AD-A243 279

DTIC
ELECTE
DEC 6 1991
S C D

(2)

AD_____

CONTRACT NO: DAMD17-90-C-0106

TITLE: PATHOBIOL[illegible] OF HIV IN THE HUMAN MONOCYTE-MACROPHAGE

PRINCIPAL INVESTIGATOR: Jerome E. Groopman, M.D.

CONTRACTING ORGANIZATION: New England Deaconess Hospital
185 Pilgrim Road
Boston, MA 02215

REPORT DATE: October 24, 1991

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

91-17153

91 12 [illegible] 029

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a REPORT SECURITY CLASSIFICATION Unclassified	1b RESTRICTIVE MARKINGS
2a SECURITY CLASSIFICATION AUTHORITY	3 DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release distribution unlimited
2b DECLASSIFICATION/DOWNGRADING SCHEDULE	
4 PERFORMING ORGANIZATION REPORT NUMBER(S)	5 MONITORING ORGANIZATION REPORT NUMBER(S)
6a NAME OF PERFORMING ORGANIZATION New England Deaconess Hospital 6b OFFICE SYMBOL (If applicable)	7a NAME OF MONITORING ORGANIZATION
6c ADDRESS (City, State, and ZIP Code) 185 Pilgrim Road Boston, MA 02215	7b ADDRESS (City, State, and ZIP Code)
8a NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b OFFICE SYMBOL (If applicable)	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER Contract No. DAMD17-90-C-0106
8c ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21702-5012	10 SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO: 63105A PROJECT NO: 3M2 3105DH29 TASK NO: AD WORK UNIT ACCESSION NO: WUDA33554C

11 TITLE (Include Security Classification)
Pathobiology of HIV in the Human Monocyte-Macrophage

12 PERSONAL AUTHOR(S)
Groopman, Jerome E.

13a TYPE OF REPORT	13b TIME COVERED	14 DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Annual	FROM 90/09/28 TO 91/09/27	91/10/24	8

16 SUPPLEMENTARY NOTATION

17 COSATI CODES			18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	HIV, Monocytes, Host defense, RAI, AIDS, Viral Tropism, Cytokine
06	03		
06	13		

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

In the past year, we have made considerable progress in addressing specific aims of our proposal. We have studied the interaction of HIV with monocyte-macrophages with particular emphasis on comparing these interactions with those that occur in human T lymphocytes. We have found that there are cellular transcription factors which interact with the HIV LTR independent of NF-kappa B which could be important in virus expression in different cells. The role of cytokines inducing the activity of such transcription factors is under investigation. We have also found that a newly identified cytokine, the kit ligand (KL)/stem cell factor (SCF), protects myeloid and erythroid progenitor cells from the suppressive effects of cytokines such as tumor necrosis factor. While KL/SCF can amplify the proliferative response of hematopoietic progenitors, it does not increase HIV replication in target cells. KL/SCF is an attractive candidate cytokine to be used therapeutically in patients with AIDS. During the first year project, we have also identified sequences within the negative regulatory element (NRE) of the HIV LTR which could interact with the family of transcription factors termed ARP. We are characterizing the expression of the ARP genes in different cells to determine whether they may influence viral tropism.

20 DISTRIBUTION/AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	
22a NAME OF RESPONSIBLE INDIVIDUAL Mrs. Virginia M.Miller	22b TELEPHONE (Include Area Code) 301-663-7325 22c OFFICE SYMBOL SGRD-RMI-S

DD Form 1473, JUN 86 Previous editions are obsolete SECURITY CLASSIFICATION OF THIS PAGE

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

_____Where copyrighted material is quoted, permission has been obtained to use such material.

_____Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

_____Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

_____In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

XX For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

XX In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

PI Signature Joanne E. Goodman Date 10/24/91

Contract No. DAMD17-90-C-0106

(September 28, 1991 - September 27, 1991)

Pathobiology of HIV in the Human Monocyte-Macrophage

TABLE OF CONTENTS

Accession For
NTIS GRA&I ☒
DTIC TAB ☐
Unannounced ☐
Justification
By
Distribution/
Availability Codes
Dist | Avail and/or Special
A-1

INSPECTED 4

INTRODUCTION

The human immunodeficiency virus (HIV) infects a number of different cell targets including the monocyte-macrophage. Infection of monocyte-macrophages is important in the pathogenesis of neurological syndromes associated with HIV and may also contribute to the impairment in host defense seen in these patients. Furthermore, the monocyte-macrophage is an important immune effector cell, working to present antigen to T cells as part of the immune response, as well as an important cell source of regulatory proteins called cytokines.

HIV is able to infect the human monocyte-macrophage; we have studied this interaction throughout our contract with the Department of Defense. Our previous work focused on dysregulated cytokine production in HIV infected monocyte-macrophages. We now have addressed viral and cellular factors which may influence viral tropism in monocyte-macrophages as well as further explore the role of cytokines in HIV biology. The methodology for the work performed during the first year was presented in the proposal (section 4 "Description of Proposed Research"). A series of cell biological and molecular biological experiments were performed, aimed at identifying cellular transcription factors which could interact with the HIV LTR and modulate expression of virus. We also performed studies to determine the applications of cytokines to protect progenitor cells from myelosuppressive effect of cytokines frequently elaborated in HIV-infected patients such as TNF.

BODY

Our initial studies performed in the first quarter of the year demonstrated that certain cytokines may cause transactivation of the HIV LTR independent of the NF-kappa B structure. This was most dramatically seen in viruses with deletion or point mutations in the two NF-kappa B sequences present in the HIV-1 LTR that have been transfected into the human megakaryocytic cell line CMK. Despite these NF-kappa B mutations, there was transactivation of the HIV LTR in the CMK megakaryocytic cells in response to induce such as the phorbol diester PMA. Similarly, GM-CSF treatment of THP-1 monocytic cells transfected with these NF-kappa B mutants

showed transactivation as well. This work was further pursued during the course of the year and we recently published (see APPENDIX, Sakaguchi et al) our findings that there are as yet uncharacterized cellular transcription factors present in certain cells, including megakaryocytes and monocytes, that are able to interact near the TATA box of the HIV LTR and significantly transactivate the HIV LTR in the absence of NF-kappa B responsive sequences. Ongoing work is designed to better characterize these cellular transcription factors and to determine their tissue distribution. Such factors could be important in activating latent virus within monocyte-macrophages as well as determining the efficiency of infection in different cell types.

We have studied as well the effects of HIV infection on surface expression of myeloid antigens. We did extensive studies and found that CD14 and CD11 were not changed in the monocytic cell line U937 following HIV infection. Further work demonstrated that only the CD4 structure and the HLA-DR structures were downmodulated following HIV infection. Based on these results, we decided not to further pursue studies since our hypothesis that HIV infection may alter the surface expression of important adhesion molecules and thereby impair monocyte function was not supported by our studies.

The newly identified KL/SCF is a cytokine produced by mesenchymal cells including bone marrow stromal fibroblasts. Two forms of KL/SCF arise via differential mRNA splicing, a transmembrane form which is present on the surface of the fibroblasts and a soluble secreted molecule. The predominant form of KL/SCF in bone marrow stromal fibroblasts is the transmembrane form; we found, using a radioreceptor assay, that less than 10 ng/ml of soluble KL/SCF was produced by bone marrow stromal fibroblasts. KL/SCF markedly increases myeloid and erythroid progenitor response to later acting growth factors such as G-CSF, GM-CSF and erythropoietin respectively. We studied the effects of KL/SCF on HIV replication in target monocyte-macrophages. We found that using either monocyte tropic or T cell tropic isolates (BAL or 9533 as the monocyte tropic isolates and HIV IIIB as the T cell tropic isolate) that there was no upregulation of virus replication with exposure to the KL/SCF. This suggested that the cytokine could be safely used in AIDS or ARC patients. To better define its potential role in the biology of HIV interaction with myeloid cells, we found that KL/SCF markedly increased the responsiveness of the progenitor cells and maintained normal progenitor numbers even in the presence of suppressive factors such as tumor necrosis factor alpha. It has been hypothesized that TNF alpha is elaborated in HIV infected individuals either due to HIV infection

itself or to concomitant opportunistic infections seen in such patients. If TNF alpha contributes to impaired myelopoiesis in AIDS, KL/SCF may overcome its effects.

Because human megakaryocytes have the c-kit receptor which serves as the KL/SCF receptor, we studied whether HIV infected megakaryocytes were altered in terms of their production of virus in the presence of KL/SCF. No increase in virus production was noted in these studies following exposure to KL/SCF.

Recently, we have focused on the ARP family of transcription factors which appear capable of interacting with regions of the negative regulatory element (NRE) of the HIV LTR. By gel shift assays, there appears to be strong binding of ARP to the NRE. We found that T cells produced very little ARP. We have recently transfected ARP in human T cells to determine whether production of the protein will reduce virus production. Initial studies using an infectious clone of HIV-1, WI-3, revealed that ARP expressing T cells had much lower levels of virus replication compared to T cells transfected with same plasmid but not expressing ARP. These studies are now being extended to the role of ARP in monocyte-macrophages and megakaryocytes with respect to differential regulation of virus production.

We have thus, in the past year of work, addressed both viral and cellular factors which may regulate replication of HIV in different cell types as well as pursued new avenues of research in cytokine biology in HIV.

CONCLUSION

Considerable progress has been made in the past year of our studies. We have found that there are responsive sequences in the HIV LTR to previously unidentified cellular transcription factors distinct from NF-kappa B. We have also identified a new family of transcriptional factors termed ARP which are capable of binding to the negative regulatory element of the HIV LTR and which may be important in suppressing virus replication. We are thus positioned to pursue studies on both positive and negative signals that regulate virus transcription. Our studies in cytokine biology focused on a newly recognized growth factor, the kit ligand/stem cell factor, which is produced by bone marrow fibroblasts and may be important in amplifying progenitor responsiveness to later

acting growth factors as well as regulating megakaryocytopoiesis. These studies of KL/SCF should be important in obtaining information with regard to the potential clinical application of this cytokine in the therapy of patients with AIDS or ARC as well as determining whether there is disturbance of its physiologic role which could account for impaired myeloid cell development in AIDS.

APPENDIX

1. Sakaguchi M, Sato T, Groopman JE. Human immunodeficiency virus infection of megakaryocytic cells. Blood. 1991; 77:481-485.

2. Sakaguchi M, Zenzie-Gregory B, Groopman JE, Smale ST, Kim S. Alternative pathway for induction of human immunodeficiency virus gene expression: Involvement of the general transcription machinery. J Virol. 1991; 65:5448-5456.

Human Immunodeficiency Virus Infection of Megakaryocytic Cells

By Mamoru Sakaguchi, Takeyuki Sato, and Jerome E. Groopman

The human immunodeficiency virus (HIV) is capable of infecting certain cells of hematopoietic lineage, particularly monocyte-macrophages and T lymphocytes. Recently, the possibility that cells of megakaryocytic lineage are susceptible to HIV infection has been raised. We have characterized infection of the permanent megakaryocytic cell line CMK by HIV in vitro. CMK cells were easily infected by HIV type 2 (HIV-2), producing significant amounts of virus in culture. Infection appeared to be mediated by the CD4 surface antigen on CMK cells. Three different strains of HIV-1 were able to minimally infect CMK cells, suggesting there may be isolates of HIV tropic for megakaryocytes. Infection of CMK cells led to downregulation of the CD4 surface antigen but no discernable change in expression of megakaryocyte-associated proteins glycoprotein Ib and glycoprotein IIb/IIIa. These observations support the likelihood that megakaryocytes are susceptible to HIV infection, and cell lines of megakaryocytic origin may provide a useful model to study effects of the retrovirus on megakaryocyte function.

© 1991 by The American Society of Hematology.

PROFOUND DEFECTS in hematopoiesis have been observed in some patients infected with the human immunodeficiency virus (HIV).[1-5] Defining the cellular targets of HIV may yield insights into the pathogenesis of dysregulated hematopoiesis due to this retrovirus. HIV is capable of infecting hematopoietic cells that bear the CD4 surface antigen, specifically T lymphocytes and monocyte-macrophages.[6-9] Recently, populations of immature bone marrow myeloid progenitors[10] as well as nonhematopoietic bone marrow stromal fibroblasts[11] have been reported to be susceptible to HIV infection in vitro. We were intrigued by the observations that certain patients with HIV and thrombocytopenia responded to 3'-Azido-3'-deoxythymidine (AZT) therapy with marked increases in their platelet counts.[12-14] This suggested that the retrovirus might directly interfere with megakaryocytopoiesis. Zucker-Franklin and Cao,[15] using in situ hybridization, detected HIV sequences in bone marrow megakaryocytes from HIV infected individuals. Another recent study found significant expression of the CD4 antigen on the marrow megakaryocyte.[16] The establishment of permanent cell lines of megakaryocytic lineage allows for direct assessment of infection of these cells by HIV. Using the CMK megakaryocytic cell line,[17,18] derived from a patient with megakaryoblastic leukemia, we have studied their susceptibility of these cells to infection by HIV. CMK cells can be infected with HIV, and this infection appears to occur via virus interaction with the CD4 surface receptor. This system may provide a model for exploring the consequences of HIV infection of megakaryocytes.

