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Infection with the Intracellular Protozoan Parasite *Theileria parva* Induces Constitutively High Levels of NF-κB in Bovine T Lymphocytes

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The intracellular protozoan parasite *Theileria parva* causes a lymphoproliferative disease of T cells in cattle and uncontrolled lymphocyte proliferation in culture. We have identified and characterized in infected cells the transcriptional activator, NF-κB, whose recognition motifs have been identified in several gene enhancers important for lymphocyte-specific gene expression. NF-κB is normally constitutively activated in nuclear extracts derived from B cells and can be induced in T cells and nonlymphoid cells by phorbol esters. *Theileria*-infected lymphocytes contained constitutively high levels of activated NF-κB in nuclear fractions and inactive NF-κB in cytoplasmic fractions. The inactive cytoplasmic precursor could be activated by treatment of extracts with deoxycholate, which was shown previously to dissociate NF-κB from an inhibitor, IκB. Treatment of lymphocyte extracts with 3 mM GTP stimulated NF-κB binding to its recognition motif in vitro, thereby distinguishing it from a related nuclear factor, H2-TF1. Selective killing of the parasite, which left the host cells intact, resulted in a rapid loss of NF-κB from the nuclear fractions and a slower loss from the cytoplasmic fractions. In parasitized cells, NF-κB could not be further stimulated by treatment with 12-*O*-tetradecanoylphorbol-13-acetate whereas in cells treated to remove the parasite, this compound stimulated elevated levels of NF-κB. We propose that high levels of activated NF-κB are maintained by the presence of the parasite in infected T cells. Similarly, we propose that the high levels of inactive cytoplasmic precursor are a result of increased synthesis due to the presence of the parasite.

The intracellular protozoan parasite *Theileria parva* causes a severe and usually fatal disease in cattle known as East Coast fever. The disease is transmitted when infected ticks, harboring infective sporozoites in their salivary glands, take a bloodmeal on a susceptible animal. The injected sporozoites invade lymphocytes and differentiate into schizonts to form the pathogenic stage of the infection. The parasite divides rapidly in the cytoplasm of the lymphocytes of the host and simultaneously induces a massive lymphoproliferation which later in the disease is followed by lymphocytolysis and death of the host (25, 32). The proliferating lymphocytes gradually displace the normal lymphoid tissue, resulting in a condition similar to a multicentric lymphosarcoma (32). Infected lymphocytes, collected by lymph node biopsy, carry T-cell surface markers and are easily established in laboratory cultures as permanently transformed lymphoblastoid cell lines (11, 29). Infected lymphocytes can be grown in soft agar, form tumors in nude mice, and can grow in culture with wholly artificial serum supplements. Lymphocyte transformation is dependent on the continuous presence of the parasite in the cytoplasm. A unique and interesting feature of these cell cultures is that their transformed phenotype can be reversed by selective elimination of the parasite after drug treatment, leaving the host cells viable but no longer proliferating (24). The reversibility of cell transformation offers unusual opportunities in the study of cell proliferation and transformation.

Our approach to understanding the parasite-induced proliferation has been to characterize gene expression in the parasite as well as in infected lymphocytes. We would like to determine what gene regulatory processes are influenced by the presence of the parasite that result in uncontrolled cell growth. We have previously described the constitutive expression of interleukin-2 receptors (IL-2R) on the surface of *Theileria*-infected T cells (14) and, along with others, have suggested that proliferation may occur via an autocrine mechanism whereby the cells produce and consume an interleukinlike growth factor (12, 19).

Regulatory processes in gene expression, including IL-2R gene expression, are mediated by interaction of *trans*-acting protein factors of transcription that bind to *cis*-acting elements of gene enhancers (reviewed by Ptashne [36]). Some of these transactivating factors, such as steroid receptors and NF-κB, are themselves activated from preexisting pools dormant in the cytoplasm (8, 30). Recently, the nuclear factor NF-κB, crucial for expression of the kappa light-chain immunoglobulin gene (41), has been shown to play a role in the expression of the IL-2R(α) chain as well as IL-2 during activation of T lymphocytes (23, 28). NF-κB is important in the activation of several other eucaryotic genes as well. NF-κB-binding sequences are found in the human immunodeficiency virus (HIV) enhancer (33, 35), in the simian virus 40 enhancer (49), in the cytomegalovirus enhancer (10), and upstream of the major histocompatibility complex class I (4, 48) and class II (9) genes. NF-κB-binding activity is constitutively present in mature B cells and some T-cell lines and can be induced in cells by lymphoid and nonlymphoid origin by agents such as lipopolysaccharides, cycloheximide, phorbol esters, and DNA-damaging agents (40, 46). Furthermore, the presence of a related nuclear factor, H2-TF1, which

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binds a sequence similar to NF-κB in lymphoid and nonlymphoid cells and also regulates MHC class I gene expression, has been described (5).

The established regulatory properties of NF-κB in lymphoid cells and the potential role of this factor in constitutive expression of the IL-2R gene in *Theileria*-infected T cells led us to characterize the NF-κB-like proteins in parasite-infected cells. We present data suggesting that the continuous presence of the parasite in the cytoplasm of *Theileria*-infected cells results in constitutively high levels of NF-κB activity in the nucleus as well as high levels of the inactive form in the cytoplasm.

MATERIALS AND METHODS

Cell lines and cultures. We used *Theileria*-infected bovine T lymphocytes of the cloned cell line Tp(M)803 (20). Cultures were grown at 37°C in Leibovitz L15 medium containing 10% heat-inactivated fetal bovine serum (GIBCO, Paisley, Scotland), 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.1), L-glutamine (20 μg/ml), benzylpenicillin (100 U/ml), and streptomycin sulfate (100 μg/ml). This medium is subsequently referred to as cL15. Cells were initially seeded at 1.25×10^5/ml (day 0) and grown in cL15 for different times. Cells were passaged on day 4, when cell density was approximately 1.5×10^6/ml.

Elimination of the parasite from infected cells. The theilericidal drug BW 720c is a hydronaphthoquinone derivative with potent broad-spectrum antiprotozoal activity. BW 720c, a gift from Coopers Animal Health, Ltd., Berkhamsted, United Kingdom, was used at a concentration of 50 ng/ml. The properties of this drug, which interferes with parasite mitochondrial electron transport, have been described elsewhere (24). The compound is not toxic for mammalian cells even at concentrations much higher than those used in this study.

ConA-stimulated lymphocytes. The isolation of bovine lymphocytes and the generation of concanavalin A (ConA)-stimulated lymphoblasts have been described previously (31).

