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Coexpression of NF-κB/Rel and Sp1 transcription factors in human immunodeficiency virus 1-induced, dendritic cell–T-cell syncytia

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ABSTRACT **Productive infection of T cells with human immunodeficiency virus 1 (HIV-1) typically requires that the T cells be stimulated with antigens or mitogens. This requirement has been attributed to the activation of the transcription factor NF-κB, which synergizes with the constitutive transcription factor Sp1 to drive the HIV-1 promoter. Recently, we have found that vigorous replication of HIV-1 takes place in nonactivated memory T cells after syncytium formation with dendritic cells (DCs). These syncytia lack activated cells as determined by an absence of staining for Ki-67 cell cycle antigen. The expression and activity of NF-κB and Sp1 were, therefore, analyzed in isolated T cells and DCs from humans and mice. We have used immunolabeling, Western blot analysis, and electrophoretic mobility shift and supershift assays. T cells lack active NF-κB but express Sp1 as expected. DCs express high levels of all known NF-κB and Rel proteins, with activity residing primarily within RelB, p50, and p65. However, DCs lack Sp1, which may explain the failure of HIV-1 to replicate in purified DCs. Coexpression of NF-κB and Sp1 occurs in the heterologous DC–T-cell syncytia that are induced by HIV-1. Therefore, HIV-1-induced cell fusion brings together factors that upregulate virus transcription. Since DCs and memory T cells frequently traffic together *in situ*, these unusual heterologous syncytia could develop in infected individuals and lead to chronic HIV-1 replication without ostensible immune stimulation.**

Human immunodeficiency virus 1 (HIV-1) replicates in cultured T cells that have been stimulated by antigens or mitogens (1–4). Stimulation activates NF-κB or Rel transcription factors (5), which can then drive corresponding NF-κB sites in the HIV-1 promoter (6). The HIV-1 promoter also contains essential Sp1 sites (6, 7), but Sp1 factors are expressed constitutively in the nuclei of most cells (8). However, antigenic activation of T cells may not be essential for HIV-1 replication in infected individuals. For example, these individuals lose T-cell memory for antigens to which they are no longer being exposed (9, 10). Recent data on the substantial rate of HIV-1 replication *in vivo* also suggests some stable chronic stimulus for virus replication (11, 12). The need for T-cell activation for HIV-1 replication became of interest to us when we identified a system in which mixtures of dendritic cells (DCs) and small-sized T cells, carrying a memory phenotype, supported vigorous infections with HIV-1 (13). The T lymphocytes did not show critical features of cell activation since there was an absence of the Ki-67 cell cycle antigen, surface interleukin 2 receptors, and HLA-DR antigens (13, 14). When these mixtures of DCs and T cells were exposed to several different isolates of HIV-1, the newly synthesized HIV-1 gag proteins and virions were confined to heterologous syncytia (DCs + T cells) that were induced by the virus (13). These permissive syncytia were formed with all seven HIV-1 isolates that we tested, including those that were classified as nonsyncytium inducing in standard test cell lines. We show herein that the formation of these DC–T-cell syncytia brings together NF-κB or Rel proteins via the DC with Sp1 via the nonactivated T cell. The capacity of HIV-1 to induce heterologous syncytia may lead to chronic unremitting virus replication in the absence of standard stimuli.

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MATERIALS AND METHODS

Antibodies. Antibodies to the NF-κB/Rel family and to Sp1 were purchased from Santa Cruz Biotechnology; anti-Ki-67 (MIB-1) was from AMAC (Westbrook, ME); anti-HIV-1 p24 (15) was provided by The AIDS Research and Reagent Program.

Cell Preparations. Mononuclear cells were isolated by flotation on Ficoll/Hypaque and separated into cell subsets as described (16). Mixed leukocyte reactions were carried out with 1×10^5 responding T cells and 3×10^3 allogeneic DCs. Skin cell emigrants were prepared as described (14). Mouse cells (DCs, T cells, B cells, and thioglycollate-elicited macrophages) were prepared by standard methods. Bone marrow DCs were grown with granulocyte–macrophage colony-stimulating factor from progenitors as described (17).

Infection of Cell Suspensions. Mixtures of allogeneic blood DCs and T cells (mixed leukocyte reaction) or 1×10^5 viable skin cell emigrants were added to wells of a 96-well round-bottom well plate. Live HIV-1_{IIIB} was added for 1.5 h at 37°C at a multiplicity of infection of 0.05–0.1 (13). The cells were then washed and cultured for 5–6 days.

