Polyamines alter sequence-specific DNA–protein interactions

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ABSTRACT

The polyamines are abundant biogenic cations implicated in many biological processes. Despite a plethora of evidence on polyamine-induced DNA conformational changes, no thorough study of their effects on the activities of sequence-specific DNA binding proteins has been performed. We describe the *in vitro* effects of polyamines on the activities of purified, representative DNA-binding proteins, and on complex protein mixtures. Polyamines at physiological concentrations enhance the binding of several proteins to DNA (e.g. USF, TFE3, Ig/EBP, NF-IL6, YY1 and ICP-4, a herpes simplex virus gene regulator), but inhibit others (e.g. Oct-1). The degree of enhancement correlates with cationic charge; divalent putrescine is ineffective whereas tetravalent spermine is more potent than trivalent spermidine. Polyamine effects on USF and ICP-4 result from increased rate of complex formation rather than a decreased rate of dissociation. DNase I footprint analysis indicated that polyamines do not alter DNA–protein contacts. Polyamines also facilitate formation of complexes involving binding of more than one protein on a DNA fragment.

INTRODUCTION

Polyamines, putrescine ($\text{H}_2\text{N}(\text{CH}_2)_4\text{NH}_2$), spermidine ($\text{H}_2\text{N}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}_2$) and spermine ($\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}_2$), are simple linear aliphatic compounds that are positively charged at physiologically relevant ionic and pH conditions. They are present in almost all cells in millimolar concentrations and together with magnesium ions they account for the majority of the intracellular cationic charges (1–3). Polyamines have been implicated in many physiological functions including DNA replication, transcription, translation, post-translational protein modifications and membrane structure (for review see 1,2,4). Some of these effects are polyamine specific, while others are due to the general cationic nature of these compounds. Irrespective of the specificity of their effects, polyamines are indispensable cellular components because their depletion, either by gene disruption or inhibitors of their biosynthesis, results in severe defects in cell growth (3,5–10).

Charge neutralization of intracellular polyanions (i.e. DNA and RNA) may be among the most important physiological roles of

polyamines. The anionic phosphates of DNA are among the primary targets for charge neutralization by intracellular cations. Polyamines bind to DNA and neutralize these charges (11), although hydrogen bonding, hydrophobic interactions and possible site-specificity have also been invoked in the binding of these compounds to DNA (for review see 2). X-ray crystallographic data and studies based on energy minimization calculations, indicate that spermine is localized along the edge of the major groove of DNA (12–16). Polyamine binding has profound effects on DNA structure causing transitions from B to both A and Z forms (17–20) and at higher concentrations condensation of either naked DNA (21–26) or chromatin (27,28). Binding of spermine also induces a 25° bend along the helical axis of the DNA (15,29). These polyamine-induced DNA conformational changes affect the activities of enzymes involved in DNA metabolism (30–33) and they would also be expected to alter the binding activities of sequence-specific DNA-binding proteins. Surprisingly, with one exception (34), no detailed studies have been reported on this latter subject.

The aim of the present work was to study polyamine effects on the DNA-binding activity of ICP-4, the major regulatory protein of herpes simplex virus type 1 (HSV-1). The physiological relevance of this study stems from the observation that spermidine and spermine along with ICP-4 are integral parts of HSV virions (35–37). Spermine, in particular, is found tightly associated with viral DNA in quantities capable of neutralizing 50% of its negative phosphate charges (36). As no nucleosomal organization of HSV-1 DNA is observed in infected cells, it can be assumed that polyamines are involved in charge neutralization of the virus DNA, even during the course of the infection.

We found that the DNA-binding activity of ICP-4 is dramatically increased in the presence of physiological concentrations of spermidine and spermine. To determine the generality of this effect we examined the activities of a battery of recombinant DNA-binding proteins, and we present here a thorough study of how polyamines affect their interaction with DNA.

MATERIALS AND METHODS

Plasmids, probes and expression of recombinant proteins

The probe containing the ICP-4 protein binding site was a 49 bp *Ava*I–*Bam*HI fragment from plasmid pIGA103 containing the sequences –17/+32 of the HSV-1 IE-3 gene cap site (38). This fragment was also end-filled using the Klenow fragment of DNA

