Vol. 160, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1989 Pages 268-275

DETECTION OF ENHANCER BINDING PROTEINS RECOGNIZING THE HUMAN IMMUNODEFICIENCY VIRUS LONG TERMINAL REPEAT BY IN SITU GEL RETARDATION

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Received January 31, 1989

We have examined the formation of a DNA "enhancer" - protein complex occurring in situ. Oligonucleotides corresponding to the human immunodeficiency virus (HIV) core enhancer sequence were synthesized, annealed and radiolabeled. The DNA was electroporated either into Jurkat cells or into fresh human peripheral blood T lymphocytes. After the appropriate incubation time and stimulation with various mitogenic agents, cells were lysed and the lysates were electrophoresed on a native polyacrylamide gel. The specific protein-nucleic acid complexes which we obtained were apparently identical to those observed with the "classical" in vitro gel mobility shift assay: one complex seems to be constitutive and the other is induced by mitogens. Additionally competition experiments using "cold" oligonucleotides demonstrated binding specificity in situ. We recommend this novel method for studying DNA-binding proteins and their activation since it requires as few as 10^6 cells, may use primary tissue isolates, and furthermore, allows the rapid assessment of cellular activation signals involved in the post-transcriptional modification of trans-acting factors.

Genes transcribed by polymerase II in eukaryotic cells are primarily regulated by enhancers and promoters. Enhancers are defined as sequences of DNA that can increase transcription in an orientation independent manner both

ABBREVIATIONS USED:

HIV LTR, Human immunodeficiency virus long terminal repeat; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 23-acetate; MONO HIV, 16 mer oligonucleotide corresponding to the HIV LTR enhancer element; TANDEM HIV, 31 mer oligonucleotide corresponding to the HIV LTR enhancer element.

0006-291X/89 $1.50

upstream and downstream and at variable distances from the start site of transcription (1-3). Both enhancers and promoters consist of multiple short binding sites for trans-acting nuclear factors. Some of these trans-acting factors are constitutively active in various cell types (4-7), but others are tissue-specific (8-10) or are inducible in response to extracellular stimuli which modulate gene expression (11-13). We have focused our study on this latter class of factors induced in T cells by external signals.

It is well known that human immunodeficiency virus (HIV) production from latently infected T lymphocytes can be induced by mitogenic compounds which activate cells to proliferate or to differentiate (14,15). One of the trans-acting factors which stimulates viral transcription is a cellular protein called NF-kB (6). This protein is constitutively activated only in mature B cells which transcribe k (kappa) light chain genes, but it can be induced in pre B cells by LPS stimulation and in Hela or T cells by phorbol ester treatment (11). NF-kB binding sites have been found in the regulatory regions of many genes including the HIV enhancer (16), SV40 enhancer (17), the cytomegalovirus enhancer (18), upstream of major histocompatibility complex class I genes (19-20) and in the interleukin 2 receptor alpha chain gene (21).

Currently the two most widely used techniques for the study of protein-nucleic acid interactions are the gel mobility shift assay (22-23) and the DNase I footprinting assay (24). These are useful assays with reasonable specificity and resolution but also some disadvantages. They require large cell cultures, lengthy preparation of protein extracts and are difficult to utilize with primary cells. Here we present a rapid and convenient method to investigate the specific interaction between the HIV core enhancer sequence and trans-acting factors in Jurkat cells, a CD4+ human T leukemia cell line, and in freshly human peripheral blood T lymphocytes.

MATERIALS AND METHODS

Oligonucleotides: The complementary oligonucleotide sequences corresponding to the binding domains of the HIV LTR enhancer element (5' - ACAAGGGACTTTCCGCTGGGGACTTTCCAGC-3' TANDEM; 5' - ACAAGGGACTTTCCGC - 3' MONO) and of the negative regulatory element: (5' - TAGCATTTCATCACA - 3' NRE) were synthesized on a Biosearch multiple column DNA synthesizer model 8700, purified on acrylamide-urea gels followed by electroelution and ethanol precipitation. The two complementary strands were annealed (25) and after ethanol precipitation, followed by several washes, the double stranded oligonucleotides were resuspended in ultrapure water. The double stranded oligonucleotides were end-labeled with [gamma ^{32}P] ATP (3000 Ci/mmol; New England Nuclear) and T4 polynucleotide kinase (BRL) and purified twice on a G25 column.

