Inhibition of Ets-1 DNA Binding and Ternary Complex Formation between Ets-1, NF-κB, and DNA by a Designed DNA-binding Ligand*

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Sequence-specific pyrrole-imidazole polyamides can be designed to interfere with transcription factor binding and to regulate gene expression, both *in vitro* and in living cells. Polyamides bound adjacent to the recognition sites for TBP, Ets-1, and LEF-1 in the human immunodeficiency virus, type 1 (HIV-1), long terminal repeat inhibited transcription in cell-free assays and viral replication in human peripheral blood lymphocytes. The DNA binding activity of the transcription factor Ets-1 is specifically inhibited by a polyamide bound in the minor groove. Ets-1 is a member of the winged-helix-turn-helix family of transcription factors and binds DNA through a recognition helix bound in the major groove with additional phosphate contacts on either side of this major groove interaction. The inhibitory polyamide possibly interferes with phosphate contacts made by Ets-1, by occupying the adjacent minor groove. Full-length Ets-1 binds the HIV-1 enhancer through cooperative interactions with the p50 subunit of NF-κB, and the Ets-inhibitory polyamide also blocks formation of ternary Ets-1·NF-κB·DNA complexes on the HIV-1 enhancer. A polyamide bound adjacent to the recognition site for NF-κB also inhibits NF-κB binding and ternary complex formation. These results broaden the application range of minor groove-binding polyamides and demonstrate that these DNA ligands are powerful inhibitors of DNA-binding proteins that predominantly use major groove contacts and of cooperative protein-DNA ternary complexes.

Pyrrole-imidazole polyamides are a novel class of small molecules that bind predetermined DNA sequences in the minor groove. Sequence-specific DNA recognition depends on side-by-side pairing of pyrrole (Py)[1] and imidazole (Im) amino acids; a Py opposite an Im targets a C·G base pair, and an Im opposite

a Py targets a G·C base pair (1, 2). Py/Py, Py/β-alanine (β), and β/β pairs binds both A·T and T·A base pairs (2, 3). Recent studies have shown that A/T degeneracy can be overcome by replacing one pyrrole ring of the Py/Py combination with 3-hydroxypyrrole (Hp), with the result that the Hp/Py prefers T·A over A·T base pairs (4). In addition to their high specificity, polyamides bind DNA with affinities comparable to or even higher than those of natural DNA-binding transcription factors. Polyamides can be designed to interfere with specific DNA-binding proteins and as a result inhibit their function. We have shown that DNA binding of transcription factor TFIIIA was inhibited by a polyamide that bound within the recognition site of zinc finger four of this nine-finger protein. TFIIIA is an important regulator of transcription of 5 S RNA genes, and as a result, transcription of 5 S RNA genes was suppressed *in vitro* as well as in cultured *Xenopus* cells with this specific polyamide (5). Mismatch polyamides, differing from the match polyamide in either the sequence of Py and Im rings or a single atom substitution, were without effect on either TFIIIA binding or transcription.

In an attempt to extend the application range of these small molecules to messenger RNA/protein-coding genes, and to a medically relevant system, specific polyamides were designed to interfere with transcription of the human immunodeficiency virus type 1 (HIV-1) (6). Transcription of HIV-1 is regulated by the 5′ long terminal repeat (LTR), which contains a series of cis-acting sequences responsible for basal and inducible viral gene expression. These sequences are well characterized and include recognition sites for upstream stimulatory factor, the E-twenty six-specific (*ets*) family of proteins, lymphoid enhancer-binding factor-1 (LEF-1), the nuclear factors NF-κB, Sp1, and the TATA box-binding protein (TBP) (reviewed in Ref. 7). TBP is indispensable for initiation of transcription, and LEF-1, considered to be an architectural protein, plays a central role in coordinating activities of multiple transcription factors. LEF-1 was shown to bend DNA, which facilitates protein-protein interactions between transcription factors bound at distant sites in enhancers (8–12). A polyamide was designed to bind a 7-base pair sequence located on each side of the TATA box and immediately upstream of the Ets-1 recognition site in the HIV-1 promoter (designated polyamide **1,** see Fig. 1). This polyamide prevented TBP and Ets-1 from binding to their recognition sites and blocked basal transcription from the HIV-1 promoter, but not from an unrelated promoter, *in vitro*. A second polyamide (designated polyamide **3,** Fig. 1), which was targeted to a sequence immediately upstream from the LEF-1-binding site and immediately downstream from the Ets-1-binding site, prevented LEF-1 from binding and blocked activated transcription *in vitro*. Polyamide **3,** however, did not inhibit Ets-1 DNA binding. Mismatch polyamides **2** and **4** had

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[1] The abbreviations used are: Py, pyrrole; HIV-1, human immunodeficiency type 1; Im, imidazole; Hp, hydroxypyrrole; LTR, long terminal repeat; USF, upstream stimulatory factor; LEF-1, lymphoid enhancer-binding factor-1; TBP, TATA box-binding protein; Dp, dimethylaminopropylamide; bp, base pair.

no effect on the DNA binding activity of TBP, LEF-1, or Ets-1. A combination of the two polyamides together effectively blocked viral transcription and replication in cultured human peripheral blood lymphocytes (6). The transcriptional activity of a variety of major T-cell-specific cellular genes (cytokine and growth factor genes) was also examined, and the expression of none of these genes was affected by the polyamides. Hence, it was concluded that inhibition of viral replication was the result of direct interference of the polyamides with transcription factor binding on the HIV-1 enhancer/promoter.

In the present study, we have focused on the differential inhibition of Ets-1 DNA binding by two distinct Py-Im polyamides and on the cooperative interaction between Ets-1 and the p50 subunit of NF-κB, which is necessary for HIV-1 enhancer function (13). The *ets* proteins are attractive candidates for polyamide targeting because of their unique mode of binding to DNA; the recognition helix of the winged helix-turn-helix domain is embedded in the major groove at the center of the DNA-binding site, and loop regions flanking the recognition helix are anchored to the phosphate backbone on both sides of the DNA recognition site (14–16). So far, we have demonstrated polyamide inhibition of DNA binding domains that exclusively contact the minor groove of DNA, such as zinc finger four of TFIIIA (5), the high mobility group domain of LEF-1, and the "saddle" of TBP (6). Recently, it was shown that a polyamide containing a basic tripeptide tail could inhibit the DNA binding activity of the basic helix-leucine zipper protein GCN4 that contacts DNA exclusively in the major groove (17). Since many transcriptional regulators contact DNA in the major groove with additional contacts in the minor groove and with the phosphate backbone, it is of interest to know the mechanism by which polyamides can inhibit a member of this class of proteins.