Immunolabeling of Cells. For immunocytochemistry, the slides were incubated for 1 h with the primary antibody (0.3 μg/ml), and bound antibody was detected by horseradish peroxidase-conjugated secondary antibody and diaminobenzidine. For immunofluorescence, cells were incubated with the primary antibody (0.3 μg/ml) followed by fluorescein isothiocyanate-coupled anti-mouse or Texas red-coupled anti-rabbit immunoglobulin.

Western Blot Analysis. Western blot analysis was carried out by using 10 μg of cell lysates. Primary antibody (0.1 μg/ml) was added in 5% (vol/vol) milk. Proteins were visualized with ECL.

DNA–Protein Binding [Electrophoretic Mobility Shift Assay (EMSA)] Assay. The EMSA was performed as described (18). The sequences of the oligonucleotides were as follows: SP1, 5′-ATTCGATCGGGGCGGGGCGAGC-3′; SP1 mutant, 5′-ATTCGATCGGTTCGGGGCGAGC-3′; NF-κB, 5′-AGTTGAGGGGACTTTCCCAGGC-3′; NF-κB mutant, 5′-AGTTGAGGCGACTTTCCCAGG-3′ (mutations are underlined). Nuclear and cytosolic fractions were prepared by a

Abbreviations: HIV, human immunodeficiency virus; EMSA, electrophoretic mobility shift assay; DC, dendritic cell.

standard protocol (18, 19). From 3 to 10 μg of proteins was used for each reaction.

***In Vitro* Transcription.** The template and the conditions of the assay were as described (20). The template carries the HIV-1 promoter sequences from positions −109 to −8 followed by the adenoviruses major late initiator element from positions −7 to +9 and a 379-bp G-free cassette. Nuclear extracts (25–75 μg) were used in these reactions. Recombinant Sp1 was from Promega.

RESULTS

HIV-1 Replicates in Mixtures of DCs and T Cells in the Absence of Cell Proliferation. It was first essential to extend the evidence that T-cell proliferation is not required for HIV-1 replication in mixtures of DCs and ostensible nonactivated T cells from skin. Skin was chosen since in many respects it resembles the lining of the organs involved in sexual transmission of HIV-1, and both DCs and T cells chronically traffic in cutaneous afferent lymphatics (21). HIV-1 was added to skin cell emigrants or antigen-stimulated blood cells. Both cultures became infected, as monitored by the release of p24 antigen and reverse transcriptase into the culture medium (13, 22), but it was not necessary to add a stimulus to the skin cultures (13). HIV-1-infected syncytia from both blood and skin expressed p24 antigen (Fig. 1 *A* and *C*). However, the nuclei of the skin syncytia lacked the Ki-67 antigen that is expressed in the nuclei of cycling cells (23), whereas the nuclei of the blood cell syncytia were all Ki-67-positive (compare Fig. 1 *B* with *D*). In two experiments, 100% of the nuclei (85 counted) in skin syncytia lacked Ki-67, whereas 94% of the nuclei (57 counted) in blood-cell syncytia expressed Ki-67. Therefore, standard T-cell activating stimuli, which are required for HIV-1 replication in isolated blood cells (1, 2, 4, 22), are unlikely to be needed for replication in these mixtures of DCs and memory T cells.

Expression of NF-κB and Sp1 Proteins in Leukocytes. Given the essential role for NF-κB (Rel family) and Sp1 sites in the regulation of the HIV-1 promoter, we set out to study the expression of Rel and Sp1 factors in different subsets of

