Polyamine-mediated Conformational Perturbations in DNA Alter the Binding of Estrogen Receptor to Poly(dG-m⁵dC).poly(dG-m⁵dC) and a Plasmid Containing the Estrogen Response Element

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The binding of estrogen receptor (ER) to the upstream regions of estrogen-responsive genes, the estrogen response elements (ERE), is of fundamental importance in the regulation of gene expression by estradiol. Multiple cell-specific factors affect ER–ERE binding and modulate the responses of estradiol. We studied the role of polyamines in the recognition of ER, a ligand-activated transcription factor, with a left-handed Z-DNA forming polynucleotide as well as with a plasmid containing ERE. Polyamines are cellular organic cations with multiple functions in cell growth and differentiation. Polyamines induce Z-DNA conformation in alternating purine–pyrimidine sequences. To understand the role of polyamine-induced DNA conformational transition in ER–DNA interaction, we studied the binding of partially purified rabbit uterine ER to poly(dG-m⁵dC).poly(dG-m⁵dC). The induction of Z-DNA in the polynucleotide was monitored by circular dichroism and ultraviolet spectroscopic measurements. Binding of ER to poly(dG-m⁵dC).poly(dG-m⁵dC) increased from 15% to approx. 50–60% in the presence of 7.5 mM putrescine, 0.5 mM spermidine or 0.25 mM spermine. Maximal binding of ER to the polynucleotide was observed near the midpoint of the B-DNA to Z-DNA transition of the polynucleotide. N^1-acetyl spermidine and N^1-acetyl spermine facilitated the B-DNA to Z-DNA transition and the binding of ER although they were less effective than the unacetylated analog. $Co(NH_3)_6^{3+}$, a trivalent inorganic cation, also provoked the B-DNA to Z-DNA transition of the polynucleotide and increased its binding to ER. At higher polyamine concentrations, there was an inhibition of ER binding to the polynucleotide. In the presence of polyamines, the binding of ER to a plasmid containing ERE was 2–3-fold higher than that to a control plasmid devoid of ERE. Polyamine-induced facilitation of ER–ERE binding was also confirmed by gel mobility shift assay. Our data indicate that conformational perturbations, similar to that of the early stages of B-DNA to Z-DNA transition, are important in the recognition of ER and ERE.

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INTRODUCTION

The diverse effects of the female sex hormone, estradiol, on the physiology of vertebrates is mediated mainly through a specific protein, the estrogen receptor (ER) [1–3]. ER is a ligand-activated transcription factor that stimulates the transcription of an array of estrogen-responsive genes by recognizing a short stretch of DNA called the estrogen response element (ERE) in the upstream regulatory regions of these

genes [4,5]. Molecular genetic approaches have identified the consensus ERE sequence as GGTCATnnnATGACC. Although the presence of this 15 bp consensus ERE is sufficient to confer estrogen responsiveness in transfected cells [4], transcriptional regulation of estrogen-responsive genes depends on the cell-specific microenvironment [6,7]. Thus, transfection of ER gene to an ER-negative cell line resulted in estradiol-induced growth inhibition of this cell line [8]. In contrast, estradiol stimulates the proliferation of most cell types expressing ER naturally [1]. Recent studies indicate that polyamines, the cellular organic cations with multiple functions, might be important regulators of ER function [9–11].

Polyamines—putrescine, $(H_2N(CH_2)_4NH_2)$, spermidine $(H_2N(CH_2)_4NH(CH_2)_3NH_2)$, and spermine $(H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2)$—are present in all cells and their levels are exquisitely regulated in normal cells [12]. Polyamines stimulate cell proliferation, cell differentiation, DNA replication, and modulate the activity of enzymes such as topoisomerase as well as those involved in their own synthesis and catabolism [13,14]. Among the various sites of interaction of polyamines in the cell, DNA is a prime target because of the negative charge on its backbone structure. Molecular modelling [15,16], X-ray crystallography [17], and solution studies [18] have provided evidence for polyamine–DNA interactions. Thus polyamines are shown to induce and stabilize unusual structures such as A-DNA [19], Z-DNA [20,21], triplex DNA [22,23] and bent DNA (24,25).

We and others have observed a critical role for polyamines in estrogenic action in different experimental models of breast cancer [26–30]. Estrogens were unable to exert their growth stimulatory effect in the presence of DL-α-difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC), that suppresses polyamine biosynthesis [31]. Our studies on ER binding to a conformationally labile polynucleotide, poly(dA-dC).poly(dG-dT) showed that putrescine, spermidine, and spermine could enhance the binding of ER to these sequences [10]. Studies using poly(dA-dC).poly(dG-dT) could not, however, resolve questions on the role of Z-DNA in ER recognition because the Z-DNA conformation induced in this polynucleotide is atypical in that it is not recognized by monoclonal anti-Z-DNA antibodies. Furthermore, it is relevant to characterize how ER–ERE binding would be affected by polyamines when ERE is part of a plasmid, as a model for chromosomal loops that facilitate protein–DNA interactions during transcriptional stimulation of gene expression. Therefore, we examined the binding of ER to poly(dG-m⁵dC).poly(dG-m⁵dC), a polynucleotide that undergoes a facile B-DNA to Z-DNA transition and plasmids containing 1 or 4 ERE inserts. Our data show that within physiologically compatible ionic conditions, polyamines modulate the binding of ER to ERE as well as to other unique DNA conformations such as the early stages of the B-DNA to Z-DNA transition.

EXPERIMENTAL

Polynucleotide, oligonucelotides, antibodies, and other chemicals

Poly(dG-m⁵dC).poly(dG-m⁵dC) was purchased from Pharmacia, Inc. (Piscataway, NJ). The polynucleotide was dissolved in a buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 150 mM NaCl. This polynucleotide solution was dialyzed three times against a buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol (DTT), and then used in ER–DNA binding assays. RNase A of bovine pancreas was obtained from Worthington Biochemicals (Freehold, NJ). The enzymatic activity of RNase A was 5400 units per mg protein. [³H]Estradiol was purchased from New England Nuclear (Boston, MA). Unlabeled diethylstilbestrol, DNA–cellulose, and reagents for buffer preparation were obtained from Sigma Chemical Co. (St Louis, MO). Putrescine–2HCl, spermidine–3HCl, and spermine–4HCl as well as the acetylated spermidine and spermine were obtained from Sigma Chemical Co. (St Louis, MO.). $Co(NH_3)_6Cl_3$ was obtained from Aldrich Chemical Co. (Milwaukee, WI).