Ets-1, like most other *ets* proteins, functions in association with other proteins. Complex formation between *ets* proteins and other factors can release the autoinhibitory effects on DNA binding of full-length Ets proteins (reviewed in Refs. 18 and 19). It was shown that Ets-1 physically and functionally interacts with AP-1 in normal and activated T-cells (20). Ets-1 does not bind the minimal T-cell receptor α-gene by itself, but it binds DNA cooperatively with a factor called CBFα2 (11). Recently, Ets-1 was identified as a factor that physically associates with the POU homeodomain protein GHF-1/Pit-1 to fully reconstitute prolactin promoter activity (21). The p50 subunit of NF-κB was shown to associate with Elf-1, and this association plays a role in regulating cell type-specific and inducible expression of the interleukin 2 receptor α-chain gene (22). Significantly, physical interactions between *ets* and NF-κB/NFAT proteins were shown to play an important role in their cooperative activation of the HIV-1 enhancer in T-cells (13). We show here that full-length Ets-1 and the p50 subunit of NF-κB bind DNA cooperatively and form a ternary complex on the HIV-1 enhancer. This cooperative binding is also effectively inhibited by polyamide **1,** which may be an important contributing factor to the highly effective shut down of the HIV-1 promoter observed *in vivo* (6). Taken together, these findings demonstrate that the application range of the small Py-Im polyamides may be broader than anticipated. Polyamides can inhibit DNA-binding domains that make both major and minor groove DNA contacts, provided that the polyamide interferes with the minor groove binding portion, and polyamides can inhibit protein-protein-DNA ternary complexes, which may be an important consideration in designing future therapeutic drugs.

EXPERIMENTAL PROCEDURES

Polyamides—Polyamides were synthesized by solid phase methods as described previously (23).

Ets-1 Protein Purification—Recombinant full-length Ets-1 and the ΔN331 deletion polypeptide were expressed in bacteria and purified as described previously (24, 25). The high affinity deletion construct, ΔN331, contains the Ets-1 DNA-binding domain without the autoinhibitory regions and binds DNA with a 23-fold higher affinity than native, full-length Ets-1 (25). Purified proteins were stored in 20 mM sodium citrate, pH 5.3, 1 mM EDTA, 500 mM KCl at 4 °C.

Gel Mobility Shift Assays—Three sets of complementary oligonucleotides were synthesized (Genosys Biotechnologies, Inc.) for use as double-stranded probes in gel mobility shift experiments. A 43-bp oligonucleotide corresponded to positions −160 to −117 of the HIV-1 enhancer and contained the binding sites for Ets-1, LEF-1, and polyamides **1** and **3** in their natural sequence context. A 38-mer corresponded to positions −160 to −144 of the HIV-1 enhancer, followed immediately by an NF-κB-binding site (Fig. 4*A*). This artificial probe contained the binding sites for polyamide **1** and **3** and Ets-1 in their natural sequence context, followed by the first NF-κB recognition site located at position −104 to −92 of the HIV-1 enhancer. A 73-mer oligonucleotide corresponded to positions −160 to −92 of the HIV-1 enhancer and contained the binding sites for polyamides **1** and **3,** Ets-1, LEF-1, and NF-κB in their natural sequence context plus four additional bases at the 3′ end. Equimolar amounts of two complementary oligonucleotides were combined and end-labeled with [γ-³²P]ATP and T4 polynucleotide kinase. After labeling, unincorporated nucleotides were removed using a Qiaquick nucleotide removal kit (Qiagen). The labeled oligonucleotides were annealed and used in gel mobility shift experiments at a final concentration of 1 fmol/20 μl (50 pM). A 143-bp polymerase chain reaction (PCR) product, corresponding to nucleotide positions −175 to −33 of the HIV-1 LTR, was also used in gel mobility shift experiments. Binding reactions were carried out in a 20-μl reaction volume with 25 mM Tris/HCl, pH 7.9, 6 mM MgCl₂, 65 mM KCl, 10 mM dithiothreitol, 0.5 mM EDTA, 10% (v/v) glycerol, and 100 μg/ml bovine serum albumin. The reactions also contained 0.5 μg of poly(dI)·poly(dC) as a nonspecific competitor. Ets-1, ΔN331, and NF-κB p50 (Promega Corp.) were diluted in binding buffer immediately before adding to the samples, which were then incubated on ice or at ambient temperature for 20 min. The bound and free DNA were resolved on a 6% non-denaturing polyacrylamide gel containing 44 mM Tris borate, pH 8.3, and 1 mM EDTA. The gels were dried and exposed to Kodak Biomax film. The results were quantitated using an LKB Laser densitometer or with a Molecular Dynamics PhosphorImager equipped with ImageQuant software.

DNase I Footprint Experiments—The probe used in DNase I footprint experiments was a 250-bp *Eco*RV/*Bgl*II restriction fragment from the plasmid pHIV LTR-CAT (obtained from Dr. K. A. Jones) (26), which was singly end-labeled at the 3′ end at the *Bgl*II site with the Klenow fragment of DNA polymerase. Thus, the top strand was labeled. DNase I digestions were carried out in a 50-μl reaction containing 50 pM labeled DNA and 1 μg of poly(dI)·poly(dC) in the same binding buffer that was used for gel mobility shift experiments. The DNA was incubated with polyamides for 20 min at ambient temperature, followed by addition of ΔN331, and incubation for 20 min on ice. DNase I digestion was allowed to proceed in the presence of 2.5 mM CaCl₂ and 5 mM MgCl₂ for 30 s at ambient temperature with 66 × 10⁻³ or 33 × 10⁻³ units of DNase I (Roche Molecular Biochemicals) in the samples with or without protein, respectively. Reactions were stopped by the addition of 0.2% SDS and 10 mM EDTA, extracted with phenol/chloroform, and precipitated with ethanol prior to electrophoresis on a 6% denaturing polyacrylamide gel containing 8.3 M urea, 88 mM Tris borate, pH 8.3, and 2 mM EDTA. The dried gels were exposed to Kodak Biomax film with DuPont Cronex Lightning Plus intensifying screens at −80 °C.

RESULTS

Polyamide-binding sites within the HIV Enhancer—A set of four Py-Im polyamides **1**-**4** (Fig. 1) was synthesized by solid phase methods, and their structures have been published (6). Binding affinities for each polyamide were previously determined by quantitative DNase I footprinting experiments (6). Polyamide **1** (ImPy-β-ImPy-γ-ImPy-β-ImPy-β-Dp; where β is β-alanine, γ is γ-aminobutyric acid, and Dp is dimethylaminopropylamide) binds the sequence 5′-AGCTGCA-3′ with an equilibrium dissociation constant (K_d) of 0.05 nM, whereas the mismatch polyamide **2** (ImIm-β-ImIm-γ-PyPy-β-PyPy-β-Dp) exhibits 100-fold lower affinity for binding that site. Polyamide
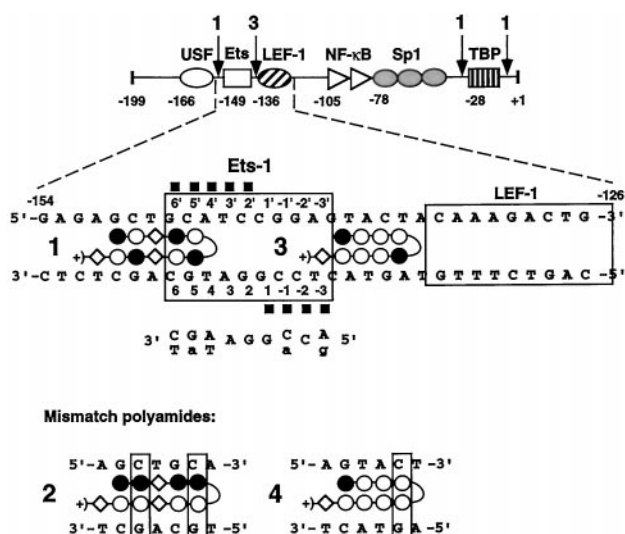
FIG. 1. **Polyamide and transcription factor-binding sites in the HIV-1 promoter/enhancer.** *Top*, DNA-binding sites for transcription factors and polyamides **1** and **3** (indicated by *numbers* above *vertical arrows*) in the HIV-1 promoter/enhancer. Nucleotide positions are shown relative to the major start site for transcription (+1). *Middle*, the double-stranded DNA sequence encompassing the Ets-1 and LEF-1 recognition sites and flanking sequences is shown. Polyamides are represented schematically between the two DNA strands at their respective binding sites. The *closed* and *open circles* represent Im and Py rings, respectively; the *diamonds* represent β-alanine (β), and the *curved lines* represent γ-aminobutyric acid (γ). The Ets-1- and LEF-1-binding sites are *boxed*. DNA binding by Ets-1 involves two types of sequence-specific DNA recognition; in the center of the site, amino acids within the recognition helix directly contact the 5′-GGA(A/T)-3′ core in the major groove, whereas backbone phosphates are contacted on the two flanks (*closed squares*) (15, 16, 30, 31, 37). The selected consensus for an optimal Ets-1-binding site is indicated below the duplex sequence (35). The bases of the Ets-1-binding site are numbered, starting with 1 for the first G of the GGA core (16). *Bottom*, binding models for mismatch polyamides **2** and **4,** where the mismatches are *boxed*.