Plasmids. The two HIV-chloramphenicol acetyltransferase (CAT) plasmids used in this work are shown in Fig. 1. Plasmid −121/+232 HIV-CAT contained the HIV type 1 (HIV-1) enhancer with two repeats of the κB elements and the promoter region of the enhancer upstream of the bacterial CAT gene in pBLCAT5. In −76/+232 HIV-CAT, the region containing the κB motifs was deleted. The original plasmids containing the HIV-1 long terminal repeat (LTR) (33) were gifts of D. J. Capon. HIV sequences from these plasmids were recloned into pBLCAT5 (46).

Plasmid pILCAT2/2+, containing two repeats of the 5'-flanking region (−293 to −7) of the mouse IL-2 gene upstream of herpes simplex virus thymidine kinase promoter (42), was a gift of E. Serfling. Plasmids pIL2R(α)CAT, containing a part of the 5'-flanking region of the human IL-2R(α) gene (−478 to +106) upstream of the CAT gene, and pIL2R*(α)CAT, a variant with four point mutations in the NF-κB-binding site of this region (28), were a kind gift of G. Nabel.

Cell transfection and CAT assays. *Theileria*-infected cells growing in log phase were transfected by the DEAE-dextran method (21, 37), with minor modifications. For each transfection, 5×10^6 cells were pelleted gently, washed with prewarmed TBS buffer (25 mM Tris hydrochloride [pH 7.4], 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$) and suspended in 1.1 ml of DNA–TBS–

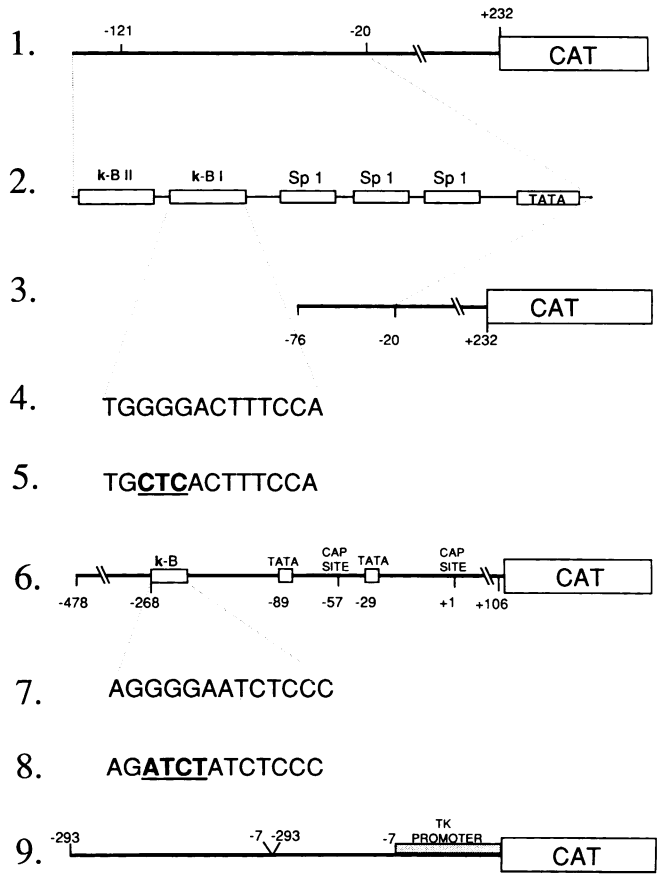

FIG. 1. Structures of the HIV-CAT plasmids used to assay κB-enhanced CAT transcription. Constructs: 1 and 2, −121/+232 HIV-CAT, containing DNA sequences from the HIV-1 LTR surrounding the HIV RNA start (+1) placed upstream the bacterial CAT gene; 3, −76/+232 HIV-CAT, containing a deletion in the region of two κB repeats; 4, sequence of the first κB repeat from the HIV enhancer; 5, mutated variant of no. 4; 6 and 7, pIL2R(α)CAT, containing the human IL-2R(α) gene upstream sequence with one κB motif; 8, sequence of the mutated κB motif from plasmid pIL2R*(α)CAT; 9, pILCAT2/2+, containing two repeats of the 5'-flanking region (−293 to −7) of the mouse IL-2 gene.

DEAE-dextran solution containing 10 μg of plasmid DNA and 250 μg of DEAE-dextran (Pharmacia, Uppsala, Sweden) per ml. The cell suspension was incubated at 37°C for 30 min. The cells were then treated with 10% dimethyl sulfoxide for 3 min, washed twice with prewarmed TBS, suspended in 20 ml of cL15, and transferred to tissue culture flasks. In some experiments, cells were treated approximately 26 h after transfection with 50 or 100 ng of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (Sigma Chemical Co., St. Louis, Mo.), 5 μg of ConA per ml, or both. In all cases, cells were harvested approximately 44 h after transfection. Cell extracts containing 400 μg of total protein were assayed for CAT activity. Quantitation of CAT assays was performed by liquid scintillation counting of the appropriate area from chromatography plates (22).

Purification and labeling of oligonucleotides. Oligonucleotides were synthesized on a Pharmacia gene assembler and purified for full-length product on a 20% polyacrylamide–8 M urea gel. Oligonucleotides were end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (30).

Preparation of nuclear and cytosol extracts and electrophoretic mobility shift assay (EMSA). Nuclear and cytosolic

extracts were prepared as described by Dignam et al. (18) from cultures containing 2×10^8 cells incubated either in medium alone or in medium with TPA (50 ng/ml) or BW 720c (50 ng/ml). The binding reactions were carried out by incubating end-labeled DNA (20,000 cpm) with 0.8 to 6 μg of nuclear or cytosolic protein and 2 μg of poly(dI-dC) in a buffer containing 10 mM HEPES (pH 7.9), 60 mM KCl, 4% Ficoll, 1 mM dithiothreitol, and 1 mM EDTA (7). After 30 min at room temperature, the reaction mixtures were loaded onto a 4% polyacrylamide gel in 0.25× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) and electrophoresed at 10 V/cm for 2 h at room temperature. For competition experiments, a 200-fold molar excess of cold oligonucleotide was added to the reaction mixture before the addition of labeled DNA.

Treatment of cytosol fractions with the dissociating agent 0.6% sodium deoxycholate (DOC) in the presence of 1.2% Nonidet P-40 (NP-40) was performed during the binding reaction as described by Baeuerle and Baltimore (2).

