NF κ B-dependent gene expression. We demonstrate evidence suggesting that redox regulation of NF κ B by Trx might be exerted at a step after dissociation of the inhibitory molecule I κ B, a cytosolic-anchoring protein for NF κ B. To examine the effect of Trx in intact cells, we performed transient assay with a chloramphenicol acetyltransferase-expressing plasmid under the control of human immunodeficiency virus (HIV) long terminal repeat and an effector plasmid expressing human Trx. The promoter activity from HIV long terminal repeat was greatly augmented by co-transfected the Trx-expressing plasmid, whose effect was dependent on the NF κ B-binding sites. These findings have suggested that cysteine residue(s) of NF κ B might be involved in the DNA-recognition by NF κ B and that the redox control mechanism mediated by Trx might have a regulatory role in the NF κ B-mediated gene expression. These results may also provide a clue to understanding of the molecular process of AIDS pathogenesis and its possible biochemical intervention.

Nuclear factor κ B (NF κ B)¹ is an inducible cellular transcription factor that regulates a wide variety of cellular and viral genes (for review, see Lenardo and Baltimore (1989) and

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¹ The abbreviations used are: NF κ B, nuclear factor κ B; Trx, thioredoxin; LTR, long terminal repeat; HIV, human immunodeficiency virus; IL-2R α , interleukin-2 receptor α chain; IL-2, interleukin-2; TNF, tumor necrosis factor; PKC, protein kinase C; ADF, adult T-cell leukemia-derived factor; NAC, *N*-acetyl-L-cysteine; EMSA, electrophoretic mobility shift assay; DOC, deoxycholate; BrdU, bromodeoxyuridine; 2-ME, 2-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; TrxR, thioredoxin reductase; GSH, glutathione; DTT, dithiothreitol; I κ B, inhibitory molecule; CAT, chloramphenicol acetyltransferase.

Greene (1990)). One of the physiological roles of NF κ B is to control gene expression of several cellular genes that are critical for immunological function of T-cells such as those encoding interleukin-2 receptor α chain (IL-2R α) and IL-2 (Bohnlein *et al.*, 1988; Cross *et al.*, 1989; Molitor *et al.*, 1991; Jamieson *et al.*, 1991). It has been noted that the promoter region of human immunodeficiency virus type 1 (HIV-1) also contains two NF κ B-binding sites (called " κ B" sites) (Dinter *et al.*, 1987; Nabel and Baltimore, 1987; Kawakami *et al.*, 1988; Okamoto *et al.*, 1989, 1990). Previous studies have revealed that the κ B sites are essential in transcriptional activation of HIV by various extracellular stimuli and several heterologous viral trans-activators (Gendelman *et al.*, 1986; Dinter *et al.*, 1987; Nabel and Baltimore, 1987; Bohnlein *et al.*, 1988; Nabel *et al.*, 1988; Okamoto *et al.*, 1989; Israel *et al.*, 1989; Ensoli *et al.*, 1989; Hazan *et al.*, 1990; Schreck *et al.*, 1991). Therefore, it has been suspected that various factors that activate NF κ B can also activate HIV replication and thus facilitate disease progression of acquired immunodeficiency syndrome (AIDS).

NF κ B appears to exist as a major heterodimer composed of two subunits with molecular mass values of 50 and 65 kilodaltons (called p50 and p65, respectively). cDNAs coding for p50 and p65 have been cloned (Kieran *et al.*, 1990; Ghosh *et al.*, 1990; Meyer *et al.*, 1991; Ruben *et al.*, 1991). Sequence analysis of p50 and p65 has revealed remarkable homology for over 300 amino acids at the amino-terminal end of both proteins to the oncogene *v-rel*, its cellular homologue *c-rel*, and the *Drosophila* maternal effect gene *dorsal* (reviewed by Gilmore (1990)). Deletion analyses of p50 gene revealed that the conserved NH₂-terminal region, called NRD (for NF κ B, *rel*, *dorsal*) domain (Henkel *et al.*, 1992), has the capability to recognize and bind to the κ B sequence (Kieran *et al.*, 1990; Ghosh *et al.*, 1990) although no known DNA-binding motif has been identified.

In the cytosol, NF κ B is in an inactive form bound to an inhibitory molecule, I κ B, and cannot bind DNA (Baeuerle and Baltimore, 1989; Urban and Baeuerle, 1990; Zabel and Baeuerle, 1990). Activation of cells with appropriate stimuli results in dissociation of NF κ B from I κ B and its translocation to nucleus. External stimuli include signals from the T-cell receptor and tumor necrosis factor (TNF) (Okamoto *et al.*, 1989; Israel, 1989; Hazan *et al.*, 1990; Meichle *et al.*, 1990; Jamieson *et al.*, 1991; Schreck *et al.*, 1991). Induction of NF κ B by way of the T-cell receptor has been shown to be dependent on protein kinase C (PKC) (Hazan *et al.*, 1990). However, TNF-induced NF κ B activation has been found to be mediated by none of the major signal-transducing kinases such as PKC, protein kinase A, or Ca²⁺-regulated kinases (Meichle *et al.*, 1990; Feuillard *et al.*, 1991) (see Camussi *et al.* (1991) and Fiers (1991) for review). Similarly, Hazan *et al.* (1990) have demonstrated that translocation of NF κ B to the nucleus is

not a sufficient condition for activating the κ B-dependent gene expression from the HIV promoter. Involvement of another biochemical pathway has thus been suspected.

Since the identification of adult T-cell leukemia-derived factor (ADF), initially reported to be an inducer of IL-2R α , as a cellular reducing catalyst thioredoxin (Trx) (Tagaya *et al.*, 1989; Wakasugi *et al.*, 1990), it has been implicated that ADF, or Trx, might activate NF κ B by a thiol-redox control mechanism (called "redox regulation"; reviewed by Holmgren (1985, 1989) and Bauskin *et al.* (1991)). An intracellular signaling pathway involving dithiol-disulfide exchange reactions of cysteine residues on protein molecule by redox regulation has been implicated to play a part in transducing the TNF signal (Schreck *et al.*, 1991; Molitor *et al.*, 1991) (reviewed by Camussi *et al.* (1991) and Fiers (1991)). We and others have recently reported that the oxidoreductive condensation of NF κ B regulates its DNA binding activity (Toledano and Leonard, 1991; Molitor *et al.*, 1991; Tsuboi *et al.*, 1991; Okamoto *et al.*, 1992; Matthews *et al.*, 1992) and presented initial observations that a cellular reducing catalyst Trx might be involved (Okamoto *et al.*, 1992; Matthews *et al.*, 1992).

In this study, we have attempted to clarify the biochemical actions of Trx in the signaling pathway that regulates the NF κ B activity and the NF κ B-mediated gene expression using HIV as a model system. We demonstrate evidence suggesting that redox control mechanism may well be serving as an alternative or ancillary pathway for controlling the NF κ B activity in addition to the known pathways involving phosphorylation.

MATERIALS AND METHODS

Cell Culture, Preparation of Nuclear and Cytosolic Extracts, and Protein Purification—Peripheral blood mononuclear leukocytes were isolated from heparinized venous blood obtained from healthy individuals after centrifugal separation on Histopaque (Sigma) gradients. Collected mononuclear cells were washed with RPMI 1640 medium and cultured for 3 days in AIM-V medium (GIBCO) with stimulation by plate-coated anti-CD3 murine monoclonal antibody (OKT3, Janssen-Kyowa, Co., Tokyo, Japan). The cells were then transferred to a gas-permeable culture bag (SteriCell, Du Pont) and cultivation was continued with AIM-V supplemented with insulin (0.2 units/ml), heat-treated human serum (1%; type-compatible), and recombinant IL-2 (700 Jurkat units/ml, Shionogi Pharmaceutical Co., Osaka, Japan). Cytofluorometric analyses were carried out sequentially to determine the lymphocyte subpopulations in culture (representative data are shown in Fig. 1A). After 10 days of culture, the total cell number reached $0.5\text{--}2.0 \times 10^{10}$ and cells were still in the growing phase. Flow cytometric examination of the cultured primary lymphocytes revealed 82.2% CD3-positive, 39.6% CD4-positive, 42.8% CD8-positive, 22.7% CD25(IL-2R α)-positive, and only 0.8% CD16-positive. The cells were harvested, and nuclear and cytosolic extracts were prepared according to the method of Dignam *et al.* (1983). The crude nuclear extract was fractionated on heparin-Sepharose and DEAE-Sepharose columns (Wu *et al.*, 1988; Okamoto and Wong-Staal, 1986) (Fig. 1B). The soluble cytosolic extract, S100, was fractionated to obtain NF κ B/I κ B complex (Ghosh and Baltimore, 1990).