Oligonucleotides were purchased from Oligos, Etc. Inc. (Wilsonville, OR). The extended ERE (5'CCA<u>GGTCAGAGTGACC</u>TGAGCTAAAATAA-CACATTCAG) from vitellogenin gene was used as the oligonucleotide ERE and as the sequence inserted in pGEM-7Zf(+) plasmid, as described by Peale *et al.* [32] and Klinge *et al.* [33]. We used the control plasmid and two plasmids with either 1 or 4 ERE inserts for this study. Detailed studies were conducted with the plasmid with 4 ERE inserts because of the cooperative binding of ER to multiple EREs in plasmids [33]. Previous studies also showed that the AT flanking region facilitated the binding of ER to this plasmid [32] . Plasmids were purified by polyethylene glycol precipitation method as described previously [34]. Anti-ER monoclonal antibodies (309) were obtained from NeoMarkers, Fremont, CA.

CD spectroscopic measurements

Poly(dG-m⁵dC).poly(dG-m⁵dC) was dissolved in a buffer containing 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, and 150 mM NaCl. This solution was diluted to 50 μg/ml concentration using 10 mM Tris–HCl (pH 7.5) buffer containing 150 mM NaCl and 1 mM DTT and dialyzed three times from the same buffer. Small volumes (5–10 μl) of polyamine stock solutions were added to the polynucleotide and incubated at 22°C for 1 h. The CD spectra of poly(dG-

m^{5}dC).poly(dG-m^{5}dC) were recorded with a Jasco J41 spectropolarimeter. The molar ellipticity was calculated from the equation, $[\theta] = \theta/cl$, where θ is the relative intensity, c is the molar concentration of polynucleotides, and l is the pathlength of the cell in centimeters. In CD spectroscopy, the inversion of the spectrum indicates B-DNA to Z-DNA transition [20,21].

UV absorbance measurements

We further used changes in the absorbance ratio, A$_{260/295}$ as a measure of the B-DNA to Z-DNA transition in poly(dG-m^{5}dC).poly(dG-m^{5}dC) [21]. Polynucleotide solution was dialyzed against 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl and 1 mM DTT and diluted to 50 μg/ml concentration in the same buffer. Small volumes (5–10 μl) of polyamine stock solutions were added to the polynucleotide and incubated at 22 C for 1 h to attain equilibrium. The absorbance of the solution was recorded at 260 and 295 nm using a Beckman DU 64 spectrophotometer [35].

Preparation of cytosolic ER and its partial purification

Immature rabbits (5–6 lbs, New Zealand White) were used for the preparation of ER. Rabbits were sacrificed by an injection of Nembutal (Abbot Laboratories, Chicago, IL), and uteri were removed. The tissue was minced and homogenized at 4 C in 10 vol of a buffer containing 10 mM Tris–HCl, (pH 7.4), 1 mM EDTA, and 1 mM DTT (TED buffer) using a Polytron PT-10 homogenizer (Brinkmann Instruments, Westbury, NY) [36]. The tissue homogenate was centrifuged at 105,000 *g* for 60 min to obtain cytosol. The cytosol was incubated with 5 nM [^{3}H]estradiol for 2 h at 4 C and purified by sucrose gradient centrifugation. The receptor content of the cytosol was determined by dextran-coated charcoal (DCC) assay, as described earlier [36]. Cytosolic ER was partially purified by preparative sucrose gradient centrifugation [37]. Cytosol labeled with [^{3}H]estradiol was incubated with RNase A at 100 μg/ml for 1 h at 4°C to transform the receptor to the DNA binding form [36]. Unbound [^{3}H]estradiol was removed by DCC treatment and the cytosol (5 ml) was layered on top of 10–30% linear sucrose gradients and centrifuged at 135,000 *g* in a SW 28.1 rotor for 20 h at 4°C. Subsequently, 1 ml fractions were collected and the radioactivity of 100 μl aliquots of each fraction was determined to locate the receptor peak. The peak fractions were pooled and stored as 1 ml aliquots at −70 C. Partial purification of ER by density gradient centrifugation, followed by binding to DNA–cellulose and washing, resulted in a 23-fold purification of ER. The specific activity of ER eluted by 0.5 M NaCl was 16.2 ± 0.5 pmol/mg protein, as determined by the method of Lowry *et al.* [38] with bovine serum albumin as the standard.

DNA–cellulose competitive elution assay

DNA–cellulose was obtained from Sigma Chemical Co. and its DNA content was determined by diphenylamine assay [39]. The DNA–cellulose suspension was aliquoted into a series of Eppendorf tubes (200 μl/tube), each tube containing 20 μg DNA. The tubes containing the DNA–cellulose suspension were centrifuged at 10,000 *g* for 5 min and the supernate was removed. Aliquots (200 μl) of partially purified ER solution were added to DNA–cellulose pellets in the tubes and incubated at 4°C for 60 min with frequent mixing. The reaction mixture was centrifuged at 10,000 *g* for 5 min and the supernate was removed. After washing the pellet twice with 1 ml TED buffer, the receptor bound to DNA–cellulose was eluted by incubating and mixing with 200 μl of polynucleotide/plasmid solution in 10 mM Tris–HCl (pH 7.5) and 1 mM dithiothreitol (TD buffer) for 60 min at 25°C. Aliquots of polynucleotide/plasmid solution were incubated with varying concentrations of polyamines for 60 min at 22°C prior to its addition to the DNA–cellulose–ER pellet. The reaction mixtures were then centrifuged and the radioactivity of the supernate was determined. A solution of 0.5 M NaCl in 10 mM Tris–HCl buffer (pH 7.5) was used to quantitatively elute total ER bound to DNA–cellulose, representing 100% of the receptor available for elution with the polynucleotide/plasmid. ER extracted by 0.5 M NaCl varied from 12,000 to 15,000 cpm. The background radioactivity due to the dissociation of [^{3}H]estradiol from the receptor was determined by eluting the DNA–cellulose pellet with 200 μl of TD buffer with the appropriate concentration of polyamines. This background (10–15%) was subtracted before calculating the percentage of receptor eluted by the polynucleotide. Previous studies showed that this assay was sensitive to changes in eluting DNA, as indicated from ER elution by a polynucleotide that does not undergo conformational transition in the presence of spermidine [10].

