does not appear to be a property unique to *C. albicans* since identical results were obtained with another species of candida (*C. glabrata*). We suspect that the initial polymorphonuclear response is in some way involved, but this opinion is based primarily on the dramatic histological differences between the control tumors and those induced by *C. albicans*–infected cells, rather than on an understanding of how the leukocytic infiltrate potentiates tumor growth. The presence of large numbers of leukocytes could facilitate tumor spread by degrading the normal tissue surrounding the tumor. Alternatively, leukocyte products or tumor cell products, or both, could facilitate tumor cell proliferation directly or could enhance formation of the tumor vascular bed. The mechanism underlying the altered in vivo behavior of the *C. albicans*–infected tumor cells is likely to be a local rather than systemic effect since the presence of *C. albicans*–infected tumor cells had no effect on the growth of tumors produced by uninfected tumor cells at distant sites in the same mice. Our work supports the concept that microbial cell infection of tumor cells can alter their behavior, and it provides an experimental model in which to probe the mechanism by which the presence of such particles affects tumor cell behavior as well as the consequences of infection.

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22. Supported in part by American Cancer Society grant IM-432.

28 July 1987; accepted 14 October 1987

Activation of the HIV-1 LTR by T Cell Mitogens and the Trans-Activator Protein of HTLV-I

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To investigate the mechanism by which immune activation augments replication of the human immunodeficiency virus type 1 (HIV-1) in infected T cells, four different classes of T cell mitogens were evaluated for their effects on the HIV-1 long terminal repeat (LTR). Phytohemagglutinin (PHA), a mitogenic lectin; phorbol 12-myristic 13-acetate, a tumor promoter; ionomycin, a calcium ionophore; and *tat*-1, the trans-activator protein from the human T cell leukemia/lymphoma virus type I (HTLV-I) each stimulated the HIV-1 LTR. Studies of deleted forms of the LTR supported a central role in these responses for the HIV-1 enhancer, which alone was sufficient for mitogen inducibility, but also suggested that other 5′ positive and negative regulatory elements contribute to the overall magnitude of the response. Synergistic activation of the HIV-1 LTR (up to several thousandfold) was observed with combinations of these mitogens and the HIV-1–derived *tat*-III protein. Cyclosporin A, an immunosuppressive agent, inhibited PHA-mediated activation of the HIV-1 LTR but was without effect in the presence of the other mitogens. Thus, HIV-1 gene expression and replication appear to be regulated, via the HIV-1 LTR, by the same mitogenic signals that induce T cell activation.

INFECTION WITH HUMAN IMMUNODEFICIENCY virus type 1 (HIV-1) in vivo may be associated with an asymptomatic interval lasting from months to years before the development of frank disease (*1*). Under selected conditions of culture in vitro, HIV-1–infected CD4$^+$ T cell lines can be maintained for long periods in the absence of detectable virus production (*2*). The events that trigger the transition from low-level or latent infection to productive viral replication remain poorly defined. Immune activation of infected T cells can augment HIV-1 replication (*2, 3*) perhaps through effects on regulatory elements located within the long terminal repeat (LTR) of HIV-1 (*4*). Here we show that four different classes of T cell mitogens [phytohemagglutinin (PHA), phorbol 12-myristic 13-acetate (PMA), ionomycin, and *tat*-1 (*5*)] can activate the HIV-1 LTR and define the cis-acting LTR sequences that mediate these responses. We also examine the interactions of these mitogenic stimuli with each other, with the HIV-1–encoded trans-activator protein (*tat*-III), and with the immunosuppressive agent cyclosporin A (CyA).