The schematic diagram of protein fractionation of the nuclear and cytosolic (S100) extracts is shown in Fig. 1B. Briefly, approximately 5 ml (8–12 mg of protein/ml) of crude nuclear extract in the D buffer of Dignam *et al.* (1983) was applied to a 20-ml heparin-Sepharose column and eluted by buffer D containing 0.4 M KCl. After dialysis, this eluate fraction was applied to a 10-ml DEAE-Sepharose column. The flow-through, 0.1 M, 0.15 M, 0.2 M, and 0.3 M KCl eluate fractions were concentrated to the initial cell pack volume, dialyzed against the starting buffer without DTT, and stored in liquid nitrogen. The S100 cytosolic fraction was first passed through a DEAE-Sepharose column in the presence of 0.28 M KCl to remove nucleic acids. The flow-through fraction was dialyzed and loaded onto another DEAE-Sepharose column, and protein was eluted by a continuous KCl gradient. The cytosolic NF κ B/I κ B complex was detected by electrophoretic mobility shift assay (EMSA) in the presence of detergent

mixture containing 1.2% Nonidet P-40, and 0.8% deoxycholate (DOC). The DEAE fractions containing this complex were further fractionated through phosphocellulose, heparin-Sepharose, and DEAE-Sepharose columns. The 0.2 M KCl eluate from the last DEAE column was concentrated by ultrafiltration (Centriprep, Amicon Corp.) and was further fractionated by a glycerol gradient centrifugation (no DTT was added in the buffer). For the experiments in Fig. 5, preparation and fractionation of the cytosolic NF κ B was carried out using deaerated and N₂-saturated buffers and no artificial reductant was added. NF κ B activities were recovered in the 0.2 M KCl eluate from the DEAE-Sepharose column chromatography of the nuclear extract (DE0.2) (Fig. 2, A and B) and in two fractions obtained from the glycerol gradient centrifugation of the column-fractionated cytosolic extract (fractions 2 and 3) (Figs. 2C and 5A). These NF κ B preparations did not contain any detectable level of Trx as revealed by immunoblotting using the specific antiserum.

Oligonucleotides—Oligodeoxynucleotides for the κ B binding site used for DNA-binding assays as probes or competitors were synthesized on a DNA synthesizer (type 380A, Applied Biosystems) by the phosphoramidite method and purified on OPC cartridge (Applied Biosystems) according to the instructions provided by the manufacturer. The sequences of the double-stranded oligonucleotide encompass the κ B motif from the HIV-1 or IL-2R α enhancers. DNA sequences of these synthetic oligonucleotides for κ B binding sites and of the mutant were as follows.

HIV κ B wild type: GATCTAGGGACTTCCGCTGGGACTTCCAG
HIV κ B mutant: GATCTActcACCTTCGGCTGctcACTTCCAG
IL-2R α κ B: GATCTCAGGGAACTCCCTCTCCTTTATGGCGTAGCG

Electrophoretic Mobility Shift Assay (EMSA)—The double-stranded κ B oligonucleotide, HIV κ B wild type, was end-labeled using [α -³²P]dATP and Klenow polymerase. Binding reactions of the DNA probe with protein were performed at 30 °C for 10 min in a total volume of 10 μ l of buffer containing 22 mM HEPES-KOH (pH 7.9), 60 mM KCl, 1 mM MgCl₂, 0.02 mM EDTA, 5% (v/v) glycerol, 0.1% Nonidet P-40, 1.0 μ g of poly(dI-dC), and 0.1 ng (20,000–30,000 cpm) of the labeled probe. The DNA-protein complexes were resolved on nondenaturing 6% polyacrylamide gels. Electrophoresis was performed with 0.5 × TBE buffer (4.5 mM Tris, 4.5 mM boric acid, 0.1 mM EDTA, pH 8.0) at 4 °C. Upon EMSA, NF κ B usually gave two retarded bands, as reported previously (Bohlein *et al.*, 1988; Meyer *et al.*, 1991; Okamoto *et al.*, 1992). Since the lower band was first seen with a smaller amount of the NF κ B fractions (DE0.2 or fraction 3), the upper and lower bands might represent tetramer and dimer complexes, respectively (see, for example, Baeuerle and Baltimore (1989)). The intensity of the upper band relative to the lower band varied among the different preparations and might reflect variations during the protein extraction procedures. For the competition experiments, 50-fold molar excess of unlabeled HIV κ B wild-type (wt), HIV κ B mutant (mut), or IL-2R α oligonucleotides were preincubated with the protein on ice for 5 min before adding the radioactive probe.

UV Cross-linking—All the procedures were essentially as described by Cereghini *et al.* (1988). The bromodeoxyuridine (BrdU)-substituted HIV κ B oligonucleotides, where the thymidine residues were substituted by BrdU, were custom-synthesized by Takara Shuzo Co., Ltd. (Kyoto, Japan). BrdU substitution did not change the specificity or the affinity for NF κ B binding as measured by EMSA (not shown). The BrdU-substituted κ B probe was end-labeled with [α -³²P]dATP by Klenow polymerase and incubated with 2 μ l each of the DEAE-Sepharose fractions of the nuclear extract, flow-through, DE0.1, DE0.15, DE0.2, or DE0.3 in EMSA buffer at 30 °C for 25 min. For competition assays, DE0.2 (containing NF κ B) was premixed on ice for 5 min with 50-fold molar excess of unlabeled HIV κ B (wt), HIV κ B (mut), or IL-2R α oligonucleotides before addition of the labeled probe. After DNA-protein binding, the samples were placed 5 cm distant from a UV lamp (253.7 nm, using a sterilization lamp, GL15, Toshiba, Japan) and irradiated on ice for 45 min. After addition of 10 μ l of sampling buffer (2.5% SDS, 0.65 mM DTT, 0.5 M sucrose) for SDS-polyacrylamide gel electrophoresis (PAGE), the samples were boiled for 5 min, separated on a 10% SDS-PAGE gel, and subjected to autoradiography.

Oxidoreductive Reagents and Reactions—Recombinant human thioredoxin (Trx), also known as adult T-cell leukemia-derived factor (ADF) (Tagaya *et al.*, 1989), its mutant with a single amino acid replacement of cysteine 31 by serine (Mitsui *et al.*, 1992), and thioredoxin reductase (TxR) purified from rat liver were generous gifts from Dr. Mitsui, Basic Research Laboratories, Ajinomoto Co., Inc., Kawasaki, Japan. Trx and its mutant were purified to homogeneity,

incubated with 1 mM DTT for 16 h at 4 °C, dialyzed against a buffer containing 20 mM HEPES-KOH (pH7.9), 60 mM KCl, 0.1 mM EDTA, and 5% glycerol, and stored in liquid nitrogen ("fully reduced form"). Some were stored at -20 °C to be naturally oxidized. Measurement of free sulphydryl (SH) contents using 5,5'-dithiobis revealed that average free SH contents of the fully reduced and the "naturally oxidized" Trx were 84 and 32%, respectively.

Chemical oxidation of the thiols on NF κ B was performed by treating the nuclear and the cytosolic fractions containing NF κ B by diamide, an inorganic catalyst of oxidation of dithiols ((SH)₂) to generate disulfides (-S-S-), on ice for 5 min in the EMSA buffer. Reversion of the disulfides to dithiols was carried out by treating with various reducing reagents. Reactions with Trx system, containing various concentrations of human Trx (naturally oxidized form), TxR, and 1 mM NADPH were performed at 25 °C for 5 min. This reaction condition was sufficient to fully reduce the naturally oxidized Trx as examined by insulin reduction assay (Luthman and Holmgren, 1982). Other reducing agents, DTT, 2-ME, glutathione (GSH), and glutathione reductase, were purchased from Sigma.

Chloramphenicol Acetyltransferase (CAT) Assay—Jurkat or COS-1 cells were transfected with a reporter plasmid, HIV κ B (wt) or HIV κ B (mut). In HIV κ B (mut), the κ B sequence GGGACTTTC was substituted by CTCACCTTCC (as described by Nabel and Baltimore (1987)). Ten micrograms of the reporter plasmid was transfected to Jurkat or COS-1 cells in combination with 10 μ g of the Trx-expressing plasmid, SR α ADF (Tagaya *et al.*, 1989) (which was a kind gift from Dr. J. Yodoi, Virus Research Institute, Kyoto University, Kyoto, Japan) or the vector control, SR α , by the methods previously described (Chen and Okayama, 1987; Mori *et al.*, 1990). One microgram of RSV-gal plasmid was also co-transfected as an internal control. Cells were harvested 24–60 h after the medium change following the transfection, and cell extracts were prepared by sonication and heat treatment at 60 °C for 10 min. Protein concentrations were determined by the method of Bradford (using a Bio-Rad protein assay). CAT activity was determined as described previously (Okamoto *et al.*, 1989; Mori *et al.*, 1990). CAT enzyme reactions were performed with 70 μ g of protein in a reaction mixture of 100 μ l. Incubation was carried out at 37 °C for 8 h. Reaction products were analyzed by thin-layer chromatography followed by autoradiography. Quantitation of the CAT enzyme activity (% conversion) was calculated based on the measurement of radioactive signals using an image analyzer (Fuji Film Co., Tokyo, Japan). To adjust the transfection efficiency, quantitation of the β -galactosidase gene product was carried out by immunoblot analysis using the specific antiserum.