Analytical sucrose gradient centrifugation

The receptor solution was layered on top of linear sucrose gradients (10–30%) containing TED buffer in 3.8 ml polyallomer tubes. The gradients were centrifuged at 256,000 *g* for 3 h to determine the sedimentation profile of ER bound to the polynucleotide [36]. Fractions were collected from the bottom of the gradient by gravity flow. The radioactivity of the fractions was determined by scintillation counting. ^{14}C-labeled protein, bovine serum albumin (4.5S), as well as 28S and 18S RNAs were used as external markers in parallel gradients.

Gel mobility assay

Complementary ERE oligonucleotides were dissolved in 10 mM Tris–HCl (pH 8), 200 mM NaCl

and equimolar solutions were allowed to anneal for 2
h after boiling for 10 min. After annealing, the oligo-
nucleotide solution was dialyzed 3 times against the
same buffer and was end-labelled with ^{32}P-γ-ATP
using a DNA 5′ end labelling kit from Boehringer
Mannheim. Approximately 20,000 cpm of end-labeled
oligonucleotide was mixed with 10 μl of ER (50
fmol). ER for the gel mobility assay was prepared
from Chinese hamster ovary (CHO) cells transfected
with recombinant human ER gene. ER from CHO
cells was used for gel mobility assay because of the
higher level of ER in the cellular extract than that
from the rabbit uterine ER preparation. CHO cells
expressing ER was obtained from Professor Geoffrey
Greene of the University of Chicago (Chicago, IL).
These cells were maintained in phenol red free
DMEM with 50% F12 medium, charcoal-treated
serum, 100 units/ml of penicillin, 100 μg/ml strepto-
mycin, and 0.4 mM sodium pyruvate. Cellular extract
was prepared by sonicating 25 × 10^6 cells in 2 ml of
a buffer containing 50 mM Tris–HCl (pH 7.4), 50
mM NaCl, 0.4 M KCl, 1.5 mM EDTA, 1 mM β-
mercaptoethanol, and 10% glycerol. Prior to the gel
mobility assay, polyamines were incubated with the
labeled probe for 1 h at 22°C. The labeled probe, ER
and 5 × binding buffer were mixed to give a final con-
centration of 10 mM Tris–HCl, 150 mM KCl, 10%
glycerol, 1 mM dithiothreitol, and 10 μg/ml poly(dI-
dC).poly(dI-dC) (Pharmacia, Piscataway, NJ). The
binding reaction was allowed to proceed for 1 h at
4°C and then loaded on a 6% polyacrylamide gel.
Electrophoresis was performed at 100 V for 3 h. The
gel was dried and exposed to Kodak Biomax MR-1
film for autoradiography for 24–48 h. Intensity of the
DNA–protein complex was quantified using a Bio-
Rad Laser densitometer, using films exposed for 24 h.

RESULTS

Effect of polyamines on ER binding to poly(dG-m^{5}dC).
poly(dG-m^{5}dC)

An unresolved question in studies on ER–DNA
interaction is whether ER recognizes an altered ter-
tiary structure of DNA or it induces altered structures
leading to recognition of RNA polymerase and other
transcription factors, stimulating the transcription of
responsive genes. Since our previous studies indicated
enhanced binding of ER to polynucleotides with the
potential to form Z-DNA [10,40], we wished to
examine the association of ER binding to Z-DNA for-
mation. In order to define the role of polyamine-
induced B-DNA to Z-DNA transition in ER–DNA
interactions, we selected ionic conditions at which ER
or other components of the receptor do not induce Z-
DNA in the polynucleotide [41]. For this purpose,
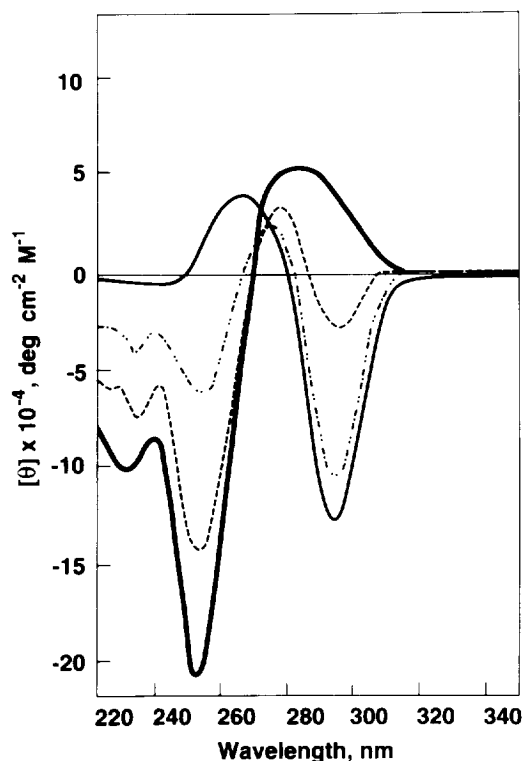DNA–cellulose elution experiments were established

Fig. 1. CD spectra of poly(dG-m^{5}dC).poly(dG-m^{5}dC) in the
presence of spermidine. Polynucleotide solution was incu-
bated with 0 (thick continuous line), 1 (dashed line), 1.5
(dotted and dashed line) and 2 mM (thin continuous line)
spermidine for 1h at 25°C, in 10 mM Tris–HCl (pH 7.5) buffer
containing, 1 mM dithiothreitol and 150 mM NaCl (TDS buf-
fer). CD spectra were recorded with a JASCO J41 spectro-
polarimeter.

in 10 mM Tris–HCl (pH 7.4), 1 mM dithiothreitol,
and 150 mM NaCl (TDS buffer).