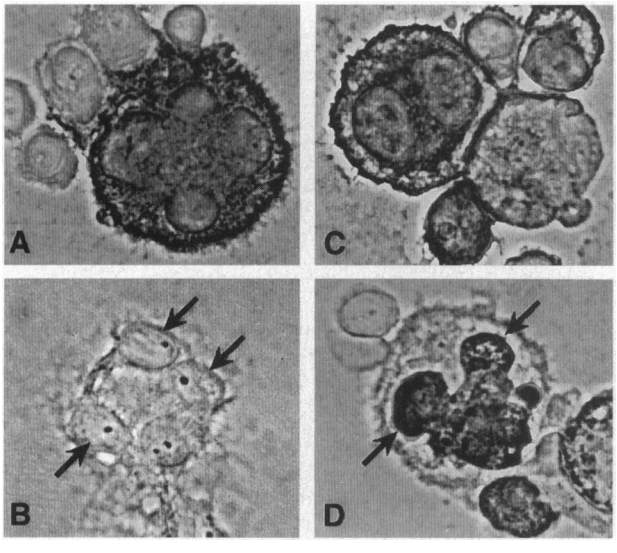

FIG. 1. HIV-1-infected syncytia contain cycling cells from blood and noncycling cells from skin. Mixtures of allogeneic blood DCs and T cells (mixed leukocyte reactions) or syngeneic DCs and T cells from skin were infected with HIV-1_{IIIB}. Cytocentrifuged cells were prepared after 5 days of infection and immunoperoxidase-stained for p24 and Ki-67. Skin syncytia were stained for HIV p24 antigen (*A*) or Ki-67 cell cycle antigen (*B*). Blood mixed leukocyte reaction syncytia were stained for HIV p24 (*C*) or Ki-67 (*D*). Arrows indicate nuclei.

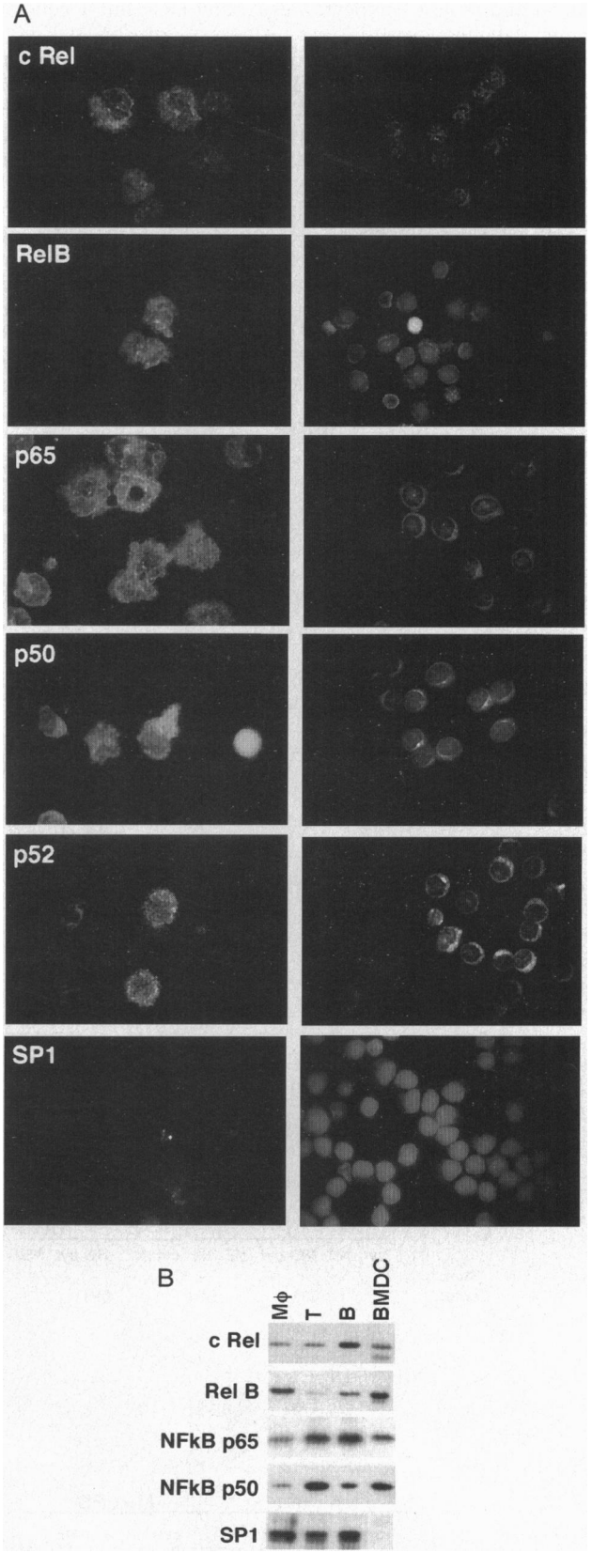

FIG. 2. Expression of NF-κB/Rel proteins and Sp1 in T cells and DCs. (*A*) Immunofluorescence. Cytocentrifuged DCs (*Left*) and T cells (*Right*) were immunolabeled with antibodies to Rel proteins or Sp1. (*B*) Immunoblot analysis. Total cell lysates (10 μg) from subsets of mouse leukocytes, peritoneal macrophages (Mφ), spleen (T cells and B cells), and bone marrow DCs (BMDC) were probed with antibodies to the indicated Rel family and Sp1 proteins. Bound antibodies were visualized with peroxidase anti-immunoglobulin, followed by ECL.

human and mouse leukocytes, especially DCs and T cells by immunolabeling.