To confirm that mitogenic stimulation enhanced HIV-1 gene expression, we performed in vitro RNA transcription assays with HIV-1–infected Jurkat T cells before and after combined stimulation with PHA plus PMA (Fig. 1A). As monitored with *tat*-III complementary DNA (cDNA) (which contains 5′ sequences common to all HIV-1 transcripts), these mitogens induced HIV-1 gene transcription within 4 hours. As expected in activated T cells, these mitogens

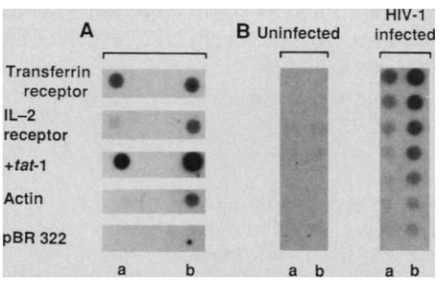

Fig. 1. (**A**) Nuclear transcription analysis of mitogen-induced gene expression in HIV-1–infected Jurkat T cells. Jurkat T cells were infected as described (*2*) and cloned 7 days later. At the time of analysis (after 50 days in culture), no RT activity was detectable. Nuclei were isolated after 4 hours of culture in the (a) absence or (b) presence of PHA (1 μg/ml) plus PMA (25 ng/ml) (*24*). In vitro RNA transcription was performed as described (*25*), using a time point previously shown to be within the linear range for Jurkat cells (*26*). The ^{32}P-radiolabeled nuclear RNA was hybridized to cDNAs immobilized on nitrocellulose filters as described (*26*). (**B**) Dot-blot analysis of HIV-1 RNA levels in infected CD4$^+$ peripheral blood lymphocytes after mitogen activation. CD4$^+$ lymphocytes were purified from normal donors by affinity rosetting, activated with PHA, and infected with HIV-1 (*2*). Cultures were maintained in RPMI 1640 with 10% fetal calf serum and supplemented with 10% partially purified human IL-2 (*24*). Six days after infection, uninfected and HIV-1–infected cultures were washed and incubated with (a) medium or (b) medium supplemented with PHA (1 μg/ml) plus PMA (25 ng/ml) for 18 hours. Total cytoplasmic RNA was isolated, serially diluted twofold, immobilized on nitrocellulose filters (*27*), and hybridized to ^{32}P-radiolabeled *tat*-III cDNA.

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also induced transcription of the interleukin 2 (IL-2) receptor (Tac) gene and moderately increased expression of the actin gene. In contrast, the constitutive transcription of the transferrin receptor gene was not altered. With purifed $CD4^+$ peripheral blood lymphocytes infected in vitro with HIV-1, PHA plus PMA also augmented cytoplasmic HIV-1 RNA as measured by a quantitative dot blotting technique (Fig. 1B).

The full-length LTR and a nested series of 5′ deletion mutants were linked to the chloramphenicol acetyltransferase (CAT) gene (*6*) and transfected into Jurkat T cells. In these leukemic cells, a single agent (PHA, PMA, ionomycin, or cotransfection with an expression vector encoding the *tat*-1 protein of HTLV-1) was sufficient to induce IL-2 receptor gene expression (*7–9*). In contrast, a combination of two of these signals (for example, PHA plus PMA, *tat*-1 plus PHA, or *tat*-1 plus PMA) is required to activate IL-2 gene expression (*8, 9*).

As shown in Fig. 2A, the HIV-1 LTR was stimulated by each of these T cell mitogens (ionomycin, PHA, PMA, or *tat*-1). Results with the −117 deletion mutant of the HIV-1 LTR are shown as examples of these responses (*10*). To define the cis-acting LTR sequences responsible for mitogen inducibility, we performed the transfections with the full-length LTR and all of the 5′ deletion mutants (Fig. 2B).

A negative regulatory element (NRE) appeared to be located between −671 and −278, because deletion of this region resulted in increased basal and mitogen-stimulated HIV-1 LTR-CAT activity. Rosen *et al.* (*11*) mapped an NRE between −340 and −185 that affects basal activity. Our data show that this NRE also controls the magnitude of the induced responses and that its 3′ border may not extend beyond −278.