We first examined the conformational status of
poly(dGm^{5}dC).poly(dG-m^{5}dC) in TDS buffer in the
presence of different concentrations of polyamines
using CD spectroscopy. Figure 1 shows the typical
changes in CD with increasing concentrations of sper-
midine. Significant changes in CD toward the Z-con-
formational state began to occur in the presence of 1
mM spermidine. Further shift to Z-DNA spectrum
was observed at 1.5 mM concentration. CD spectrum
was completely inverted, representing B-DNA to Z-
DNA transition at 2 mM spermidine. Thus the ionic
conditions selected for ER binding assay are appropri-
ate for the B-DNA to Z-DNA transition of poly
(dGm^{5}dC).poly(dG-m^{5}dC), under the influence of
polyamines.

In the next set of experiments, we measured the
ability of ER to bind to poly(dG-m^{5}dC).poly(dG-
m^{5}dC) in the presence of different concentrations of
polyamines. Z-DNA formation was studied in parallel
samples by recording the absorbance ratio, A_{260}/A_{295},
as a function of polyamine concentration. Figure 2
(A, B and C) shows our results on the effects of
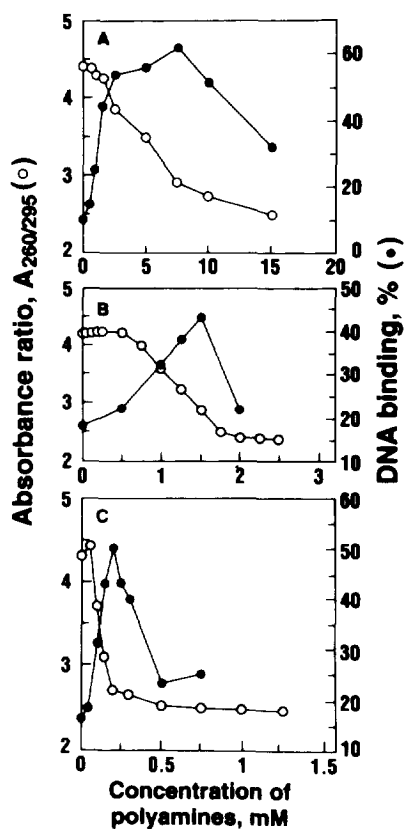putrescine, spermidine, and spermine, respectively, on

Fig. 2. Effects of putrescine (A), spermidine (B), and spermine (C) on B to Z transition of poly(dG-m⁵dC).poly(dG-m⁵dC) and ER–DNA binding. B to Z transition was monitored by changes in the absorbance ratio ($A_{260/295}$) after 1 h incubation of the polynucleotide in TDS buffer with polyamines. ER binding was determined by competitive elution of ER from DNA–cellulose by the polynucleotide (50 μg/ml) in TDS buffer with increasing concentrations of polyamines. Background elution of ER by the buffer and appropriate concentrations of polyamines were determined and subtracted before calculating the percentage of binding. 100% represents total ER eluted from the DNA–cellulose by 0.5M NaCl after subtracting the background elution. Data are the mean from 3 triplicate measurements. Standard deviations of DNA-cellulose binding experiments were about 5%. Variation in absorbance was less than 3%.

the B-DNA to Z-DNA transition of poly(dG-m⁵dC).poly(dG-m⁵dC) in TDS buffer. The concentrations of polyamines at the midpoint of B-DNA to Z-DNA transition were 5 mM putrescine, 1.25 mM spermidine, and 0.15 mM spermine. These values compare with 2 mM putrescine, 35 μM spermidine, and 2 μM spermine reported in previous studies in a buffer containing 1 mM sodium cacodylate and 0.15 mM EDTA [20,35,42]. Increased requirement of polyamines to induce the Z-DNA conformation in the present study is a consequence of high level of NaCl in the buffer [42] and the midpoint values are consistent with that obtained from CD spectroscopy. Figure 2 also shows the competitive elution of ER by poly(dG-m⁵dC).poly(dG-m⁵dC) complexed with

different concentrations of polyamines. The ability of ER to bind to poly(dG-m⁵dC).poly(dG-m⁵dC) peaked at concentrations slightly higher than the midpoint of transition, 7.5 mM putrescine, 1.5 mM spermidine or 0.2 mM spermine. However, there was a decrease in ER binding at the next higher concentration of polyamines studied, 10 mM putrescine, 2 mM spermidine, or 0.25 mM spermine. Experiments using an enzyme-linked immunosorbent assay showed that the polynucleotide recognized a monoclonal anti-Z-DNA antibody even at the concentrations at which an inhibition of ER binding was observed (results not shown). These data indicated a correlation of high affinity binding of ER to the early stages of B-DNA to Z-DNA transition of the polynucleotide and suggested that ER might be recognizing a unique conformational state of poly(dG-m⁵dC).poly(dG-m⁵dC).

In another series of experiments, we examined whether the ER eluted by polynucleotide in the presence of 150 mM NaCl and 250 μM spermine is stable as a high molecular weight species. We used sucrose density gradient centrifugation for this purpose. The ER–polynucleotide complexes were loaded on 10–30% sucrose gradient and centrifuged for 3 h. The free receptor, extracted by 0.5 M NaCl, sedimented at the very top of the gradient under these conditions (Fig. 3). The ER–polynucleotide complex formed in the presence of spermidine migrated in a region heavier than the peak of free polynucleotide. It is also evident from this figure that the polynucleotide is polydisprese, a reason for the overlap of the ER–polynucleotide complex with the heavier fractions of the polynucleotide. These results demonstrate that under our elution conditions, ER remains complexed with the polynucleotide.