All Rel family proteins were readily identified in the cytoplasm and nuclei of HLA-DR-positive DCs from human blood, but Sp1 was not detectable (Fig. 2*A Left*). Previously only *Drosophila* cells were known to lack Sp1 (24). Contrasting findings were made in T cells from human blood. Cells stained weakly and primarily in the cytoplasm with antibodies to the Rel family members, but Sp1 was abundant in all T-cell nuclei (Fig. 2*A Right*).

Both cytofluorography and immunocytochemistry for Rel and Sp1 gave the same results on mouse spleen DCs and T cells, as presented above for human blood, i.e., high levels of Rel proteins and low levels of Sp1 in DCs and vice versa in T cells. We used immunoblot analysis to detect these proteins in different cell subsets from mice (Fig. 2*B*) and human blood (data not shown). Purified DCs that were generated from proliferating bone marrow progenitors (17) contained all Rel proteins but lacked Sp1. T and B cells from spleen and macrophages elicited into the peritoneal cavity had abundant Sp1 (Fig. 2*B*).

Analysis of NF-κB/Rel and Sp1 Binding Activities in Blood Cells. The activity of these transcriptional control proteins was then assessed with a consensus NF-κB oligonucleotide and EMSA applied to several subsets of leukocytes. Data are shown for human blood leukocytes, but comparable findings were made with mouse cells (data not shown). The NF-κB activity was primarily localized to the nucleus vs. cytoplasm in every cell type. In each case, the activity was ablated by a wild-type competing oligonucleotide but not by a mutated sequence (Fig. 3*A*). NF-κB activity was greatly enhanced by stimulation of T cells (phytohemagglutinin and phorbol 12-myristate 13-acetate) and monocytes (lipopolysaccharide), but B cells and DCs had readily detectable NF-κB activity in the