RESULTS

The κB motif of the HIV-1 enhancer mediates high-level transcription of the CAT gene transfected into *Theileria*-infected bovine T lymphocytes. To probe for the presence of *trans*-acting factors that are activated by the parasite, we transfected into *Theileria*-infected lymphocytes chimeric plasmids containing different regulatory regions of the HIV LTR, of the IL-2R(α) gene, and of the IL-2 gene (Fig. 1). Each of these controlling regions was coupled to the reporter CAT gene. Plasmid −121/+232 HIV-CAT (Fig. 1, no. 1) contained both repeats of the NF-κB-binding sequence; −76/+232 HIV-CAT (Fig. 1, no. 3) had the two κB sequences deleted. Plasmid −121/+232 HIV-CAT showed a much higher basal level of CAT expression than did the κB-deleted plasmid, −76/+232 HIV-CAT (Fig. 2A; compare lanes 1 and 7). Levels of expression directed by the κB sequences were increased 10- to 16-fold in separate transfection experiments. Similar increased levels of CAT expression were directed by plasmid pIL2R(α)CAT, containing the κB motif, in comparison with CAT expression in cells transfected with pIL2R*(α)CAT, which contained the mutated κB-site and thereby interfered with NF-κB binding (Fig. 2C, lanes 23 and 28). These results show that *Theileria*-infected bovine T lymphocytes contained *trans*-acting factors that specifically enhanced CAT transcription via the NF-κB-binding sequences from the HIV LTR or the IL-2R(α) gene promoter regions.

T-cell lines have been shown to respond to activators such as ConA and the phorbol ester TPA. Studies have shown that these agents operate along fairly well defined signal transduction pathways leading to the activation of calcium mobilization and of protein kinase C. Antigenic and mitogenic activation of T cells results in the induction of T-cell growth factors and their receptors via activation of transactivating factors of transcription, leading to cell proliferation (15; reviewed by Isakov et al. [26]). Mitogen-activated transcription in T cells can be blocked by cyclosporin A (CsA) (44). To characterize the regulatory pathways utilized by *T. parva* that lead to cell proliferation, we measured the response of infected lymphocytes to these growth modifiers. The effects of TPA, ConA, and CsA on CAT expression directed by the HIV enhancer as well as by the IL-2R(α) promoter region were tested in *Theileria*-infected cells transfected with these plasmids.

TPA tested at two concentrations, 50 and 100 ng/ml, had no effect on stimulating increased CAT activity directed by the HIV enhancer (Fig. 2A; compare lane 1 with lanes 2 and 3). CsA had no inhibitory effect on CAT activity when used alone (lane 4) or together with TPA (compare lane 1 with lane 4 and lane 4 with lanes 5 and 6). Treatment by ConA also had no effect on the CAT activity in these cells whether measured in the presence (Fig. 2B, lane 15) or absence (lane

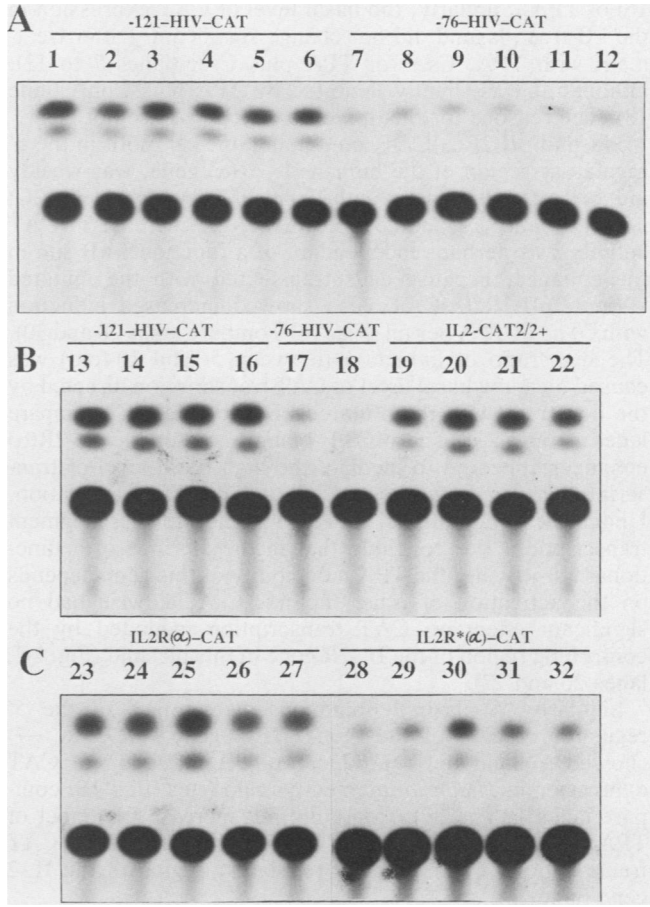

FIG. 2. (A) CAT activity directed by HIV-CAT plasmids in bovine T lymphocytes infected by *T. parva*. Infected lymphocytes were transfected with the HIV-CAT plasmids depicted in Fig. 1 by using DEAE-dextran and maintained for 44 h before extracts were prepared. TPA (50 or 100 ng/ml) and CsA (1 μg/ml) were added 16 h before harvesting. CAT activity was measured by determining the amount of acetylated chloramphenicol produced from [^{14}C]chloramphenicol that separated on thin-layer chromatographs. The cell were transfected by plasmids −121/+232 HIV-CAT (lanes 1 to 6) and −76/+232 HIV-CAT (lanes 7 to 12). Cells were either not treated (lanes 1 and 7) or treated with TPA at 50 (lanes 2 and 8) or 100 (lanes 3 and 9) ng/ml, with CsA (lanes 4 and 10), with TPA (50 ng/ml) plus CsA (lanes 5 and 11), or with TPA (100 ng/ml) plus CsA (lanes 6 and 12). (B) CAT activity directed by HIV-CAT plasmids or pILCAT2/2+. *Theileria*-infected lymphocytes were transfected with either of two HIV-CAT plasmids or with pILCAT2/2+ (see Fig. 1). TPA (50 ng/ml) and ConA (5 μg/ml) were added 16 h before harvest. Extracts for CAT assays were prepared from cells transfected by plasmids −121/+232 HIV-CAT (lanes 13 to 16), −76/+232 HIV-CAT (lanes 17 and 18), and pILCAT2/2+ (lanes 19 to 22). Cells were either not treated (lanes 13, 17, and 19) or treated with TPA (lanes 14 and 20), TPA plus ConA (lanes 15, 18, and 21), or ConA (lanes 16 and 22). (C) CAT activity directed by pIL2R(α)CAT (lanes 23 to 27) and pIL2R*(α)CAT (lanes 28 to 32). Cells were treated with TPA at 50 (lanes 24 and 30) or 100 (lanes 25, 31, and 32) ng/ml, CsA (lane 26), or CsA plus TPA (50 ng/ml) (lane 27) or were untreated (lanes 23, 28, and 29).

16) of TPA. Similarly, the basal level of CAT expression in the κB-free plasmid did not change significantly after treatment with TPA, CsA, or TPA plus CsA (lanes 7 to 12), although it was slightly inhibited by TPA plus ConA (lane 18). The reasons for this inhibition by ConA are not clear.