RESULTS

Preparation and Identification of the Nuclear and the Cytosolic NF κ B—We prepared nuclear and cytosolic extracts from primary human lymphocytes after short term culture. After 10 days in culture, the cells were still growing while CD25- (or IL-2R α)-positive cells were about 20% and rapidly decreasing (Fig. 1A). Thus, we expected NF κ B in both the nucleus and the cytoplasm at this stage and harvested cells for preparation of nuclear and cytosolic extracts (Fig. 1B). The DNA binding activity was detected by EMSA in the nuclear fractions eluted from DEAE-Sepharose with 0.1 M (DE0.1) and 0.2 M KCl (DE0.2) (Fig. 2A, lanes 3 and 5). Cold competition experiments with oligonucleotide DNAs containing the wild-type or the mutant κ B sequences were performed to determine the sequence specificity of these DNA-binding proteins. The κ B-binding activity in the DE0.2 fraction was specifically competed by preincubation with the unlabeled κ B oligonucleotides but not with the mutant κ B oligonucleotide (data not shown). In contrast, similar experiments with DE0.1 revealed no sequence specificity to the κ B sequence. Incubation of DE0.2 with a monoclonal antibody to NF κ B (Rel homology region) (c-rel/NF κ B cross-reactive mouse monoclonal antibody, American Bio-Technologies Inc., Cambridge, MA) abolished the DNA binding activity in EMSA (data not shown). We carried out UV cross-linking experiment to identify the κ B-binding protein. After incubation with the probe, the DNA-protein complex was cross-linked by UV irradiation and resolved on SDS-PAGE. As shown in Fig. 2B, a single

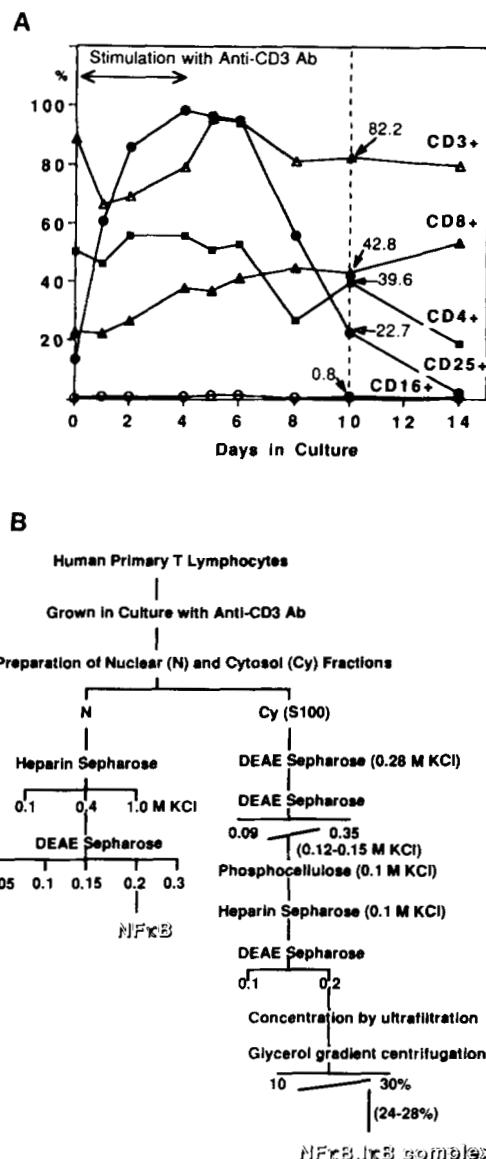


FIG. 1. Lymphocyte culture and preparation of the nuclear and cytoplasmic NF κ B. *A*, cytofluorometric analyses of the primary lymphocytes in culture. *B*, diagram for the preparation of κ B-binding proteins. The nuclear (*N*) and cytosol (*Cy*) extracts were fractionated to yield NF κ B and NF κ B/I κ B fractions as described under "Materials and Methods."

protein of approximately 50 kDa was detected with DE0.2 (lane 5). Cold competition with unlabeled oligonucleotides confirmed the specificity to the κ B sequence of this 50-kDa protein (Fig. 2B, lanes 7–9). From these observations, as well as the similarities of the gel retardation pattern reported previously (Bohnlein *et al.*, 1988; Ghosh *et al.*, 1990; Meyer *et al.*, 1991), we concluded that this 50-kDa DNA-binding protein is the DNA-binding subunit (p50) of NF κ B. Since cysteine-pyrimidine pairs are known to be the major product after UV cross-linking (Smith and Aplin, 1966), the efficient cross-linking of p50 to DNA can be ascribed to the cysteine residue(s) at or in proximity of the DNA-binding interface of p50. We could not detect the p65 subunit of NF κ B by the present UV cross-linking conditions, probably because of the low DNA-binding affinity for p65.

The NF κ B/I κ B complex in the cytosolic fractions was examined by EMSA in the presence of detergent mixture (final concentrations: 1.2% Nonidet P-40 and 0.8% DOC) to phys-