MATERIALS AND METHODS

Cell lines. The CMK cell line,[17,18] derived from a patient with megakaryoblastic leukemia, was the generous gift of Dr T. Sato (Chiba University, Japan). Three different clones of the CMK cells were available for study. These clones were termed CMK, CMK-6, and CMK11-5. The CMK cell lines were carried in RPMI 1640 medium containing 10% fetal bovine serum (GIBCO, Grand Island, NY), L-glutamine, penicillin, and streptomycin. The cells were shown to be free of mycoplasma.

Characterization of CMK cells. The three CMK clones were characterized for expression of cell surface markers of megakaryocytic, erythroid, lymphoid, and myeloid lineage. This was done by flow cytometry using specific monoclonal antibodies (MoAbs) to platelet glycoprotein (GP) Ib (Amac, Inc, Westbrook, ME), platelet GP IIb/IIIa (Amac, Inc), the erythroid marker glycophorin A (Amac, Inc), and the myeloid marker CD15 (anti-Leu M1; Becton Dickinson Immunocytometry Systems, Mountain View, CA). The detection of the surface CD4 protein was performed using the murine MoAb Leu3a (Becton Dickinson) and of the surface CD34 antigen using the MoAb MY10 (Becton Dickinson). The cell surface expression of HLA-DR antigen was analyzed using anti-HLA-DR MoAb (Becton Dickinson). All samples were analyzed using a FACS analyzer (Becton Dickinson). CMK cells were also analyzed by Northern blot for expression of the GF-1 gene, a transcription factor specific for megakaryocytic and erythroid cells, as described.[19,20]

Virus stocks. HIV-1 IIIB and HIV-2 ROD were obtained from Dr R.C. Gallo (National Cancer Institute, Bethesda, MD)[21] and Dr J.C. Cherman (Institut Pasteur, Paris, France),[22] respectively. The HIV-2 DOU strain was derived from a West African man with acquired immunodeficiency virus (AIDS) and provided by Dr Y. Perol (Hopitaux de Paris, France). Virus stocks were propagated in H9 cells as previously described.[23] Cells were harvested at peak infectivity and virus stocks were made by the shaking method of Vujcic et al[23] and stored in aliquots at −70°C in 50% fetal bovine serum. HIV-1 Ba-L (provided by Dr M. Popovic, National Cancer Institute, Bethesda, MD)[24] and HIV-1 9533 (isolated in our laboratory) were grown in primary macrophages as described.[24] Tissue culture supernatants from primary macrophages were harvested at peak infectivity and stored in aliquots at −70°C in 60% fetal bovine serum. The tissue culture infectious dose (TCID), a measure of infectious titer of virus, was determined by a terminal dilution assay in susceptible H9 T cells or peripheral blood monocyte-macrophages.

HIV infection of CMK cells. Infection of CMK cells was performed as described previously.[25] Briefly, 60 μL of CMK, CMK-6, or CMK11-5 cells (5×10^6/mL) was incubated for 1 hour at 37°C with 60 μL of 10^2 to 10^4 50% tissue culture infectivity doses ($TCID_{50}$) of HIV and 60 μL of complete media. One hundred fifty microliters of this mixture containing cells and virus was then

From the Division of Hematology/Oncology, The Robert Mapplethorpe Laboratory for AIDS Research, New England Deaconess Hospital, Harvard Medical School, Boston, MA; and the Department of Pediatrics, Chiba University School of Medicine, Chiba, Japan.

Submitted March 1, 1990; accepted October 3, 1990.

Supported in part by grants from the National Institutes of Health: HL42112; HL43510; HL33774; HL41374; A124475; and DAMD 17-87-C-7017; and the Ministry of Health and Welfare, Japan

Address reprint requests to Jerome E. Groopman, MD, Chief, Division of Hematology/Oncology, New England Deaconess Hospital, 110 Francis St, 4A, Boston, MA 02215.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1784 solely to indicate this fact.

© 1991 by The American Society of Hematology.

0006-4971/91/7703-0009$3.00/0

transferred to 2 mL of media and cultured in 24-well plates (Costar, Cambridge, MA) at 37°C in 5% CO_2. Control cultures consisted of mock infected CMK cells not exposed to virus. Positive control cultures used to assure the infectivity of virus within the frozen stocks consisted of H9 or CEM T cells or peripheral blood monocyte-macrophages. Cultures were periodically monitored for productive HIV infection by measuring supernatant reverse transcriptase (RT) activity, and by quantitation of cells expressing HIV specific proteins by indirect immunofluorescence (IFA), as described below. Cell viability in these cultures was assessed by trypan blue exclusion. In some experiments, expression of cell surface antigens was measured by flow cytometry before and after HIV infection.

RT assay. The assay for HIV RT activity was performed as previously described.[26] Virus was concentrated from 1 mL of cell-free tissue culture supernatant by precipitation with 0.5 mL of polyethylene glycol overnight at 4°C.

IFA. HIV antigens on the cells were detected by IFA on methanol-fixed cells using the serum of an AIDS patient.[21] Serum from a seronegative donor was used as a negative control.

Blocking experiments. To determine if HIV used the CD4 surface receptor to infect CMK cells, cultures were established in the presence of Leu3a, an anti-CD4 MoAb (Becton Dickinson) or with soluble recombinant CD4 (Genentech, South San Francisco, CA) as previously described.[25,26] Productive HIV infection was monitored by RT and IFA.

RESULTS

Phenotypic characterization of the three different clones of CMK showed different degrees of maturity (Table 1). CMK-6 cells expressed the lowest amounts of megakaryocytic differentiation markers, particularly GPs Ib and IIb/IIIa, while CMK11-5 had the most mature surface phenotype. The parent CMK clone was intermediate in stage of maturation between the CMK-6 and CMK11-5 clones. In addition to expression of markers of megakaryocytic lineage, the CMK cells expressed the erythroid marker glycophorin A, as well as the CD34 antigen. The CD4 surface antigen was found on CMK cells at all three levels of maturation. CMK cells were strongly positive for GF-1 expression by Northern blot (data not shown).

The CMK megakaryocytic cell line proved highly susceptible to HIV-2 ROD infection. Comparable kinetics of infection of CMK-6, CMK, and CMK11-5 are shown in Fig 1. High levels of productive infection were achieved with peak RT activity greater than 10×10^6 cpm/mL and IFA for

Table 1. Surface Antigen Characterization of the Three CMK Clones

MoAb	CMK-6 Positive Cells (%)	CMK Positive Cells (%)	CMK11-5 Positive Cells (%)
GP Ib (CD42b)	0.3* (0.2)	3.0 (1.0)	8.0 (0.9)
GP IIb/IIIa (CD41)	12.7 (3.1)	52.6 (3.3)	69.5 (6.1)
Glycophorin A	2.5 (0.5)	37.0 (7.2)	47.3 (4.8)
Leu3a (CD4)	90.5 (1.2)	91.1 (2.5)	66.8 (7.5)
My10 (CD34)	1.3 (0.3)	6.7 (0.5)	4.9 (0.5)
Leu M1 (CD15)	65.9 (6.5)	20.7 (4.0)	1.3 (0.4)
HLA-DR	ND	31.2 (5.6)	ND

IFA were performed as described in the text. Values in parenthesis represent the standard error (SE).

Abbreviation: ND, not done.

*Values represent the averages of two separate experiments.

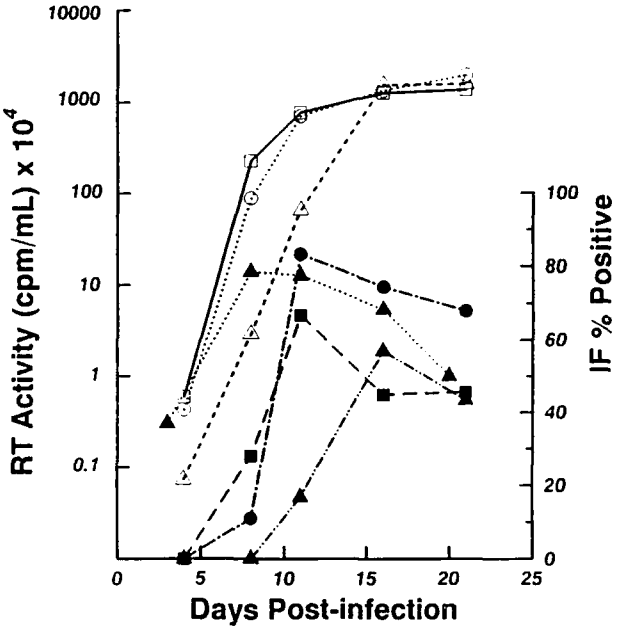


Fig 1. Characteristics of HIV-2 ROD infection of three CMK clones (CMK-6, CMK, and CMK11-5 cells). As described in the text, CMK cells were infected with 10^2 $TCID_{50}$ of HIV-2 ROD. RT activity and immunofluorescence (IF) positive cells were monitored every 3 to 4 days. RT activity of ROD infection ([-□-)], CMK; [----△----], CMK-6; [····○····], CMK11-5) and DOU infection [····▲····], CMK11-5) is shown. IF assay of ROD infection ([---■---], CMK; [---▲---], CMK-6; [---●---], CMK11-5).

HIV antigens greater than 50% after 21 days of culture. The less mature CMK-6 cells were somewhat less susceptible to HIV-2 ROD infection, with a lag in appearance of RT in the supernatant. Peak virus production was comparable among the three CMK clones. Similar kinetics of infection were seen with the HIV-2 DOU strain (Fig 1).

CMK megakaryocytic cells were significantly less susceptible to infection with the HIV-1 isolates (IIIB, Ba-L, 9533) despite challenge of the three different CMK clones with high titer virus inocula (10^4 $TCID_{50}$). Productive infection was barely detectable by RT or IFA. Cultures were sustained for more than 28 days without a change in HIV-1 production (data not shown). In some experiments, uninfected H9 T-lymphoid cells were added to HIV-1 IIIB infected CMK cultures. After cocultivation with H9 T cells, there was a rapid increase in detectable infection after 20 days of cocultivation (Fig 2).

To determine whether HIV entered CMK cells via the CD4 surface structure, we challenged CMK cells with HIV-2 ROD in the presence of anti-Leu3a MoAb, which binds at or near the HIV binding site on CD4. There was no detectable infection of CMK cells with HIV-2 ROD in cultures containing the anti-Leu3a MoAb (5 μg/mL). Similarly, addition of soluble recombinant CD4 at concentrations of 100 μg/mL completely inhibited productive infection of CMK cells by HIV-2 ROD (Fig 3)

Infection of CMK cells with HIV-2 ROD led to reduced expression of the surface CD4 and HLA-DR antigens, but no apparent change in expression of the megakaryocytic markers GP Ib or GP IIb/IIIa or the erythroid marker

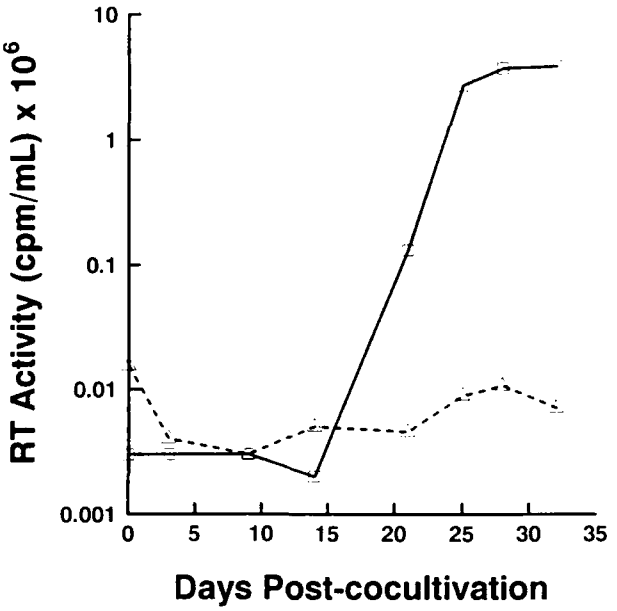


Fig 2. Recovery of HIV-1 IIIB from CMK cells after cocultivation with noninfected H9 T cells. CMK cells (2×10^5/mL) were initially challenged with HIV IIIB (10^4 $TCID_{50}$). Three months after challenge CMK cells were cocultivated with noninfected H9 cells (2×10^5/mL). RT activity was monitored at 3- to 4-day intervals. Symbols: (□), CMK cells challenged with HIV-1 IIIB and later cocultivated with H9 uninfected cells; (△), CMK cells challenged with HIV-1 IIIB without cocultivation.

glycophorin A (Table 2). There was no significant loss of cell viability or gross cytopathic effect in CMK cells after HIV infection.

