Biotinylated probes in the electrophoretic mobility shift assay to examine specific dsDNA, ssDNA or RNA–protein interactions

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Gel retardation or electrophoretic mobility shift assay (EMSA) is a useful method for visualizing specific interactions between DNA-binding proteins and DNA. DNA-binding proteins are involved in a variety of cellular processes ranging from transcription and replication to recombination and viral integration. Typically, ^{32}P -labeled DNA probes containing the sequence bound by the protein of interest are used in mobility shift assays, but a non-radioactive method using DNA labeled with digoxigenin-dUTP has previously been described (1). While hybridization of biotinylated probes has been employed in Southern or Northern analysis, use of biotin-labeled probes directly for studying protein–polynucleotide interactions with EMSA has not previously been described. We have tested a range of DNA sizes, from a 63mer Oct-1 binding site to a 690 bp dsDNA sequence containing the HIV-1 long terminal repeat (LTR), as well as biotinylated ssDNA and RNA probes, in this non-radioactive EMSA (Fig. 1). Biotinylation of RNA, ssDNA or dsDNA probes is easily performed, and the resultant probe is stable and sensitive, with none of the hazards of handling and disposing of radioactively-labeled oligonucleotide probes. EMSA can be performed in a standard fashion, with only a few adaptations required for detection of the biotinylated probe, as described below.

Synthetic oligonucleotides were either end-labeled or synthesized *in vitro*, with incorporation of biotin-11-UTP (Sigma Chemical Company, St Louis, MO) into RNA or biotin-16-dUTP (Boehringer-Mannheim Corporation, Indianapolis, IN) into ssDNA or dsDNA during the synthesis reaction as follows.

EBNA-1 and Oct-1 DNAs are 82mer and 63mer sequences containing the binding sites for Epstein–Barr virus nuclear antigen (EBNA-1) and Oct-1 protein, respectively, obtained from Pharmacia LKB Biotechnology Inc., Piscataway, NJ (Band Shift Kit). The interleukin-2 (IL-2) enhancer *Cla*I, *Hind*III fragment was isolated and purified from the vector 15cxCAT, a kind gift from Dr Gerald Crabtree (2). DNAs containing 5' overhangs were each incubated with 40 μM biotin-16-dUTP, 20 μM each dATP, dCTP and dGTP, in 10 mM Tris–HCl pH 7.5, 10 mM MgCl_2 , 50 mM NaCl, 5 mM β -mercaptoethanol and 5 U of the Klenow fragment of DNA polymerase I and in a total volume of 50 μl at 37°C for 1 h.

HIV-1 dsDNA PCR products were generated from a template derived from a plasmid containing the HIV-1 long terminal repeat (LTR) and the primer binding site (PBS), (pNLgag, a kind gift

from Dr Barbara Felber, NCI) as follows: the DNA including LTR and PBS was cut out from pNLgag using restriction enzymes and then eluted from the gel. The purified LTR (5' U3-R-U5-PBS 3') was used as a template in polymerase chain reactions (PCR), along with synthesized primers complementary to + or – strands of the U3, R or PBS regions of the LTR. These primers also contained either a T₇ or Sp6 RNA polymerase site ssDNA. These unique primers were used to generate and amplify dsDNA PCR products that would also incorporate sites for bacteriophage DNA-dependent RNA polymerases on either end. Because the dsDNA PCR products [LTR A (239 bp) and LTR B (690 bp)] incorporated DNA-dependent RNA polymerase sites on either end, we could use them as templates to synthesize RNA in either orientation. T₇ or Sp6 RNA polymerase were used with LTR A or B template in RNA synthesis reactions to generate the corresponding labeled or unlabeled RNA. The non-biotinylated LTR A or LTR B-derived RNA could then be used as template(s) to generate the corresponding, complementary, biotinylated, ssDNA HIV constructs. To make ssDNA, RNA was synthesized *in vitro* using unlabeled ribonucleotides, and then the original PCR-generated dsDNA template was removed with DNase treatment, followed by phenol–chloroform extraction and ethanol precipitation. The purified non-labeled RNA templates were then used to synthesize the labeled, complementary ssDNA in an *in vitro* reaction containing RNA template, biotin-16-dUTP (Boehringer Mannheim) and dNTPs, along with the corresponding, complementary DNA oligonucleotide primer and Moloney murine reverse transcriptase (Stratagene, La Jolla, CA) as described (3). The RNA template could then be removed from the biotinylated ssDNA by RNase H and RNase A treatment. All biotinylated dsDNA, ssDNA or RNA were spun over a Sephadex G-50 column equilibrated in water to remove unincorporated biotin-dUTP or unincorporated biotin-UTP, respectively.