3 (ImPyPyPy-γ-ImPyPyPy-β-Dp) binds the sequence 5′-AG-TACT-3′ with a K_d of 0.06 nM, and the mismatch polyamide **4** (ImPyPyPy-γ-PyPyPyPy-β-Dp) binds the same sequence with a 33-fold lower affinity.

Polyamide **1**-binding sites are found in the HIV-1 promoter and enhancer immediately flanking the TATA element and partially overlapping the upstream side of the Ets-1-binding site, at nucleotide positions −153 to −147 (relative to the start site for transcription at +1) (Fig. 1). The consensus recognition site for Ets-1 is located at nucleotide positions −149 to −141 (Fig. 1). DNase I footprinting revealed additional binding sites for polyamide **1** located within adjacent vector DNA sequences (see Fig. 2C). Polyamide **3** binds to a site at nucleotide positions −141 to −136 within the HIV-1 enhancer, adjacent to the recognition sequence for LEF-1 (−135 to −126), and partially overlapping the downstream side of the Ets-1 recognition site (Fig. 1). Additional recognition sites for polyamide **3** are found in the HIV-1 promoter/enhancer and coding sequence (see Ref. 6 and Fig. 2C). Therefore, both polyamides have the potential to block DNA binding by Ets-1 in addition to LEF-1 and TBP.

Differential Inhibition of Ets-1 DNA Binding by Polyamides 1 and 3—We previously reported that the DNA binding activity of the isolated high affinity DNA binding domain of Ets-1 was specifically inhibited by polyamide **1** but not by polyamide **3** (6). We wished to explore the basis for this differential inhibition in detail. A set of two complementary oligonucleotides corresponding to position −160 to −117 was synthesized and end-labeled followed by annealing of both strands. This 43-bp probe contained the binding sites for polyamide **1,** Ets-1, polyamide **3,** and LEF-1. Gel mobility shift experiments were done with a

deletion peptide ΔN331, which contains the DNA-binding domain but lacks the autoinhibitory regions present in the full-length protein, and as a result binds DNA with substantially higher affinity than full-length Ets-1 (24, 25). The reported K_d for ΔN331 binding to a synthetic Ets site, termed SC1, is 8.5 pM, a 23-fold higher affinity than that observed for the full-length protein binding the same DNA probe (25). When ΔN331 (at a concentration of 12 nM) was added to the labeled, 43-bp HIV-1 oligonucleotide (at a concentration of 50 pM), approximately 80% of the probe was converted into a protein-DNA complex (Fig. 2*A, lane 2*). In separate protein titration experiments with both the SC1 and HIV-1 probes, we found that ΔN331 had an ~10-fold lower affinity for the HIV-1 probe compared with the SC1 probe (data not shown). This difference in binding affinity is likely due to the different sequences of the Ets-1 sites within these two probes rather than to the length of the two DNA probes (23 bp for the SC1 probe *versus* 43 bp for the HIV-1 probe and three nucleotide differences within the respective 9-bp Ets-1 sites (25)). When polyamides were preincubated with the DNA probe before adding ΔN331, polyamide **1** prevented Ets-1·DNA complex formation (Fig. 2*A, lanes 3–10*). In contrast, polyamide **3** had no effect on Ets-1 DNA binding, even at a concentration as high as 200 nM (Fig. 2*B* and Ref. 6). When both polyamides were combined, the degree of inhibition was very similar to that observed with polyamide **1** alone (data not shown). Quantitation of these gel mobility shift experiments revealed that polyamide **1** inhibited Ets-1 ΔN331·DNA complex formation by 50% at a concentration of approximately 6 nM, and nearly complete inhibition was achieved between 50 and 200 nM polyamide (Fig. 2*B*). Polyamide **3,** and the two mismatch polyamides **2** and **4,** had virtually no effect on ΔN331 DNA binding in the same concentration range (Fig. 2*B* and Ref. 6).

Polyamide 1 Prevents Ets-1 DNA Binding, Whereas Polyamide 3 Coexists with Ets-1 on Overlapping DNA-binding Sites—We employed DNase I footprinting to visualize the DNA sequence contacts made by polyamides **1** and **3** and by ΔN331. A labeled DNA fragment derived from the HIV-1 enhancer was incubated with each polyamide either alone or followed by addition of ΔN331 and a further 30-min incubation. As expected, both polyamides bound their target sites within the HIV-1 enhancer with high affinity (Fig. 2*C, lanes 3–5* and *11–13*). Additional sites for polyamide **1** are present in upstream vector DNA sequence (*lane 5*), and additional sites for polyamide **3** are located both in the vector and in the HIV-1 enhancer sequence (*lane 13* and see Ref. 6). The Ets-1 footprint is characterized by two DNase I-hypersensitive sites appearing in the center and at the 5′ boundary of the footprint (*lanes 6* and *14*). These hypersensitive sites disappear with the addition of 20–100 nM polyamide **1** (Fig. 2*C, lanes 7–9*) but remain unchanged with the addition of polyamide **3** (*lanes 15–17*). The simultaneous presence of polyamide **3** and ΔN331 results in a broadening of the footprint, corresponding to a combined footprint created by ΔN331 and polyamide **3** (*lanes 15–17*), whereas the Ets-1 footprint is replaced by the polyamide **1** footprint (*lanes 7–9*).