Polyamine interconversion and catabolism are triggered by acetylation through the action of the enzyme, spermidine/spermine acetyl transferase (SSAT) [43]. Consequently the ability of acetylated polyamines to support ER–DNA interaction is important to the overall role of polyamines in estrogenic action. Our results on the effect of acetylspermidine and acetylspermine on B-DNA to Z-DNA transition as well as ER binding to poly(dG-m⁵dC).poly(dG-m⁵dC) are shown in Fig. 4 (A and B). There was a concentration-dependent increase in ER binding to the polynucleotide up to a concentration that stabilized Z-DNA, but ER binding decreased at higher polyamine concentrations. ER binding to the polynucleotide occurred after the polyamine concentration exceeded the midpoint of transition, 10 mM for acetyl-spermidine and 1.5 mM for acetylspermine. Maximal binding of ER occurred at 25–40 mM N¹-acetylspermidine and 2–3 mM acetylspermine. These concentrations are about 20- and 10-fold higher than the corresponding values for spermidine and spermine, respectively.
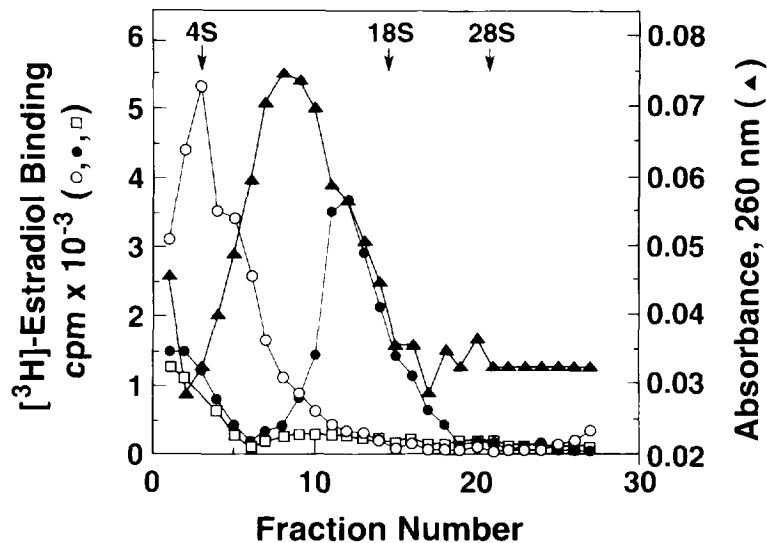
Fig. 3. Sucrose density gradient centrifugation profile of ER–polynucleotide complexes. [³H]ER was eluted by 10 mM Tris–HCl (pH 7.5) buffer containing 0.5 M NaCl (○) or TDS buffer with 250 μM spermine (□) or TDS buffer with 250 μM spermine and poly(dG-m⁵dC).poly(dG-m⁵dC) (50 μg/ml) (●). The sedimentation profile of poly(dG-m⁵dC).poly(dG-m⁵dC) in the absence of ER (△) is also shown. Samples were loaded on 10–30% linear sucrose gradients and centrifuged for 3 h in an SW 60 rotor at 256,000 g. Bovine serum albumin and ribosomal RNA markers were centrifuged in parallel gradients. Similar results were obtained in two separate experiments.
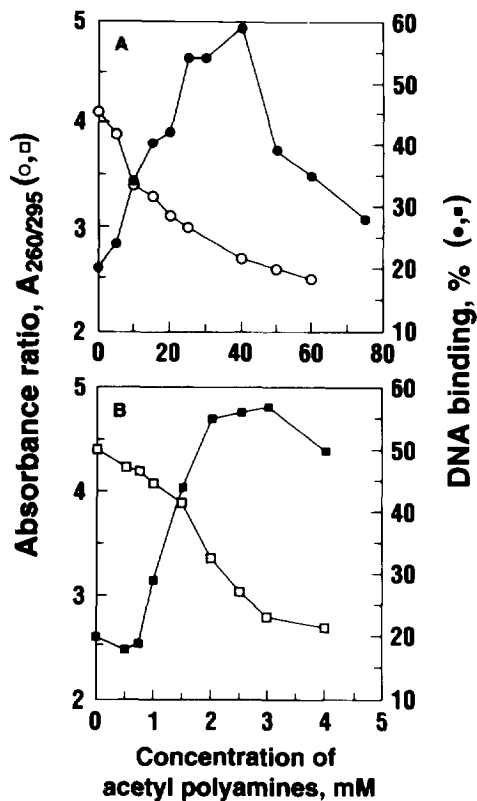
Effect of Co(NH₃)₆³⁺ on B-DNA to Z-DNA transition and ER binding

The inorganic trivalent cation, Co(NH₃)₆³⁺ is known to induce and stabilize Z-DNA conformation of poly(dG-m⁵dC).poly(dG-m⁵dC) [35,42]. We therefore used this compound to delineate the ionic and structural influences of polyamines on ER–DNA
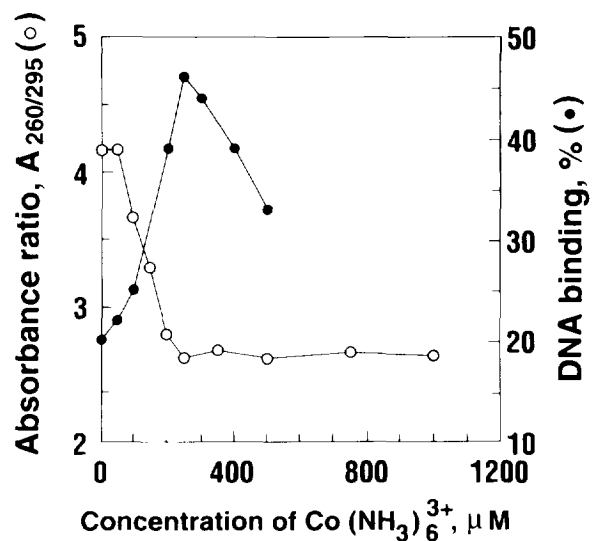

Fig. 4. Effects of N¹-acetylspermidine (A) and N¹-acetyl spermine (B) on the B-DNA to Z-DNA transition of poly(dG-m⁵dC).poly(dG-m⁵dC) and its ability to bind to ER. Experimental details are as described in the legend to Fig. 2 and in Materials and Methods. Data are the mean of 2 triplicate experiments. Standard deviations in ER elutions were within 6%.