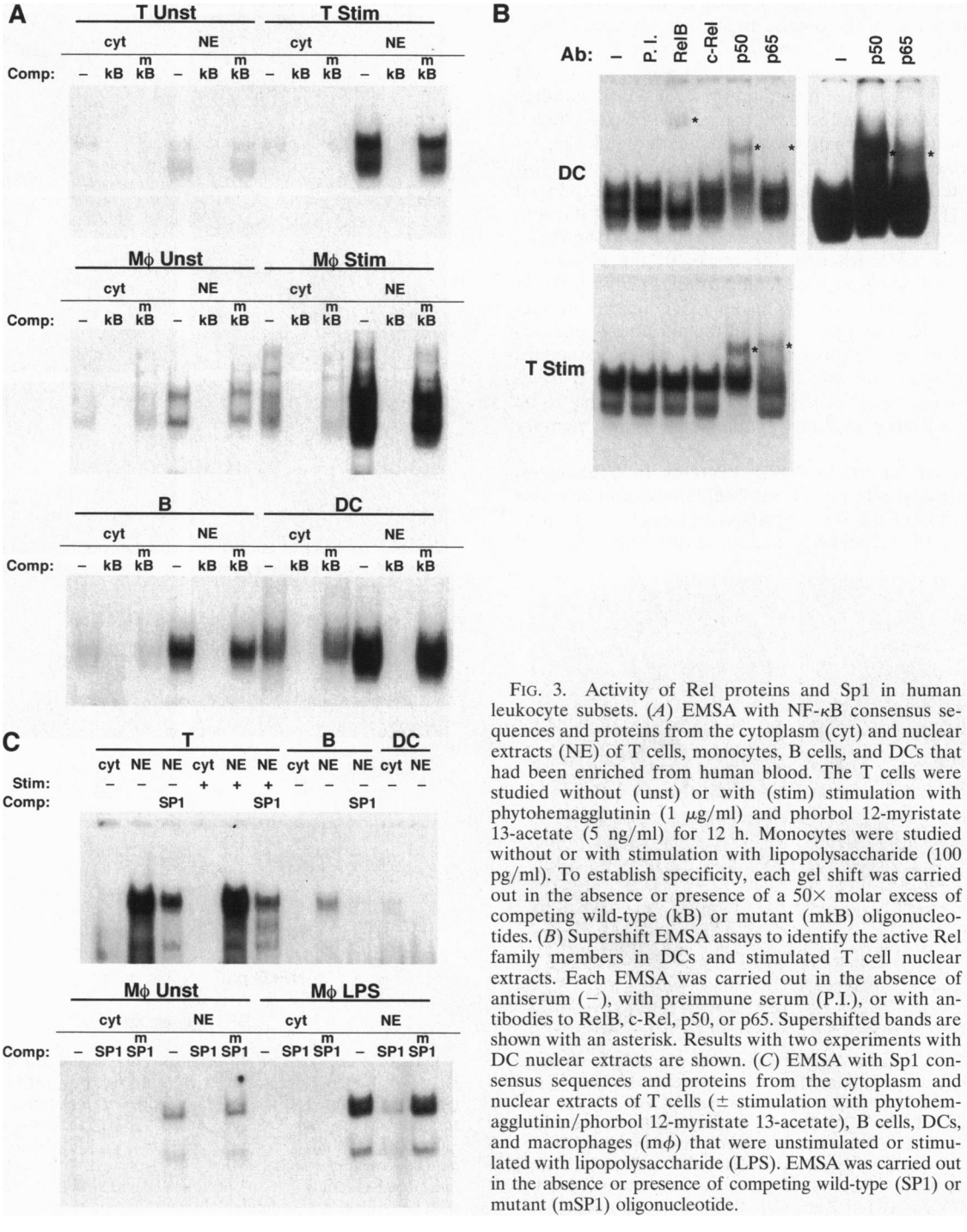

FIG. 3. Activity of Rel proteins and Sp1 in human leukocyte subsets. (*A*) EMSA with NF-κB consensus sequences and proteins from the cytoplasm (cyt) and nuclear extracts (NE) of T cells, monocytes, B cells, and DCs that had been enriched from human blood. The T cells were studied without (unst) or with (stim) stimulation with phytohemagglutinin (1 μg/ml) and phorbol 12-myristate 13-acetate (5 ng/ml) for 12 h. Monocytes were studied without or with stimulation with lipopolysaccharide (100 pg/ml). To establish specificity, each gel shift was carried out in the absence or presence of a 50× molar excess of competing wild-type (kB) or mutant (mkB) oligonucleotides. (*B*) Supershift EMSA assays to identify the active Rel family members in DCs and stimulated T cell nuclear extracts. Each EMSA was carried out in the absence of antiserum (–), with preimmune serum (P.I.), or with antibodies to RelB, c-Rel, p50, or p65. Supershifted bands are shown with an asterisk. Results with two experiments with DC nuclear extracts are shown. (*C*) EMSA with Sp1 consensus sequences and proteins from the cytoplasm and nuclear extracts of T cells (± stimulation with phytohemagglutinin/phorbol 12-myristate 13-acetate), B cells, DCs, and macrophages (mϕ) that were unstimulated or stimulated with lipopolysaccharide (LPS). EMSA was carried out in the absence or presence of competing wild-type (SP1) or mutant (mSP1) oligonucleotide.

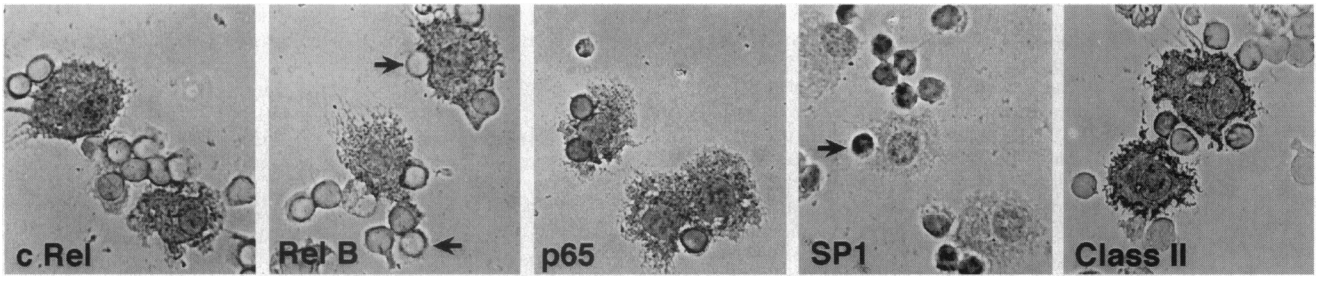

FIG. 4. Expression of Rel and Sp1 proteins in mixtures of DCs and T cells from skin. Explants of skin were cultured for 4 days and emigrate cells were cytocentrifuged and stained with immunoperoxidase for c-Rel, RelB, p65, Sp1, and major histocompatibility complex class II. The large DCs are readily distinguished from the small T cells, some of which are indicated with arrows.