Cell Culture: Jurkat cells, a CD4+ human T leukemia cell line, were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and

standard concentrations of L-glutamine and antibiotics. Stimulation was carried out with phytohemagglutinin (PHA-Burrough's Welcome) at 1 ug/ml and phorbol 12-myristate 13-acetate (PMA-Sigma) at 50 ng/ml for 3 hrs. Fresh human peripheral blood T lymphocytes were purified by Ficoll/Hypaque centrifugation followed by a nylon wool column and Percoll density-gradient centrifugation (26) and maintained in culture in the same medium as Jurkat cells.

Oligonucleotide Transfection and In Situ Gel Retardation Assay: $4x10^6$ cpm of double stranded oligonucleotide (specific radioactivity: $2\text{-}5x10^8$ cpm/ug) were electroporated into Jurkat cells or resting peripheral blood lymphocytes using a Bio-Rad cell electroporator (500 uF, 200 volts). Several concentrations of poly dI:dC (polydeoxyinosinic deoxycytidylic acid - Sigma) were added as a non-specific carrier to avoid non-specific binding. For competition experiments, 1 or 5 ug of unlabeled double stranded oligonucleotides (specific and unrelated) were co-electroporated into the cells. 16 hrs after transfection cells were purified on a lymphocyte separation medium (Organon Teknica Corporation) gradient and the viable cells were maintained in culture or, when indicated, treated with PHA, (1 ug/10^6 cells) and (PMA 50 ng/10^6 cells). After 3 hrs cells were washed in PBS several times, resuspended in a small volume of 1X TBE (100mM Tris-borate pH=8.3, 2mM EDTA) (15 ul/1- $3X10^5$ for Jurkat cells and 15 ul/$2X10^6$ for resting human T cells) and lysed by three cycles of freeze/thawing. The lysates were centrifuged 5 min to eliminate particulate debris. When indicated, NaCl was added or, alternatively, lysates were treated with proteinase K (50 ug/ml, 15 min at 37°C; similar treatment of ^{32}P-labeled enhancer alone showed that the proteinase K used in this study was totally devoid of nuclease activity). Lysates were electrophoresed through a native 6% polyacrylamide gel in 0.25X TBE buffer. Electrophoresis was carried out at 10V/cm for 90 min. Gels were dried and exposed to Kodak XAR-5 film at -70°C.

Mobility Shift Assay: Total proteins were extracted as described (26). Protein extracts (10 ug) were preincubated for 10 min on ice with 20 ug of Salmon Sperm DNA in Hepes pH 7.8 20 mM/ KCl 0.6 M/DTT 10 mM/Glycerol 12.5%. 10^5 cpm of ^{32}P-labeled oligonucleotide was then added to each sample; following an additional 15 min at room temperature loading buffer was added and the mixture was analyzed by electrophoresis on native 6% polyacrylamide gels in 0.25X TBE buffer. Gels were then dried and autoradiograph performed as described above.

RESULTS AND DISCUSSION

The principal aim of this study was to examine the regulation of genes involved in T cell proliferation and differentiation, by investigating the intracellular pathways involved in DNA-binding protein activation. We have introduced radiolabeled double stranded oligonucleotides corresponding to the HIV regulatory regions into T cells by electroporation. The double stranded oligonucleotide sequences used in our experiments and their locations in the regulatory region of the HIV LTR are shown in Fig. 1. The method which we have developed for detecting DNA protein complexes formed within the cells is schematically depicted in Fig. 2. The conditions for electroporation are critical. The percentage of viable cells under some conditions of electroporation was very low, but using our optimized procedure with $2\text{-}20x10^6$ cells in 100 ul of PBS the recovery of viable cells was at least 50%.