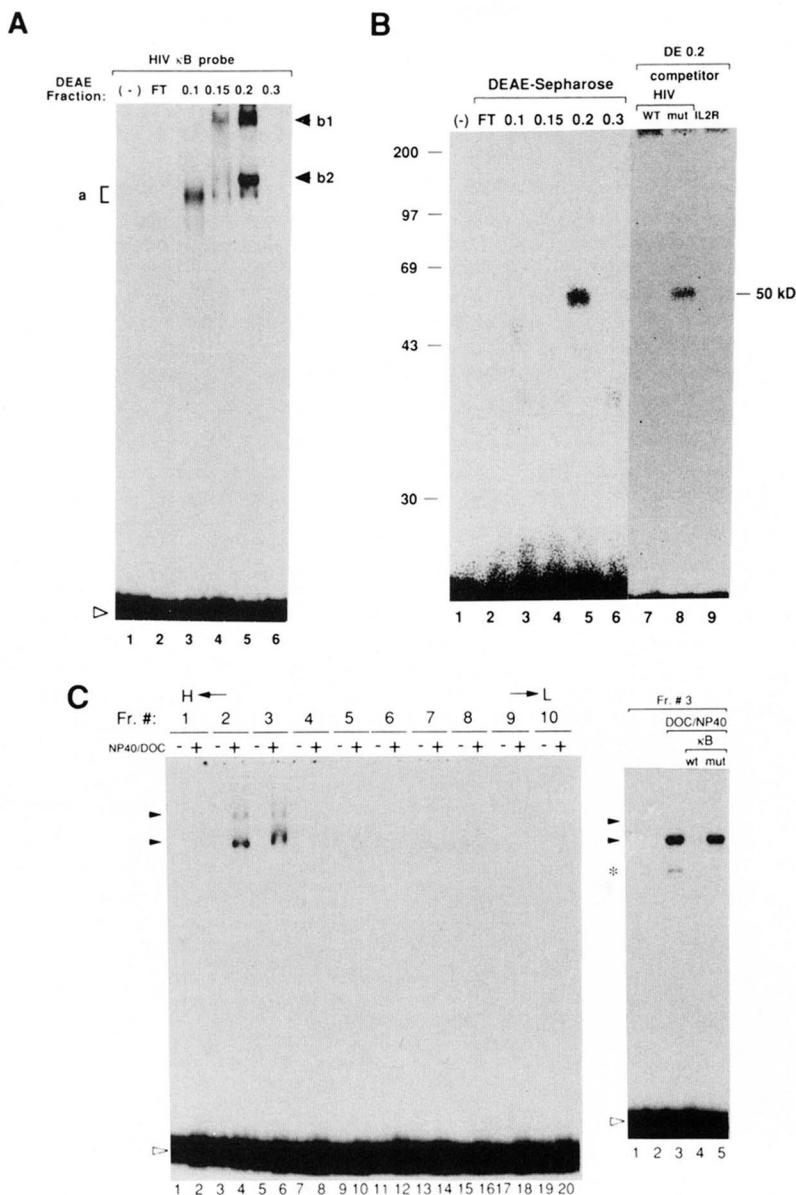


FIG. 2. Identification of nuclear and cytosolic NF κ B. *A*, DNA binding activities of the nuclear protein fractions after separation by DEAE-Sephadex column. The binding activity to the HIV κ B probe was examined by EMSA. 32 P-End-labeled oligonucleotide DNA (HIV wt) was incubated with 2 μ l of each DEAE-Sephadex fraction: flow-through (FT) and the 0.1–0.3 M KCl eluates as indicated above each lane. The position of nonspecific protein-DNA complex is indicated by *a*. Arrowheads *b*1 and *b*2 indicate the position of κ B-specific protein-DNA complexes. *B*, identification of the DNA-binding subunit of NF κ B (p50) by UV cross-linking. Each DEAE-Sephadex fraction of the nuclear protein was incubated with the 32 P-end-labeled BrdU-substituted κ B (HIV WT) DNA probe. The cross-linked samples were analyzed by 10% SDS-PAGE. Positions of molecular size makers in kDa are shown on the left. The position of the 50-kDa protein complex is indicated on the right. *Lane 1*, no protein was added; *lane 2*, flow-through (FT); *lane 3*, DE0.1; *lane 4*, DE0.15; *lane 5*, DE0.2; *lane 6*, DE0.3. Specificity of the DNA-protein interaction with DE0.2 was examined by premixing with a 50-fold molar excess of cold competitor DNAs: wild type κ B sequences (*lane 7*, HIV κ B (wild type); *lane 9*, IL-2R α) and its mutant (*lane 8*, HIV κ B (mut)). *C*, demonstration of κ B binding activity in fractions from the preparative glycerol gradient centrifugation. *Left*, NF κ B·I κ B complex was identified by adding 0.8% DOC and 1.2% Nonidet P-40. In even-numbered lanes, the detergents were added into the reaction mixture (+), but in odd-numbered lanes, they were not added (-). The neighboring two lanes are from the same fraction (fraction numbers are indicated on top). The closed triangles indicate the NF κ B·DNA complexes, and the open triangle indicates the free probe. *Right*, specificity for the κ B binding activity in the glycerol gradient fraction number 3 (Fr.#3). After treatment with DOC/Nonidet P-40, fraction 3 was premixed with 50-fold molar excess of cold competitors. Asterisk indicates the position of a nonspecific band.

ically dissociate I κ B from NF κ B. The NF κ B activity was demonstrated in two consecutive fractions from the glycerol density gradient centrifugation, fractions 2 and 3 (Fig. 2C, *left*). The mobility of DNA-protein complex was identical to the nuclear NF κ B (lower band). Cold competition experiments again demonstrated the sequence-specific DNA-binding (Fig. 2C, *right*). We also performed *in vitro* phosphorylation with purified PKC. When increasing amounts of PKC

was added to fractions 2 or 3, the activation of the κ B-binding activity was observed in a dose-dependent manner (data not shown). From these observations, we concluded that fractions 2 and 3 contained the NF κ B/I κ B complex.

Redox Regulation of the DNA Binding Activity of NF κ B by Human Thioredoxin (Trx).—The DNA binding activity of the nuclear and the cytosolic NF κ B was examined upon oxidoreductive modifications of sulfhydryls on the cysteine residues

by various oxidizing or reducing reagents. First, the effects of oxidation and reduction of the nuclear NF κ B were examined. Fig. 3 demonstrates the representative results obtained with the nuclear NF κ B. When the DE0.2 fraction containing the nuclear NF κ B was treated with diamide, the DNA binding activity was totally abolished (Fig. 3, lanes 2 and 3). However, the subsequent addition to 10 mM DTT (lane 4) or 2-ME (data not shown) fully restored the DNA binding activity of NF κ B. The effect of reduction by Trx (20 μ M, reduced form) was demonstrated with much greater efficiency (Fig. 3, lane 6).

We then examined whether Trx requires the proton recruitment in reactivating the oxidized NF κ B (Fig. 3, lanes 5, 7, and 9–13). The reducing activity of Trx appeared to require proton recruitment from NADPH through Trx-reductase (TxR), since omission of one component from the Trx system, either NADPH, TxR, or Trx itself, did not support restoration of the NF κ B activity, whereas a complete Trx system effi-

ciently restored the NF κ B activity in a dose-dependent manner for Trx (Fig. 3, lanes 11–13). In contrast, a mutant Trx (a single amino acid replacement of the catalytic active center cysteine 31 by serine) (Mitsui *et al.*, 1992) failed to recover the NF κ B activity even with the Trx-regenerating system (Fig. 3, lanes 8 and 14–16). These observations indicate that Trx restores the DNA binding activity of the oxidized NF κ B by facilitating proton transfer to the disulfides on NF κ B molecule through its catalytic action.

Redox Regulation of the Cytosolic NF κ B by Trx and the Relationship with the Signal Pathway Involving Dissociation of I κ B—Similarly, we evaluated redox regulation of the DNA binding activity with the cytosolic NF κ B (Figs. 4 and 5). In these experiments, the NF κ B/I κ B complex in a cytosolic fraction, fraction 3, was first treated by detergents (deoxycholate and Nonidet P-40) as in Fig. 2C and the effects of oxidoreductive modifications were examined. The cytosolic NF κ B, showing strong binding to the κ B DNA probe (Fig. 4A, lane 4), lost its activity by oxidation with diamide (Fig. 4, lane 5). Effects of Trx in restoring the DNA binding activity of the oxidized cytosolic NF κ B was also evident (lanes 8–10). The full components of the Trx system (Fig. 4A, lanes 8–10), as well as the reduced form of Trx (Fig. 4A, lane 7), restored the DNA-binding activity of the oxidized NF κ B. The relative activity of Trx in restoring the DNA binding activity of NF κ B was about 500-fold greater than 2-ME or DTT, since 20 μ M Trx had nearly the same activity as 10 mM DTT (compare lanes 4 and 13 in Fig. 3) or 10 mM 2-ME (compare lanes 8 and 12 in Fig. 4A). However, either oxidation or reduction alone could not dissociate the cytosolic NF κ B from the NF κ B/I κ B complex (Fig. 4A, lanes 1–3).

We then investigated the hierarchy of actions of these two modalities in regulating the NF κ B activity: dissociation of I κ B from the NF κ B·I κ B complex and the redox regulation by Trx. In lanes 3–14 of Fig. 4B, the cytosolic fraction 3 containing the NF κ B·I κ B complex was first oxidized by diamide. When we reduced the oxidized NF κ B·I κ B complex by the Trx system prior to dissociation of I κ B by the detergents, the DNA binding activity was not recovered (Fig. 4B, lanes 4–6 and 9). However, the activity of the Trx system in recovering the DNA binding activity of the oxidized NF κ B was evident when I κ B had been dissociated prior to the reduction by the Trx system (Fig. 4B, lanes 3 and 11–13). Interestingly, when we used 2-ME for reduction, the DNA binding activity of NF κ B was recovered even when we added 2-ME before treatment with the detergent (Fig. 4B, lanes 8 and 10). These differences of action of Trx and 2-ME were probably due to the differences in their accessibility to the oxidized disulfide(s) on p50. Since the molecular mass of 2-ME is only 78 daltons, whereas Trx is approximately 13,000 daltons (Tagaya *et al.*, 1989), it is likely that 2-ME, not Trx, can reach the target disulfide(s) on p50 that is incompletely masked by the associating molecule I κ B. From these results, we concluded that Trx might act on NF κ B only after the dissociation of I κ B and that this order of action might be determined based on the lack of accessibility of Trx to reach the oxidized disulfide(s) on the NF κ B when associated with I κ B.

To examine the κ B binding activity of the cytosolic NF κ B with its natural form within the intact cells, we prepared the cytosolic NF κ B·I κ B complex with all the buffers deaerated and replaced by nitrogen gas and without using any artificial reductant (performed as in Fig. 1B). Each cytosolic fraction from glycerol density gradient was first treated with Nonidet P-40 and DOC (Fig. 5A). Unlike the result in Fig. 2C, we could not detect any κ B binding activity. However, when we further reduced the proteins by the Trx system, the κ B-