Changes in LTR activity induced by PHA or PMA were maximal with the −278 construct. Further deletion to −176 was associated with a decline in this activation while basal activity remained constant. This region (−278 to −176) contains two segments highly homologous to two imperfect repeats within the 5′ regulatory regions of the genes for both IL-2 and the IL-2 receptor (*10*). Further deletion to −117 and −103, which removes one base from the first direct repeat (enhancer) of the HIV-1 LTR (*6*), produced a decline in both the basal and PHA- and PMA-induced responses. Additional deletion to −65, which eliminates both of the direct repeats and one of the three Sp1 transcription factor binding sites (*12*), but leaves the TATA box intact, was associated with a near complete loss of basal activity and mitogen inducibility.

Ionomycin stimulation of the HIV-1 LTR was constant at 1.7 to 2.5 times the basal level over the full range of deletions until −65 where the stimulatory effects disappeared. Although these effects were modest, they were observed in 21 of 21 determinations with either the full-length or deleted versions of the HIV-1 LTR.

Cotransfection with a plasmid encoding *tat*-1 produced a very different pattern of HIV-1 LTR activation. In contrast to PHA and PMA, *tat*-1 had only a modest effect on the −671, −278, and −176 deletion mutants relative to the basal response. However, marked stimulation of the HIV-1 LTR by *tat*-1 occurred with the −117 and −103 deletions. These findings raise the possibility of a second NRE located between −278 and −117 that selectively impairs activation by *tat*-1. As with PHA, PMA, and ionomycin, deletion to −65 was associated with a complete loss of *tat*-1 activation. Combined stimulation with *tat*-1 and either PHA or PMA produced additive effects, whereas PHA plus PMA yielded no greater response than that of the most active agent alone.

To determine the potential role in these induced responses of the direct repeat located at −104 to −81, an oligonucleotide corresponding to this direct repeat was ligated in either the sense or antisense orientation to the 5′ end of the herpes simplex virus thymidine kinase (TK) promoter (*13*). The HIV-1 direct repeat conferred properties of PHA, PMA, and *tat*-1 inducibility to the TK promoter (Fig. 3). In the absence of the direct repeat, these mitogens did not alter TK-promoter activity (*14*).