DISCUSSION

The megakaryocytic cell line, CMK, was highly susceptible to infection with the HIV-2 ROD and HIV-2 DOU strains in vitro. The CMK cells expressed specific megakaryocytic lineage markers, including the surface platelet GPs Ib and IIb/IIIa, and GF-1 RNA. Like most other permanent cell lines with megakaryocytic potential, ie, HEL, LAMA-84, and Dami,[27,28] CMK cells also expressed glycophorin A, an erythroid-specific protein. This suggests that megakaryocyte precursors and erythrocyte precursors may be derived from a common progenitor.[28] Although the surface phenotype of the megakaryocyte and megakaryocyte progenitor have not been extensively investigated in humans, CFU-Meg (colony-forming units-megakaryocyte) bear the HLA-DR antigen and certain myeloid antigens.[29] CMK cells appear to have the phenotype of early megakaryocyte progenitors.

Our studies with the CMK cell line provide data supporting the susceptibility of cells of megakaryocytic lineage to HIV infection. The initial study of Zucker-Franklin and Cao,[14] which used in situ hybridization techniques, showed HIV-1 RNA in marrow megakaryocytes from HIV-infected patients. This indicated the likelihood of in vivo infection of megakaryocytic cells. The subsequent report by Basch et al[16] of surface CD4 expression on mature bone marrow megakaryocytes also suggested that megakaryocytic cells would be susceptible to HIV infection, because the CD4 structure is the major receptor for HIV infection in T lymphocytes and monocyte-macrophages.[6-9] More recently, Zucker-Franklin et al,[30] using electron microscopy, identified HIV-1 particles in human megakaryocytes and platelets after in vitro incubation with virus. These three prior studies, and our work presented here, provide data using different methodologies and indicate HIV infection of cells of megakaryocytic lineage.

The high degree of susceptibility of CMK cells to infection with HIV-2 ROD and HIV-2 DOU compared with minimally detectable productive infection with three different HIV-1 strains suggests that there may be viral

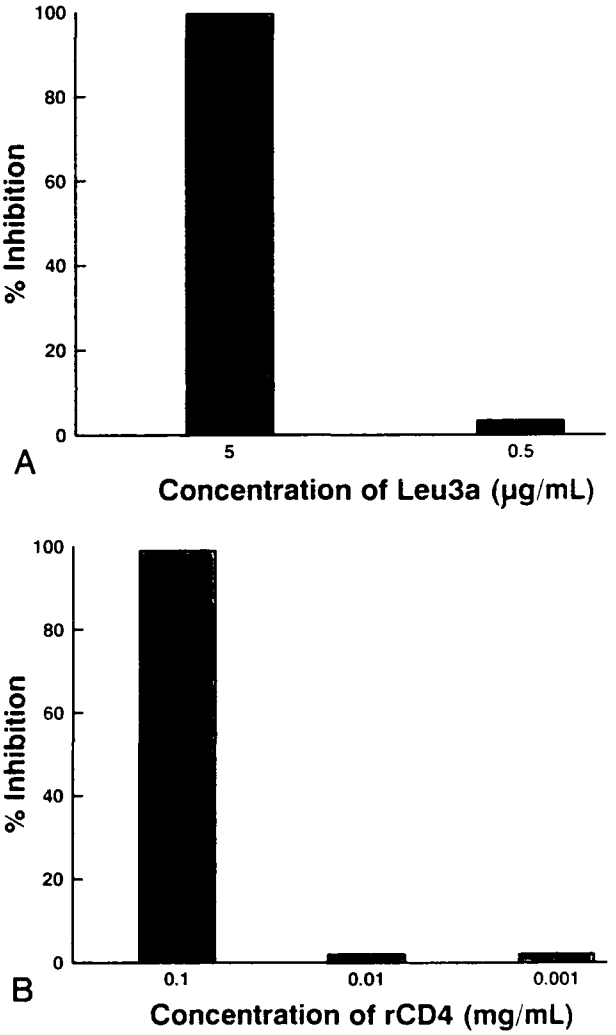


Fig 3. Inhibition of HIV-2 ROD infection of CMK cells by anti-Leu3a (A) and rCD4 (B). HIV-2 ROD virus, 100 $TCID_{50}$, in 60 μL was mixed with 60 μL of anti-Leu3a MoAb or rCD4, and incubated at 4°C for 1 hour. Then, 60 μL of CMK cells (5×10^4/mL) was added and incubated at 37°C for 1 hour. One hundred fifty microliters of this mixture was transferred to 2 mL of complete media and cultured in 24-well plates at 37°C in 5% CO_2. RT activity was measured on the seventh day. Control cultures consisted of mock infected CMK cells. Indicated concentrations of anti-Leu3a and rCD4 represent final concentration. Each experiment was performed in duplicate.

Table 2. Cytometric Analysis of CMK Cells: Effects of HIV-2 ROD Infection

MoAb	Before Infection % Positive Cells	After Infection* % Positive Cells
GP Ib (CD42b)	1.7† (1.3)	2.3 (0.9)
GP IIb/IIIa (CD41)	46.9 (9.6)	61.2 (7.9)
Glycophorin A	34.2 (3.3)	40.1 (14.5)
Leu3a (CD4)	83.6 (2.9)	1.4 (0.6)
HLA-DR	36.8 (5.1)	7.5 (0.5)

Values in parenthesis represent the standard error (SE).

*Analyses were performed 1 month after infection.

†Values represent the averages of two separate experiments.

tropism for megakaryocytic cells. HIV tropism is well described for infection of monocyte-macrophages versus T lymphocytes in vitro.[31,32] Of clinical interest, monocyte tropic HIV isolates have frequently been derived from AIDS patients with central nervous system degeneration.[31] HIV-1 IIIB is a T-lymphotropic isolate while HIV-1 BaL[24,32] and 9533 (Groopman J: unpublished data, March 1990) are monocyte tropic isolates. HIV-2 ROD propagated in our laboratory and used in these studies can infect both T cells and monocyte-macrophages. The issue of tropism of HIV isolates for megakaryocytic cells merits further work. Our initial studies suggest that less than 1 in 10^5 CMK cells was infected by HIV-1 IIIB using the polymerase chain reaction method (M. Sakaguchi: unpublished data, January 1990). Current data indicate that early events such as virus binding, entry, and/or uncoating are important determinants of HIV tropism in T cells and monocytes.[33,34] We are currently studying such early steps in HIV infection of CMK cells.

The different susceptibility of CMK cells to HIV-1 and HIV-2 could be explained by their different gene structure. For example, the VPX gene in HIV-2, not present in HIV-1, might contribute to this different susceptibility, although the effect of the VPX protein on infectivity has been controversial.[35,36] Furthermore, it would be of interest to obtain isolates of HIV-1 from patients with thrombocytopenia or bone marrow failure states, and determine if they are more capable of infecting megakaryocytic cells than HIV-1 IIIB, BaL, and 9533.

The observations reported here provide evidence for HIV infection of megakaryocytic cells, a possibility suggested by the prior in situ hybridization and ultrastructural studies and the finding of surface CD4 antigen on bone marrow megakaryocytes.[15,16,30] Because of the difficulties in obtaining highly purified populations of bone marrow megakaryocytes for in vitro study, it may be that pathologic evidence, provided by in situ hybridization or other studies, will form the major basis for determining in vivo infection.

The availability of megakaryocytic cell lines like CMK provides an in vitro model to study the effects of HIV on megakaryocyte function. Such studies may broaden our understanding of the pathogenesis of dysregulated hematopoiesis after infection with this retrovirus.

REFERENCES

1. Scadden DT, Zon LI, Groopman JE: Pathophysiology and management of HIV-associated hematologic disorders. Blood 74:1455, 1989

2. Spivak JL, Bender BS, Quinn TC: Hematologic abnormalities in the acquired immune deficiency syndrome. Am J Med 77:224, 1984

3. Castella A, Croxson TS, Mildvan D, Witt DH, Zalusky R: The bone marrow in AIDS: A histologic, hematologic, and microbiologic study. Am J Clin Pathol 84:425, 1985

4. Spivak JL, Selonick SE, Quinn TC: Acquired immune deficiency syndrome and pancytopenia. JAMA 250:3084, 1983

5. Treacy M, Lai L, Costello C, Clark A: Peripheral blood and bone marrow abnormalities in patients with HIV-related disease. Br J Haematol 65:289, 1987

6. Popovic M, Read-Connole E, Gallo RC: T4 positive human neoplastic cell lines susceptible to and permissive for HTLV-III. Lancet 2:1472, 1984

7. Klatzmann D, Champagne E, Chamaret S, Gruest J, Guetard D, Hercend T, Gluckman JC, Montagnier L: T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature 312:767, 1984

8. Klatzmann D, Barre-Sinoussi F, Nugeyre MT, Douguet C, Vilmer E, Griscelli C, Brun-Vezinet F, Rouzioux C, Gluckman JC, Chermann JC, Montagnier L: Selective tropism of lymphadenopathy associated virus (LAV) for helper inducer T lymphocytes. Science 225:59, 1984

9. Folks TM, Benn S, Rabson A, Theodore T, Hoggan MD, Martin M, Lightfoote M, Sell K: Characterization of a continuous T-cell line susceptible to the cytopathic effects of the acquired immunodeficiency syndrome (AIDS)-associated retrovirus. Proc Natl Acad Sci USA 82:4539, 1985

10. Folks TM, Kessler SW, Orenstein JM, Justement JS, Jaffe ES, Fauci AS: Infection and replication of HIV-1 in purified progenitor cells of normal human bone marrow. Science 242:919, 1988

11. Scadden DT, Zeira M, Woon A, Schieve L, Ikeuchi K, Lim B, Groopman JE: HIV infection of human bone marrow stromal fibroblasts. Blood 76:317, 1990

12. Hymes KB, Greene JB, Karpatkin S: The effect of azidothymidine on HIV-related thrombocytopenia. N Engl J Med 318:516, 1988

13. Gottlieb MS, Wolfe PR, Chafey S: Case report: Response of AIDS related thrombocytopenia to intravenous and oral azidothymidine (3'-Azido-3'deoxythymidine). AIDS Res Hum Retroviruses 3:109, 1987

14. Pottage JC, Benson CA, Spear JB, Landy AL, Kessler HA: Treatment of human immunodeficiency virus-related thrombocytopenia with zidovudine. JAMA 260:3045, 1988

15. Zucker-Franklin D, Cao Y: Megakaryocytes of human immunodeficiency virus infected individuals express viral RNA. Proc Natl Acad Sci USA 86:5595, 1989

16. Basch R, Kouri Y, Karpatkin S: Human megakaryocytes have a CD4 receptor on their surface. Blood 74:767, 1989 (abstr)

17. Sato T, Fuse A, Eguchi M, Hayashi Y, Ryo R, Adachi M, Kishimoto Y, Teramura M, Mizoguchi H, Shima Y, Komori I, Sunami S, Okimoto Y, Nakajima H: Establishment of a human leukemic cell line (CMK) with megakaryocytic characteristics from a Down's syndrome patient with acute megakaryoblastic leukemia. Br J Haematol 72:184, 1989

18. Komatsu N, Suda T, Moroi M, Tokuyama N, Sakata Y, Okada M, Nishida T, Hirai Y, Sata T, Fuse A, Miura Y: Growth and differentiation of a human megakaryoblastic cell line, CMK. Blood 74:42, 1989

19. Martin DI, Tsai SH, Orkin SH: Increased gamma globin

expression in a nondeletion HPFH mediated by an erythroid-specific DNA-binding factor. Nature 338:435, 1989

20. Tsai SH, Martin DI, Zon LI, D'Andrea AD, Wang GG, Orkin SH: Cloning of cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells. Nature 339:446, 1989