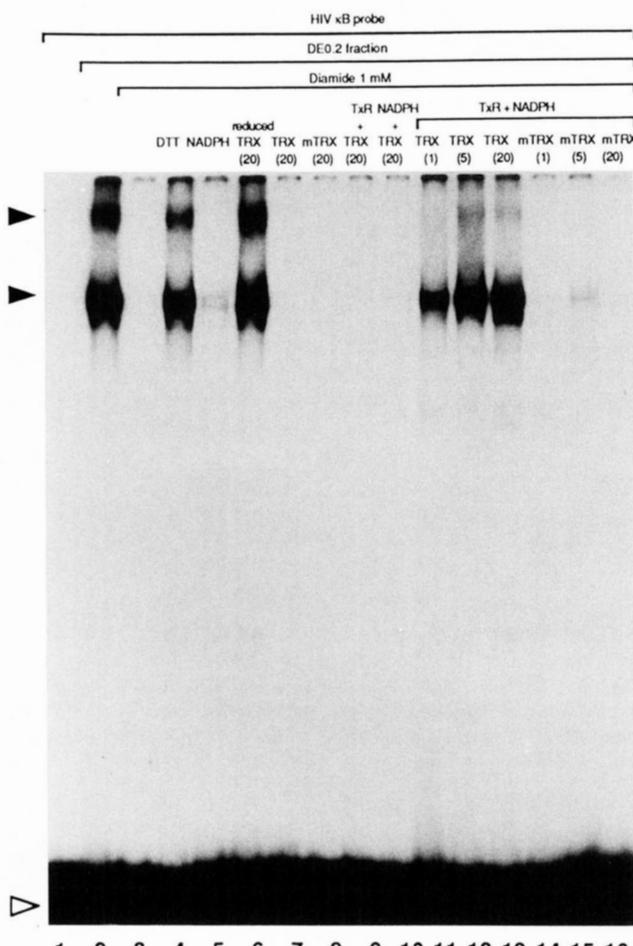


FIG. 3. Oxidoreductive modulations of the DNA binding activity of NF κ B and the effect of Trx system. The nuclear NF κ B (DE0.2 from Fig. 2) was treated with the oxidizing agent diamide, and activities of various reducing agents were examined for the ability to restore the DNA binding activity. Oxidation was performed by 1 mM (final concentration) of diamide on ice for 5 min. Reduction with various reducing agents including the Trx system was performed at 30 °C for 5 min. The oxidized NF κ B was treated by 10 mM DTT (lane 4), 1 mM NADPH (lane 5), 20 μ M fully reduced Trx (lane 6), 20 μ M Trx (lane 7), 20 μ M mutant Trx (lane 8), Trx and Trx-reductase purified from rabbit liver (TxR) (lane 9), or Trx and 1 mM NADPH (lane 10). In lanes 11–16, various concentrations (1, 5, and 20 μ M) of Trx (lanes 11–13) and its inactive mutant (*m*Trx) (lanes 14–16) was added in addition to the fully competent Trx-reducing system, TxR and NADPH.

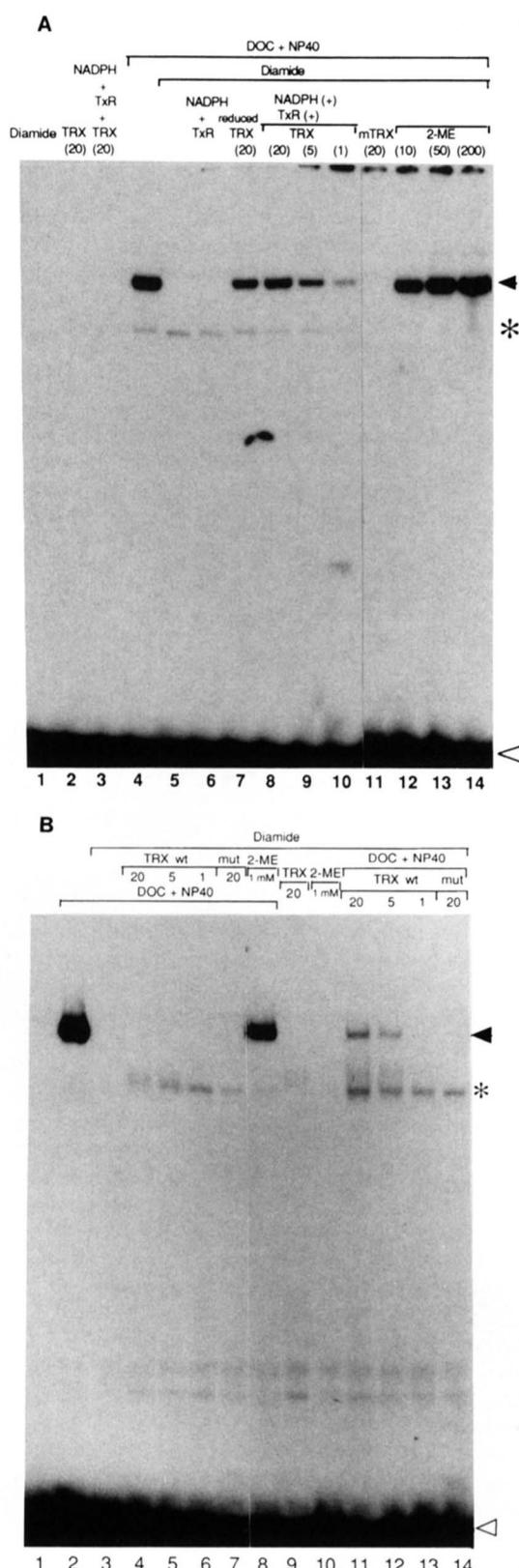


FIG. 4. Oxidoreductive modulations of the cytosolic NF κ B. *A*, a cytosolic fraction containing the NF κ B-I κ B complex (fraction 3) was treated by the detergents to dissociate I κ B from the NF κ B-I κ B complex. Oxidation by diamide and the reduction by Trx system was performed as in Fig. 3. Direct oxidation of fraction 3 by diamide (*lane 1*) or reduction by Trx (*lane 2*, Trx alone; *lane 3*, full component of Trx system) did not activate the DNA binding activity. In *lanes 4–14*, fraction 3 was first treated by the detergents and then oxidized by 1 mM diamide. Oxidized proteins were then treated by various

binding activity was demonstrated in fractions 2 and 3, and slightly in fraction 4, as demonstrated in Fig. 2C. Although we cannot exclude a possibility that NF κ B might have been oxidized during the preparation, this kind of experiment was necessary to verify the biological relevance of the redox regulation of NF κ B. We then compared the ability of various reducing catalysts, including DTT, 2-ME, Trx, and GSH, in activating the κ B binding activity (Fig. 5B). Among these reductants Trx showed the highest efficiency. In contrast, GSH even with higher concentration and together with its regenerating system did not show any activity. These results were consistent with the results obtained with the diamide-oxidized NF κ B that was prepared from the cells using DTT.

Activation of HIV Gene Expression by Trx in Cell Culture System Using Transient CAT Assay—We carried out transient CAT assay to confirm the effects of Trx on NF κ B in cell-free DNA-binding assays. We used CAT-expression plasmids under the control of HIV LTR, either wild type or its mutant (Fig. 6). The mutant construct contains substitutions in the two tandemly repeated κ B sequence, so that NF κ B can no longer recognize the promoter (Fig. 6A). Either of these plasmids was transfected to COS-1 (Fig. 6B) or Jurkat (Fig. 6C) cells in combination with a Trx-expressing plasmid, SR α ADF (Tagaya *et al.*, 1989), or with the vector control, SR α Neo. We harvested the cells 24–60 h after transfection and prepared the cell lysates for CAT enzyme assays. As demonstrated in Fig. 6 (B and C), CAT gene expression from the wild type HIV LTR was greatly stimulated (greater than 4-fold for Jurkat cells and nearly 13-fold for COS-1 cells) by co-transfection of SR α ADF plasmid. In contrast, CAT expression from the mutant HIV LTR was not stimulated by co-transfection with SR α ADF. Therefore, it was concluded that Trx could stimulate HIV gene expression through NF κ B in cultured cells.

DISCUSSION

In this report we demonstrate that the DNA binding activity of an inducible transcription factor, NF κ B, prepared from the nucleus or from the cytoplasm, is regulated by a cellular reducing catalyst Trx through redox control mechanism. Trx appears to modulate the DNA binding activity by dithiol-disulfide exchange reaction. By using the cell-free system and the transient CAT gene expression system with intact cells we were able to show that Trx activates the DNA binding activity of NF κ B and augments gene expression from HIV LTR in an NF κ B-dependent manner. Similar findings have been shown by Matthews *et al.* (1992) using transient CAT expression under HIV LTR in COS cells. They also demon-

strated that Trx activates the HIV LTR by modulating the NF κ B activity. Our results are consistent with their findings. In addition, we demonstrated that Trx activates the NF κ B activity by modulating the DNA binding activity of NF κ B. The mechanism of Trx activation of NF κ B is not clear at present. It is likely that Trx activates NF κ B by modulating the conformation of NF κ B or by modulating the interaction of NF κ B with other proteins. The exact mechanism remains to be determined.