The *tat*-III protein of HIV-1 may exert

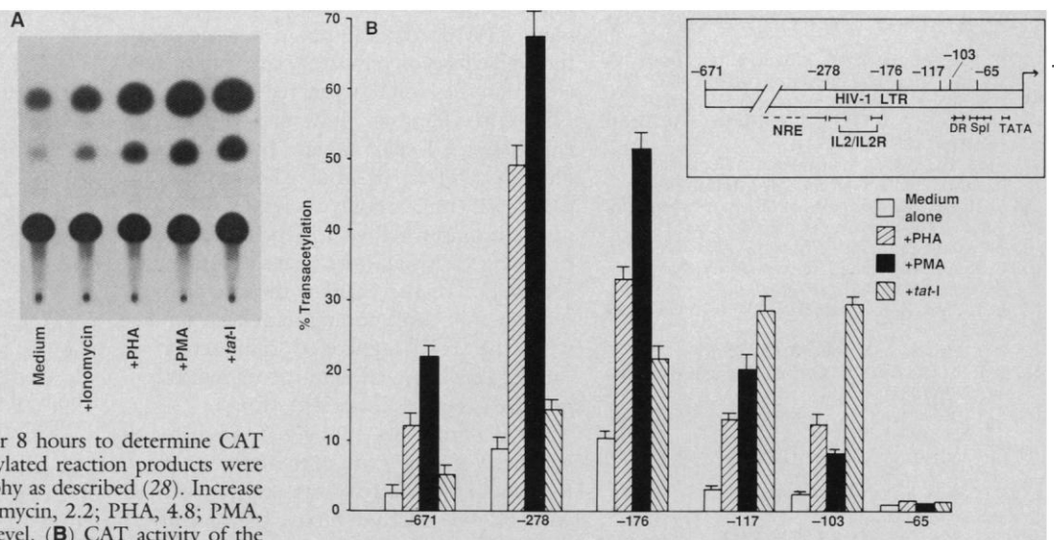

Fig. 2. (**A**) Mitogen stimulation of the HIV-1 LTR. Plasmids containing the HIV-1 LTR ligated to the CAT gene (−117 construct) (*6*) were cotransfected using DEAE-dextran into Jurkat cells (5 μg of DNA per 10^7 cells) with either a *tat*-I cDNA expression vector or a control expression plasmid containing an inactive frameshift of the *tat*-I gene (Δ-*tat*-I) as previously described (*9*). Ionomycin (0.5 μg/ml), PHA (1 μg/ml), or PMA (50 ng/ml) was added 24 hours after transfection. Extracts from 2×10^6 cells, normalized for total protein content, collected 20 to 24 hours after induction, were incubated with ^{14}C-labeled chloramphenicol for 8 hours to determine CAT activity. The acetylated and nonacetylated reaction products were separated by thin layer chromatography as described (*28*). Increase in CAT activity was as follows: ionomycin, 2.2; PHA, 4.8; PMA, 7.8; and *tat*-I, 9.5 times the basal level. (**B**) CAT activity of the HIV-1 LTR deletion mutants in the presence or absence of PHA, PMA, or *tat*-1. Values shown for each deletion construct and stimulatory agent represent the mean ± the standard error of the mean (SEM) of three to six different experiments with all experiments giving similar patterns of activity. The average activity (normalized as in Fig. 2A) of each transfected DNA in the absence of inducer was compared to the average increase in activity produced by the indicated stimulant. The ratios of stimulated versus basal activity for each deletion and mitogen are as follows (mean ± SEM with two to three plasmid preparations for each HIV-1 LTR-CAT construct): at −671, ionomycin 1.95 ± 0.2, PHA 4.4 ± 0.6, PMA 7.9 ± 0.5, *tat*-1 1.9 ± 0.4; at −278, ionomycin 1.7 ± 0.2, PHA 5.5 ± 0.3; PMA 7.5 ± 0.4, *tat*-1 1.7 ± 0.1; at −176, ionomycin 1.9 ± 0.1; PHA 3.2 ± 0.2, PMA 5.0 ± 0.2, *tat*-1 2.1 ± 0.2; at −117, ionomycin 2.5 ± 0.3, PHA 4.2 ± 0.3; PMA 6.5 ± 0.7, *tat*-1 9.2 ± 0.7; at −103, ionomycin 2.0 ± 0.2, PHA 4.9 ± 0.5, PMA 3.3 ± 0.1, *tat*-1 11.7 ± 0.5; at −65, ionomycin 1.2 ± 0.1, PHA 1.5 ± 0.2, PMA 1.0 ± 0.2, *tat*-1 1.3 ± 0.1. The inset of the HIV-1 LTR indicates the position of the TATA box, Spl binding sites (*12*) direct repeats (DR), sequences homologous to the IL-2 and IL-2 receptor promoters (IL-2/IL-2R) (*10*), negative regulatory element (NRE), and the deletion mutants studied.

both transcriptional and post-transcriptional effects on HIV-1 gene expression (*15, 16*), and sequences extending from −29 to +54 within the LTR appear to be involved in this response (*11, 16*). To study the potential interplay between the mitogens and *tat*-III, an expression vector encoding the *tat*-III gene product was cotransfected with the full-length HIV-1 LTR-CAT plasmid into Jurkat T cells. As shown in Table 1, combinations of the mitogenic agents (ionomycin, PHA, or PMA) and *tat*-III produced synergistic stimulation of the HIV-1 LTR. Similar synergism was observed with combinations of *tat*-I and *tat*-III.