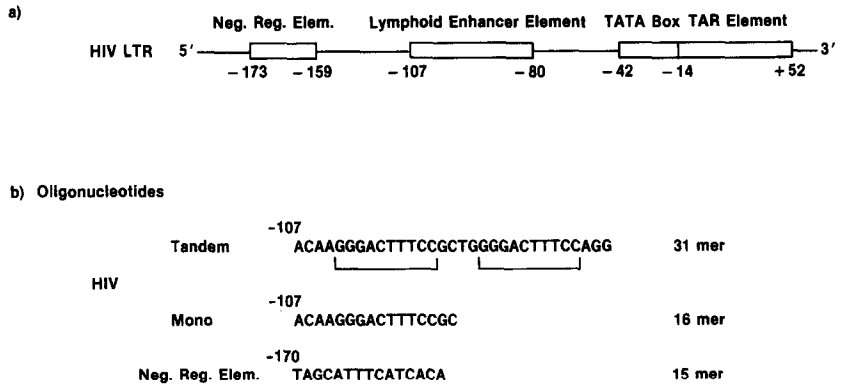

Figure 1 - Oligonucleotides used and their location in the HIV LTR.

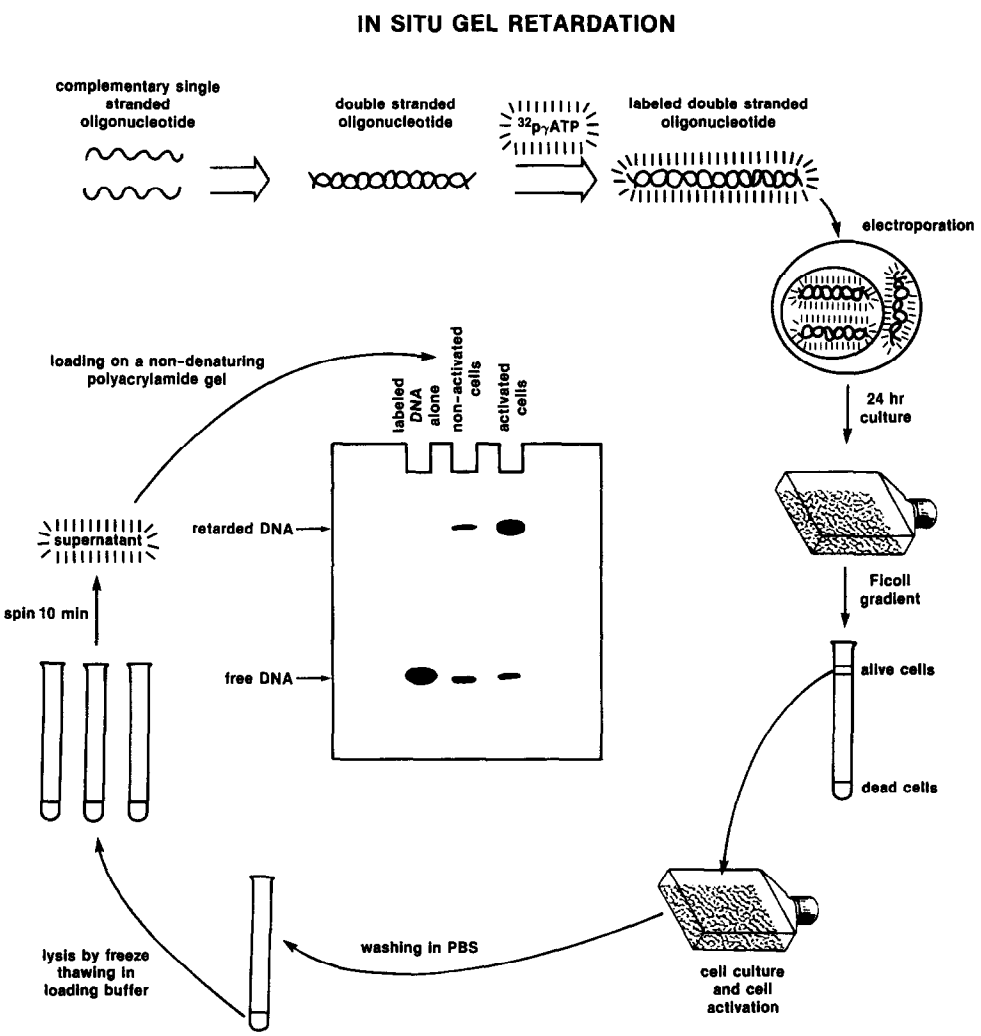

Figure 2 - Panel describing the protocol for the in situ DNA-binding proteins detection assay.