Order of Addition Determines the Inhibitory Activity of Polyamide 1—An order of addition experiment was performed to determine whether polyamide **1** could disrupt a preformed Ets-1·DNA complex (Fig. 3). Various concentrations of polyamide **1** were added to the DNA probe, in separate reactions, either 20 min before ΔN331, simultaneously with ΔN331, or after incubation of the probe with ΔN331 for 30 min. After an additional 20 min incubation, the reactions were subjected to nondenaturing gel electrophoresis, and the fraction of DNA in the Ets-1·DNA complex was determined by phosphorimage
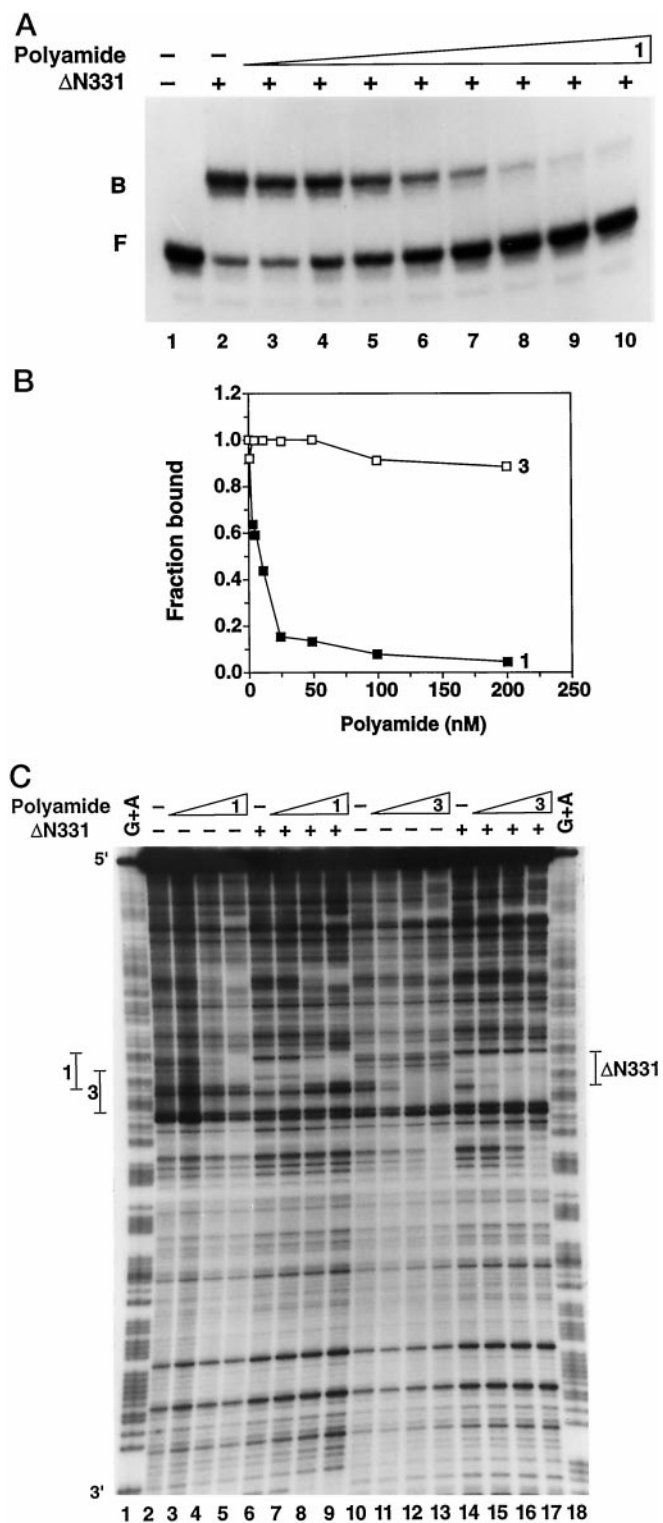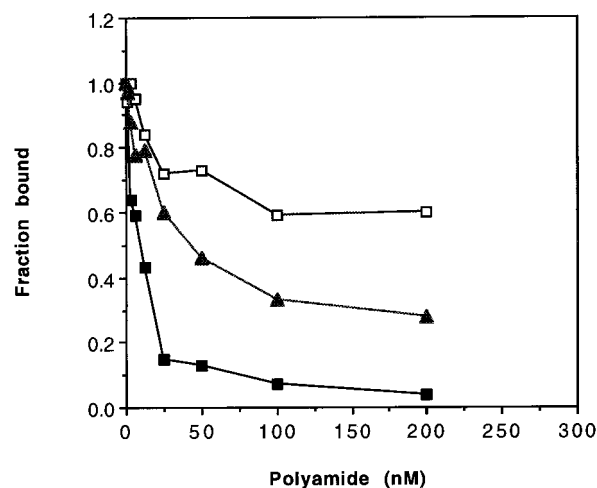
FIG. 3. **Order of polyamide 1 addition determines the inhibitory activity of the polyamide on ΔN331 DNA binding.** Gel mobility shift assays were carried out as described in the legend to Fig. 2, where the indicated concentrations of polyamide **1** were added to the DNA 20 min prior to adding ΔN331 (*closed squares*), or added simultaneously with ΔN331 (*closed triangles*), or ΔN331 was incubated with the DNA for 30 min prior to the addition of polyamide (*open squares*). After a subsequent 20-min incubation, the reaction mixtures were subjected to nondenaturing gel electrophoresis. The dried gel was subjected to phosphorimage analysis, and the fraction of DNA in the Ets-1·DNA complex in the presence of polyamide was plotted as a function of polyamide concentration, normalized to the amount of DNA in complex in the absence of polyamide (a value of 1.0).

analysis. Fig. 3 shows the results of these experiments in graphical form. Polyamide **1** is substantially more inhibitory when added to the DNA prior to ΔN331 than when added either simultaneously with ΔN331 or after formation of the ΔN331·DNA complex. These results likely reflect the similar affinities of ΔN331 and polyamide **1** for their respective target sites in the HIV-1 enhancer (see "Discussion").

Full-length Ets-1 Binds DNA Cooperatively with the p50 Subunit of NF-κB—The previous binding experiments were done with the high affinity deletion peptide ΔN331, which consists of the Ets-1 DNA-binding domain. Full-length Ets-1 binds to the HIV-1 probe with extremely low affinity, due to the presence of autoinhibitory regions in the protein (25). Ets-1 has been shown to form partnerships with a number of different proteins, which appears to counteract these negative auto-inhibitory effects (reviewed in Refs. 18 and 19). A recent report showed that Ets-1 physically interacts with NF-κB/NFAT proteins (13), and other studies have shown that the HIV-1 NF-κB sites are bound by heterodimers of p50/p65 and p50/RelB, and by p50 homodimers (reviewed in Ref. 7). This prompted us to analyze whether Ets-1 can bind cooperatively with the p50 subunit of NF-κB and form a ternary protein-protein-DNA complex. To this end we constructed a synthetic double-stranded oligonucleotide that contained the binding sites for Ets-1 and polyamides **1** and **3** in the natural sequence context and, in addition, a binding site for p50 in close proximity to the Ets-1-binding site (Fig. 4A). In a gel mobility shift assay, this probe was bound by full-length Ets-1 with very low affinity; at

FIG. 2. **Inhibition of Ets-1 DNA binding by polyamide 1.** *A,* polyamide **1** titration on the ΔN331·DNA complex. Autoradiogram of a representative gel mobility shift assay showing the inhibitory effect of polyamide **1** on ΔN331 binding is shown. Positions of the free probe (*F*) and bound probe (*B*) are indicated. The concentration of ΔN331 was constant (12 nM in *lanes 2–10*), and the concentration of the 43-bp probe (position −160 to −117) was 50 pM. The polyamide concentrations were 1.56, 3.125, 6.25, 12.5, 25, 50, 100, and 200 nM in *lanes 3–10*, respectively. The polyamide was preincubated with the DNA probe for 20 min at room temperature before addition of the protein, followed by 20–30 min incubation on ice. *B,* graphical representation of the decrease of the fraction of bound probe as a function of the polyamide concentration. *Closed squares* represent the data points obtained for polyamide **1**; *open squares* represent data for polyamide **3.** *C,* DNase I footprint titration