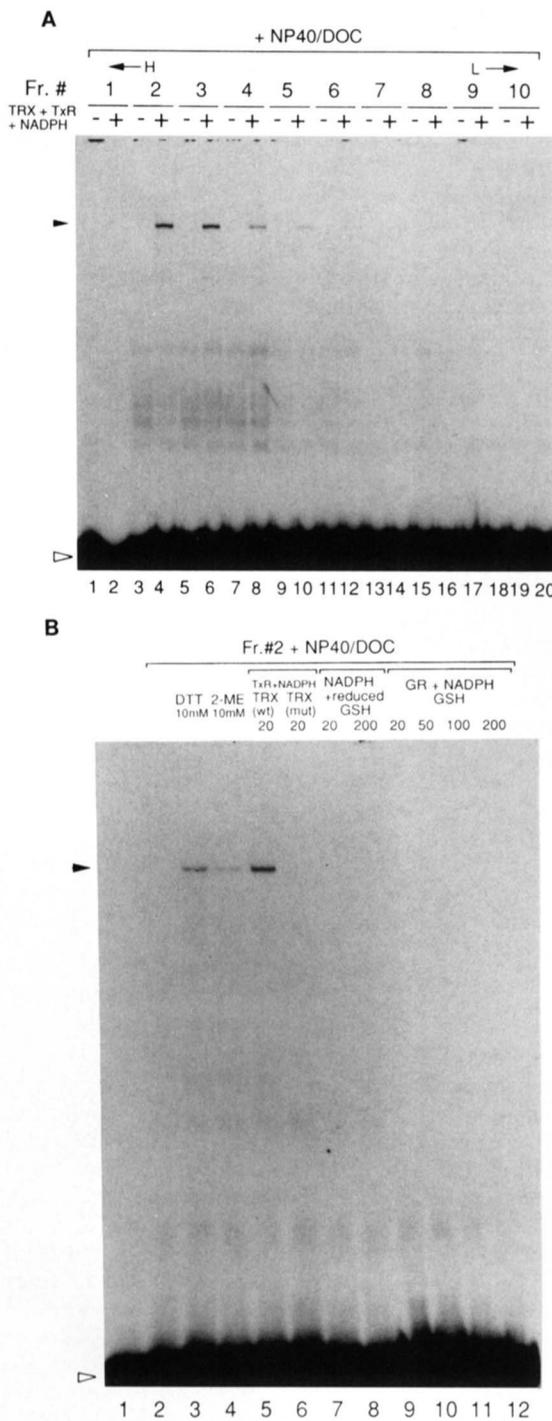


FIG. 5. Oxidoreductive modulations of the cytosolic NF κ B with its natural redox status. *A*, the κ B binding activity in fractions from the glycerol density gradient centrifugation prepared with buffers deaerated and replaced by nitrogen gas. Each cytosolic fraction was first treated by the detergents to dissociate I κ B before reduction by the Trx system. In even numbered lanes, the cytosolic NF κ B was reduced by the Trx system (+); in odd numbered lanes, it was not reduced (-). The closed triangle indicates the NF κ B-DNA complex, and the open triangle indicates the free probe. *B*, comparison of reducing activities of various reductants in activating the cytosolic NF κ B. The DNA binding activity was examined by EMSA. Reduction with the Trx system or the glutathione (GSH) system was performed at 30 °C for 5 min. Lanes 2–12, cytosolic fraction 2 was first treated by the detergents to dissociate I κ B from the NF κ B-I κ B complex and then various reductants were added: no reductant added (lane 2), 10 mM DTT (lane 3), 20 mM 2-ME (lane 4), full component of Trx system with 20 μ M Trx (lane 5), with 20 μ M (lane 6), fully reduced GSH (lane 7, 20 μ M; lane 8, 200 μ M), full component of GSH system

strated that effects of the cotransfection of Trx (ADF)-expression plasmid are dependent on the κ B sequence within the reporter plasmid, which supports our present findings. *In vitro* biochemical analyses were able to dissect this phenomenon in greater detail and revealed that redox regulation of NF κ B activity appeared to be exerted after dissociation of I κ B from NF κ B. These findings not only demonstrate the presence of a novel modality in the regulation of NF κ B-mediated gene expression, but also provide evidence that cysteine residue(s) on NF κ B might be involved in its interaction with the target DNA sequence.

In this study, we have attempted to clarify in greater detail how Trx regulates NF κ B. We prepared the cytosolic NF κ B/I κ B from primary human lymphocytes because cell lines are considered to have wide variety of differences in the responsiveness to oxidoreductive reagents that might have evolved during long term culture. We have demonstrated evidence suggesting that the redox regulation of NF κ B by Trx might be exerted after the dissociation of I κ B. Next, we prepared the cytosolic NF κ B retaining the redox status of the cells in culture without using artificial reductants and found that the cytosolic NF κ B might require further reduction to acquire its full DNA binding activity. This *in vitro* observation could explain why gene expression from HIV LTR can be activated solely by the co-transfection of the Trx-expressing plasmid (this study; Matthews *et al.*, 1992). We also found that among various reductants, either physiological or artificial, Trx showed the greatest effect in activating the DNA binding activity of NF κ B. These observations support the possibility that a cellular redox control system such as Trx has a regulatory role in controlling the activity of NF κ B.

The present observations raise the importance of the redox-reactive cysteine(s) in the DNA-binding by NF κ B. Fig. 7*A* illustrates the amino acid sequence alignment of the putative DNA-binding domain, or NRD domain (Henkel *et al.*, 1992), of the members of the *rel* oncogene family. Among the amino acid residues, 3 cysteines within this region (amino acid positions 62, 119, and 273 in p50) are conserved. We then attempted to locate the specific target cysteine by inspecting the electrostatic microenvironment of each cysteine, since it was indicated that the reactivity of a cysteine was enhanced by the neighboring basic amino acids through formation of a thiol-anion base pair (Kallis and Holmgren, 1980; Snyder *et al.*, 1981). The cysteine at amino acid position 62 in p50 appeared to have the most and the only basic environment as shown in Fig. 7*B* and was considered to be the target of the redox regulation (see also Mathews *et al.* (1992)).

In apparent contrast to our observations demonstrating the potentiation of NF κ B binding by reducing agents, several groups have reported that the TNF-mediated NF κ B-dependent activation of gene expression from the HIV-1 LTR in intact cells was efficiently inhibited by treating the cells with *N*-acetylcysteine (NAC), a precursor for GSH, or another thiol compound pyrrolidine derivative of dithiocarbamate (Staal *et al.*, 1990; Roederer *et al.*, 1991; Schreck *et al.*, 1991). Since GSH even with a higher concentration did not restore the activity of the diamide-oxidized NF κ B (Fig. 5*B*), the effect of NAC could not be ascribed to its reducing activity but to the indirect effects on other cellular reducing system such as the Trx system. We have recently found that the amount of Trx is greatly increased in primary lymphocytes and T-cell lines by treatment with hydrogen peroxide or TNF and that pretreatment of the cells with NAC prohibits the induction

with GSH concentrations of 20 (lane 9), 50 (lane 10), 100 (lane 11), and 200 μ M (lane 12).

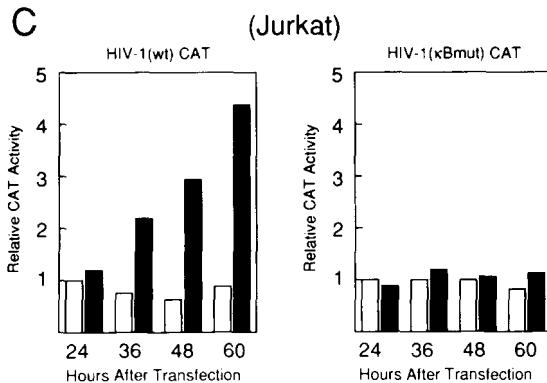
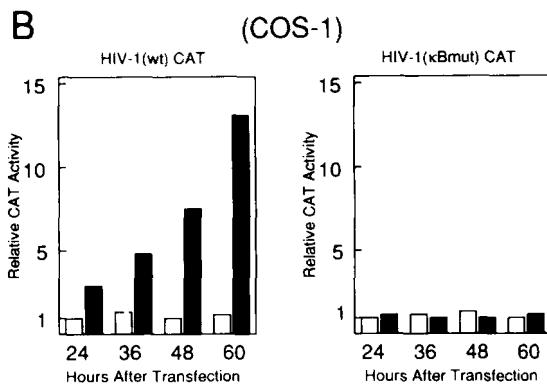
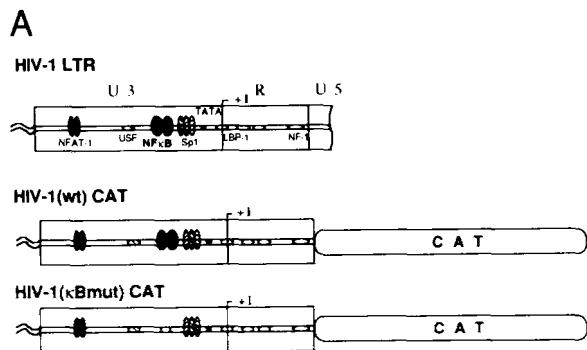


FIG. 6. Augmentation of gene expression from HIV-1 LTR by co-transfection of Trx gene: evidence of redox regulation in intact cells. A, schematic diagrams of the HIV-1 LTR-driven CAT plasmids. Cis-regulatory elements within the HIV-1 LTR are shown. In the mutant (\times Bmut), each copy of the tandemly repeated \times B sequence was replaced by the NF κ B nonbinding sequence. B and C, results of CAT assays with the HIV-1 LTR-CAT constructs in COS-1 (B) and Jurkat (C) cells. Cells were transfected by the reporter plasmid in combination with either SR α ADF or the control plasmid. SR α ADF is an effector plasmid that expresses human Trx (also known as ADF) (Tagaya *et al.*, 1989). The numbers indicated below each lane are the time (in hours) of cell harvest after the medium change following transfection. A fixed amount (70 μ g of protein) of cell lysate was used for CAT enzyme assays. The efficiency of transfection was monitored by co-transfection of β -galactosidase expression plasmid under the control of RSV LTR.

of Trx production upon such treatment.² Transient oxidative stress within the cells elicited by hydrogen peroxide, TNF or others, might induce the production of Trx and the induced Trx would then activate NF κ B that had been oxidized by reactive oxygen intermediates (which probably dissociates I κ B by either a direct or indirect mechanism at the same time).