21. Gallo RC, Salahuddin SZ, Popovic M, Shearer GM, White M, Foster P, Markham PD: Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science 224:500, 1984

22. Clavel F, Guetard D, Brun-Vazinet F, Chamaret S, Rey M, Santos-Ferreia AD, Laurent AG, Dougnet C, Kattama C, Rauzioux C, Klatzmann D, Champalimaud JL, Montagnier L: Isolation of a new retrovirus from West African patients with AIDS. Science 233:343, 1986

23. Vujcic LK, Shepp DH, Klutch M, Wells MA, Hendry RM, Wittek AE, Krilov L, Quinnan GV: Use of a sensitive neutralization assay to measure the prevalence of antibodies to the human immunodeficiency virus. J Infect Dis 157:1047, 1988

24. Gardner S, Markovits P, Markovitz DM, Kaplan MH, Gallo RC, Popovic M: The role of mononuclear phagocytes in HTLV-III/LAV infection. Science 233:215, 1986

25. Byrn RA, Sekigawa I, Chamow SM, Johnson JS, Gregory TJ, Capon DJ, Groopman JE: Characterization of in vitro inhibition of human immunodeficiency virus by purified recombinant CD4. J Virol 63:4370, 1989

26. Groopman JE, Benz PM, Ferriani RA, Mayer K, Allan JD, Weymouth LA: Characterization of serum neutralization response to the human immunodeficiency virus (HIV). AIDS Res Hum Retroviruses 3:71, 1987

27. Hoffman R: Regulation of megakaryopoiesis. Blood 74: 1196, 1989

28. Greenberg SM, Rosenthal DS, Greely TA, Tantravahi R, Handin RI: Characterization of a new megakaryocytic cell line: The Dami cell. Blood 72:1968, 1988

29. Janowska-Wieczorek A, Mannoni P, Turner AR, McGann LE, Shaw ARE, Turc JH: Monoclonal antibody specific for granulocytic lineage cells and reactive with human pluripotent and committed haematopoietic progenitor cells. Br J Haematol 58:159, 1984

30. Zucker-Franklin D, Seremetis S, Zeng ZY: Internalization of human immunodeficiency virus type 1 and other retrovirus by megakaryocytes and platelets. Blood 75:1920, 1990

31. Koyanagi Y, Miles S, Mitsuyasu RT, Merrill JE, Vinters HV, Chen ISY: Dual infection of the central nervous system by AIDS viruses with distinct cellular tropisms. Science 236:819, 1987

32. Perno CF, Yarchoan R, Cooney DA, Hartman NR, Gartner S, Popovic M, Hao Z, Gerrard TL, Wilson YA, Jonson DG, Broder S: Inhibition of human immunodeficiency virus (HIV-1/HTLV-III Ba-L) replication in fresh and cultured human peripheral blood monocyte-macrophages by azidothymidine and related 2', 3'-dideoxy nucleosides. J Exp Med 168:1111, 1988

33. Cordonnier A, Montagnier L, Emerman M: Single amino-acid changes in HIV envelope affect viral tropism and receptor binding. Nature 340:571, 1989

34. Kim S, Ikeuchi K, Groopman JE, Baltimore D: Factors affecting cellular tropism of HIV. J Virol 1990 (in press)

35. Henderson LE, Sowder RE, Copeland TD, Benveniste RI, Oroszland S: Isolation and characterization of novel protein (X orf product) from SIV and HIV-2. Science 241:199, 1988

36. Guyader M, Emerman M, Montagnier L, Peden K: Vpx mutants of HIV-2 are infectious in established cell lines but display a severe defect in peripheral blood lymphocytes. EMBO J 8:169, 1989

JOURNAL OF VIROLOGY, Oct. 1991, p. 5448–5456
0022-538X/91/105448-09$02.00/0
Copyright © 1991, American Society for Microbiology
Vol. 65, No. 10

Alternative Pathway for Induction of Human Immunodeficiency Virus Gene Expression: Involvement of the General Transcription Machinery

MAMORU SAKAGUCHI,[1] BEATRICE ZENZIE-GREGORY,[2] JEROME E. GROOPMAN,[1] STEPHEN T. SMALE,[2] AND SUNYOUNG KIM[3]*

Divisions of Hematology/Oncology[1] and Infectious Diseases,[3] New England Deaconess Hospital, and Harvard Medical School, Boston, Massachusetts 02215, and Howard Hughes Medical Institute and Department of Microbiology and Immunology, University of California-Los Angeles School of Medicine, Los Angeles, California 90024[2]

Received 8 May 1991/Accepted 16 July 1991

Human immunodeficiency virus type 1 (HIV-1) is viable and mitogen inducible in the absence of its binding sites for the inducible transcription factor NF-κB. We have investigated alternative mechanisms for induction of HIV-1 transcription. Using transient transfection assays, we found that transcription from an HIV-1 LTR containing mutant κB sites was activated 10- to 20-fold in a variety of human cell types by the phorbol ester phorbol myristate acetate (PMA). The promoter elements conferring this inducibility were localized to the region downstream of nucleotide −70, which contains the TATA and TAR elements and binding sites for transcription factors Sp1 and LBP-1. Synthetic promoters containing only Sp1 sites and a TATA element were also induced in transfection experiments as well as in in vitro transcription experiments with T-cell nuclear extracts. Moreover, promoters containing a TATA box in the absence of Sp1 sites or Sp1 sites in the absence of a TATA box were equally inducible in vitro, as was an RNA polymerase III promoter. The activities of RNA polymerases II and III and of the 38-kDa TATA-binding protein transcription factor IID (TFIID), were not induced by PMA, but electrophoretic mobility shift assays revealed a highly inducible protein-DNA complex that interacted specifically with the TATA sequence. This protein-DNA complex appeared to be much larger than that found with the 38-kDa human TFIID expressed in bacteria. Taken together, these data suggest that a component of the general transcription machinery, and possibly a TFIID-associated protein, is induced in T cells by PMA. This induction may be important for augmenting HIV expression and for the pathogenesis of AIDS.

Gene expression of human immunodeficiency virus type 1 (HIV-1) is regulated both by cellular factors and by its own viral gene products, including the *tat* and *rev* proteins (reviewed in references 10 and 29). One of the major routes for modulating HIV-1 expression appears to be through the control of transcription initiation from the long terminal repeat (LTR). Transcriptional control sequences in the LTR interact with several cellular DNA-binding proteins, including Sp1, NF-κB, LBP-1, NFAT-1, and the TATA-binding protein, transcription factor IID (TFIID) (reviewed in reference 16). Indeed, genetic and virologic studies of HIV strains containing mutations in the binding sites for some of these proteins have confirmed that they influence viral growth and replication (13a, 21, 25).

The accepted model of viral kinetics in HIV-infected individuals predicts that during the early phase of infection, virus replicates rapidly in the absence of antibodies to HIV, causing an early viremia (reviewed in reference 22). This stage is followed by a long period of latency, clinically characterized as the asymptomatic phase, during which virus production is very low or undetectable. In general, the progression to AIDS coincides with an increase in virus titer in the peripheral blood. It is not clear whether this is caused by a sudden insurgence of virus in vivo or whether the dramatic rise in virus titer is due to a weakening of the immune system. Whatever the mechanism, it appears that there is a tight correlation between viral replication and the development of AIDS. Therefore, study of the transition from nonproductive to productive (or low- to high-level) replication is important for understanding how latent virus is activated and thus for unraveling the pathogenetic mechanism of HIV. For this reason, identification and characterization of factors that activate viral gene expression, especially upon cellular induction, have been major components of the molecular studies of HIV.

Among the many cellular transcription factors, NF-κB (20, 40) has been shown to be a potent activator of HIV transcription and to be highly inducible in T cells stimulated with a variety of agents, including phorbol ester (20, 27). Therefore, it has been proposed that NF-κB or related proteins (4) might be involved in the activation of dormant HIV-1 provirus in vivo, resulting in the transition from latent to productive infection. However, it has recently been shown that the DNA sequence elements in the LTR that interact with NF-κB sites are not essential for the growth or infectivity of HIV in certain cell lines and that virus lacking these sites could still be induced by mitogen (13a, 21, 25). These studies suggest that factors other than NF-κB play a role in induction of HIV expression.

In this report, we have examined the possibility that inducible cellular factors other than NF-κB might regulate HIV transcription. Our data demonstrate that transcription from an LTR containing mutated or no κB sites was highly induced by treatment of cells with phorbol ester. The induced activator appears to be a component of the general

* Corresponding author.

transcription machinery and possibly a protein that interacts with either the TATA box or TFIID.

MATERIALS AND METHODS

Plasmids and construction of LTR mutants. The wild-type LTR-chloramphenicol acetyltransferase (CAT) plasmid, p938, contains the HIV-1 LTR from pUR-III (34) and was described previously (19). Plasmid p942 is identical to p938 but contains point mutations at both of the κB sites of the LTR. This was constructed by replacing the *Bgl*II fragment of p938 with that of pUR-III containing mutations at both κB enhancer sites (19, 27). The *tat* expression vector, pCMV-Tat, was provided by D. Trono (Salk Institute) and consists of the first exon of *tat* on a *Sal*I-*Kpn*I fragment from HXB2 cloned into the polylinker linker site (*Xho*I) of the expression vector pCPLK (45).

Deletion mutations were constructed by standard methods as described by Maniatis et al. (26). All plasmids used in these experiments had an SP65 backbone and were identical except for various mutations or deletions described. To construct deletion derivatives, *Taq*I (−120), *Hae*III (−70), *Ban*II (−46), and *Pvu*II (−20) sites of the LTR region were converted to *Bam*HI sites, and then *Bam*HI-*Hin*dIII fragments of the LTR were ligated into the *Bgl*II-*Hin*dIII backbone of p942. Therefore, the 5′ endpoint of all of the deletions is the *Bgl*II site (−488) of the HIV LTR.

The CAT plasmids containing the herpes simplex virus *tk* gene TATA box with or without multimerized Sp1-binding sites were described previously (7) (obtained from Al Courey, University of California, Los Angeles).

Plasmids IV, V, and VI were described previously (42, 43). (The inductions shown with plasmids V and VI were also observed with similar plasmids that delete a binding site for transcription factor AP-1, which is located adjacent to the Sp1 sites in V and VI. This single AP-1 site did not influence the levels of either uninduced or induced transcription in vitro [data not shown]. It was not surprising that a single AP-1 site had no influence on the strong in vitro transcription activated by five Sp1 sites. However, this result served as an important control because it also ruled out the possibility that a cryptic AP-1 site elsewhere in the plasmid was influencing the induction observed.)

Transfections and CAT assays. Cells (1×10^7 to 2×10^7) were transfected with 1 μg of HIV LTR-CAT and 2 μg of *tat* expression plasmids (for cotransfection), using DEAE-dextran (13, 44). Cells were grown in RPMI 1640 containing 10% (CMK) or 20% (H9) fetal bovine serum; 12 to 15 h later, half of the cell population was treated with phorbol myristate acetate (PMA; Sigma) at 50 ng/ml. Cell extracts were prepared 15 to 18 h postinduction, and CAT activity was determined according to standard methods (12), using equivalent amounts of protein. The degree of conversion of [^{14}C]chloramphenicol to its acetylated forms was determined by cutting out appropriate spots and quantitating the amount of radioactivity by liquid scintillation.

Gel retardation assays. Cellular extracts were prepared as described by Baeuerle and Baltimore (2). Approximately 15×10^6 cells were harvested, washed with ice-cold phosphate-buffered saline, and transferred to Eppendorf tubes. Cells were then lysed in a *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer containing 20mM (HEPES; pH 7.9), 0.35 M NaCl, 20% glycerol, 1% Nonidet P-40, 1 mM $MgCl_2$, 1mM dithiothreitol (DTT), 0.5 mM EDTA, 0.1 mM EGTA, 1% aprotinin (Sigma), and 1 mM phenylmethylsulfonyl fluoride. After lysis and extraction for 10 min on ice, particulate material was removed by centrifugation (Microfuge) for 15 min at 4°C. Amounts of protein in the resulting supernatants were quantitated by using bicinchoninic acid (Micro BCA protein assay reagent 23235; Pierce).