experiment with polyamides **1** and **3** in the absence (−) or presence (+) of 9.6 nM ΔN331 protein. Polyamides were incubated with the radiolabeled probe prior to addition of ΔN331. The polyamide concentrations were 0 nM (*lanes 2, 6, 10*, and *14*), 4 nM (*lanes 3, 7, 11*, and *15*), 20 nM (*lanes 4, 8, 12,* and *16*), and 100 nM (*lanes 5, 9, 13*, and *17*). *Lanes 1* and *18* show a G + A sequencing ladder. The regions protected by polyamides and by ΔN331 are indicated alongside the sequencing ladder. Note the two DNase I-hypersensitive sites are characteristic of the ΔN331 footprint.
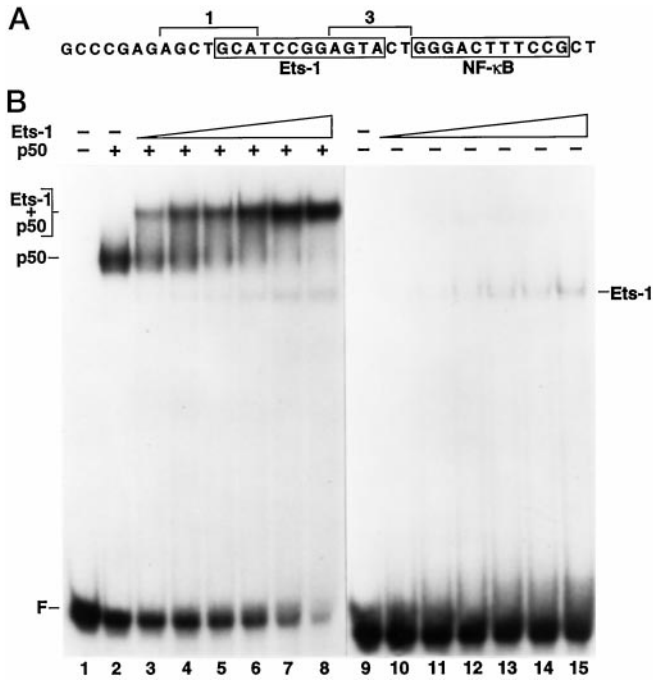
FIG. 4. **Full-length Ets-1 and NF-κB p50 bind DNA cooperatively and form a ternary complex.** Gel mobility shift experiment with a 38-bp double-stranded oligonucleotide derived from the HIV-1 enhancer and containing the recognition sites for polyamide 1, Ets-1, and NF-κB. The p50-binding site was moved close to the Ets-1-binding site to facilitate interaction *in vitro. A,* sequence of the top strand of the synthetic 38-bp probe with Ets-1, NF-κB, and polyamide-binding sites are indicated. *B,* gel mobility shift assay with a constant amount of p50 (6 nM, 5.8 ng/20 μl) and increasing concentrations of full-length Ets-1, compared with full-length Ets-1 in the absence of p50. A *plus* or *minus sign* at the *top* of the lanes indicates the presence or absence of protein. The Ets-1 concentrations were 8.5, 17, 34, 68, 136, and 272 nM in *lanes 3–8* and *10–15*, respectively. The positions of the protein-DNA complexes and the free probe (*F*) are indicated.

the highest protein concentration used (272 nM), only 6% of the probe was bound (Fig. 4*B, lanes 10–15*). To examine whether p50 and Ets-1 bind the HIV-1 enhancer in a cooperative fashion, increasing concentrations of Ets-1 were added to a constant but limiting amount of p50 (6 nM). Coincubation of p50 and Ets-1 yielded a complex that migrated at a slower mobility than that formed with either p50 or Ets-1 alone (Fig. 4*B, lanes 3–8*). Significantly, ternary complex formation was detectable at the lowest concentration of Ets-1 used, which did not yield a complex with Ets-1 alone (compare *lanes 3* and *10* in Fig. 4*B*). Furthermore, at the maximal concentration of Ets-1 used in the presence of p50, approximately 90% of the probe was shifted into the ternary complex (*lane 8*), whereas less than 10% of the probe was shifted when Ets-1 was used in the absence of p50 at the same concentration (*lane 15*), and only 50% of the probe was shifted when p50 was used alone (*lane 2*). When this same experiment was repeated at a lower concentration of p50 (3 nM), only 13% of the probe was converted into the p50·DNA complex in the absence of Ets-1 (Fig. 5, *lane 3*). The addition of full-length Ets-1 had an even more dramatic effect on DNA binding under these conditions (Fig. 5, *lane 4*). These data indicate that DNA binding by Ets-1 and p50 is highly cooperative.

Inhibition of Ternary Complex Formation by Polyamide 1—We next examined whether the cooperative DNA binding activity by combined p50 and Ets-1 proteins would still be susceptible to polyamide inhibition. When the DNA probe was preincubated with polyamides prior to incubation with proteins, the DNA binding activity of p50 was not inhibited by either polyamide 1 or the mismatch polyamide 2 (Fig. 6, *lanes 3–6*). The small degree of DNA binding by full-length Ets-1 was

FIG. 5. **Cooperative interaction of Ets-1 and p50 at low concentrations of p50.** A gel mobility shift assay with the 38-bp oligonucleotide probe was carried out as described in the legend to Fig. 4, at a p50 concentration of 3 nM and at an Ets-1 concentration of 136 nM, where indicated. The positions of protein-DNA complexes and free probe (*F*) are indicated.

inhibited by polyamide 1 but not by polyamide 2 (Fig. 6, *lanes 13–16*). When p50 and Ets-1 were added to the DNA probe after preincubation with polyamide 1, ternary complex formation was prevented, whereas p50 was still able to bind the probe (Fig. 6, *lanes 8* and *9*). The mismatch polyamide 2 failed to inhibit ternary complex formation (*lanes 10* and *11*). These results confirm that Ets-1 is an integral part of the ternary complex, and ternary complex formation can be prevented by blocking Ets-1 access to its recognition site.

A titration experiment showed that the polyamide concentration required for 50% inhibition of the ternary complex was approximately 3–6 nM. Complete inhibition was achieved with a polyamide concentration of 100–200 nM (data not shown). This concentration range is similar to the concentration of polyamide 1 required for preventing the high affinity deletion peptide ΔN331 from binding to its recognition site (see Fig. 2*B*).

We also tested whether ternary complex formation could be prevented by blocking p50 binding with polyamide 3. Polyamide 3 binds immediately upstream of the p50-binding site in the 38-mer DNA probe (Fig. 4*A*), and preincubation of the probe with polyamide 3 blocks binding of p50 and, hence, ternary complex formation (Fig. 7). However, a much higher concentration of polyamide 3 than polyamide 1 is required to block ternary complex formation. As before, Ets-1 binding is not affected by polyamide 3, and curiously, the DNA binding affinity of full-length Ets-1 appears to be modestly enhanced (approximately 2–3-fold) in the presence of polyamide 3. We do not have an explanation for this small level of enhanced binding.