A

57

p50 ... RPRYV[RE]GSHGGLPGA-SS-EKNNKSY[PQVK]CNVGP[AKV]VQLVTNG-KN
p65 ... RPRYV[RE]GRSGASGIPGH-RS-TDTTKTHPTIKCNYGPGTVRISLVTKD-PP
p49/p100 ... RPRYV[RE]GRSGASGIPQH-NS-TDNNTRTYPSINIMNYGGRKVRITLVTKN-DP
c-rel dorsal ... RPRYV[RE]GRSGASGIPGVNSTPE-NK-TYPTIEV[CYKG]-RAVV-VVSCVTKD

107

p50 1HL-HAHSVLVGKH-[RE]EDG[CTVTAGPKD-M-VVGPANLGIDV]VTKKKVFET
p65 HRP-HPHELWKGD-[RE]RDGYTAEELCPDR-C-HISVQNLGIDVVKRKDLBEA
p49/p100 PRA-HAHSVLVGKH-[RE]ELGICAVSVGPKD-M-TAQFNLGLV[RE]VTKKKNMGT
c-rel dorsal YKP-HPHDLWKGD-[RE]DGFPEAEFGNBR-R-PLFQNLGIDVVKKKKEVKA
TPYKPHPHNIVLGKEDG-XKGVCTLEINS-ETMRAV-PSNLGIDVKKR-D-

154

p50 ... TEACIRGYNPGILLYVHPDLAYLQAE[G]GG-D[RE]QLCDRE
p65 ... QTNNNPPQ-----VP1EEQRGDY
p49/p100 M1QKLQQRBLBSRPGQLT[RE]A-----E-QRELEQA
c-rel dorsal I1T[RE]I-KAGINPFN-----VP-IXQLDIE
dorsal IEA-A-----LKAEE-IRVDP-----KT-GFSK

194

p50 KEL[RE]QAA[LQQTKEHDLVSRMLMTAFLPDS]-CSFTRRLPEVVSQDIAVYD
p65 ... DL-NAVFLCPQVTVRQDPS-GRP-LRLPPVLPHP1FDN
p49/p100 KELVMDL-S1VRLRPSAFI[RASD]-GSFSLPLPKVTSQPIHDN
c-rel ... DCDL-NVVELCQVFLPDEK-GNCTTALPPVVSNPYIDN
dorsal RF-QPS-S-[RE]LNSV-LRCFQVFM-ESEQKGRFTSPLPPVVS&P1FDK

244 285

p50 KAPNASNLKIV-RMDRTAGC-VTG-GEE-IYLL[DOKVQKDDI]QIR-
p65 RAPTAA[RE]LIC-RVNRNSCG-CLG-GDE-[RE]FL[DOKVQKDDI]EVY-
p49/p100 KSPGASHNLKIS-RMOKTAGS-VRG-GDE-VYL[DOKVQKDDI]EVY-
c-rel RAPNTA[RE]LIC-RVNRNCGS-VRG-GDE-[RE]FL[DOKVQKDDI]EVY-
dorsal KA-MSDVLIC-RLC-S-[RE]CATVFGMTQI[LRE]EKVAKEDISV-

B

local pI

R	F	R	Y	V	C	E	G	P	S	H	9.1
+	+	-	-	-	-	-	-	-	-	-	
L	V	G	K	H	C	E	D	G	I	C	5.2
-	-	-	-	-	-	-	-	-	-	-	
E	I	Y	L	L	C	D	K	V	Q	K	6.2
-	-	-	-	-	-	-	-	-	-	-	

FIG. 7. Putative target cysteine in the p50 subunit of NF κ B for redox regulation. A, amino acid sequence homology in the DNA-binding domains among the members of *rel* oncogene family. The sequences are taken from human p50 (Kieran *et al.*, 1990), p65 (Ruben *et al.*, 1991), p49/p100 (Schmid *et al.*, 1991), c-rel (Brownell *et al.*, 1989), and dorsal (Steward, 1989). The indicated numbers are the amino acid sequence position of p50 subunit of NF κ B (Kieran *et al.*, 1990). Positions of the conserved cysteines (or histidines) are marked by shadowed boxes. B, locations of charged amino acids and estimated isoelectric points (local pI) of the cysteine-containing peptides of p50. The pI value was calculated with each 11 amino acids sequence described here. Both positively and negatively charged amino acids are indicated.

The presence of large amount of oxygen scavengers such as GSH might block the induction of Trx by neutralizing reactive oxygen intermediates and thus could intervene the activation of NF κ B. Additionally, the results in Fig. 5 indicated that the cytosolic NF κ B might need to be reduced to acquire the DNA binding activity (Fig. 5). It is, therefore, suggested that the PKC cascade might also stimulate cellular reducing system such as Trx.

Regulation of the DNA binding activity by the redox control mechanism is also known for Fos-Jun DNA interaction (Abate *et al.*, 1990). Xanthoudakis *et al.* (1992) have recently identified Ref-1 protein as a cellular reductant. They have shown that Ref-1 stimulates the DNA binding activity of some other transcription factor. However, considering the relatively low efficiency of Ref-1 on reducing NF κ B (Xanthoudakis *et al.*, 1992), it is unlikely that Ref-1 has a major role in the redox regulation of NF κ B. It is possible that various intracellular reductants might have differential roles in the redox regulation of various target proteins.

Redox regulation is intrinsic for cells, especially for lymphocytes and macrophages (reviewed by Ziegler (1985), Holm-

² T. Okamoto, T. Masutani, T. Hayashi, and J. Yodoi, manuscript in preparation.

gren (1989), and Liebermann and Baltimore (1990)). These cells appear to utilize this metabolic pathway in responding to the various extracellular stimuli, including those elicited during cell-cell communication or by infection with microbes. Although the ability of NF κ B to respond to the cellular redox status may not be unique to this protein, regulation of HIV replication through NF κ B appears to endow this virus with various biological features that are probably favorable for its life cycle during disease progression and for the virus spread. HIV may have acquired the responsiveness to NF κ B through mutations and selections of the LTR sequence before it becomes fully pathogenic. Indeed, accumulation of oxidative stress in the HIV-infected individuals has been demonstrated by decreased serum and cellular glutathione (GSH) levels (Buhl *et al.*, 1979; Roederer *et al.*, 1991), which is in contrast to the increasing virus load and the elevated TNF level (Lahdevirta *et al.*, 1988). Thus, pathological processes in the HIV-infected individuals may be linked to the shift of the redox status, and it is likely that Trx level is elevated although it is yet to be documented.

In view of therapeutic interventions, one can envisage the application of reductants, such as NAC and other thiol compounds, to restore the redox status to prevent AIDS development and its progression. It remains to be examined whether Trx plays a part in disease progression *in vivo* and whether reversion of the redox status is effective in blocking the induction of Trx.

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Addendum—During preparation of this manuscript, Matthews *et al.* (1992) reported the results of site-directed mutagenesis of putative cysteines and concluded that cysteine 62 of p50 is involved in the redox regulation by Trx, thus confirming our consideration here.