Two binding conditions for the electrophoretic mobility shift assay (EMSA) were used. For Sp1 and the inducible TATA factor(s), the binding reaction mixture contained, in a total volume of 15 μl, 3 μg of poly(dI-dC), the radioactive probe (5,000 to 10,000 cpm in Tris-EDTA), and 5 to 10 μg of cellular extracts (generally 0.5 to 1 μl) in the binding buffer (10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1 mM DTT, 20 μg of bovine serum albumin, 1 mM EDTA ([pH 8.5], 5% glycerol) (1). After incubation for 30 min at room temperature, samples were loaded onto 5% polyacrylamide gels and run with 0.5× TBE (0.045 M Tris, 0.045M borate, 0.001 M EDTA) at 150 V for 1.5 to 2 h. For purified TFIID, binding was carried out according to the method of Kao et al. (18). Basically, the reaction mixture contained, in a total volume of 15 μl, 3 μg of poly(dI-dC), the radioactive probe (5,000 to 10,000 cpm in Tris-EDTA), and 5 to 10 μg of cellular extracts (generally 0.5 to 1 μl) in the binding buffer (12 mM HEPES [pH 7.9], 10% glycerol, 5 mM $MgCl_2$, 60 mM KCl, 1 mM DTT, 50 μg of bovine serum albumin, 0.5 mM EDTA, 0.05% Nonidet P-40). After incubation for 30 min at 30°C, the binding reaction mixtures were loaded onto 5% polyacrylamide gels and run with 0.5× TBE at 100 V for 2 to 3 h.

Radioactive probes were prepared from oligonucleotides as described by Sen and Baltimore (40). The nucleotide sequences of the oligonucleotides (synthesized by Genosys, The Woodlands, Tex.) used were as follows: wild-type Sp1 oligonucleotide:

```
GATCTGCCTGGGCGGGACTGGGGAGTGGCG
    ACGGACCCGCCCTGACCCCTCACCGCCTAG
```

mutant Sp1 oligonucleotide:

```
GATCTGCCTGTTCGGGACTGGTTAGTGGCG
    ACGGACAAGCCCTGACCAATCACCGCCTAG
```

wild-type TATA oligonucleotide:

```
GATCTGCATATAAGCAG
    ACGTATATTCGTCCTAG
```

mutant TATA oligonucleotide:

```
GATCTGCAGAGAAGCAG
    ACGTCTCTTCGTCCTAG.
```

In vitro transcription reactions and analysis of RNA polymerase and TFIID activities. Extract preparation and in vitro transcription reactions were as described previously (42, 43). A total of 10^9 CEM T cells (at 5×10^5 cells/ml) were induced for 24 h with PMA (50 ng/ml) in 1-liter roller bottles. A similar number of cells were not induced. Extracts were prepared simultaneously. Protein pellets following ammonium sulfate precipitation were suspended in 2.4 ml of HM.1 (25 mM HEPES [pH 7.9], 20% glycerol, 12.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 100 mM KCl) and dialyzed against HM.1. Protein concentrations were determined by Bradford assay and were found to be 1.8 mg/ml for the uninduced extract and 2.3 mg/ml for the induced extract. Equal amounts of protein were used for each comparison. TFIID activity was heat inactivated by the method of Nakajima et al. (28). HeLa TFIID was partially purified by chromatography on phosphocellulose as described previously (38). RNA polymerase III transcription with the adenovirus VAI gene (plasmid pVAI) was performed by the method of Clark and Dasgupta (6), using a runoff transcription assay with

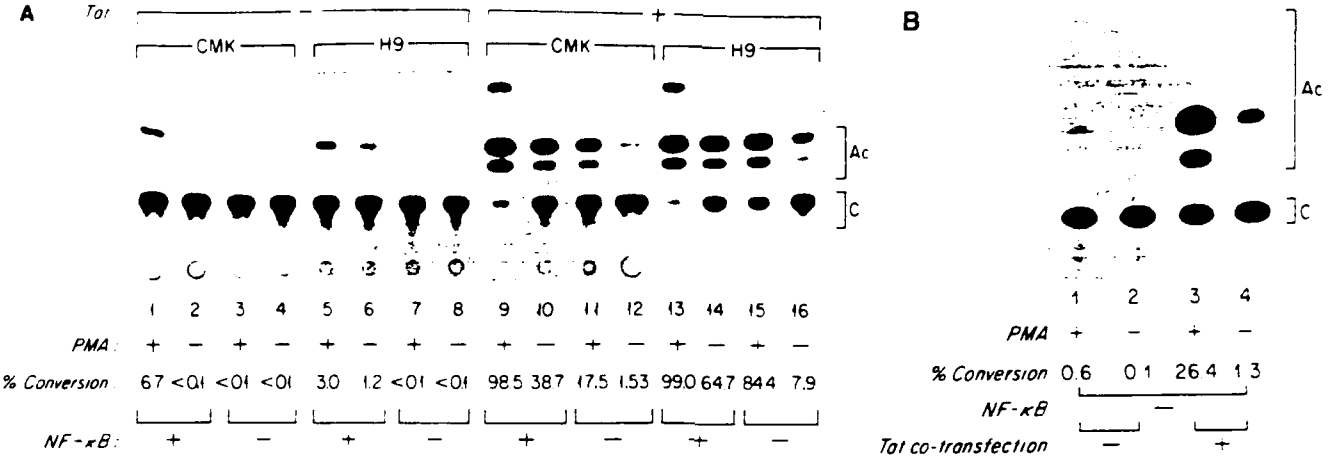


FIG. 1. Induction of HIV-1 transcription in the absence of binding sites for NF-κB. (A) CMK or H9 cells were transfected with HIV-1 LTR-CAT constructs containing wild type (+) or mutated (−) κB sites in the presence (+) or absence (−) of a *tat* expression vector. One half of each transfected population was induced with PMA (50 ng/ml) for 15 to 18 h (+ lanes). CAT activity was measured by determining the amount of acetylated chloramphenicol (AC) produced from [^{14}C]chloramphenicol (C). The values shown are from one representative of at least three independent assays (also see Fig. 2). (B) CMK cells were transfected and induced as described above. When cells were transfected only with the HIV LTR-CAT plasmid containing mutated κB sites, 100 μg of protein was incubated with [^{14}C]chloramphenicol for 3 h at 37°C (lanes 1 and 2); 40 μg of protein was incubated for 40 min when a *tat* expression vector was cotransfected (lanes 3 and 4).

[α-^{32}P]GTP. The RNA product of 247 nucleotides was analyzed by electrophoresis on an 8% denaturing acrylamide gel. Western immunoblot analysis of TFIID was performed with a rabbit anti-human TFIID primary antibody, a biotinylated anti-rabbit immunoglobulin G secondary antibody, and then peroxidase-conjugated streptavidin (BioGenex, Inc.). Chemiluminescence was detected with the Amersham ECL gene detection reagent. Nonspecific RNA polymerase activities were determined by the method of Schwartz et al. (39), using α-amanitin (Sigma) at 2 μg/ml to inhibit RNA polymerase II activity and at 100 μg/ml to inhibit RNA polymerase III.

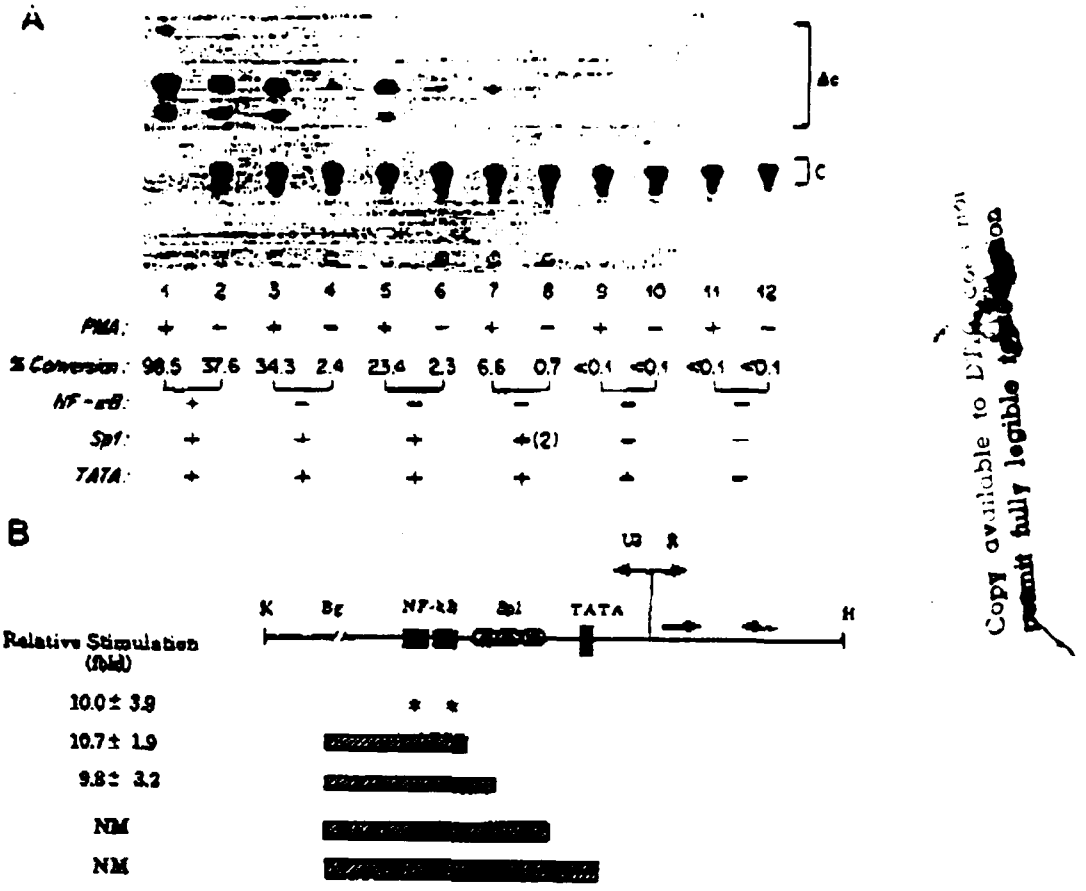
RESULTS

Induction of HIV-1 transcription in the absence of binding sites for NF-κB. To investigate NF-κB-independent mechanisms for induction of HIV-1 transcription, we performed transient transfections with HIV LTR-CAT fusion constructs containing wild-type or mutated κB sites (Fig. 1A). It previously was shown that the mutations used in these constructs abolish NF-κB binding (27). After transfection into the human T-lymphoid cell line H9 (31) or the human megakaryocytic cell line CMK (36), both constructs resulted in low or undetectable levels of CAT activity (Fig. 1A, lanes 2, 4, 6, and 8). However, treatment with the phorbol ester PMA following transfection induced readily detectable CAT activity with the wild-type but not the mutant LTR construct (lanes 1, 3, 5, and 7).

As expected, cotransfection with a *tat* expression vector resulted in a dramatic increase in CAT activity from both constructs, although the mutations in the κB sites significantly decreased overall promoter strength (Fig. 1A; compare lanes 2 and 10 and lanes 6 and 14 for the wild type and lanes 4 and 12 and lanes 8 and 16 for the mutant construct). Treatment with PMA following cotransfection with *tat* produced higher levels of CAT activity with the wild-type construct (lanes 9 and 13) and also increased the level of expression from the mutant LTR (lanes 11 and 15). This induction, of at least 10- to 20-fold, was observed in other human cell lines tested, including the T-lymphoid cell lines CEM and the monocytic cell line U937 (data not shown). In the T-lymphoid line Jurkat, which is frequently used for studies of HIV expression, the degree of induction was marginal. This increase in LTR activity, despite the absence of functional κB sites, implied that factors other than NF-κB were induced in these cells by treatment with PMA.

***tat* is not involved in the observed transcriptional induction.** It has been reported that transactivation of the LTR by *tat* requires protein kinase C, raising the possibility that the increase in CAT activity of the mutant LTR might be due simply to an increase in *tat* activity rather than to the induction of cellular transcription factors (15). However, because we treated the transfected cells with PMA (50 ng/ml) for relatively short periods (16 to 18 h), *tat* is unlikely to be responsible. Under these conditions, *tat* transactivation of the LTR has been reported to be unaffected by PMA (15). To confirm this hypothesis, we tested whether CAT activity was induced when cells were transfected only with the mutant LTR-CAT construct and without a *tat* expression vector. Because the level of CAT activity was very low in these circumstances, we used higher amounts of protein than usual and extended reaction times for the CAT enzyme assay (Fig. 1B, lanes 1 and 2). PMA treatment of cells transfected with only the mutant LTR construct increased CAT activity by at least sixfold. (The background level of activity usually resulted in 0.1% conversion of [^{14}C]chloramphenicol to its acetylated forms with our experimental conditions. Thus, the induction actually may be greater than sixfold.) This result demonstrates that the induction observed was not a result of increased *tat* activity, although a minor influence of *tat* cannot be ruled out. This conclusion is supported by the results presented below.