Cooperative Binding and Polyamide Inhibition of Ets-1 and NF-κB to the Native HIV-1 Enhancer Sequence—Since the DNA probe used in the previous gel mobility shift experiments was an artificial construct, we next tested whether full-length
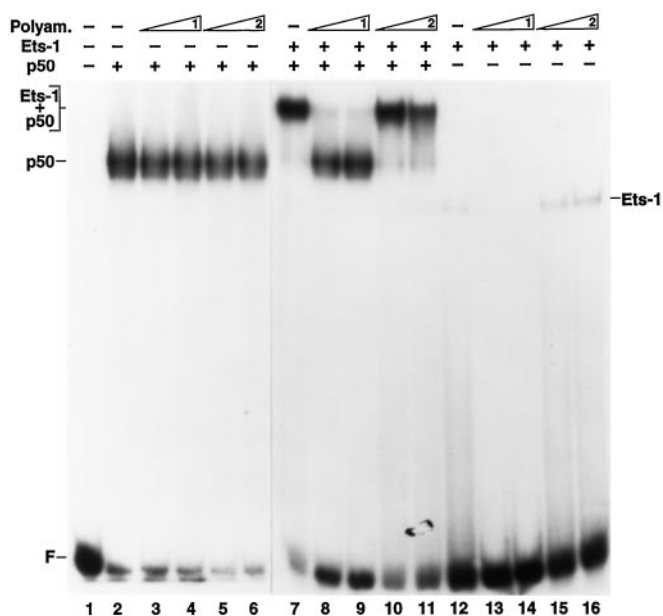
FIG. 6. **Specific inhibition of ternary complex formation by polyamide 1.** A gel mobility shift assay with the 38-bp oligonucleotide probe was carried out as in Fig. 4. *Lane 1* contained no added protein; *lanes 2–6* contained p50; *lanes 7–11* contained both p50 and Ets-1; and *lanes 12-16* contained Ets-1. The protein concentrations were 136 nM full-length Ets-1 and 6 nM p50. Polyamide **1** was preincubated with the probe for 20 min at ambient temperature at a concentration of 100 nM (*lanes 3, 8,* and *13*) and 200 nM (*lanes 4, 9* and *14*); mismatch polyamide **2** was added at 100 and 200 nM in *lanes 5, 10, 15* and *6, 11, 16*, respectively. The positions of protein-DNA complexes and the free probe (*F*) are indicated.

Ets-1 and the p50 subunit of NF-κB could bind cooperatively to the native HIV-1 enhancer sequence and form a ternary complex. A double-stranded 73-bp oligonucleotide, corresponding to positions −160 to −92 of the HIV-1 enhancer and containing the binding sites for polyamides **1** and **3**, Ets-1, LEF-1, and NF-κB, was used as a probe in gel mobility shift experiments. As before, in the absence of p50, Ets-1 bound to the 73-mer with very low affinity (Fig. 8*A, lanes 2–5*). In contrast, coincubation of a constant concentration of p50 and increasing concentrations of Ets-1 yielded a complex that migrated with a slower mobility than that formed with p50 alone, and the intensity of this ternary complex increased with increasing Ets-1 concentrations (Fig. 8*A, lanes 6–10*). These data indicate that full-length Ets-1 can bind the native HIV-1 enhancer cooperatively with NF-κB even though the NF-κB and Ets-1 sites are separated by 35 bp in the native HIV-1 sequence. Additionally, polyamide **1** inhibits ternary complex formation by preventing Ets-1 binding without affecting p50 binding (Fig. 8*B, lanes 5–7*). Polyamide **3,** which binds downstream of the Ets-1 recognition site and 30–36 bp upstream of NF-κB in the native HIV-1 sequence, does not significantly inhibit ternary complex formation (Fig. 8*B, lanes 8–10*). The −160 to −92 probe contained only one binding site for NF-κB, whereas two sites are present in the native HIV-1 enhancer sequence (Fig. 1). To assess whether polyamide **1** could also inhibit ternary complex formation on the full native enhancer sequence, we used a 143-bp DNA probe corresponding to nucleotide positions −175 to −33 of the HIV-1 enhancer/promoter sequence. Similar to the results for the 73-bp probe (Fig. 8*B*), we find that polyamide **1** can inhibit formation of the Ets-1·NF-κB·DNA complex with this longer probe containing two NF-κB sites (Fig. 9). As before, in the absence of p50, full-length Ets-1 binds very weakly to this DNA probe (*lanes 2–4*). In the absence of Ets-1, p50 forms two complexes on this probe (*lane 5*). The addition of a 100-fold

molar excess of unlabeled 143-bp HIV-1 DNA to the p50-labeled DNA complex reveals that the faster migrating species is nonspecific, whereas the slower migrating species is the specific p50-DNA complex (*lane 6*). Interestingly, the nonspecific complex is not observed in the presence of Ets-1 (*lanes 7–9*) and reappears when Ets-1 binding is inhibited by polyamide **1** (*lane 12*). It seems likely that Ets-1 can recruit p50 from the nonspecific complex into the specific ternary Ets-1·p50·DNA complex.

DISCUSSION

Differential Inhibition of Ets-1 Binding by Two Polyamides Flanking the Ets-1 Recognition Site in the HIV-1 Enhancer— Ets-1 is an important regulator of many promoters in lymphoid cells, including the HIV-1 long terminal repeat. We show here that Ets-1 can be inhibited from binding to its recognition sequence in the HIV-1 enhancer by a small Py-Im DNA-binding ligand. Polyamides bind in the minor groove of DNA and, significantly, can inhibit minor groove binding proteins by binding to a recognition site that is adjacent to but does not coincide with the recognition site of the DNA-binding protein (6). This is a crucial requirement for achieving specificity in inhibiting binding of ubiquitous transcription factors to certain promoters. We speculate that polyamides, which bind B-type DNA, can inhibit minor groove binding proteins such as TBP and LEF-1 because polyamide-bound DNA is likely resistant to the extensive bending and unwinding that accompanies DNA binding by these transcription factors (12, 27–29). In contrast, we document here that a Py-Im polyamide can also inhibit the DNA binding activity of Ets-1, a winged helix-turn-helix protein that primarily contacts DNA in the major groove with additional adjacent phosphate contacts.

The Ets-1-binding site in the HIV-1 enhancer is flanked on both sides by polyamide-binding sites, upstream by polyamide **1** and downstream by polyamide **3**. Both polyamide-binding sites partially overlap with the protein-binding site. However, only polyamide **1** blocks Ets-1 binding, whereas polyamide **3** has no effect, despite the fact that polyamide **3** binds even closer to the GGA recognition core than does polyamide **1** (Fig. 1). This differential polyamide inhibition of Ets-1 DNA binding can be explained within the context of the high resolution model of ETS domain-DNA interactions. The ETS domain displays a structural motif known as a winged helix-turn-helix which includes three α helices and a β sheet (14–16, 30, 31). Significantly, DNA contacts are confined to one face of the DNA helix. Helix 3 of the helix-turn-helix motif contacts the 5′-GGA(A/T)-3′ core within the major groove. A β sheet "wing" and a turn separating helices 2 and 3 are tethered to the phosphate backbone on both sides of the major groove contacts made by the recognition helix (16) (Fig. 1). Polyamide **3,** which does not disrupt Ets-1 binding, interacts in the minor groove just beyond the phosphate contacts made by the wing. Polyamide **3** binds the symmetrical DNA sequence 5′-AGTACT-3′ and can conceivably bind in either orientation, that is either with its terminal β-alanine proximal to the Ets-1 site or its "hairpin turn" γ-aminobutyric acid proximal to the Ets-1 site. Depending on the orientation of the polyamide, the β-alanine amide will form a hydrogen bond to the O-2 of T^{-3} or the γ-aminobutyric acid amide will form a hydrogen bond to the N-3 of $A^{-3'}$ (32) (see Fig. 1). Although *ets* proteins make a phosphate contact at T^{-3}, neither of these potential polyamide contacts is sufficient to block Ets-1 from binding. On the other hand, an Im/Py pair and a Py/Im pair of polyamide **1** interact with $G^{6'}/C^6$ and $C^{5'}/G^5$, thus interfering with the phosphate contacts made by the ETS domain to nucleotides $G^{6'}$ and $C^{5'}$ (Fig. 1). Since the polyamides do not make phosphate contacts with DNA, it is likely that inhibition arises from a steric blockage of the minor groove by the polyamide. These results indicate that targeting