absence of experimental stimulation (Fig. 3*A*). The NF-κB binding activity detected in DCs was not inhibited when extracts of unstimulated T cells were added (data not shown). This indicates that the DC NF-κB binding activity is dominant over that of the T cell.

Supershift assays were carried out to identify the active NF-κB factors in human blood DCs and stimulated T cells (Fig. 3*B*). In standard NF-κB EMSA assays, the p65 and p50 components of the heterodimer migrate as separate species. Antibodies to p50, p65, and Rel-B produced a supershift of the corresponding NF-κB species from DCs, whereas strong supershifts in stimulated T cells were observed with anti-p50 and anti-p65.

For Sp1 EMSA assays, activity was noted in unstimulated T cells and stimulated macrophages but was weak in B cells and not detectable in DCs (Fig. 3*C*). The lack of detectable binding of DC nuclear extracts to the Sp1 consensus sequence suggested that Sp2 and Sp3 (25) were also absent in these extracts.

Detection of NF-κB/Rel and Sp1 Proteins in Skin Cells. Next, we evaluated the expression of NF-κB/Rel and Sp1 in populations of cells isolated from skin. These contain DCs and T cells that form tight conjugates and are permissive to HIV-1 (13). For both mouse and human skin, the DCs are large and irregularly shaped, while the T cells are small and round. The DCs are known to be major histocompatibility complex class II-bright and CD3-negative, while the T cells are class II negative and CD3-positive. In cells from mouse and human skin, the large DCs showed strong nuclear and cytoplasmic labeling with antibodies to all the Rel family members (c-Rel, RelB, and p65, shown here, but also p50 and p52, not shown), but no labeling with anti-Sp1 (Fig. 4). In contrast, the T cells showed weak labeling with all anti-Rel antibodies except p65, but strong nuclear labeling with anti-Sp1.

Syncytia of HIV-1-Infected Skin Cells Coexpress NF-κB/Rel and Sp1 Transcription Factors. The studies were then repeated on HIV-1-infected human skin cells. These cultures contained numerous syncytia. Prior work had shown that the syncytia are heterologous (double labeling with the CD1a marker for DCs and the CD3 marker for T cells) and are the major site of viral replication (13). Fig. 5 shows three typical syncytia that were double labeled for the Rel proteins (visualized by fluorescein isothiocyanate-conjugated antibody) and Sp1 (visualized by Texas red-conjugated antibody). These syncytia show RelB, p50, and p65 cytoplasmic and nuclear staining, p50 being the strongest. Sp1 is in a fraction of the