Plasmid pIL2R(α)CAT, containing one κB motif in the 5′ regulatory region of the human IL-2R(α) gene, was weakly induced after stimulation with 100 ng of TPA per ml (Fig. 2C; compare lanes 23 and 25). However, the increase in CAT activity was perhaps independent of a functional κB site in the enhancer because cells transfected with the mutated variant, pIL2R*(α)CAT, also showed increased induction with 50 ng of TPA per ml (Fig. 2C; compare lanes 28 and 30). The high ratio of induction (between 5- and 10-fold) was caused by a low basal level of CAT transcription directed by the construct with the mutated κB site (Fig. 2C; compare lanes 23 and 28). The NF-κB-binding site of the IL-2R(α) enhancer appeared to mediate the high basal level of transcription in *Theileria*-infected cells without TPA stimulation. Under these conditions, TPA could not further augment transcription. We conclude that in the absence of a functional NF-κB site, the TPA inducibility of this gene depends on the activation of other factors. CsA likewise had no significant effect on CAT transcription mediated by the controlling region of the IL-2R gene in this plasmid (Fig. 2C, lanes 26 and 27).

Similarly, a plasmid containing two copies of the 5′ regulatory region of the mouse IL-2 gene (−293 to −7) showed a slight but reproducible TPA induction of CAT expression in *Theileria*-infected lymphocytes (Fig. 2B; compare lanes 19 and 20). ConA did not increase the effect of TPA (lane 21) and did not change the basal level of CAT transcription directed by the regulatory region of the IL-2 gene (lane 22).

Consequently, both the IL-2 and IL-2R(α) gene regulatory regions, unlike the HIV enhancer, could be induced by TPA in *Theileria*-infected T cells, and this TPA inducibility was independent of NF-κB-binding activity. To conclude, however, that factors other than NF-κB enhance the IL-2R gene in response to TPA stimulation will require a more quantitative analysis of this observation. NF-κB or an NF-κB-like factor is fully activated in *Theileria*-infected cells and is not further activated by TPA and ConA. CsA cannot block because presumably there is no interference beyond the activated transcription factors. It is possible that the parasite directly activates NF-κB without influencing protein kinase or the TPA pathway, unlike the situation in other T-cell lines in which NF-κB activation is dependent on TPA treatment (28, 47). There is as yet no direct proof for the involvement of protein kinase C in activation of NF-κB or other *trans*-acting factors.

Binding of nuclear proteins from extracts of *Theileria*-infected T cells to the κB motif of the HIV enhancer. The results of the functional studies described above focused our attention on the κB sequence of the HIV enhancer. To measure directly the level and location of NF-κB-like proteins in T cells infected by *T. parva*, we used EMSA and two different double-stranded oligonucleotides as radioactive probes. The first oligonucleotide was the κB motif (repeat I) of the HIV enhancer (Fig. 1, no. 4). The second oligonucleotide (mκB) contained three point mutations in the κB motif (Fig. 1, no. 5). This mutation has been shown to enhance neither transcriptional activity in transfected cells nor NF-κB binding in vitro (34, 46). Incubation of the radiolabeled κB oligonucleotide with nuclear extracts from *Theileria*-infected T cells isolated on day 3 after passage produced two

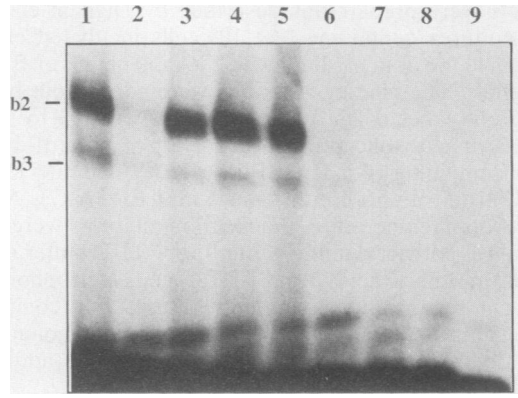

FIG. 3. Presence and specificity of a factor binding to the κB-like sequence from *Theileria*-infected T lymphocytes 3 days after seeding. Shown are analyses of binding site specificity and competition studies, using EMSA with the radiolabeled κB oligonucleotide from the HIV-1 enhancer (lanes 1 to 5) or the mκB oligonucleotide (lanes 6 to 9). (These are the oligonucleotides depicted in Fig. 1, no. 4 and 5, respectively.) Nuclear extracts (4 μg of protein) from infected lymphocytes (lanes 1 to 4 and 7 to 9) or from infected lymphocytes treated with 50 ng of TPA per ml for 16 h (lanes 5 and 6) were incubated alone or in the presence of unlabeled competitors, the κB oligonucleotide (lanes 2, 6, and 9), or the mκB oligonucleotide (lanes 3 and 8). b2 and b3 are the two main retardation bands.

discrete, electrophoretically retarded DNA-protein complexes (b2 and b3 in Fig. 3). The b2 complex was the major component. Formation of these complexes was inhibited by preincubation with a 200-fold molar excess of unlabeled κB oligonucleotide but was not inhibited by the unlabeled mκB oligonucleotide (Fig. 3, lanes 2 and 3, respectively). Treatment of the cells with TPA for 16 h did not alter the EMSA patterns (Fig. 3; compare lanes 4 and 5). Extracts from cells treated for shorter times (30 min or 2 h) with TPA also did not change the EMSA patterns (data not shown). As suggested by results of the CAT experiments, it appears that there was no effect of TPA on the activation of the NF-κB-related proteins in T cells infected by the parasite. Incubation of the radiolabeled mκB oligonucleotide with nuclear extracts from *Theileria*-infected cells did not produce any significant DNA-protein complexes (Fig. 3, lanes 6 to 9). This experiment clearly shows the presence and specificity of NF-κB-related proteins in extracts from bovine T cells infected with the parasite *T. parva*.