To confirm that mitogen activation of HIV-1 LTR-CAT activity corresponded to an increase of HIV-1 LTR-CAT RNA appropriately initiated within the HIV-1 LTR, we performed ribonuclease protection studies with RNA isolated from the transfected Jurkat T cells stimulated with PHA or PMA in the presence or absence of *tat*-III (Fig. 4). The increased intensity of the 430-nucleotide protected band, which represents correct initiation within the LTR (*10*), after PHA and PMA stimulation, paralleled the observed increase in HIV-1 LTR-CAT activity. Cotransfection of the *tat*-III cDNA also produced an increase in steady-state HIV-1 LTR-CAT RNA, indicating that this viral protein exerts some effects at a pretranslational level. PHA and PMA further increased the amount of specific RNA in the presence of *tat*-III. However, the increase in HIV-1 LTR-CAT RNA in the presence of the *tat*-III protein did not fully match the magnitude of the increase in CAT enzyme activity, suggesting possible additional effects of the *tat*-III protein at a post-transcriptional level. The same transcription initiation site within the HIV-1 LTR appeared to be used in the presence or absence of the *tat*-III protein.

The capacity of CyA to block mitogen-induced activation of the HIV-1 LTR was investigated because this drug has been proposed as a treatment for AIDS patients on the basis of its capacity to block many steps in T cell activation (*17*). We showed previously that CyA prevents expression of transfected IL-2 promoter-CAT plasmids in Jurkat T cells costimulated with PHA plus PMA and that these specific inhibitory effects can be largely circumvented by cotransfection of *tat*-1 (*9*). CyA inhibited 70 to 75% of the PHA-induced activation of the HIV-1 LTR (*18*). In sharp contrast, HIV-1 LTR activation mediated by PMA or *tat*-1 proved resistant to the inhibitory effects of CyA. In addition, as in the case of the IL-2 promoter–CAT plasmid, cotransfection of *tat*-1 prevented the inhibitory effects of CyA on PHA activation of the HIV-1 LTR.

Our data suggest that, in many respects, the HIV-1 LTR is regulated like a T-cell activation gene. Its response to single mitogenic signals in Jurkat T cells resembles the IL-2 receptor gene (*7–9*). These effects of mitogens on the HIV-1 LTR appear to be specific, because other cellular and viral promoters are unaffected by these stimuli (*9*). The inhibition of PHA activation of the HIV-1 LTR by CyA is similar to its effects on interferon-γ and IL-2 gene expression (*9, 17*). However, these studies also suggest that clinical use of CyA to block conversion from low-level to active infection by interrupting T cell activation may fail depending on the nature of the T cell stimulant.

The ability of the HIV-1 direct repeat to impart mitogen inducibility to the neutral TK promoter supports a central role for the direct repeat in these mitogen responses and is consistent with the recent studies of Nabel and Baltimore (*4*). These investigators have shown that mutation of the direct repeat, which is identical in sequence to a region within the κ-light chain enhancer, prevents both the binding of a mitogen-inducible nuclear protein (NF-κB) and the activation of the HIV-1 LTR by combinations of