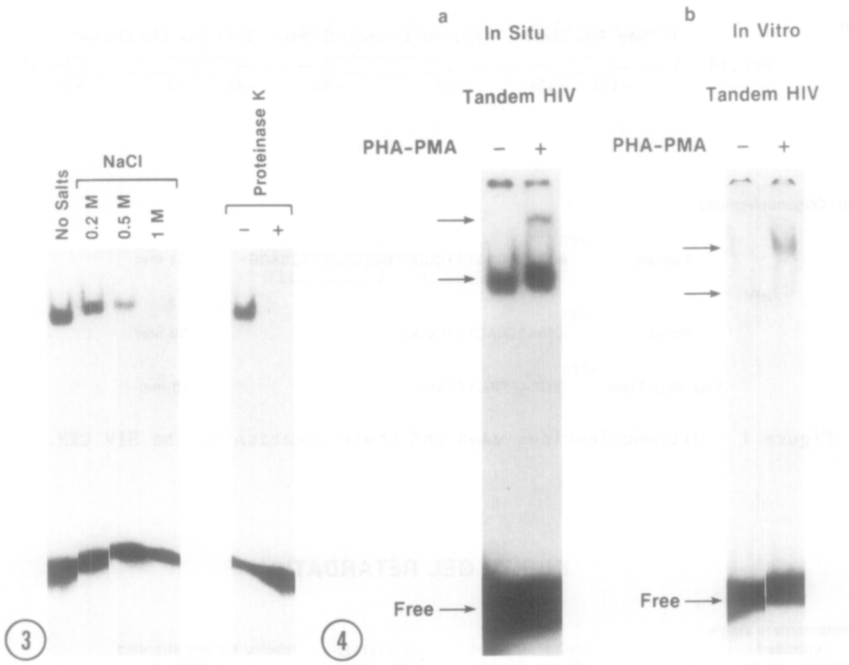

Figure 3 - MONO HIV transfected in fresh human peripheral blood T lymphocytes. Increased salt concentrations were added to the lysates or, where indicated, they were treated with proteinase K (50 ug/ml) at 37 C for 15 min before loading them on a 6% native polyacrylamide gel.

Figure 4 - Comparison of in situ gel retardation (a) and in vitro gel retardation (b) with double stranded oligonucleotide corresponding to the TANDEM HIV. Cells were treated either with no stimulant (-) or PHA-PMA (+) for 3 hrs.

When ^{32}P labeled double stranded synthetic MONO or TANDEM HIV "enhancer" oligonucleotides were electroporated into peripheral blood T cells the oligonucleotides remained undegraded over 1-2 days (data not shown) and, interestingly, a portion of the ^{32}P-labeled oligonucleotide was retarded in gel electrophoresis indicating that it bound proteins in the cell. To confirm this observation we have treated the lysates from normal peripheral blood T lymphocytes electroporated with ^{32}P labeled MONO with various concentrations of NaCl and with proteinase K. We found that 0.5 M or 1 M NaCl completely dissociated the oligo-protein interaction and the complex was completely absent in the presence of proteinase K; in contrast, incubation at 37°C for 15 min did not dissociate the complex (Fig. 3). These results indicate that the retarded radioactivity was due to the synthetic oligonucleotide and not to the incorporation of free ^{32}P, from degraded MONO into other cellular components, and that the shift in mobility was due to binding of proteins since the retarded band was disrupted by agents affecting DNA-protein interactions.