In vitro activation of κB motif-specific DNA-binding proteins in cytoplasmic extracts. In pre-B cells and nonlymphoid cells, NF-κB is activated posttranslationally (40). NF-κB resides in the cytoplasm in an inactive form. Treatment of a cytosolic extract with 0.6% DOC in the presence of 1.2% NP-40 leads to the dissociation of the complex NF-κB and its cytoplasmic inhibitor IκB (2, 3). We used this technique to look for the presence of a cytoplasmic precursor of NF-κB in *Theileria*-infected T cells. Treatment of cytoplasmic extracts from *Theileria*-infected cells with these detergents resulted in a large increase in the observable binding of protein to the κB oligonucleotide (Fig. 4; compare bands b0 and b1 in lanes 4 and 5 and in lanes 6 and 7). It is interesting that ConA-stimulated bovine lymphocytes contained a large amount of NF-κB-related proteins in the cytosol that also produced two DNA-protein bands, b0 and b1, in EMSA. Treatment of cytosolic extracts from these cells with detergents slightly increased their binding to the κB oligonucleotide (Fig. 4; compare lanes 1 and 2). DOC and NP-40 together had no

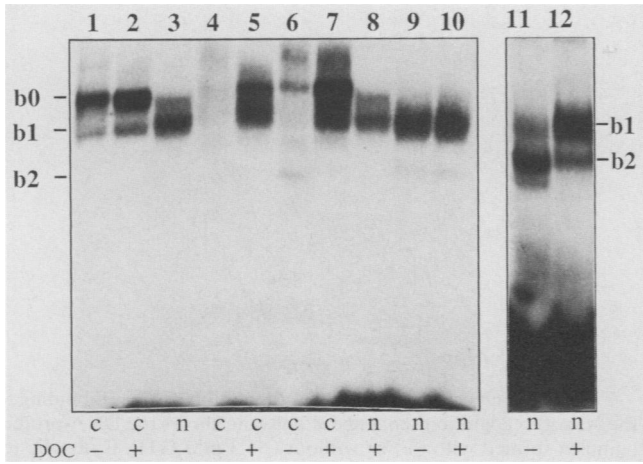

FIG. 4. Effects of dissociating agents on the binding of NF-κB from cytosolic (c) and nuclear (n) fractions of bovine T lymphocytes infected by *T. parva* or stimulated by ConA. Cell-free activation of an NF-κB precursor was prepared by treatment with (+) or without (−) 0.6% DOC in the presence of 1.2% NP-40. The DNA-binding reactions were performed in the presence of 2 μg of poly(dI-dC) and 2.5 μg of protein from cytosol or nuclear extracts. The subcellular fractions used for EMSA were the cytosolic fraction of ConA-stimulated lymphocytes (lanes 1 and 2), the cytosolic fraction of *Theileria*-infected lymphocytes prepared 3 days after passage (lanes 4 and 5), the same fractions prepared 5 days after passage (lanes 6 and 7), nuclear extracts from ConA-stimulated lymphocytes (lanes 3 and 8), nuclear extracts prepared on day 1 after passage from *Theileria*-infected lymphocytes (lanes 9 and 10), and the same extracts prepared on day 1 after passage (lanes 11 and 12). b0, b1, and b2 are the three main retardation bands.

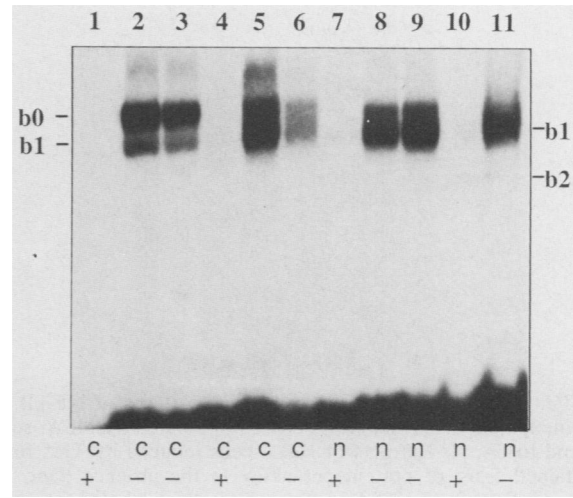

FIG. 5. Specificity of NF-κB binding for cytoplasmic and nuclear extracts from *Theileria*-infected bovine lymphocytes and extracts from ConA-stimulated lymphocytes. Subcellular fractions were analyzed by EMSA. Each assay consisted of 2.5 μg of protein from nuclear (n) or cytoplasmic (c) extracts (except for lane 6; see below) incubated with the radiolabeled κB oligonucleotide preincubated in the presence (+) or absence (−) of a 200-fold molar excess of unlabeled κB oligonucleotide. Lanes: 1 to 3, cytoplasmic extracts from ConA-stimulated lymphocytes; 4 to 6, cytoplasmic extracts prepared on day 3 after seeding from *Theileria*-infected lymphocytes; 6, same as lane 5 but with 0.8 instead of 2.5 μg of protein; 7 to 9, nuclear extracts from ConA-stimulated lymphocytes; 10 and 11, nuclear extracts prepared from *Theileria*-infected cells on day 1 after seeding.

additional effect on binding to the DNA probes of nuclear proteins from extracts of ConA-stimulated lymphocytes or from extracts of *Theileria*-infected lymphocytes that were prepared on day 1 after passage. These nuclear extracts produced a major common band, b1, and different minor bands, b0 in ConA-stimulated lymphocytes and b2 in *Theileria*-infected cells (Fig. 4; compare lanes 3 and 8 and lanes 9 and 10).

The NF-κB-like factor was fully activated in nuclei of these cells, since the intensity of band b1 did not increase after DOC treatment. Unlike the case with ConA-stimulated lymphocytes, activation by DOC of nuclear extracts prepared 3 days after seeding of *Theileria*-infected cells in vitro had a noticeable effect on the pattern of NF-κB binding to its recognition sequence (Fig. 4, lanes 11 and 12). The intensity of the minor band, b1, was consistently increased by DOC. Band b1 was also produced by nuclear extracts isolated on day 1 after seeding, although no effect by DOC was observed. This finding suggests that the appearance of a DOC-sensitive band in the nuclei prepared on day 3 was probably not due to systematic contamination from a DOC-sensitive complex in the cytoplasm. The intensity of the major band, b2, was variable after DOC treatment in different EMSA experiments, possibly because of the sensitivity to small differences in DOC concentration during treatment of the extracts. Thus, there was an appreciable amount of inactivated NF-κB present in nuclear extracts from *Theileria*-infected cells beginning from day 3 after passage that could be reactivated by detergent treatment.

Figure 5 shows the results of EMSA experiments measuring the specificity of protein complexes that bound the NF-κB oligonucleotide motif. All of the extracts tested contained proteins that bound to NF-κB sequences. Cytoplasmic extracts from ConA-stimulated cells (Fig. 5, lane 1 compared with lanes 2 and 3), nuclear extracts from the same cells (lane 7 compared with lanes 8 and 9), and extracts from *Theileria*-infected lymphocytes (lane 4 compared with lanes 5 and 6 as well as lane 10 compared with lane 11) produced bands in EMSA that could be abolished by excess cold κB oligonucleotides. Formation of the bound complexes could not be prevented by addition of excess nonradioactive mutated oligonucleotide (data not shown).