Localization of the *cis*-acting element conferring PMA inducibility. To localize the DNA sequence element responsive to PMA in the κB mutant plasmid, deletions of the LTR were tested for their abilities to direct PMA-inducible transcription (Fig. 2A). Deletion of the nucleotide sequences upstream from the κB sites did not significantly affect inducibility (data not shown). Mutations or complete dele-



Copy available to DTIC does not permit fully legible reproduction

FIG. 2. Localization of the DNA sequence element responsive to phorbol ester. (A) Each mutant DNA (1 μg) was cotransfected into CMK cells with 2 μg of rat expression plasmid, treated with PMA, and subjected to CAT analysis as described for Fig. 1. The autoradiograph shown is one representative transfection, and the absence (−) or presence (+) of the major transcription elements of the HIV LTR such as κB sites, Sp1 sites, and TATA box is indicated (also see Fig. 2B). There are three Sp1-binding sites in the HIV LTR, and +2 indicates the deletion of one Sp1 site (the most distal from the mRNA start site). (B) Sequences deleted from the LTR are indicated by the shading. The 5′ endpoint of all of the deletions is the BglII site of the LTR, and their 3′ endpoints are as described below. Asterisks indicate point mutations introduced into the κB sites as described by Nabel and Baltimore (27). NM, the magnitude of stimulation could not be calculated because of undetectable levels of CAT activity. The relative stimulation (fold) is the ratio of CAT conversion following PMA induction to that without induction. Means and standard deviations are based on three or more independent transfection assays.

tion of the κB sites significantly lowered LTR activity relative to the wild-type plasmid (Fig. 2A; lane 4 for point mutations and lane 6 for deletion), but CAT activity remained highly inducible by PMA (compare lane 2 with lanes 3 and 5). Removal of one of the three Sp1 sites significantly decreased basal CAT activity (compare lanes 6 and 8), but the remaining activity was still inducible by 10-fold (lanes 7 and 8). When all three Sp1 sites were deleted, CAT activity was undetectable (compare lanes 9 and 10). Overall, non-NF-κB factors activated the HIV LTR 10- to 15-fold upon PMA induction (Fig. 2B). Similar results were obtained for CEM and H9 cells (data not shown). This analysis indicates that Sp1 itself or proteins interacting with the nucleotide sequences downstream from the Sp1 sites are induced by PMA.

Sp1 is not induced by mitogen. The minimal region of the LTR responding to PMA induction contained two Sp1 sites, a TATA box, and regions near the transcription start site and within the transcribed leader that are important for HIV-1 expression. None of these regions bind proteins known to be induced by PMA (16). However, it was recently reported that Sp1 can be induced following infection with simian virus 40 (35) and that Sp1 can be phosphorylated (14). To our knowledge, Sp1 has not been tested for inducibility by mitogen. Therefore, we investigated this possibility. We transfected cells with an Sp1 test plasmid containing a CAT gene fused to a promoter incorporating multimerized consensus Sp1-binding sites upstream from the herpes simplex virus *tk* gene TATA box (7). It has previously been shown that the activity of this promoter depends on the presence of Sp1 (7). As a negative control, a similar plasmid lacking the Sp1 sites was used. Both plasmids were transfected into CMK cells, and transient expression of the CAT enzyme was used to measure the inducibility by PMA. In the absence of PMA induction, both plasmids gave a background conversion of chloramphenicol to its acetylated forms of 0.1% (Fig. 3A, lane 2 and 4). In most human lymphoid or myeloid cell lines tested, these promoters resulted in similarly low levels of CAT activity (lane 2). However, when cells were treated with PMA, CAT activity was induced to detectable levels with the test plasmid containing the Sp1 site (Fig. 3A; compare lanes 1 and 2). Only background levels were detected in the absence of the Sp1 sites. Similar results were obtained for the human T-cell line H9. These data suggested

Copy available to DTIC does not permit fully legible reproduction

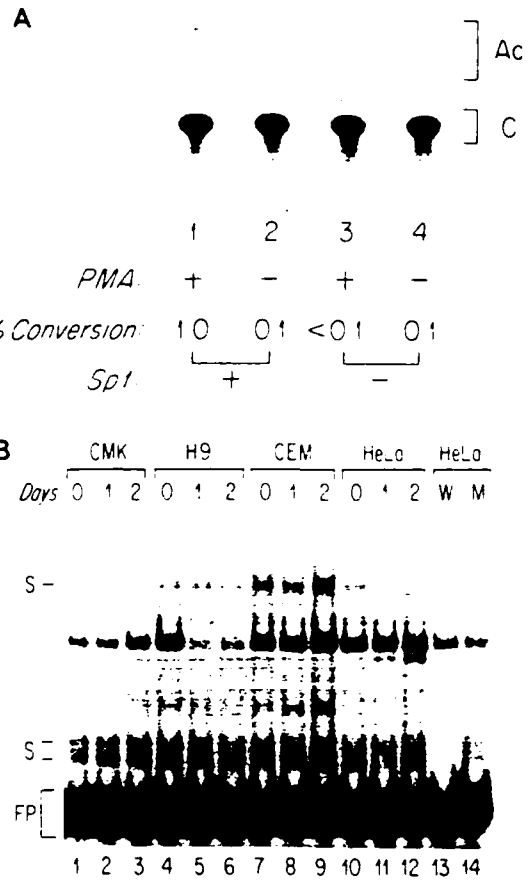


FIG. 3. Effect of PMA induction on Sp1. (A) Transient transfection assay with Sp1 test plasmids. CMK cells were transfected with Sp1 test plasmids carrying the truncated promoter of the herpes simplex virus *tk* gene with (+) or without (−) inserted Sp1-binding-site oligonucleotides. (B) EMSA with ^{32}P-labeled Sp1 oligonucleotide. Nuclear extracts from a variety of human cell lines, either uninduced (day 0) or stimulated (days 1 and 2) with PMA, were incubated with a ^{32}P-labeled oligonucleotide whose sequence was taken from the Sp1-binding sites of the HIV LTR (−70 to −46). Sp1-specific DNA-protein complexes were verified by competition assay which involved incubating the protein sample from lane 10 with unlabeled wild-type (W) or mutated (M) oligonucleotide (lanes 13 and 14). S, specific DNA-protein complex; FP, free probe.

that the PMA induced either Sp1 activity or the activity of a protein required for basal transcription from a TATA box. Also, because this experiment did not involve a *tat* expression vector, it confirmed our observation that induction of transcription from an LTR containing defective κB sites did not result simply from increased *tat* activity.

To test whether Sp1-binding activity was induced by PMA, we performed EMSA. Nuclear extracts from a variety of cell lines, either prior to or following stimulation with PMA, were incubated with a double-stranded ^{32}P-labeled oligonucleotide probe, corresponding to the −70 to −46 region of the HIV-1 LTR. The same sequence has been shown to interact with Sp1 by DNase I footprinting analysis (17). As expected, there were significant basal levels of Sp1 in uninduced CMK, H9, and HeLa cells (Fig. 3B, lanes 1, 4, 7, and 10). Induction of these cells with PMA did not yield significant increases in amounts of electrophoretically retarded DNA-protein complexes. On the contrary, amounts of DNA-protein complexes decreased upon induction in most cell types. This result suggested that the DNA-binding activity of Sp1 was not increased by treatment with phorbol ester. Thus, the transcriptional induction observed might be due to induction of a component of the general transcription machinery.

In vitro analysis of PMA-inducible transcription. In vitro transcription experiments were used to further analyze transcriptional induction by PMA. These experiments were necessary because we could not detect transcription in vivo from a TATA box in the absence of Sp1 sites. Moreover, in vitro analyses allowed us to assay for activities of individual components of the general transcription machinery.

Nuclear extracts were prepared from CEM cells either with or without a 24-h induction by PMA. The protein concentrations of the extracts were similar and were adjusted to equivalence with buffer. In vitro transcription experiments were performed with three different synthetic promoters to define the DNA sequence elements responsible for PMA inducibility. Plasmid V (Fig. 4A, lanes 1 to 4) contained Sp1 sites upstream from a consensus TATA element derived from the adenovirus major late promoter. Plasmid IV (lanes 5 to 8) contained the TATA box in the absence of Sp1 sites but in the presence of a transcriptional initiator (Inr) element at the start site (42). This element enhances transcription from the TATA element and is thought to interact with a known component of the general transcription machinery (e.g., TFIID, TFIIB, or RNA polymerase II [32, 43]). Plasmid VI (lanes 9 to 12) contained the Sp1 sites and the Inr element but lacked a TATA element. Detailed characterization of these promoters and of the proteins required for their activities have been reported (32, 43) (see also Materials and Methods).

Comparison of the activities of these promoters in the CEM nuclear extracts showed that transcription from all three was induced to a similar degree by PMA (Fig. 4A). Laser densitometry revealed that transcription from plasmid V was induced by fivefold, from plasmid IV by fourfold, and from plasmid VI by sixfold. Somewhat stronger inductions were observed with CMK cells, but no inductions were found with HeLa cells (data not shown). These results confirm that Sp1 is not responsible for the observed PMA induction and also demonstrate that a TATA box is not required. However, these results do not rule out the induction of the TATA-binding protein, TFIID, because TFIID is known to be required for transcription from plasmid VI as well as from other promoters that lack TATA elements (32, 43).

Because of the general nature of the PMA inducibility observed, we tested in the CEM extracts the RNA polymerase III-transcribed adenovirus VAI gene (11). We found that specific RNA polymerase III transcription was also strongly induced (ninefold) by PMA in CEM cells (Fig. 4B). These results demonstrate that one or more general factors required for specific transcription by RNA polymerases II and III are induced in PMA-treated T cells. Based on the multiplicity of events that occur in activated T cells (8), these results may not be surprising (see Discussion).

Analysis of general transcription factors. The induction of RNA polymerase II transcription could result from the induction of a number of general transcription factors. These include RNA polymerase II itself or TFIID. Alternatively, other general transcription factors (5), such as TFIIB, TFIIE, or TFIIF, might be induced by PMA. Finally, a general effect on elongation of transcription by RNA polymerase II might be responsible. To begin to identify the

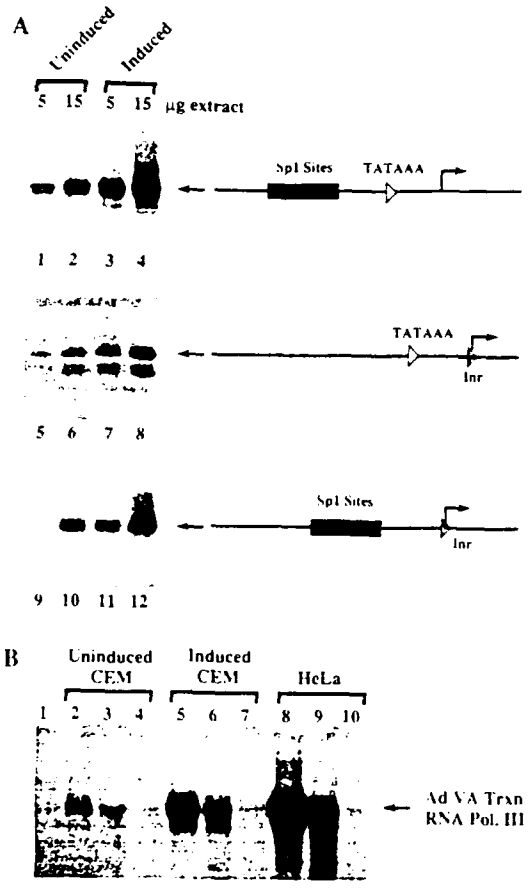


FIG. 4. In vitro analysis of PMA-inducible transcription. (A) Three different plasmids were tested for promoter activity and inducibility in nuclear extracts prepared from CEM cells (lanes 1, 2, 5, 6, 9, and 10) or from CEM cells induced for 24 h with PMA (lanes 3, 4, 7, 8, 11, and 12). Characterization of these plasmids was described previously (43) (see also Materials and Methods), and the promoters contain either Sp1 sites and a TATA box (plasmid V; lanes 1 to 4), a TATA box and an Inr element (plasmid IV; lanes 5 to 8), or Sp1 sites and an Inr element (plasmid VI; lanes 9 to 12). Reaction mixtures contained 900 ng of template DNA and 5 or 15 μg of nuclear extract, as indicated. RNA synthesized in vitro was analyzed by primer extension with an SP6 promoter primer. cDNA products were either 79 (lanes 5 to 12) or 70 (lanes 1 to 4) nucleotides. (B) Specific transcription of the adenovirus VAI gene, transcribed by RNA polymerase III (Ad VA Trxn RNA Pol. III), was tested by a runoff transcription assay. The reactions included 300 ng of template DNA and either no extract (lane 1) or 2 μg (lanes 2, 5, and 8), 10 μg (lanes 3, 6, and 9), or 20 μg (lanes 4, 7, and 10) of nuclear extract. Extracts were from untreated CEM cells, PMA-treated CEM cells, or HeLa cells, as indicated. The labeled RNA product was 247 nucleotides and was analyzed on an 8% denaturing polyacrylamide gel.

inducible protein(s), we have begun to analyze the levels and activities of individual components.