Fig. 5. Effect of Co(NH₃)₆³⁺ on B to Z transition of poly(dG-m⁵dC).poly(dG-m⁵dC) and its ability to bind to ER. Experimental details are as described in the legend to Fig. 2 and in Materials and Methods. Data are the mean of 2 triplicate experiments. Standard deviations in ER elutions were within 5%.

binding and in B-DNA to Z-DNA transition. Figure 5 shows the effect of $Co(NH_3)_6^{3+}$ on the B-DNA to Z-DNA transition as well as on the ER binding of poly(dG-m^{5}dC).poly(dG-m^{5}dC). The midpoint concentration of this compound in B-DNA to Z-DNA transition was 150 μM, whereas the peak level of ER binding was observed at 250 μM level. Thus the high affinity interaction of ER with poly(dG-m^{5}dC).poly(dG-m^{5}dC) occurred not only in the presence of polyamines but also in the presence of other cations capable of inducing the B-DNA to Z-DNA transition. The subsequent inhibition of ER binding at higher concentrations of $Co(NH_3)_6^{3+}$ was more gradual compared to that of spermidine and spermine. With spermine, there was a sharp decrease from 50% ER binding at 200 μM to 23% at 500 μM concentration. In contrast, with $Co(NH_3)_6^{3+}$, the maximal binding of ER (45%) occurred at 250 μM, and a decrease to 32% occurred at 500 μM. Thus the trend in stimulation of ER binding of poly(dG-m^{5}dC).poly(dG-m^{5}dC) by $Co(NH_3)_6^{3+}$ was similar to that polyamines, even though spermidine and sper-

Fig. 7. Relative binding affinity of ER for control ($\bigcirc$) and ERE-containing ($\bullet$) plasmids. ER binding was determined by DNA–cellulose competition assay using different concentrations of the indicated concentrations of plasmids under the following conditions: (A) 10 mM Tris–HCl (pH 7.5), 1 mM DTT; (B) 10 mM Tris–HCl (pH 7.5) 1 mM DTT and 150 mM KCl; and (C) 10 mM Tris–HCl (pH 7.5), 1 mM DTT, 150 mM KCl, and 150 μM spermidine. Data are the mean of 3 triplicate experiments. Standard deviations in ER elutions were within 5%.

mine were more efficient inhibitors at higher concentration.

ER binding to plasmids with ERE inserts

In the next set of experiments, we used pGEM-7Zf(+) plasmid with one and 4 ERE inserts [32,33] to study the effect of polyamines on ER–ERE interaction. Control and ERE plasmids were used in DNA–cellulose elution assay to determine the effects of polyamines. Figure 6 shows typical results of our experiments, presented as percentage of ER eluted from DNA–cellulose by the plasmids in the presence of polyamines. Putrescine, spermidine and spermine (panels A, B, and C, respectively) increased the binding of ER to ERE plasmid (with 4 inserts) over a relatively narrow concentration range. Surprisingly, the concentration of putrescine, spermidine, and spermine that facilitated ER–ERE interaction is in the range of 100–200 μM concentration. Effects of polyamines on the binding of ER to the ERE plasmid was higher than that on the control plasmid. Percent ER binding to ERE plasmid in the presence of 200 μM
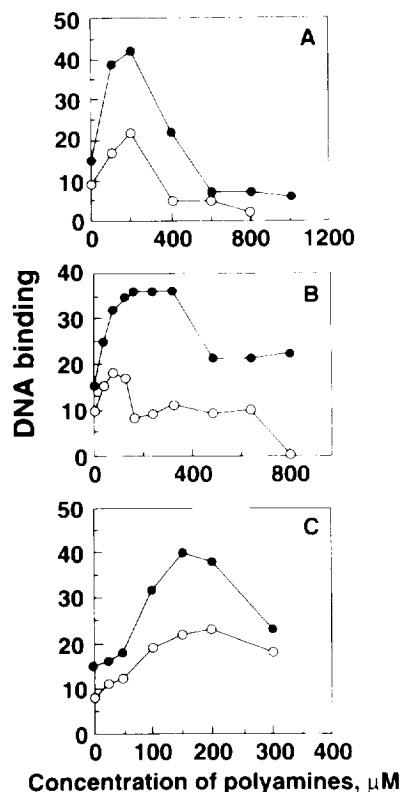
Fig. 6. Effects of putrescine (A), spermidine (B), and spermine (C) on the binding of ER to plasmid (5 μg/ml) containing 4 ERE (38 bp sequence) inserts ($\bullet$) in TDS buffer. Results with control plasmid in the same buffer are also shown ($\bigcirc$). The ability of plasmid DNA to bind to ER was determined by DNA–cellulose competitive elution assay in the presence of increasing concentrations of polyamines as described in Materials and Methods. Data are the mean of 3 triplicate experiments. Standard deviations are in ER elutions were within 5%.
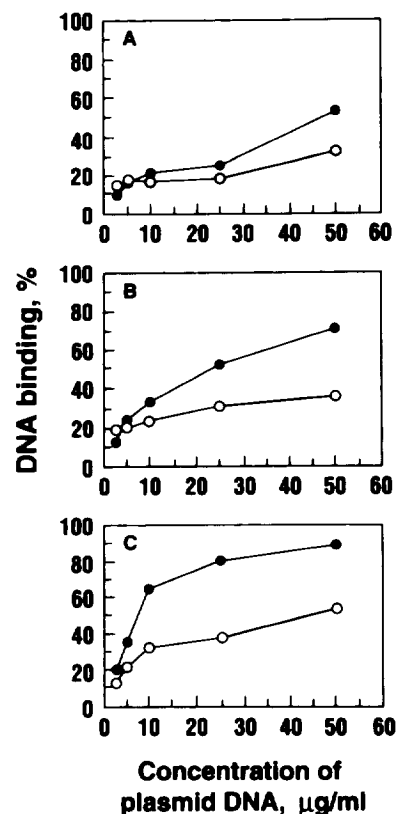
spermidine was 38%, whereas that of control plasmid was 10%. Similar results reaching a maximal binding of 32% were obtained for the plasmid with one ERE insert in the presence of 200 μM spermidine. These experiments on ERE and control plasmids were conducted in 10 mM Tris–HCl (pH 7.5), 1 mM EDTA and 150 mM KCl following a report by Murdoch et al. [44] that 150 mM KCl provided maximal difference in the binding affinity of ERE compared to non-specific DNA. The background elution of ER by Tris buffer containing 150 mM KCl was in the range of 5–10% of the total ER present on the DNA–cellulose. Our results indicate that polyamines provide additional discrimination between ER binding to specific and non-specific sequences.