An NF-κB-related factor has been shown to be induced in HeLa cells after treatment with TPA (41). We compared the binding properties of extracts from HeLa cells and bovine lymphocytes. Extracts of TPA-treated HeLa cells produced a DNA-protein complex, b0, that was similar to the main band from ConA-stimulated lymphocytes (Fig. 6, lanes 1 and 3). On the other hand, a more complex set of band retardations was produced with extracts of *Theileria*-infected T lymphocytes. This observation suggests the existence of different forms of NF-κB. There was a difference in the EMSA band pattern of nuclear extracts from *Theileria*-infected cells that depended on the age of the culture after seeding. The difference may reflect a change in the rate of culture growth. One band, b1, was characteristic of nuclear extracts prepared on day 1 after passage, whereas two bands, b1 and b2, were observed in extracts prepared 3 days after passage (Fig. 6, lanes 4 and 5). Hence, the EMSA experiments with extracts from *Theileria*-infected lymphocytes revealed three main DNA-protein complexes that resulted from binding of the NF-κB-like protein to its recognition site. Bands b0 through b3 from *Theileria*-infected cell extracts have been shown to comigrate in a consistent relationship with each other, although the total pattern of the

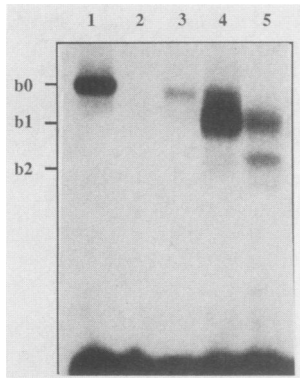

FIG. 6. Comparison of the band shift patterns of NF-κB from bovine lymphocytes and HeLa cells. For details of EMSA, see the legend to Fig. 4. Extracts of HeLa cells induced by TPA for 3 h contained 5 μg of protein per assay in the absence (lane 1) or presence (lane 2) of a 200-fold molar excess of unlabeled competitor. Cytosolic extracts of ConA-stimulated lymphocytes (lane 3) and nuclear extracts prepared 1 and 3 days after passage of *Theileria*-infected lymphocytes (lanes 4 and 5) contained 2.5 μg of protein per assay. b0, b1, and b2 are the main retardation bands.

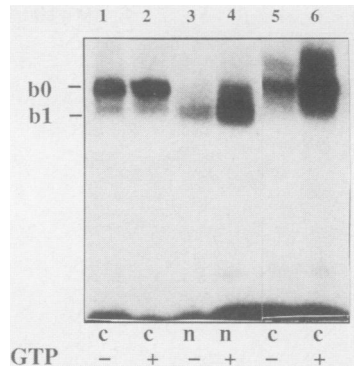

FIG. 7. Nucleoside triphosphate stimulation of NF-κB binding to the NF-κB recognition sequence. Each lane shows the DNA-protein complex formed with (+) or without (−) 3 mM GTP. Cytosolic (c) and nuclear (n) extracts treated in vitro with 0.6% DOC contained 0.8 μg of protein per reaction. Lanes: 1 and 2, cytosolic extracts from ConA-stimulated lymphocytes; 3 and 4, nuclear extracts prepared on day 1 after passage from *Theileria*-infected lymphocytes; 5 and 6, cytosolic extracts prepared on day 5 after passage from *Theileria*-infected lymphocytes. b0 and b1 are the main retardation bands.

bands varied under different experimental conditions. We could not distinguish in these experiments whether the different bands resulted from different proteins binding to the oligonucleotide or whether one protein produced multiple bands due to different states of modification.

The data presented above support the proposal that *Theileria*-infected cells contain substantial amounts of NF-κB-like proteins in their nuclei and precursors of these proteins in their cytoplasm. The data also suggest that some NF-κB can be bound in a DOC-sensitive complex in the nuclei. The cytoplasmic components b0 and b1 became more evident after treatment with detergents that dissociated the precursor-inhibitor complex. However, a small amount of band b0 could be seen without detergent treatment, especially in *Theileria*-infected cells left to grow for 5 days without passage (Fig. 4, lanes 6 and 7). The release of NF-κB from its inhibitor appeared to operate at the maximal rate, since no further increase in NF-κB binding of nuclear extracts from TPA-treated cells could be detected. The phorbol ester-induced signal transduction pathway to other transcription factors, however, still functioned, which suggests that *T. parva* does not utilize the TPA pathway observed in other cells (28). Alternatively, the existence of a large pool of the cytoplasmic precursor of NF-κB suggests that the limiting step in the activation of NF-κB may be the association of NF-κB with other factors in the nucleus or possibly an additional modification of NF-κB in the nucleus. It is possible that these processes are controlled by *T. parva*. It is also clear from these data that there is an excess of NF-κB synthesis in parasite-infected lymphocytes, resulting in an abundance of the cytoplasmic precursor. The excess synthesis and abundance of NF-κB in infected lymphocytes is in sharp contrast to levels found in pre-B or non-B cells.

GTP-stimulated binding of NF-κB from *Theileria*-infected cells to the κB motif of the HIV enhancer. GTP has been shown to substantially stimulate the binding of purified NF-κB to its recognition sequence. This property has been used to distinguish purified NF-κB from a closely related nuclear factor, H2-TF1, whose binding is not influenced by GTP even though the factor binds a DNA sequence motif very similar to κB (5, 27). We tested the influence of GTP on extracts from lymphocytes to determine whether *Theileria*-infected lymphocytes express an NF-κB with properties similar to those of purified NF-κB. Treatment of nuclear and cytoplasmic fractions with *Theileria*-infected lymphocytes with 3 mM GTP strongly increased the DNA-binding properties of the cellular extracts for bands b0 and b1 (Fig. 7, lanes 3 to 6). However, GTP treatment was not able to change the DNA-binding properties of extracts from ConA-stimulated lymphocytes (lanes 1 and 2). These data support the idea that the NF-κB-like proteins observed in *Theileria*-infected cells (bands b0 and b1) are similar to purified NF-κB. The presence of protein complexes not influenced by GTP may suggest the existence of NF-κB proteins or related proteins with different state of modification.

Changes in NF-κB-binding activity after elimination of the parasite. Having established that *Theileria*-infected lymphocytes constitutively express and activate NF-κB, we attempted to determine for what length of time the stimulatory effect of the parasite remained after removal of the parasite by drug treatment. By treating the *Theileria*-infected cells with the drug BW 720c, which selectively kills the parasite, leaving the host cell intact, we could determine the fate of activated NF-κB. The effect of BW 720c on the growth of *Theileria*-infected cells has been described earlier (19). Cell growth and rates of [^{3}H]thymidine incorporation during the first 4 days of drug treatment remained approximately the same in both drug-treated and untreated cell cultures. After passage, however, the drug-treated cultures stopped growing. Only in the presence of added IL-2 will the drug-treated cells grow for an additional 4 to 7 days (19).