We first determined the relative levels of RNA polymerase activities in the uninduced and induced extracts (Table 1). These experiments measured the incorporation of tritiated nucleotides into RNA synthesized from total herring sperm DNA (39). Because the three RNA polymerases efficiently begin transcription from single-stranded nicks and gaps, or from double-stranded breaks in the absence of other general transcription factors, this assay could be used to determine

TABLE 1. Total RNA polymerase activities in uninduced and PMA-induced CEM cells

Prepn	Incorporation of [^{3}H]UTP into RNA (cpm)	
	Uninduced	PMA induced
No protein	180	180
4 μg of CEM extract	1,340	1,250
10 μg of CEM extract	4,170	3,020
10 μg of CEM extract + 2 μg of α-amanitin per ml	3,560	2,750
10 μg of CEM extract + 100 μg of α-amanitin per ml	3,380	2,250

the total RNA polymerase activity within the extracts. The results from duplicate experiments (Table 1) demonstrate that the total RNA polymerase activity from the induced extract was significantly lower (8 to 30%, depending on whether 4 or 10 μg was used) than from the uninduced extract. In addition, inhibition experiments with α-amanitin revealed that the individual activities of RNA polymerases I, II, and III were not significantly different in the two extracts. α-Amanitin at 2 μg/ml is known to specifically inhibit RNA polymerase II activity, and 100 μg/ml inhibits both RNA polymerases II and III. RNA polymerase I is resistant to α-amanitin. Thus, neither RNA polymerase II nor III appears to be responsible for the induced specific transcription observed. These results additionally rule out a general effect on transcriptional elongation, which would have been revealed in this assay as well.

We next tested for the levels of TFIID, the TATA-binding protein (37). As mentioned above, this protein is absolutely required for transcription from promoters that both contain and lack TATA boxes (32, 37, 43). By Western blot analysis with rabbit antisera directed against the human 38-kDa TFIID protein (18, 30) (Fig. 5A), no induction of TFIID protein was observed. Moreover, in vitro transcription experiments showed that the activity of TFIID was not induced (Fig. 5B). Depletion of TFIID activity by heat treatment of the nuclear extracts resulted in strong decreases in transcriptional activity from plasmid IV (Fig. 5B, lanes 2 and 6). When a partially purified HeLa TFIID fraction was added back to the heat-treated extracts, the degree of induction was similar to that before heat treatment (lanes 3, 4, 7, and 8). If the heat-labile TFIID were responsible for the induction, HeLa TFIID would have increased transcription by similar amounts from both the induced and uninduced heat-treated extracts. Thus, the protein responsible for PMA induction did not correspond to the heat-labile TFIID.

PMA induction of a protein or protein complex interacting with the TATA box. Despite the foregoing evidence that TFIID is not induced by PMA, EMSA studies revealed a surprising result. We directly tested by EMSA whether a protein or protein complex interacting with the TATA sequence was induced by PMA. Cellular extracts from human cells (Fig. 6A, lanes 2 to 4) or cloned, 38-kDa human TFIID purified from *Escherichia coli* (lane 1) were incubated with an oligonucleotide probe corresponding to the TATA box (−33 to −20) of the HIV LTR. The bacterially expressed TFIID protein formed a specific band (S2) with the TATA oligonucleotide, whereas crude cellular extracts formed a DNA-protein complex (S1) whose electrophoretic mobility was slower. This result is consistent with a current hypoth-

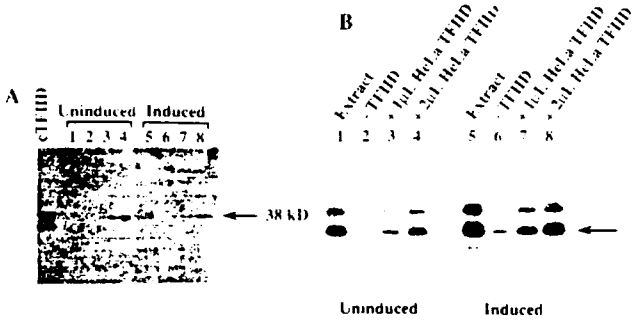


FIG. 5. Analysis of TFIID in uninduced and PMA-induced extracts. (A) The amounts of the 38-kDa TFIID protein in the uninduced and induced CEM extracts was analyzed by Western blot, with rabbit antisera directed against the human TFIID protein expressed in bacteria (see Materials and Methods). The lane labeled cTFIID contains an *E. coli* extract expressing this protein. The largest band corresponds to the full-length protein, and the lower-molecular-weight bands are thought to be degradation products. Increasing amounts of uninduced and induced extract were tested. (B) In vitro transcription experiments with TATA/Inr plasmid IV were performed to measure the TFIID activities in the uninduced and PMA-induced extracts. This experiment was performed by heat-treating the extracts to inactivate the labile TFIID, followed by reconstitution with 1 or 2 μl of partially purified TFIID from HeLa cells, as indicated. If the heat-labile TFIID in the CEM extracts was responsible for the PMA induction, the signal in lane 8 would have been equivalent to the sum of the signals from lanes 4 and 6.

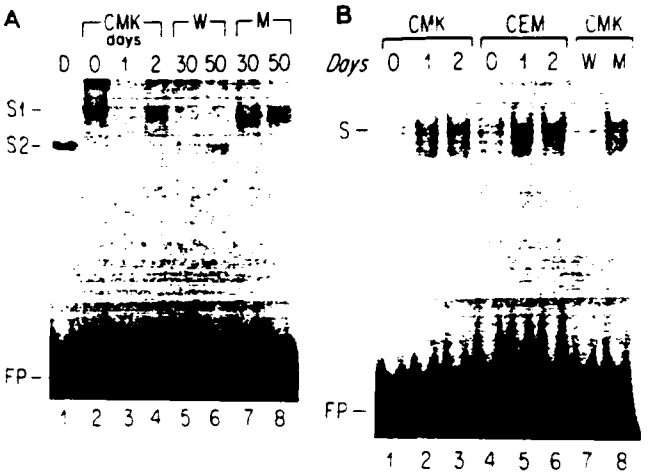


FIG. 6. EMSA with ^{32}P-labeled TATA oligonucleotide. (A) Cloned TFIID purified from *E. coli* (lane 1) and nuclear extracts prepared from CMK cells (lane 2 to 8) were analyzed by EMSA. The cloned TFIID protein forms a single specific band (S2) with the TATA oligonucleotide, whereas crude nuclear extract formed a DNA-protein complex (S1) whose electrophoretic mobility was slower. Specificities of DNA-protein complexes were verified by competition assays, using the protein sample from lane 2 with two different concentrations (30 and 50 ng) of unlabeled wild-type (W) or mutated (M) oligonucleotide. (B) Inducible factors other than TFIID interact with the TATA box. Nuclear extracts from CMK cells, either uninduced (day 0) or stimulated (days 1 and 2) with PMA, were incubated with a ^{32}P-labeled TATA oligonucleotide whose sequence corresponds to the TATA box of the HIV LTR (−33 to −20). Specificities of DNA-protein complexes were verified by competition assays by incubating the protein sample used in lane 2 with unlabeled wild-type (W) or mutated (M) oligonucleotide (lanes 7 and 8).

esis that TFIID may interact with other proteins in vivo (23, 32, 43), slowing down its mobility in gel shift analyses. These complexes were specific for the TATA element because they were abolished by the unlabeled wild-type TATA sequence but not by a mutant oligonucleotide which was identical to the wild-type probe used except for two G's substituting the T's of TATA (lanes 5 to 8). The amounts of these DNA-protein complexes did not increase with PMA induction in most cells that we have tested (lanes 2 to 4). Similar results were obtained with human T-cell lines such as CEM and H9.

We also performed EMSA with different binding conditions (see Materials and Methods), the most significant differences being the inclusion of 5 mM $MgCl_2$ in the binding reaction and incubation of the binding reaction at 30°C rather than at room temperature (Fig. 6B). With these conditions, even high concentrations of cloned TFIID protein did not bind to the TATA oligonucleotide (data not shown). However, an electrophoretically retarded DNA-protein complex was readily observed with crude extracts, and this complex was specifically abolished by the unlabeled wild-type TATA sequence but not by a mutant oligonucleotide (Fig. 6B, lanes 7 and 8). Most interestingly, the amounts of this DNA-protein complex increased more than 10-fold upon treatment of cells with PMA, as determined by laser densitometry analysis (lanes 1 to 3 for CMK and lanes 4 to 6 for CEM). These results suggest that cellular proteins interacting with the TATA sequence are highly inducible and that activation of an LTR containing defective κB sites by phorbol ester might be due to the increase in this factor(s). This protein-DNA complex may or may not contain the 38-kDa TATA-binding protein.

DISCUSSION

alyzed NF-κB-independent pathways for in-/-1 transcription. These studies were necessary because HIV strains with mutations in the binding sites for the inducible transcription factor NF-κB were found to be viable and mitogen inducible (13a, 21, 25). Transient transfection assays in both myeloid and T-cell lines demonstrated that PMA strongly induced transcription from an HIV LTR lacking κB sites. The DNA sequence elements responsive to PMA were localized to the region downstream of nucleotide −70. In vitro transcription experiments suggested that no particular sequence element was responsible for the PMA induction of RNA polymerase II transcription and that specific transcription by RNA polymerase III was also PMA inducible. These results suggest that the activities of components of the general transcription machinery are responsible for the observed inductions. These components do not appear to include an RNA polymerase or the 38-kDa TATA-binding protein, TFIID. However, our studies revealed that a protein or protein complex interacting with the TATA box is highly inducible by PMA. This complex might be responsible for the observed inducible transcription by RNA polymerase II.

Although the data implicated a TATA-associated protein in the PMA induction, we do not know what protein(s) are involved in this protein-DNA complex. It might include the 38-kDa TFIID protein in association with another protein or might instead be a novel TATA-binding protein. The latter possibility is unlikely because no functional alternative TATA-binding proteins have been identified. By contrast, the idea that the 38-kDa TFIID is part of a multisubunit protein or multiprotein complex is already widely accepted

Not only does TFIID from cellular extracts migrate as a very high molecular weight protein when analyzed by gel filtration chromatography (33), but functional studies have demonstrated that the cloned TATA-binding protein could only partially substitute for extensively purified TFIID from HeLa cells (30, 32, 43). Moreover, TFIID is one target for transcriptional transactivation by the adenovirus E1a protein, which recently was found to form a tight complex with TFIID (3). The proteins in the EMSA complex will need to be purified to determine whether TFIID is included, because the purified anti-TFIID immunoglobulin G used for the Western analysis could not inhibit or supershift the EMSA complex observed even with bacterially expressed TFIID (data not shown).

The induction of transcription from a promoter lacking a TATA box is consistent with the hypothesis that a TFIID-associated protein is responsible. It has been established that TFIID is essential for transcription from promoters that lack TATA elements and that subunits in addition to the 38-kDa TATA-binding subunit are likely to be required for complete TFIID function (32, 43).

Although the EMSA data implicated a putative TATA-binding protein in PMA induction, the functionally induced protein could instead be one of the other general transcription factors (5), such as TFIIA, TFIIB, TFIIE, or TFIIF. In addition, although nonspecific RNA polymerase II activity decreased upon induction, a polymerase subunit that is required only for specific initiation might be induced. The RNA polymerase III transcription factor responsible for induction of adenovirus VAI transcription must also be defined. Because nonspecific RNA polymerase III activity was not induced, the most likely candidates are TFIIIB and TFIIIC (reviewed in reference 11).