In the next series of experiments, we quantitated the relative binding affinity of control and ERE plasmids for ER binding in the presence and absence of polyamines. Since the concentration of the plasmid (at constant polyamine concentration) required to elute 50% of the receptor (EC_{50}) is proportional to the affinity of the plasmid for the receptor, the EC_{50} values could serve as a measure of the relative binding affinity of the plasmid for ER. Figure 7 (A, B, and C) shows the concentration dependent elution of ER
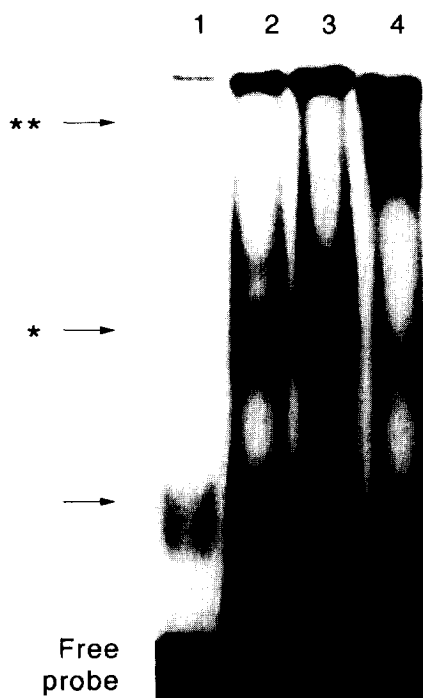
Fig. 8. Electrophoretic mobility shift assay to examine the effect of spermidine in facilitating ER–ERE interaction. The labeled ERE oligonucleotide was incubated at 25 C with 250 μM spermidine for 1h. The lanes are as follows: (1) ERE incubated with heat denatured ER; (2) ER–ERE reaction mixture without spermidine; (3) ER–ERE reaction mixture with 250 μM spermidine; and (4) ER–ERE reaction mixture with 250 μM spermidine and 2 μl of anti-ER antibody. Arrows indicate retarded bands representing DNA–proteins complexes. Specific binding (*) as well as antibody supershifted complexes (**) are also shown.

from DNA–cellulose by ERE (with 4 inserts) and control plasmids under various conditions. The relative affinity of ER for ERE and control plasmids was similar in the absence of salt (TD buffer) (panel A). Addition of 150 mM KCl increased ER binding to the ERE plasmid with little effect on the control plasmid (panel B). Addition of 150 μM spermidine further increased the binding affinity of ER to the ERE plasmid (panel C). The EC_{50} values for ERE and control plasmids were 7.5 and 50 μg/ml, respectively, in the presence of 150 μM spermidine. These results confirm that spermidine facilitates ER binding to ERE in the presence of physiologically relevant ionic concentrations of other salts.

ER binding to the ERE oligonucelotide

We next examined the ability of polyamines to enhance ER binding to ERE using an electrophoretic mobility shift assay. ERE oligomer was labeled with ^{32}P-ATP and then incubated with 250 μM spermidine. This concentration showed maximal binding in preliminary experiments designed to determine the optimal spermidine concentration that facilitated ER–ERE binding in gel mobility assay. As shown in Figure 8, 250 μM spermidine showed a 3-fold increase in the intensity of the band representing ER–ERE complex. In a parallel lane, we used ER binding reaction mixture incubated with an anti-ER monoclonal antibody (Neomarkers, Fremont, CA). We found that this antibody was effective in "supershifting" or retarding the mobility of the ER–ERE complex. A second band of protein–DNA complex was found in all samples including the labeled probe incubated with heat-denatured (65°C for 10 min) ER. This band was not supershifted by anti-ER antibody and was attributed to non-specific protein binding. Thus the shifted band that has increased intensity in the presence of spermidine represents the specific ER–ERE complex.

DISCUSSION

Results of this study demonstrate that physiological levels of polyamines facilitate ER binding both to synthetic polynucleotides that undergo the B-DNA to Z-DNA transition as well as to a consensus ERE sequence. Polyamine-induced conformational transition of the polynucleotide to the Z-DNA form under the conditions of the ER binding assay was confirmed by both CD and UV spectroscopy. Polyamines enhanced ER–ERE binding in a dose-dependent manner, with higher concentrations inhibiting binding. The binding of ER to the Z-DNA forming polynucleotide correlated with the midpoint of transition, but further increase in polyamine concentrations led to the inhibition of ER binding. The modulatory effects of polyamines on ER–ERE interaction may be a mechanism by which polyamines influence gene regulation. Cellular concentrations of polyamines are

reported to be in the millimolar range [45], a portion of which is bound to cellular macromolecules or sequestered into different cellular compartments [46]. Thus the concentration range that facilitates ER binding to a plasmid containing ERE sequences can be expected to have a regulatory role on estrogenic action in gene expression.