REFERENCES

- Abate, C., Patel, L., Rauscher, F. J., III, and Curran, T. (1990) *Science* **249**, 1157–1161.
- Baeuerle, P. A., and Baltimore, D. (1989) *Genes & Dev* **3**, 1689–1698.
- Bauskin, A. R., Alkalay, I., and Ben-Neriah, Y. (1991) *Cell* **66**, 685–696.
- Bohnlein, E., Lowenthal, J. W., Siekevitz, M., Franzia, B. R., and Greene, W. C. (1988) *Cell* **53**, 827–836.
- Brownell, E., Mittereder, N., and Rice, N. R. (1989) *Oncogene* **4**, 935–942.
- Buhl, R., Holroyd, K. J., Mastrangeli, A., Cantin, A. M., Jaffe, H. A., Wells, F. B., Saltini, C., and Crystal, R. G. (1979) *Lancet* **ii**, 1294–1296.
- Camussi, G., Albano, E., Tetta, C., and Bussolino, F. (1991) *Eur. J. Biochem* **202**, 3–14.
- Cereghini, S., Blumenfeld, M., and Yaniv, M. (1988) *Genes & Dev* **2**, 957–974.
- Chen, C., and Okayama, H. (1987) *Mol. Cell. Biol.* **7**, 2745–2752.
- Cross, S. L., Halden, N. F., Lenardo, M. J., and Leonard, W. J. (1989) *Science* **244**, 466–468.
- Dignam, J. P., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475–1489.
- Dinter, H., Chiu, R., Imagawa, M., Karin, M., and Jones, K. A. (1987) *EMBO J.* **6**, 4067–4071.
- Ensoli, B., Lusso, P., Schachter, F., Josephs, S. F., Rappaport, J., Negro, F., Gallo, R. C., and Wong-Staal, F. (1989) *EMBO J.* **8**, 3019–3027.
- Feuillard, J., Gouy, H., Bismuth, G., Lee, L. M., Debre, P., and Korner, M. (1991) *Cytokine* **3**, 257–265.
- Fiers, W. (1991) *FEBS Lett.* **285**, 199–212.
- Gendelman, H. E., Phelps, W., Feigenbaum, L., Ostrove, J. M., Adachi, A., Howley, P. M., Khoury, G., Ginsberg, H. S., and Martin, M. A. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 9759–9763.
- Ghosh, S., and Baltimore, D. (1990) *Nature* **344**, 678–682.
- Ghosh, S., Gifford, A. M., Riviere, L. R., Tempst, P., Nolan, G. P., and Baltimore, D. (1990) *Cell* **62**, 1019–1029.
- Gilmore, T. D. (1990) *Cell* **62**, 841–843.
- Greene, W. C. (1990) *Annu. Rev. Immunol.* **8**, 453–475.
- Hazan, U., Thomas, D., Alcamí, J., Bachelerie, F., Israel, N., Yssel, H., Virelizier, J. L., and Arenzana-Seisdedos, F. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 7861–7865.
- Henkel, T., Zabel, U., Zee, K. V., Muller, J. M., Fanning, E., and Baeuerle, P. A. (1992) *Cell* **8**, 1121–1133.
- Holmgren, A. (1985) *Annu. Rev. Biochem.* **54**, 237–271.
- Holmgren, A. (1989) *J. Biol. Chem.* **264**, 13963–13966.
- Israel, N., Hazan, U., Alcamí, J., Munier, J., Arenzana-Seisdedos, F., Bachelerie, F., Israel, A., and Virelizier, J. L. (1989) *J. Immunol.* **143**, 3956–3960.
- Jameson, C., McCaffrey, P. G., Rao, A., and Sen, R. (1991) *J. Immunol.* **147**, 416–420.
- Kallis, G.-B., and Holmgren, A. (1980) *J. Biol. Chem.* **255**, 10261–10265.
- Kawakami, K., Scheidereit, C., and Roeder, R. G. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 4700–4704.
- Kieran, M., Blank, V., Logeat, F., Vandekerckhove, J., Lottspeich, F., Le Bail, O., Urban, M. B., Kourilsky, P., Baeuerle, P. A., and Israel, A. (1990) *Cell* **62**, 1007–1018.
- Lahdevirta, J., Maury, C. P. J., Teppo, A.-M., and Repo, H. (1988) *Am. J. Med.* **85**, 289–291.
- Lenardo, M., and Baltimore, D. (1989) *Cell* **58**, 227–229.
- Liebermann, T. A., and Baltimore, D. (1990) *Mol. Cell. Biol.* **10**, 2327–2334.
- Luthman, M., and Holmgren, A. (1982) *Biochemistry* **21**, 6628–6633.
- Matthews, J. R., Wakasugi, N., Virelizier, J.-L., Yodoi, J., and Hay, R. T. (1992) *Nucleic Acids Res.* **20**, 3821–3830.
- Meichle, A., Schutze, S., Hensel, G., Brunsing, D., and Kronke, M. (1990) *J. Biol. Chem.* **265**, 8339–8343.
- Meyer, R., Hatada, E. N., Hohmann, H. P., Haiker, M., Bartsch, C., Rothlisberger, U., Lahm, H. W., Schlaeger, E. J., Van Loon, A. P., and Scheidereit, C. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 966–970.
- Mitsui, A., Hirakawa, T., and Yodoi, J. (1992) *Biochem. Biophys. Res. Commun.* **186**, 1220–1226.
- Molitor, J. A., Ballard, D. W., and Greene, W. C. (1991) *New Biol.* **3**, 987–996.
- Mori, S., Takada, R., Shimotohno, K., and Okamoto, T. (1990) *Jpn. J. Cancer Res.* **81**, 1124–1131.
- Nabel, G., and Baltimore, D. (1987) *Nature* **326**, 711–713.
- Nabel, G. J., Rice, S. A., Knipe, D. M., and Baltimore, D. (1988) *Science* **239**, 1288–1302.
- Okamoto, T., and Wong-Staal, F. (1986) *Cell* **47**, 29–35.
- Okamoto, T., Matsuyama, T., Mori, S., Hamamoto, Y., Kobayashi, N., Yamamoto, N., Josephs, S. F., Wong-Staal, F., and Shimotohno, K. (1989) *AIDS Res. Hum. Retroviruses* **5**, 131–138.
- Okamoto, T., Benter, T., Josephs, S. F., Sadaie, M. R., and Wong-Staal, F. (1990) *Virology* **177**, 606–614.
- Okamoto, T., Ogiwara, H., Hayashi, T., Mitsui, A., Kawabe, T., and Yodoi, T. (1992) *Int. Immunol.* **4**, 811–819.
- Roederer, M., Raju, P. A., Staal, F. J. T., Herzenberg, L. A., and Herzenberg, L. A. (1991) *AIDS Res. Hum. Retroviruses* **7**, 563–567.
- Ruben, S. M., Dillon, P. J., Schreck, R., Henkel, T., Chen, C. H., Maher, M., Baeuerle, P. A., and Rosen, C. A. (1991) *Science* **251**, 1490–1493.
- Schmid, R. M., and Perkins, N. D., Duckett, C. S., and Nabel, G. J. (1991) *Nature* **352**, 733–736.
- Schreck, R., Rieber, P., and Baeuerle, P. A. (1991) *EMBO J.* **10**, 2247–2258.
- Shirakawa, F., and Mizel, S. B. (1989) *Mol. Cell. Biol.* **9**, 2424–2430.
- Smith, K. C., and Aplin, R. T. (1966) *Biochemistry* **5**, 2125–2130.
- Snyder, G. H., Cennerazzo, M. J., Karalis, A. J., and Field, D. (1981) *Biochemistry* **20**, 6509–6519.
- Staal, F. J., Roederer, M., Herzenberg, L. A., and Herzenberg, L. A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 9943–9947.
- Steward, R. (1989) *Cell* **59**, 1179–1188.
- Tagaya, Y., Maeda, Y., Mitsui, A., Kondo, N., Matsui, H., Hamuro, J., Brown, N., Arai, K., Yokota, T., Wakasugi, H., and Yodoi, J. (1989) *EMBO J.* **8**, 757–764.
- Toledano, M. B., and Leonard, W. J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 4328–4332.
- Tsuhoi, A., Sugimoto, K., Yodoi, J., Miyatake, S., Arai, K., and Arai, N. (1991) *Int. Immunol.* **3**, 807–817.
- Urban, M. B., and Baeuerle, P. A. (1990) *Genes & Dev.* **4**, 1975–1984.
- Urban, M. B., Schreck, R., and Baeuerle, P. A. (1991) *EMBO J.* **10**, 1817–1825.
- Wakasugi, N., Tagaya, Y., Wakasugi, H., Mitsui, A., Maeda, M., Yodoi, J., and Tursz, T. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 8282–8286.
- Wu, F. K., Garcia, J. A., Harrich, D., and Gaynor, R. B. (1988) *EMBO J.* **7**, 2117–2130.
- Xanthoudakis, S., Miao, G., Wang, F., Pan, Y.-C. E., and Curran, T. (1992) *EMBO J.* **11**, 3323–3335.
- Zabel, U., and Baeuerle, P. A. (1990) *Cell* **61**, 255–265.
- Ziegler, D. M. (1985) *Annu. Rev. Biochem.* **54**, 305–329.