Upon further characterization, we found that by decreasing the number of cells and cotransfecting them with radiolabeled DNA and excess Poly dI:dC (to

decrease non-specific binding), we gained increased resolution and sensitivity. When $2x10^6$ Jurkat cells were transfected with $4x10^6$ cpm of ^{32}P-labeled TANDEM or MONO and 5 ug of Poly dI:dC; we were able to detect a constitutive lower band and an inducible upper band, representing proteins able to recognize the core enhancer sequence of the HIV-LTR. Figure 4 shows, in fact, that the in situ detection method produces the same results obtained in our laboratory and in others (16) utilizing the mobility shift assay. Three hrs of PHA-PMA stimulation before lysis induces the same "upper" complex in situ that is found with the classical gel retardation assay using extracts from stimulated Jurkat cells. Furthermore, when a mutant of the MONO oligonucleotide, which does not bind to the proteins in vitro, was electroporated into the cells no bands were detected (data not shown).

To further ensure that the intracellular binding is sequence specific we performed competition experiments with an excess of unlabeled MONO oligonucleotide or of an unrelated sequence such as the NRE (negative regulatory element) (28). These "cold" competitors were co-electroporated into Jurkat cells along with the labeled oligonucleotide. After three hrs of PHA-PMA stimulation, the cells were lysed and the lysates electrophoresed on a native polyacrylamide gel. As shown in Fig. 5, a 250-molar excess of cold MONO completely inhibited the complexes, which are not effected by the same molar excess of NRE. Analogous results were obtained with double stranded oligonucleotides corresponding to other regulatory elements such as the heat

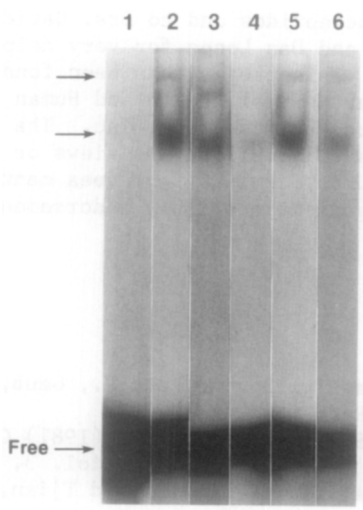

Figure 5 - Competition experiment. $4x10^6$ cpm of MONO HIV with 5 ug of poly dI:dC were transfected in $2x10^6$ Jurkat cells in the absence (lane 2) or in the presence of cold MONO HIV (50 fold excess = lane 3, 250 fold excess = lane 4), and in the presence of cold NRE (50 fold excess = lane 5, 250 fold excess = lane 6). Lane 1 shows the probe alone. Next day after transfection cells were treated with PHA-PMA for 3 hrs before lysis.

shock element (HSE) (29-30) and the CRE (cAMP responsive element) (31), whose specific DNA binding proteins were induced, in Jurkat cells, after heat shock for 20 min at 42°C or 8-bromoadenosine 3':5'-monophosphate (1mM) treatment, respectively.

This study suggests a new approach to investigate DNA-protein interactions occurring in situ and accelerates the detection of transacting factors on their sequence targets. We have used as little as 10^6 cells and detected specifically retarded bands. The method can obviously be used on freshly isolated cells and subpopulations. The in situ binding of labeled sequences can also offer an approximation of the number of proteins bound in situ. Furthermore, the increased binding with mitogenic stimulation shows that cellular activation signals are detectable and thus the technique may be suitable for the study of receptor mediated signal transduction. For example, by pretreating Jurkat cells with actinomicin D (50 ug/ml) and cycloheximide (10 ug/ml) before stimulation, we can confirm that the NF-kB like protein is already present in the cells and that its complex with the HIV core enhancer could be due to post-translational modifications or to displacement of an inhibitor (32) (data not shown). The use of protein kinase inhibitors or other modulators of second messenger systems may be useful in understanding how the activation of DNA binding proteins occurs in situ.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Leo Lee for the synthesis and purification of the oligonucleotides and to Drs. David Kelvin, Douglas K. Ferris, Joost J. Oppenheim and Dan Longo for very helpful discussion and comment on the manuscript. This project has been funded at least in part with Federal funds from the Department of Health and Human Services under contract number NO1-CO-74102 with Program Resources, Inc. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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