Polyamines facilitated ER–polynucleotide interaction at concentrations near the midpoint of the B-DNA to Z-DNA transition, but there were differences in the ability of various polyamines and $Co(NH_3)_6^{3+}$ in enhancing ER–DNA interaction. Although putrescine, spermidine, and spermine varied by one positive charge only, their optimal concentrations to enhance ER–DNA binding vary by 5-fold between putrescine and spermidine, and 7.5-fold between spermidine and spermine. Similarly, there is a 20-fold difference in the effective concentration of spermidine and N^1-acetyl spermidine and a 10-fold difference between spermine and acetylspermine in supporting ER–DNA interaction, even though acetylation removes only one positive charge from the polyamine. These differences suggest that polyamine structure, in addition to the positive charges, has a major role in modulating ER–DNA interactions. Among the trivalent cations, maximal effect on ER binding is exerted by $Co(NH_3)_6^{3+}$. The potency of $Co(NH_3)_6^{3+}$ is consistent with previous studies with this cation in B-DNA to Z-DNA transition, the apparent symmetry and steric properties of this molecule contributing to its higher efficacy in collapsing bacteriophage DNA to toroidal condensates [42]. In contrast to the structural specificity of polyamines in B-DNA to Z-DNA transition and ER binding to the polynucleotides, binding of ER to ERE plasmid was facilitated by approximately the same concentration of putrescine, spermidine, and spermine. Considering that the contact sites of monomeric ER with ERE is only 5 bp (half-palindrome) [5,6], the limited sites available for polyamine binding might be equally sensitive to putrescine, spermidine, and spermine.

The increase in ER–DNA binding near the midpoint of the B-DNA to Z-DNA transition suggests that high affinity binding is associated with a Z-DNA like conformational transition in the polynucleotide. One possibility is that the breakage of hydrogen bonds during Z-DNA formation presents a transitory opening of the double helix for ER binding. Molecular modelling studies suggest that a nucleation step in B-DNA to Z-DNA transition involves breakage of hydrogen bonding in a 3–4 bp region, causing free rotation of bases and subsequent transition to the left-handed conformation [47]. Amino acid residues of the DNA binding region of ER may form specific hydrogen bonds with nucleic acid bases during this nucleation process. ER was reported to bind preferentially to the coding strand of the ERE sequence [48,49]. A DNA-binding protein that destabilizes

DNA helix augmented binding of ER to ERE [50, 51]. X-ray crystallography of short oligonucleotides with $(CA)_n$ shows anomalous base pairing shift with mismatched major groove hydrogen bonding and a destabilization of adjacent G–C base pairing [52]. It is conceivable that similar abnormal hydrogen bonding and stacking interactions occur in the presence of polyamines in the ERE sequence, initiating a transitory opening of the helix analogous to the nucleation stage of the B-DNA to Z-DNA transition.

In addition to polyamine effects on DNA structure, stabilization of ER by polyamines might also contribute to enhanced ER–DNA binding. For example, estradiol binding to the receptor and the affinity of the ligand–receptor interactions were increased by polyamines. Our results, on the facilitation of ER–ERE binding by polyamines using DNA–cellulose competition assay, were confirmed by gel mobility shift assay. Similar results were also obtained by Sabbah et al. [53]. Our results on ERE inserted in a plasmid indicate that polyamines modulate ER binding even when the ERE forms only short stretches as it exists in the upstream regions of estrogen responsive genes. Recent studies on rat prolactin gene indicate the presence of multiple EREs spanning in 5′-flanking, 5′-untranslated and first exon regions and demonstrates the versatility of arrangements of EREs [54].

The high affinity of ER for DNA sequences that undergo B-DNA to Z-DNA transition suggests that sequences such as $(dA-dC)_n$ found in the estrogen responsive prolactin gene may have an effect on the topology and function of this region [55]. In our previous studies, a plasmid containing a 60 bp insert of $(dA-dC)_n$ eluted ER at a concentration of 1.5 $\mu g/ml$ [34], a value comparable to the value of 7.5 $\mu g/ml$ concentration determined in the present study with a plasmid containing ERE. The occupation of the Z-DNA forming region with ER could affect the availability of ER for selective activation of other genes/regions. Thus, intracellular polyamines might contribute to estrogenic responses depending on the presence of adjacent sequences in addition to the direct effect of polyamines on ERE. A down-regulation of estrogen-dependent expression of chloramphenicol acetyl transferase was observed in a pituitary cell line in the presence of $(dA-dC)_n$ inserts in the regulatory regions of this fusion gene [56] whereas similar sequences had stimulatory effects on transcription in other cellular and sequence contexts [57].

Inhibition of ER binding to DNA at high concentrations of polyamines might be a consequence of competitive interactions of polyamines and ER for available sites on DNA. Crystallographic studies of polyamine-oligonucleotide as well as ER–ERE complexes have shown preferential binding of ER and polyamines in the major groove of DNA [17,58]. Polyamines also have a preference for unusual confor-

mations such as Z-DNA and triplex DNA and drive labile blocks of DNA sequences to these conformations [20–24]. If the transient, single-stranded structure is the preferred binding site of ER, further alteration of this conformation to the Z-DNA form would disrupt ER interaction with the polynucleotide. Furthermore, polyamine-induced condensation and aggregation of DNA might also dislocate ER from its preferential binding sites on DNA [25,42]. The inhibitory effect of polyamines on ER–DNA binding might be a part of the control mechanism of estrogenic regulation of gene expression.

Our experiments depend on dissociation–association equilibria of ER established on DNA–cellulose and by elution with competing DNA. This experimental system is sensitive for quantifying affinity differences and may mimic the interaction of gene regulatory proteins with DNA, where the initial event involves the binding of the protein to non-specific, random sites on DNA [59]. The protein then slides or exchanges with specific sites on DNA until it reaches its recognition site. Thus the binding affinity of ER to specific DNA will depend on the efficiency of association and dissociation reactions. Since this process occurs *in vivo* in the presence of various cellular cations, our results represent a specific control mechanism that has not as yet been recognized.

In summary, our results provide evidence for the involvement of polyamines in facilitating ER–ERE binding in oligonucelotides and plasmids as well as in the binding of ER to polynucleotides capable of forming Z-DNA conformation. Conformational perturbations in ERE that are comparable to the B-DNA to Z-DNA transition might be involved in ER–ERE recognition. Enhanced binding of ER to ERE was followed by inhibition of this binding at higher polyamine concentration, indicating the modulatory effect of polyamines on ER–DNA interactions. The dynamics of the regulation of cellular polyamine concentrations might be functionally related to its ability to modulate DNA–protein interactions.

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