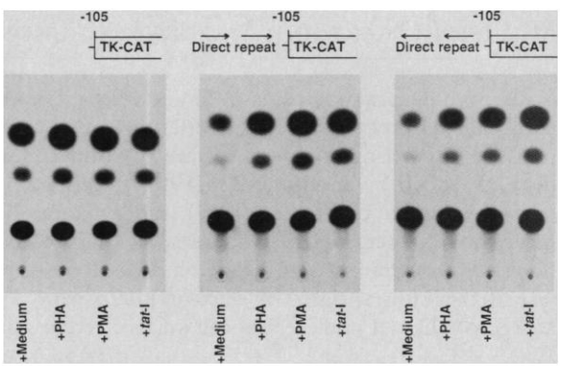

Fig. 3. Activity of the HIV-1 direct repeat. A double-stranded oligonucleotide corresponding to the sequence from −105 to −80, which contains the direct repeat of the HIV-1 LTR, was synthesized with Bam HI linkers and inserted in the sense and antisense orientation into the polylinker of TK-CAT which contains the sequence from −105 to +51 of the TK promoter (*13*). Orientation was confirmed by DNA sequencing. Basal activity and inducibility with PHA, PMA, or *tat*-1 were determined as described in Fig. 2A. The reason for the decreased basal activity in the presence of the direct repeat is unknown. The increase in CAT activity (mean of three experiments ± SEM) was as follows: TK-CAT, PHA 1.3 ± 0.1, PMA 1.1 ± 0.2, *tat*-1 1.2 ± 0.1; sense direct repeat–TK-CAT, PHA 5.2 ± 0.5, PMA 9.5 ± 0.6, *tat*-1 7.8 ± 0.1; antisense direct repeat–TK-CAT, PHA 4.3 ± 0.5; PMA 6.2 ± 0.4, *tat*-1 12.4 ± 1.3 times the basal level.

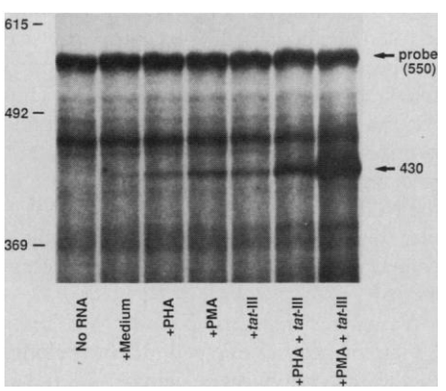

Fig. 4. Ribonuclease protection analysis of HIV-1 LTR-CAT RNA isolated from transfected Jurkat T cells. HIV-1 LTR-CAT (−278 construct) was cotransfected with either a visna virus LTR-*tat*-III expression plasmid or a plasmid containing an inactive nonproducing frameshift mutant, x-*tat*-III. This viral promoter was not induced by PHA, although its activity increased two to three times with PMA (*9*). Total RNA (50 μg) isolated from the transfected cells (*29*) was hybridized with a ^{32}P-radiolabeled negative strand RNA probe transcribed with T3 RNA polymerase from a fragment of the HIV-I LTR-CAT DNA (−117 in the LTR to the Eco RI site in the CAT gene, 550 bases) which was inserted into the Xba I–Eco RI sites of the IBI31 plasmid. Hybridization and ribonuclease digestion were as described (*30*). Messenger RNA utilizing the expected cap site within the LTR will protect 430 bases of this probe (*6, 10*). The stimulation ratios for CAT activity simultaneously measured in these transfectants were: PHA, 5.5; PMA, 6.5; *tat*-III, 120; PHA + *tat*-III, 970; and PMA + *tat*-III, 1630.

Table 1. The multiplicative effects of T cell mitogens and *tat*-III on HIV-1 LTR-CAT expression. Jurkat T cells were cotransfected with HIV-1 LTR-CAT (−671 construct) and the *tat*-III or control x-*tat*-III expression plasmids and subsequently induced with ionomycin, PHA, or PMA (see Fig. 2A). Relative CAT activity represents the ratio of the responses obtained with the various stimuli versus with medium alone. Activity in the presence of medium alone (relative CAT activity equals 1.0) was 0.5% transacetylation per 10^7 cells per hour.

Stimulant	Relative CAT activity
Medium alone	1.0
Ionomycin	1.7
PHA	5.6
PMA	8.2
tat-III	378
Ionomycin + *tat*-III	805
PHA + *tat*-III	3275
PMA + *tat*-III	9379

PHA and PMA. Since the NF-κB protein is not only restricted to lymphoid cells, but is also detectable in PMA-stimulated HeLa cells (*19*), additional positive and negative elements located further 5′ in the HIV-1 LTR might regulate expression of the LTR in a cell- or tissue-specific manner. In particular, the sequences homologous to the IL-2 and IL-2 receptor gene promoters are of interest since a segment containing these imperfect repeats is important for mitogen activation of the IL-2 promoter that is largely restricted to T cells (*20*).

The increased HIV-1 LTR activity observed with combinations of the mitogens and *tat*-III suggests that these agents act through independent mechanisms (*4*) and underscores the large changes in LTR activity that can occur when both agents are present (for example, >9000-fold increase in CAT activity in the presence of PMA and *tat*-III). Thus, even relatively weak mitogenic stimuli, such as the effect of *tat*-1 on the full-length HIV-1 LTR, may lead to marked changes in viral gene expression by triggering production of the *tat*-III protein.