FIG. 7. **Inhibition of ternary complex formation with polyamides 1 and 3 on the 38-bp probe.** Gel mobility shift assays were performed as in Fig. 6 with increasing concentrations of polyamides (*Polyam.*). *Lanes 3–10* contained polyamide **1**, and *lanes 13–20* contained polyamide **3** at the following final concentrations: 1.6, 3.1, 6.3, 12.5, 25, 50, 100, 200 nM, respectively. The positions of protein-DNA complexes and the free probe (*F*) are indicated.
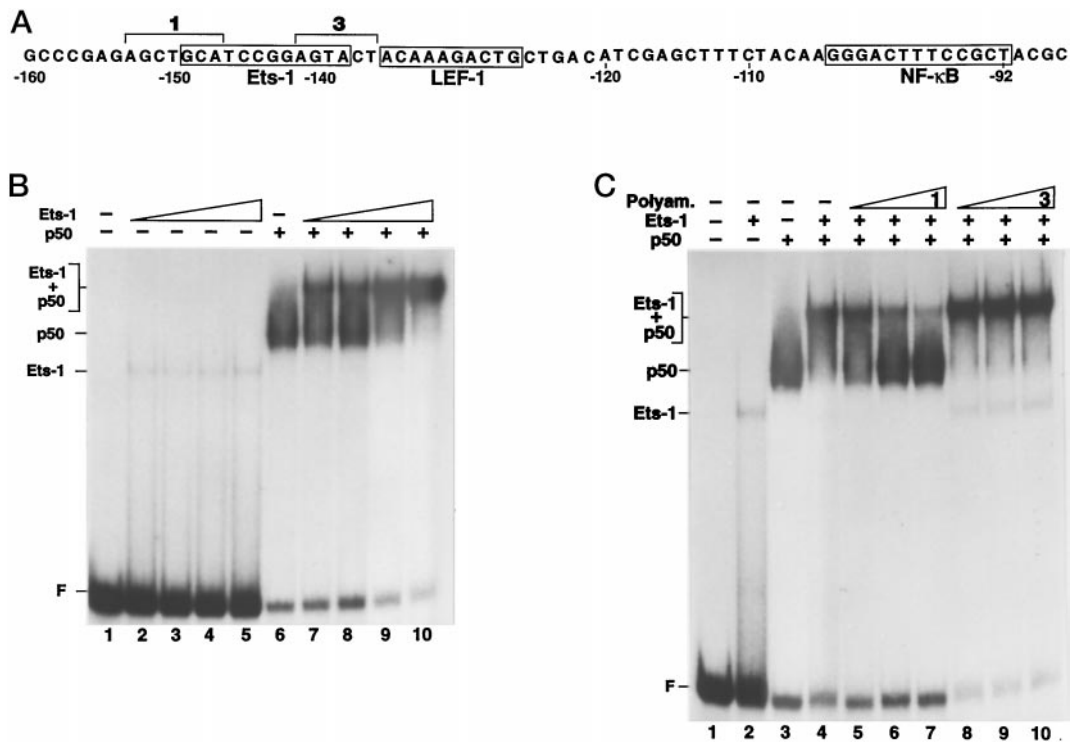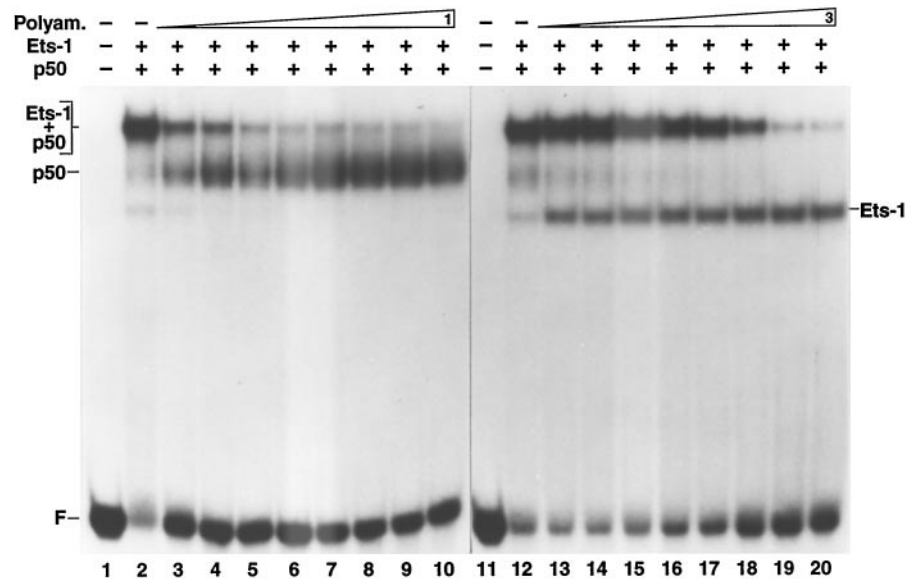

FIG. 8. **Full-length Ets-1 and NF-κB p50 form a ternary complex with the native HIV-1 enhancer sequence that is inhibited by polyamide 1.** *A*, sequence of the top strand of the 73-bp DNA probe (positions −160 to −92). *B*, gel mobility shift experiment with increasing concentrations of full-length Ets-1 in the absence of p50 (*lanes 2–5*) or in the presence of p50 (*lanes 7–10*). p50 was used at a constant concentration of 6 nM. The concentrations of Ets-1 were 17, 34, 68, and 136 nM in *lanes 2–5* and *7–10*, respectively. *C,* inhibition of ternary complex formation between Ets-1, p50, and the native HIV-1 enhancer sequence. The proteins were added at constant concentrations of 136 nM for Ets-1 and 11 nM for p50. The polyamide (*Polyam.*) concentrations were 1 nM (*lanes 5* and *8*), 10 nM (*lanes 6* and *9*), and 100 nM (*lanes 7* and *10*). Free probe (*F*) and protein-DNA complexes are indicated.

sites adjacent to protein-binding sites may not be sufficient for polyamide inhibition of certain DNA-binding proteins. This approach was successful with TBP and LEF-1, likely because these factors extensively bend and unwind the DNA upon binding, whereas polyamides bound to DNA likely prevent this type of distortion. For Ets-1, however, interference with phosphate contacts was necessary to inhibit binding. These results demonstrate the importance of taking the three-dimensional structure of the protein-DNA complex into consideration when designing polyamides to disrupt a transcription factor-DNA interaction.