Our data demonstrating the induction of an LTR with mutated or deleted κB sites was somewhat unexpected in view of previous transfection analyses reporting that similar constructs did not respond to PMA (27). One possible explanation for this discrepancy is that different cell lines were used for the experiments. We have found that the activity of the mutant LTR was indeed very weak and, at best, marginally inducible by mitogen in the human T-lymphoid Jurkat cell line (data not shown), which is widely used for HIV transcriptional studies. Recently, in vitro transcription experiments with extracts from Jurkat cells revealed an approximately threefold PMA-induction of HIV transcription in the absence of the binding sites for NF-κB (24). In our transfection experiments, we have found the mutant HIV LTR to be inducible by mitogen in multiple human cell lines, indicating that our observation is not restricted to a particular cell line.

This induction of the general transcription machinery might not be surprising, considering the events that occur during T-cell activation (reviewed in reference 8). It has been reported that this process induces more than 70 genes. Transcription factors, such as NF-κB, NFAT, c-Fos, and c-Myc, are induced in activated T cells, but a contribution by the general transcription machinery seems reasonable in light of the extent of transcriptional activation. Moreover, induction of RNA polymerase III transcription may be necessary to allow for increased processing and translation of mRNA.

If a component involved in transcription of all protein-coding genes is induced, why is the transcription of some genes not induced? The interleukin-2 (IL-2) gene as well as other genes appear to be unaffected by PMA (8, 41). Possibly, the degree of induction is determined by which transcription factor is rate limiting for each promoter. For example, an increase in a putative TFIID-associated protein may not increase IL-2 transcription upon PMA induction because another required factor, NFAT-1, has not yet been induced (8). This putative general factor may not even be limiting for basal IL-2 transcription in the absence of the NFAT-1 sites.

Our findings are consistent with studies of IL-2 receptor transcription, which is inducible by PMA in T cells (9). The element responsible for this induction has not been easily defined. Although a region containing an NF-κB site has been found in the IL-2 receptor enhancer, no mutation has been found to eliminate induction. It must be considered that these results may be due, at least in part, to the fact that a general transcription factor is PMA inducible.

The observation that our inducible factor(s) interacts with nucleotide sequences downstream from the HIV κB sites has implications for the role of NF-κB in HIV gene expression. The reported 50-fold induction of LTR activity upon PMA treatment has been attributed to an increase in NF-κB activity (27). However, since PMA appears to induce another factor, which we have shown to increase LTR activity by more than 10-fold, a significant portion of this increase might be due to this other inducible factor. The relative contributions of these two factors will need to be addressed with the intact virus in primary cells. It has been found that mutations in the NF-κB sites do not significantly affect virus replication or induction (13a, 21, 25). If a general transcription factor is indeed important for induction, we might expect that no mutations will inhibit induction unless they abolish transcriptional activity.

These possibilities have implications for the pathogenesis of AIDS. We have found NF-κB-independent induction in a variety of cell types, including T cells, megakaryocytes, and early myeloid cells, all of which have been shown to be infected with HIV in vivo. The possible induction of a general RNA polymerase II transcription factor by immune stimulation or cell differentiation might activate virus in infected cells, causing accelerated viral spread or switching of the virus from a latent state to a productive infection.

ACKNOWLEDGMENTS

We thank Al Courey for providing Sp1 test plasmids, Arnold Berk for the TFIID expression vector, Melody Clark for the RNA polymerase III transcription reagents, and Paula Cannon and Sidne Omori for reading the manuscript.

This work was supported by Public Health Service grants AI 30897 (S.K.), AI29847, HL33774, and HL42112 (J.E.G.), by Defense Department contract DMAD 17-90-C-0106 (J.E.G.), and by the Howard Hughes Medical Institute (S.T.S.).

REFERENCES

1. **Baeuerle, P. A., and D. Baltimore.** 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell 53:211–217.
2. **Baeuerle, P. A., and D. Baltimore.** 1988. IκB: a specific inhibitor of the NF-κB transcription factor. Science 242:540–546.
3. **Berk, A. (University of California, Los Angeles).** Personal communication.
4. **Bohnlein, E., J. W. Lowenthal, M. Siekevitz, D. W. Ballard, B. R. Franza, and W. Greene.** 1988. The same inducible nuclear protein regulates mitogen activation of both the interleukin-2 receptor-alpha gene and type 1 HIV. Cell 53:827–836.
5. **Buratowski, S., S. Hahn, L. Guarente, and P. A. Sharp.** 1989. Five intermediate complexes in transcription initiation by RNA polymerase II. Cell 56:549–561.
6. **Clark, M. E., and A. Dasgupta.** 1990. A transcriptionally active form of TFIIIC is modified in poliovirus-infected HeLa cells.

Mol. Cell. Biol. 10:5106–5113.
7. **Courey, A. J., D. A. Holtzman, S. P. Jackson, and R. Tjian.** 1989. Synergistic activation by the glutamine-rich domains of human transcription factor Sp1. Cell 59:827–836.
8. **Crabtree, G. R.** 1989. Contingent genetic regulatory events in T lymphocyte activation. Science 243:355–361.
9. **Cross, S. L., M. B. Feinberg, J. B. Wolf, N. J. Holbrook, F. Wong-Staal, and W. J. Leonard.** 1987. Regulation of the human interleukin-2 receptor α chain promoter: activation of a non-functional promoter by the transactivator gene of HTLV-1. Cell 49:47–56.
10. **Cullen, B. R., and W. C. Greene.** 1989. Regulatory pathways governing HIV-1 replication. Cell 58:423–426.
11. **Geiduschek, E. P., and G. P. Tocchini-Valentini.** 1988. Transcription by RNA polymerase III. Annu. Rev. Biochem. 57: 873–914.
12. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. 2:1044–1051.
13. **Grosschedl, R., and D. Baltimore.** 1985. Cell-type specificity of immunoglobulin gene expression is regulated by at least three DNA sequence elements. Cell 41:885–897.
13a. **Harrich, D., J. Garcia, R. Mitsuyasu, and R. Gaynor.** 1990. TAR independent activation of the human immunodeficiency virus in phorbol ester stimulated T lymphocytes. EMBO J. 9:4417–4423.
14. **Jackson, S. P., J. J. MacDonald, S. Lees-Miller, and R. Tjian.** 1990. GC box binding induces phosphorylation of Sp1 by a DNA-dependent protein kinase. Cell 63:155–165.
15. **Jakobovits, A., A. Rosenthal, and D. J. Capon.** 1990. Transactivation of HIV-1-directed gene expression by *tat* requires protein kinase C. EMBO J. 9:1165–1170.
16. **Jones, K. A.** 1990. HIV trans-activation and transcription control mechanisms. New Biol. 1:127–135.
17. **Jones, K. A., J. T. Kadonaga, P. A. Luciw, and R. Tjian.** 1986. Activation of the AIDS retrovirus promoter by the cellular transcription factor, Sp1. Science 232:755–759.
18. **Kao, C. C., P. M. Lieberman, M. C. Schmidt, Q. Zhou, R. Pei, and A. J. Berk.** 1990. Cloning of a transcriptionally active human TATA binding factor. Science 248:1646–1650.
19. **Kim, S., K. Ikeuchi, J. G. Groopman, and D. Baltimore.** 1990. Factors affecting cellular tropism of human immunodeficiency virus. J. Virol. 64:5600–5604.
20. **Lenardo, M., and D. Baltimore.** 1989. NF-κB: a pleiotropic mediator of inducible and tissue-specific gene control. Cell 58:227–229.
21. **Leonard, J., C. Parrot, A. J. Bukler-White, W. Turner, E. K. Ross, M. A. Martin, and A. B. Rabson.** 1989. The NF-κB binding sites in the human immunodeficiency virus type 1 long terminal repeat are not required for virus infectivity. J. Virol. 63:4919–4924.
22. **Levy, J. A.** 1988. Mysteries of HIV: challenges for therapy and prevention. Nature (London) 333:519–522.
23. **Lewin, B.** 1990. Commitment and activation at pol II promoters: a tail of protein-protein interactions. Cell 61:1161–1164.
24. **Li, Y., J. Ross, J. A. Scheppler, and B. R. Franza, Jr.** 1991. An in vitro transcription analysis of early responses of the human immunodeficiency virus type 1 long terminal repeat to different transcriptional activators. Mol. Cell. Biol. 11:1883–1893.
25. **Lu, Y., N. Touzjian, M. Stenzel, T. Dorfman, J. G. Sodroski, and W. A. Haseltine.** 1991. The NF-κB independent *cis*-acting sequences in HIV-1 LTR responsive to T-cell activation. J. AIDS 4:173–177.
26. **Maniatis, T., E. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Nabel, G., and D. Baltimore.** 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature (London) 326:711–713.
28. **Nakajima, N., M. Horikoshi, and R. G. Roeder.** 1988. Factors involved in specific transcription by mammalian RNA polymerase II: purification, genetic specificity, and TATA box-promoter interactions of TFIID. Mol. Cell. Biol. 8:4028–4040.
29. **Pavlakis, G. N., and B. K. Felber.** 1991. Regulation of expression of human immunodeficiency virus. New Biol. 2:20–31.
30. **Peterson, M. G., N. Tanese, F. Pugh, and R. Tjian.** 1990. Functional domains and upstream activation properties of cloned human TATA binding proteins. Science 248:1625–1630.
31. **Popovic, M., M. G. Sarngadharan, E. Read, and R. C. Gallo.** 1984. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science 224:910–913.
32. **Pugh, B. F., and R. Tjian.** 1990. Mechanism of transcriptional activation by Sp1: evidence for coactivators. Cell 61:1187–1197.
33. **Reinberg, D., M. Horikoshi, and R. G. Roeder.** 1987. Factors involved in specific transcription by mammalian RNA polymerase II: functional analysis of initiation factors IIA and IID, and identification of a new factor operating at sequences downstream of the initiation site. J. Biol. Chem. 262:3322–3330.
34. **Rosen, C. A., J. G. Sodroski, and W. A. Haseltine.** 1985. The location of cis-acting regulatory sequences in the human T-cell lymphotropic virus type III (HTLV-III/LAV) long terminal repeat. Cell 41:813–823.
35. **Saffer, D. J., S. P. Jackson, and S. J. Thurston.** 1990. SV40 stimulates expression of the trans-acting factor Sp1 at the mRNA level. Genes Dev. 4:659–666.
36. **Sato, T., A. Fuse, M. Eguchi, Y. Hayashi, R. Ryo, M. Adachi, Y. Kishimoto, M. Teramura, H. Mizoguchi, Y. Shima, I. Komori, S. Sunami, Y. Okimoto, and H. Nakagima.** 1989. Establishment of a human leukemic cell line (CMK) with megakaryocytic characteristics from a Downs syndrome patient with acute megakaryoblastic leukemia. Br. J. Haematol. 72:184–190.
37. **Sawadogo, M., and R. G. Roeder.** 1985. Interaction of a gene-specific transcription factor with the adenovirus *major late* promoter upstream of the TATA box region. Cell 43:165–175.
38. **Schmidt, M. C., Q. Zhou, and A. J. Berk.** 1989. Sp1 activates transcription without enhancing DNA-binding activity of the TATA-box factor. Mol. Cell. Biol. 9:3299–3307.
39. **Schwartz, L. B., V. E. F. Sklar, J. A. Jaehning, R. Weinmann, and R. G. Roeder.** 1974. Isolation and partial characterization of multiple forms of deoxyribonucleic acid-dependent ribonucleic acid polymerase in the mouse myeloma, MOPC 315. J. Biol. Chem. 249:5889–5897.
40. **Sen, R., and D. Baltimore.** 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell 46:705–716.
41. **Siebenlist, U., D. B. Durand, P. Bressler, N. J. Holbrook, C. A. Norris, M. Kamoun, J. A. Kant, and G. R. Crabtree.** 1986. Promoter region of interleukin-2 gene undergoes chromatin structure changes and confers inducibility on chloramphenicol acetyltransferase gene during activation of T cells. Mol. Cell. Biol. 6:3042–3049.
42. **Smale, S. T., and D. Baltimore.** 1989. The "initiator" as a transcription control element. Cell 57:103–113.
43. **Smale, S. T., M. C. Schmidt, A. J. Berk, and D. Baltimore.** 1990. Transcriptional activation by Sp1 as directed through TATA or initiator: specific requirement for mammalian transcription factor IID. Proc. Natl. Acad. Sci. USA 87:4509–4513.
44. **Sompayrac, L. M., and K. J. Danna.** 1981. Efficient infection of monkey cells with DNA of simian virus 40. Proc. Natl. Acad. Sci. USA 78:7575–7587.
45. **Trono, C., and D. Baltimore.** 1990. A human cell factor is essential for HIV-1 Rev action. EMBO J. 9:4155–4160.