The capacity of the trans-activator gene product of HTLV-I to stimulate the HIV-1 LTR when present in the same cell is similar to the effects of many DNA viruses (herpes simplex, papovavirus, and varicella zoster) (*21*). This finding may be of potential clinical importance in view of the incidence (27%) of dual HIV-1 and HTLV-I infection in some populations of intravenous drug abuse patients (*22*).

Note added in proof: Similar results regarding the mitogen inducibility of the HIV-1 LTR have recently been described by Tong-Starksen *et al.* (*23*).

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31. We thank S. Z. Saluhuddin for HIV-1–infected Jurkat T cells, C. Lane for HIV-1–infected CD4$^+$ peripheral blood lymphocytes, R. Randall for oligonucleotide synthesis, A. McClelland for transferrin receptor cDNA, L. Kedes for human γ-actin cDNA, M. Feinberg for *tat*-III cDNA (pHXB) and the *tat*-I and Δ-*tat*-I expression plasmids, C. A. Rosen for the *tat*-III and x-*tat*-III expression plasmids, and D. Russell for the TK-CAT plasmid. We also thank J. Hoffman for technical assistance and B. Kissell for manuscript preparation.

5 May 1987; accepted 5 October 1987

Identification of Nuclear Receptors for VIP on a Human Colonic Adenocarcinoma Cell Line

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Vasoactive intestinal peptide (VIP) is a neuropeptide with broad tissue distribution. Although its precise function is unknown, it is thought to exert its effect, at least in part, by interacting with cell surface receptors. Nuclear receptors for VIP have now been identified by specific binding of ^{125}I-labeled VIP to nuclei of a human colonic adenocarcinoma cell line (HT29) and by cross-linking of ^{125}I-labeled VIP to its receptor on intact nuclei. In contrast, ^{125}I-labeled transferrin shows only background binding to nuclei but significant binding to intact cells. Purity of the isolated nuclei was further substantiated by electron microscopy. The apparent molecular sizes of the VIP–cross-linked nuclear and cell surface receptors are similar but not identical.

PEPTIDE HORMONES INDUCE THEIR responses by interacting with cell surface receptors of their target tissue (*1*). Nuclear receptors have been reported for several polypeptide hormones (*2–4*), including epidermal growth factor (EGF) and nerve growth factor (NGF) (*4*). The biologic role that these nuclear receptors play has not been clearly defined, but evidence exists for direct action in the nucleus (*2–5*). Thus far, the putative nuclear receptors for polypeptide hormones have not been characterized well biochemically and, in the case of the NGF nuclear receptor, a different molecular size species was immunoprecipitated compared to that for the cell surface receptor (*6*).

Vasoactive intestinal peptide (VIP) is a 28–amino acid residue peptide that belongs to the secretin-glucagon family (*7*). It has been considered to be a neurotransmitter with various activities, but its precise physiologic role has not been established (*7*). After binding to its cell surface receptor, VIP causes an increase in intracellular adenosine 3′,5-monophosphate via adenylate cyclase (*8*). In the human colon adenocarcinoma cell line HT29 there is evidence for rapid internalization of VIP after binding at 37°C (*9*). The receptor for VIP has been identified by detergent solubilization (*10*), by cross-linking ^{125}I-labeled VIP to its receptor on intact cells or membrane preparations (*11, 12*), and by photoaffinity labeling (*13*).

In this study, binding of ^{125}I-labeled VIP was tested on intact HT29 cells and purified nuclei. There was similar binding of ^{125}I-labeled VIP to both HT29 cells and nuclei (Fig. 1), which was competitively displaced in the presence of unlabeled VIP. Since there is a significant amount of cell surface transferrin (Tr) receptors on HT29 cells (*14*), we compared the presence of Tr recep-

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