Binding reactions with EBNA-1 extract containing the cloned DNA-binding domain from EBNA-1 protein (Pharmacia Band-Shift Kit) or Jurkat T cell nuclear extracts prepared as described (4) and ~6 ng biotinylated probe were incubated in 20 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM DTT, 10% glycerol, 0.05% NP-40 and 50 ng poly(dI-dC) poly(dI-dC) in a total volume of 20 μl for 20 min at 22°C. After the binding reaction, 10–20 μl of the binding mixture was electrophoresed on a 5% acrylamide gel in 1× TBE buffer as described (3). Following depurination,

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Figure 1. Biotinylated dsDNA, ssDNA or RNA interaction with protein analyzed using EMSA. (A) Specificity of EBNA-1 DNA and EBNA-1 protein interaction is demonstrated by competition. Biotinylated EBNA-1 DNA (82mer) was incubated alone (lane 3) or with EBNA-1 protein extract (lanes 4–6) and in the presence of 30 ng unlabeled competitor DNA (EBNA-1 in lane 5 or Oct-1 DNA in lane 6). (B) Comparison of non-radioactive EMSA using variously-sized dsDNA probes; biotinylated IL-2 enhancer (326 bp) in the presence and absence of Jurkat nuclear extract (lanes 1 and 2); biotinylated Oct-1 DNA (63mer) in the presence (lanes 3 and 4) and absence (lane 5) of Jurkat nuclear extract; biotinylated HIV LTR B (690 bp) alone (lane 8) or in the presence of stimulated or control Jurkat nuclear extracts (lanes 6 and 7); and biotinylated EBNA-1 DNA with (lane 9) or without (lane 10) EBNA-1 protein extract. (C) Biotinylated RNA synthesized using HIV LTR A template with T7 polymerase (as described in Methods) alone (lane 3) or incubated in the presence of Jurkat nuclear extract (lane 4); biotinylated RNA synthesized from HIV LTR B alone (lane 5) and in the presence of Jurkat nuclear extract (lane 6). (D) Biotinylated ssDNA synthesized from HIV LTR B-derived RNA incubated alone (lane 2) or in the presence of Jurkat nuclear extract. Molecular weight standards were biotinylated λ HindIII digest (Promega Corporation, Madison, WI), lane 1: A, C, D or biotinylated low molecular weight standards (Gibco BRL, Gaithersburg, NM) in lane 2, A and C.

denaturation and neutralization (of gels analysing DNA only), the DNA or RNA from the gel was transferred overnight onto Biotodyne B membranes (Pall, VWR Scientific, Greenbelt, MD) using 20 $\times$ SSC buffer and Posiblot apparatus (Stratagene) or capillary transfer. We found that using the electroblot transfer as previously described for digoxigenin-labeled DNA (1) resulted in a very variable transfer of the biotinylated-DNA in the retarded complex (DNA + protein extract), with no correlation with the transfer of the biotinylated-DNA probe alone. Posiblot transfer of biotinylated DNA in the presence or absence of DNA-binding protein onto Nytran membranes also worked poorly, in as much as only the biotinylated DNA in the absence of protein extract transferred to the membrane (data not shown). The best combination for efficient and complete transfer of both biotinylated-DNA species (alone and in the presence of DNA-binding protein) was to use the combination of Posiblot apparatus transfer onto Biotodyne B membranes. This also worked well for biotinylated ssDNA and RNA as shown in Figure 1C and D. Following UV cross-linking of the DNAs (or RNA) to the membrane (UV-Stratalinker-1800, Stratagene), colorimetric detection of the biotinylated DNA or RNA probes on the membrane was performed by blocking, then incubating with Streptavidin-alkaline phosphatase, followed by nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) as described (Boehringer-Mannheim, Genius kit).

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