We find that the order of addition of polyamide **1** and Ets-1 (ΔN331) has a dramatic effect on the inhibitory action of the polyamide (Fig. 3). More pronounced inhibition is observed if the polyamide is allowed access to its DNA target prior to the addition of ΔN331 protein than if the protein-DNA complex is allowed to form prior to the addition of polyamide. An intermediate result is obtained if both polyamide and ΔN331 are added to the DNA simultaneously. This dependence on order of addition likely reflects the similar affinities of the two DNA ligands for their recognition sites: ΔN331 binds a selected high affinity site with an equilibrium dissociation constant of 8.5 pM (25) and
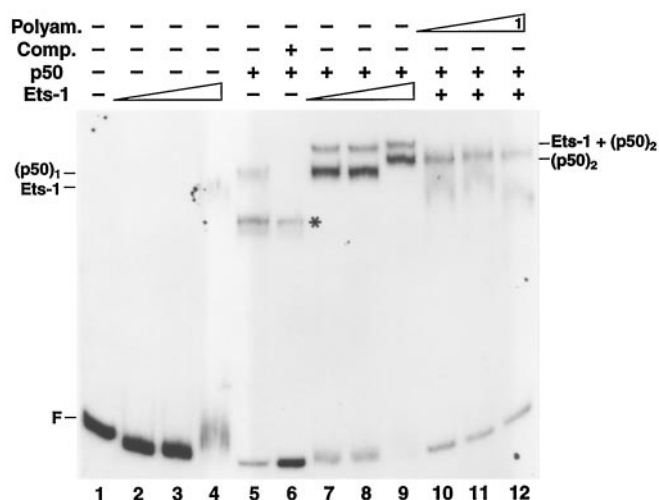
FIG. 9. **Ternary complex formation and polyamide 1 inhibition of Ets-1 and p50 on the native HIV-1 enhancer sequence.** Gel mobility shift experiment with the 144-bp probe, corresponding to nucleotide positions −175 to −33 of the HIV-1 LTR, and increasing concentrations of full-length Ets-1 in the absence of p50 (*lanes 2–4*) or in the presence of p50 (*lanes 7–9*). p50 was used at a constant concentration of 6 nM, where indicated (+). The concentrations of Ets-1 were 68, 136, 272 nM in *lanes 2–4* and *7–9*, respectively. The concentration of Ets-1 in *lanes 10–12* was 272 nM. The polyamide (*Polyam.*) concentrations were 50, 100, 200 nM in *lanes 10–12*, respectively. In *lane 6*, a 100-fold molar excess of the unlabeled probe was added as a specific competitor (*Comp.*). Free probe (*F*) and protein-DNA complexes are indicated. The complex with one p50 site occupied is denoted $(p50)_1$ and the complex with two occupied sites is denoted $(p50)_2$. The nonspecific p50-DNA complex observed in the absence of Ets-1 is indicated by an *asterisk*.

binds the HIV-1 site with approximately 10-fold lower affinity. Similarly, polyamide **1** binds its target site with a K_d of 50 pM. Given these comparable binding affinities, which ever ligand has initial access to its target site will be the primary bound species. In the context of the living cell, it is likely that transcription factors are displaced from promoter sequences during both DNA replication and during mitotic chromosome condensation (33, 34); thus, polyamides would have access to their target sites during these two phases of the cell cycle providing a window of opportunity for binding.

Ternary Complex Formation of Full-length Ets-1 and NF-κB p50 on Adjacent Binding Sites—Full-length Ets-1 binds to the HIV-1 enhancer with very low affinity. This result is in agreement with published reports showing that the DNA binding activity of many members of the *ets* family is negatively regulated by autoinhibitory regions present in the protein. It has been suggested that cooperation with other factors counteracts the autoinhibitory effect of Ets-1 (reviewed in Refs. 18 and 19). Indeed, we also show that when full-length Ets-1 is coincubated with the p50 subunit of NF-κB and a DNA probe containing binding sites for both proteins, Ets-1 binds cooperatively with p50 and forms a ternary complex that migrates with slower mobility than either protein-DNA complex alone.

The cooperativity observed between Ets-1 and p50 is most likely the result of direct physical interaction between the two proteins that are brought to close vicinity by binding to adjacent sites on the same DNA fragment. A recent report showed that Ets-1 physically interacts with NF-κB/NFAT proteins *in vitro* and *in vivo* and that this interaction requires the presence of DNA for both binding sites (13). We also found that cooperative binding between Ets-1 and p50 required a DNA probe containing a binding site for both proteins on the same fragment. We could not detect cooperative binding with oligomers that contained either the Ets-1 or p50 recognition site alone nor

with an equimolar mixture of both oligomers (data not shown).

Significantly, ternary complex formation is inhibited by polyamide **1,** which prevents Ets-1 from binding to its recognition site. It is noteworthy that inhibition of Ets-1 DNA binding in the ternary complex is achieved with the same concentrations of polyamide **1** as required for inhibition of DNA binding by the high affinity deletion peptide ΔN331. This observation suggests that full-length Ets-1 in the ternary complex binds to its DNA recognition site with a similar affinity as the isolated high affinity deletion peptide ΔN331.

The artificial probe that was used in these experiments contained a polyamide **3**-binding site upstream of the p50-binding site (Fig. 4*A*). Polyamide **3** prevented ternary complex formation as well, by preventing p50 from binding to its recognition sequence. However, the concentration of polyamide **3** required for inhibition of p50 was significantly higher than that required for polyamide **1** inhibition of Ets-1. It is likely that a higher concentration of polyamide **3** is required because of the different modes of DNA recognition and binding utilized by these two proteins (15, 35, 36). Ets-1 binds DNA as a monomer, whereas p50 binds as a dimer; furthermore, the polyamide **3**-binding site may be too distant from the minor groove contacts made by p50, which are located toward the center of the 10-base pair recognition site. The p50 dimer contacts DNA predominantly in the major groove, where the dimer grips the DNA duplex like a set of jaws, whereas minor groove contacts are limited to only two amino acid residues (36). It is therefore likely that the location of the polyamide **3**-binding site is not optimal for inhibition of the minor groove contacts made by p50.

Cooperative Binding of Ets-1 and NF-κB p50 on Non-adjacent Sites in the HIV-1 Enhancer—We show that Ets-1 binds cooperatively with NF-κB p50 not only on adjacent binding sites but also on the natural HIV-1 enhancer sequence. In the HIV-1 enhancer sequence the binding sites for Ets-1 and NF-κB are separated by approximately 35 base pairs, with an intervening LEF-1-binding site. LEF-1 is considered an architectural protein that coordinates interactions between multiple proteins. For example, LEF-1 induces a sharp bend in the TCR α-gene enhancer, which facilitates interactions between Ets-1, CBFα2, and ATF/CREB transcription factors bound at distant sites flanking the LEF-1-binding site (11). However, we found that cooperativity between Ets-1 and p50 on the HIV-1 enhancer did not require the presence of LEF-1. Since both the ETS domain and the p50 dimer induce a moderate bend in the DNA upon binding (15, 36), it is conceivable that the combined effect of the two proteins on the DNA configuration is sufficient to bring their binding sites in close proximity to allow protein-protein interaction.

Numerous physical interactions between *ets* family members and other transcription factors, including p50, have been described (reviewed in Ref. 19). However, very few studies have shown cooperative DNA binding and ternary protein-protein-DNA complex formation between Ets proteins and other transcription factors (11). Although direct physical association between Ets-1 and p50 has been demonstrated (13), this is the first report providing evidence for cooperative DNA binding and ternary complex formation by Ets-1 and p50 on the HIV-1 enhancer sequence.

Cooperative interactions between transcription factors appear to be important for regulation of gene expression in general, and it is thought that these interactions may represent targets for novel antiviral therapies. The *in vitro* experiments described in this report, together with *in vivo* assays described elsewhere (6), suggest that small Py-Im polyamides may represent the basis for developing novel therapeutic agents that target transcription.

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