Infected lymphocytes were treated with the drug at various times, and nuclear extracts were prepared for testing in EMSA. We used DOC treatment of nuclear extracts to estimate the total amount of NF-κB (activated as well as released from the inhibitor complex). The amount of NF-κB in *Theileria*-infected lymphocytes varied with time after passage (Fig. 8A, lanes 1 to 3). Levels of the factor remained maximal for actively growing cells 1 day after seeding and dropped three- to fourfold by days 3 and 5. On day 5, cells were close to stationary growth phase (Fig. 8A, lanes 1 to 3). Upon treatment with the drug BW 720c, a marked loss over time of NF-κB binding in nuclear extracts were observed

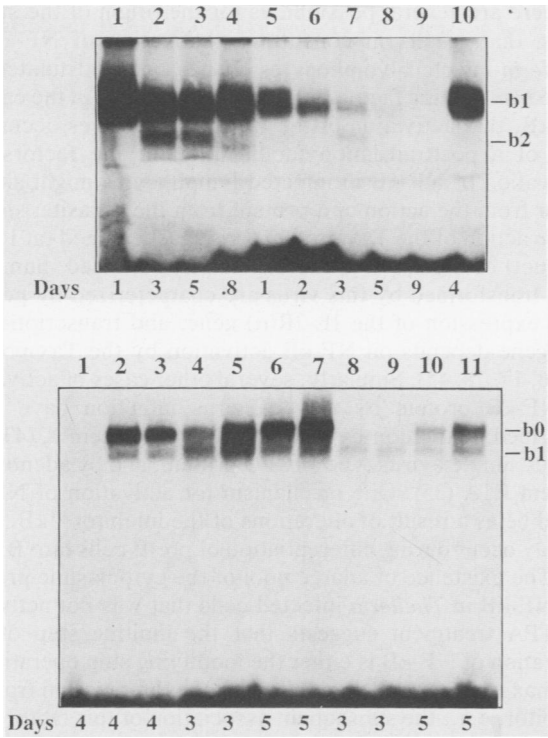

FIG. 8. (A) Influence of the theilericidal drug BW 720c and changes in NF-κB binding from nuclear extracts isolated at different time points during the growth of *Theileria*-infected cell cultures. Nuclear extracts from *Theileria*-infected T cells (lanes 1 to 9) and from ConA-stimulated lymphocytes (lane 10) were used for EMSA with the radiolabeled κB oligonucleotide. Cells were initially seeded at 1.25×10^5/ml (day 0) and grown in cL15 containing no additives (lanes 1 to 3) or BW 720c (lanes 4 to 9) for the indicated times. Cultures were passed on day 4. Cell density on day 4 was approximately 1.5×10^6/ml; on day 5, the density of the nonpassaged culture was 1.7×10^6 cells per ml. Cells treated with BW 720c and recombinant IL-2 continued to divide after passage for 7 to 9 days and were harvested at that time (lane 9). (B) Changes in the level of the NF-κB precursor in the cytosolic fraction after drug treatment. Bovine lymphocytes stimulated by ConA were used as positive controls in absence (lanes 2 and 3) or presence (lane 1) of the competitor, the unlabeled κB oligonucleotide. *Theileria*-infected cells were grown with (lanes 8 to 11) and without (lanes 4 to 7) the drug BW 720c. Extracts were prepared at the indicated times after passage. Two protein concentrations were used in the DNA-binding reactions: 2.5 (lanes 1 to 3, 5, 7, 9, and 11) and 1.2 (lanes 4, 6, 8, and 10) μg per reaction mixture.

(Fig. 8A, lanes 4 to 9). A decrease of NF-κB binding was evident at 18 h after drug addition (lane 4); binding was substantially reduced after 3 days of treatment (compare lanes 7 and 2) and was barely detectable thereafter (lane 8). Cells treated with the drug failed to grow further after passage on day 4 and had nearly undetectable levels of NF-κB at that time. Cells allowed to continue dividing in the presence of IL-2 for 7 to 9 days also lost detectable levels of NF-κB (lane 9). The loss of NF-κB closely paralleled the previously documented loss of the parasite (19) as well as the loss of parasite-specific transcripts (data not shown). It also paralleled the reduction in proliferation. Loss of NF-κB from the cytoplasmic fractions after drug treatment can be seen in Fig. 8B (lanes 4 to 11). Loss from the cytoplasm occurred to a lesser extent than from nucleus. The losses were maximal for extracts isolated from cells 3 days after drug treatment

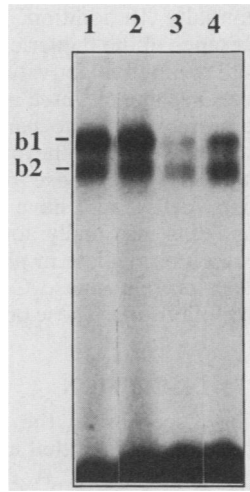

FIG. 9. Influence of TPA on levels of NF-κB in extracts from infected and cured lymphocytes. *Theileria*-infected T-cell cultures were seeded at 10^5 cells per ml and grown for 3 days before preparation of nuclear extracts (lane 1). At 12 h before harvest, a portion of the culture was treated with 50 nM TPA (lane 2). For removal of the parasite, cultures were seeded at 5×10^4 cells per ml and grown in the presence of BW 720c for 5 days before preparation of nuclear extracts (lane 3). At 12 h before harvest, a portion of the drug-treated culture was treated with 50 nM TPA (lane 4). EMSA gel analysis was performed as for Fig. 8, using 4 μg of protein in the binding reactions.

(lanes 8 and 9) but were less for extracts isolated at 5 days (lanes 10 and 11). The observed increase of NF-κB precursor binding from extracts isolated 5 days after drug treatment over the level observed for extracts isolated after only 3 days of treatment could possibly be explained if the synthesis of NF-κB were maintained for a longer period than was the parasite-stimulated activation of NF-κB from its cytoplasmic precursor.

As a comparative control for the loss of NF-κB from treated lymphocytes, extracts from infected and drug-treated lymphocytes were tested in EMSA for proteins that bind the TATTTA box motif. We observed no change in the amounts of TATTTA motif-binding proteins either as a result of different culture growth conditions or as a result of changes after drug treatment (data not shown). It appears, therefore, that killing of the parasite by the drug BW 720c specifically decreased NF-κB binding in nuclear fractions and at the same time decreased the size of the cytoplasmic precursor pool. We conclude that the presence of *T. parva* not only activates NF-κB but also increases the synthesis of NF-κB such that an excess of precursor is maintained in the cytoplasm.