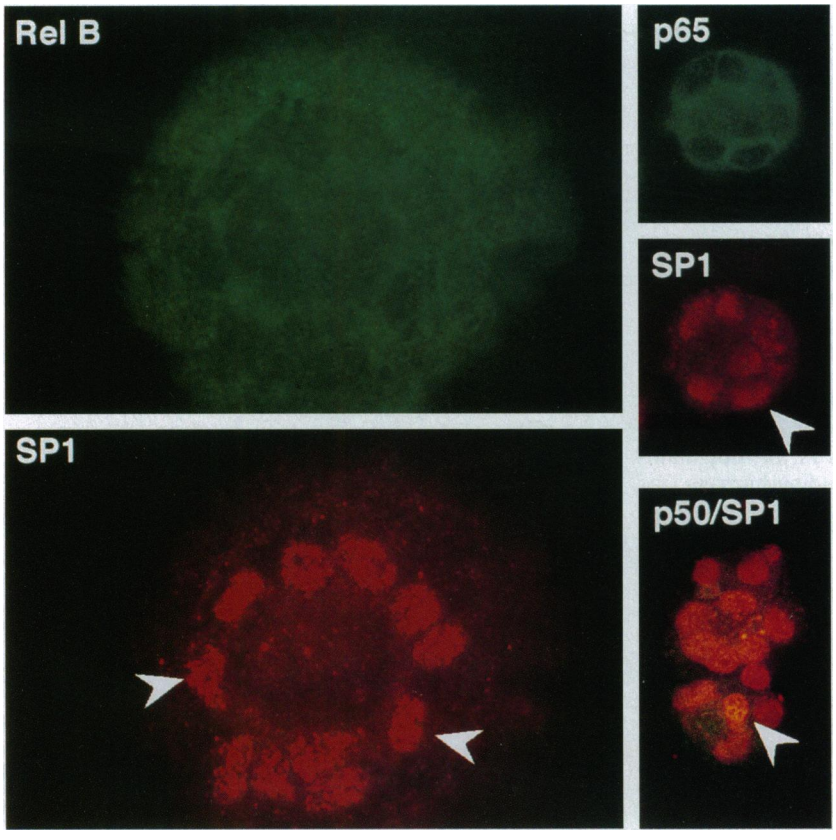

FIG. 5. Coexpression of NF-κB/Rel and Sp1 proteins in heterologous syncytia of skin DCs and T cells. Shown here is double immunofluorescent labeling of syncytia for RelB (green) and Sp1 (red) on the left or p65 (green) and Sp1 (red) on the right or superimposed staining of p50 and Sp1 on the right in the lower box. Some T-cell nuclei are indicated by arrows.

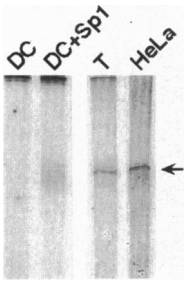

FIG. 6. Cooperation of DC extracts and Sp1 for activation of the HIV-1 promoter. Nuclear extracts (25–75 μg) from the indicated cells were tested for the ability to transactivate the HIV-1 promoter in a cell-free system. DC extracts were assayed alone or in combination with Sp1 (1 footprinting unit). The transcribed RNA was purified, resolved in a urea/PAGE gel, and autoradiographed. An arrow indicates the expected size of transcripts.

nuclei in these syncytia, presumably those derived from the T cells. The efficiency with which virus replicates in skin syncytia may be due to the colocalization of these requisite transcription control proteins for HIV-1 in the syncytia.

DC Nuclear Extracts Transactivate the HIV-1 Promoter. We have looked at the ability of DC and T-cell nuclear extracts to activate the HIV-1 promoter. Nuclear extracts from HeLa and stimulated T cells show transcriptional activity (Fig. 6). However, DC nuclear extracts needed to be supplemented with Sp1 to drive transcription *in vitro*.

DISCUSSION

Prior studies in cell lines have identified an important role for NF-κB and Sp1 proteins in the transcriptional control of the HIV-1 (6, 26, 27). In primary cells, both monocytes and T cells will support replication of HIV-1, but the cells must be stimulated, presumably to activate NF-κB and thereby initiate HIV-1 transcription. DCs contain high levels of all known Rel family members and express strong activity for NF-κB in DNA binding. This may reflect stimulation with, for example, granulocyte–macrophage colony-stimulating factor during DC development. However, DCs lack Sp1, whereas T cells and monocytes express Sp1 in an active form. These findings were made with DCs from human blood and skin, as well as mouse spleen, bone marrow, and skin. The absence of Sp1 could account for the fact that isolated DCs do not support a productive infection with HIV-1 (13, 22, 28). T cells from several sites, which also do not support productive HIV-1 infection unless stimulated, lack active Rel proteins but have abundant Sp1.

When HIV-1 infects mixtures of DCs and T cells (and possibly, when DCs conjugate to "latently" infected T cells and vice versa), expression of low levels of envelope proteins might mediate cell fusion via the CD4 molecule that can be expressed by either cell type. As a result of the formation of these unusual heterologous syncytia, high levels of active Rel and Sp1 proteins are brought together into the same cell. The HIV-1 promoter should then be stimulated strongly, leading to the marked production of virions even in the absence of standard stimuli.

DCs are present in the linings of all of the organs involved in the sexual transmission of HIV-1, while DC–T-cell conjugates are found in the afferent lymphatics of all four mammalian species that have been studied including humans (for review, see refs. 29–33). The capacity of HIV-1 to create heterologous syncytia between these two $CD4^+$ cell types can bring together requisite transcriptional control proteins for the viral promoter. To our knowledge, this phenomenon of heterologous syncytia and mixing of transcriptional control proteins has not been seen previously in viral infection (or other circumstances) and may contribute to chronic HIV-1 replication *in situ*.

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