Elimination of the parasite restores NF-κB responsiveness to TPA. To extend our observation in parasitized cells that CAT activity under the control of NF-κB promoters in the HIV-1 LTR does not respond to stimulation by TPA, and having shown that the levels of NF-κB drop rapidly after removal of the parasite by drug treatment, we decided to test the TPA responsiveness of NF-κB in cured cells. Figure 9 represents an EMSA test showing the results of treating T-cell cultures with TPA in the presence or absence of the parasite. The results shown in lanes 1 and 2 support our conclusion from the results in Fig. 2 that TPA had no observable effect on the levels of NF-κB in infected cells. The result in lane 3 demonstrates once again that upon loss of the parasite by drug treatment, the levels of NF-κB

dropped very substantially. In addition, TPA treatment of cells grown in the presence of the theilericidal drug BW 720c to remove the parasite resulted in activation of NF-κB (lane 4). Thus, NF-κB levels became elevated in response to TPA treatment only after removal of the parasite. This result demonstrates that the presence of the parasite directly or indirectly prevents the NF-κB response to TPA. The lack of response in parasitized cells could have resulted from the fact that NF-κB was being maximally activated or that the parasite fed into a step intermediate in phorbol ester transduction, e.g., between protein kinase C and activation of cytoplasmic precursor of NF-κB. These possibilities are now being investigated.

DISCUSSION

Infection of bovine T cells with the parasite *T. parva* produces immortalized cell cultures that maintain characteristics of transformed cancer cells. A detailed molecular analysis of the host-parasite relationship should allow the identification of important control processes used by the host and possibly modified or interrupted by the parasite that result in uncontrolled cell proliferation. We show here that one of the effects resulting from the presence of the parasite either directly or indirectly is the constitutive activation of the *trans*-acting factor NF-κB. We show that the factor is localized unevenly in both nuclear and cytoplasmic fractions. The nuclear form is activated, whereas the cytoplasmic form is bound to an inhibitor. The NF-κB-like proteins in infected lymphocytes differ from those in ConA-stimulated lymphocytes and HeLa cells because the GTP-stimulated binding of the protein to the NF-κB motif is found only in parasitized cells, similar to the binding reported for purified NF-κB (27). We cannot distinguish in these experiments whether ConA-stimulated lymphocytes and *Theileria*-infected lymphocytes have different NF-κB-like proteins or the same proteins in different states of modification. We show that only in infected cells can the cytoplasmic precursor-inhibitor complex be activated by DOC in vitro. This treatment has no significant effect on the factor in cytosol of ConA-stimulated lymphocytes.

Although treatment with TPA and ConA does not result in increased binding of NF-κB to the κB motif of the HIV-1 LTR and does not change κB-dependent transcription, TPA does have some stimulating effect on CAT expression under the control of 5′-flanking regions from the mouse IL-2 and human IL-2R genes. It appears that the mild stimulatory effect on the IL-2R(α) regulatory region is not related to NF-κB binding, because plasmid pIL-2R*(α)CAT, containing a mutated κB sequence, is stimulated to a greater degree. A more analytical analysis of this observation is required to confirm and understand the factors that may activate transcription. It is likely that in the IL-2 gene regulatory region (−293 to −7), TPA-activated binding of the nuclear factor AP-1 plays a role in IL-2 enhancer function (42). *Theileria*-infected lymphocytes express constitutively high levels of activated NF-κB in the nucleus. The high levels of inhibitor-bound precursor in the cytoplasm must result from increased synthesis of the factor in these parasitized cells. TPA treatment does not alter the NF-κB levels in these cells and does not increase κB-enhanced transcription, unlike the case with other T-cell lines in which NF-κB activation was found to be dependent on TPA treatment (28, 47). Stimulation of NF-κB by TPA treatment does, however, reappear upon removal of the parasite with drugs. This finding suggests that the signal transduction pathway is not altered permanently by the parasite.

There are several possibilities for the origin of the stimulation that results in constitutive activation of NF-κB in *Theileria*-infected lymphocytes. It has been postulated for some *trans*-acting factors, and is most probable in the case of NF-κB, that activation of the factors themselves occurs by way of a posttranslation modification of the factors (2). Activation of NF-κB in infected lymphocytes might simply occur from the action of a protein from the parasite, similar to the action of the Tax protein (previously named tat I gene product) of human T-cell leukemia virus. Indeed, human T cells transformed by this virus are characterized by permanent expression of the IL-2R(α) gene, and transcription of this gene depends on NF-κB activation by the Tax protein (6, 16, 17, 28, 45). Similarly, several other cases of activation of NF-κB protein by way of virus infection have been described: activation by hepatitis B virus protein X (43), by herpes simplex virus type 1 ICPO protein, and by adenovirus protein E1A (35). One mechanism for activation of NF-κB could be as a result of alterations of the inhibitor, IκB, such as may occur during differentiation of pre-B cells into B cells (3). The existence of a large pool of the cytoplasmic precursor NF-κB in *Theileria*-infected cells that was not activated by TPA treatment suggests that the limiting step of the activation of NF-κB is either the modifying step operating at the maximal rate or a step following the dissociation from its inhibitor, e.g., the subsequent association of this factor with other nuclear factors or possibly additional modifications of NF-κB in the nucleus. Once the parasite is killed, the level of active NF-κB drops dramatically and the inducibility of TPA reappears. This finding shows that the signal transduction pathway has not been altered permanently by the parasite. An important precedent for associations with multiple nuclear factors exists in the interaction of AP-1, the c-*jun* gene product, with the c-Fos protein, which results in the generation of an active AP-1-site binding complex (13, 38, 39). Preliminary evidence for the association of NF-κB with the Fos protein has been obtained (B. Stein and V. Ivanov, unpublished observations).

NF-κB is a *trans*-acting protein active in several eucaryotic enhancer regions containing the κB motif, such as the immunoglobulin kappa-chain gene enhancer (40), the IL-2R(α) gene promoter region (28), and possibly the IL-2 gene promoter region, which also contains motifs for other *trans*-acting factors (27, 42). Activation of the cytoplasmic precursor of NF-κB and subsequent binding of the protein to the κB motif in the regulatory regions are the necessary stages for expression of these genes. The constitutive expression of the IL-2R(α) gene in *Theileria*-infected lymphocytes (14, 19) could result from such an activation by NF-κB.

A question that remains unresolved is the influence of the parasite that results in increased NF-κB precursor synthesis. We have clearly observed a decrease in the size of the cytoplasmic NF-κB precursor pool as a result of parasite elimination by treatment with the drug BW 720c. This finding could be indirect evidence of the influence of the parasite on the synthesis of the NF-κB precursor or possibly parasite-mediated changes in its stability. Direct data on the influence of the parasite on NF-κB gene transcription can be measured only after cloning and sequencing of the NF-κB gene. A reasonable speculation would be that activated NF-κB regulates its own expression, as has been shown for AP-1 transcription factor (1).

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