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polymerase I and cloned in the *HincII* site of plasmid pUC19 to yield plasmid pCPC-1A. The ICP-4 binding site was recovered from pCPC-1A as a 68-bp *AvaI* fragment, end-filled and self-ligated. The ligation products were cloned in the *HincII* site of pUC19 and a plasmid containing six, concatamerized ICP-4 binding sites was isolated and designated pCPC-1B. The Oct-1 probe was a 197 bp *SmaI*-*NcoI* fragment from the IE-3 promoter (-329/-205) of HSV-1 containing the TAATGARAT element and an Oct-1 binding site (39,40). A 200 bp *DraI*-*DdeI* fragment from the murine IgH enhancer (41), containing both USF and Ig/EBP binding sites, was cloned in the *HincII* site of plasmid pUC18 to yield plasmid pUC-DD. The *DdeI* site is proximal to the *HindIII* and the *DraI* to the *EcoRI* sites of the vector. Plasmid pUC- μ E3, containing a TFE3 binding site (41), was prepared by cloning a double stranded oligonucleotide (5'-CCTTGACATGACCTGCTT-3') in the *HincII* site of pUC19. Plasmid pUC-E contains the C/EBP site from the IgH enhancer (41) and was created by cloning the double stranded oligonucleotide 5'-TCAACTCAACATTGCTCAAT-3' in the *HincII* site of plasmid pUC19. Plasmid pUC-MLU, containing an USF site, was constructed by cloning the double-stranded oligonucleotide 5'-GTGTAGGCCACGTGACCGGG-3' in the *HincII* site of pUC19. An *AvaI*-*HpaII* fragment from the myc gene promoter, containing a YY1 binding site, was cloned in the *HincII* site of pUC19 to yield pUC-myc213. The probes were end-labeled and purified as previously described (42).

The proteins Oct-1, NF-IL6 (LAP), TFE3, USF and YY1 were expressed in bacteria as glutathione S-transferase (GST) fusions (43). A *HincII*-*HindIII* fragment from the human Oct-1 cDNA was end-filled using the Klenow fragment of DNA polymerase I and cloned into the *SmaI* site of plasmid pGEX-2T (Pharmacia) to generate the Oct-1 expression plasmid. Plasmid pSCT-LAP (44) was digested with *EcoRI* and *NheI* and the insert was isolated, end-filled and cloned into the *SmaI* site of pGEX-2T. The resulting expression plasmid contains the rat NF-IL6 (LAP) coding sequence, beginning from the second methionine, fused in-frame with that of GST. The entire human USF cDNA (*AvaII*-*EcoRI*) was cloned in plasmid pGEX-2T in the same frame as GST (a gift of Cathleen Cooper, unpublished). A *BglII*-*StuI* fragment of the mouse TFE3 cDNA (base pairs 254-670 of the coding sequence) was ligated in the *SmaI* site of pGEX-2T. The resulting plasmid expresses the fusion protein containing a 138 amino acid fragment from TFE3, including the basic region, helix-loop-helix and leucine zipper domains, fused to GST. GST-YY1 expression has been described (45). The GST fusion proteins were expressed in *Escherichia coli*, purified and cleaved with thrombin as previously described (46).

Cells, viruses, nuclear extract preparation and electrophoretic mobility shift analysis (EMSA)

HeLa cells were propagated in monolayers as previously described (42). Nuclear extracts were prepared according to Dignam (47), with the modifications described in (42). The infection of HeLa cells with herpes simplex virus type-1, strain F (48), was performed as previously described (42) at a multiplicity of infection of 5 p.f.u./cell. Nuclear extracts were prepared from infected cells 6 h post-infection. The protein concentrations were determined using the Bradford method (49) and all extracts were stored frozen in aliquots at -70°C.

The study of ICP-4 complex formation with its DNA-binding site from the IE-3 cap site (-17/+32, *BamHI*-*AvaI* fragment) was performed by incubating either nuclear extracts or purified protein with the end-labeled restriction fragment for 20 min, as described (42). The EMSA analysis of the ICP-4 complexes was performed using native 4% polyacrylamide gels and a low ionic strength buffer system (50). Supershift assays were performed by adding 1 μ l anti-ICP-4 monoclonal antibody (H640, a gift of Dr L. Pereira) to the preformed DNA-protein complexes and incubating for an additional 30 min at room temperature before loading the gels. The DNA-binding assays for the other proteins tested (USF, TFE3, Ig/EBP, NF-IL6, GST-YY1 and Oct-1) were performed for 20 min at room temperature in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5% (v/v) glycerol, 1 mM EDTA, 4 mM dithiothreitol, 0.2 mg/ml bovine serum albumin in a final volume of 15 μ l. Poly(dI-dC) (200-400 ng/reaction) was included as non-specific competitor. The complexes were analyzed on native 6% polyacrylamide gels containing 0.25 $\times$ TBE as buffer.

Saturation binding analysis for the determination of the equilibrium dissociation constant (K_d) of USF was performed by incubating an appropriate amount of protein with increasing concentrations of probe DNA, ranging from 2.5×10^{-11} M to 10^{-8} M, for 20 min at room temperature. Under these conditions the binding reaction reached equilibrium (data not shown). The USF-DNA complexes were separated by EMSA and the amounts of free and protein-bound DNA were determined by β -emission spectroscopy using a Betascope 603 (Betagen). Only one USF-DNA complex was detectable, consistent with the idea that there is a 1:1 interaction between USF dimers (51) and the DNA probe. This was expected as the DNA probe contained only one USF binding site. Computer analysis of the data and the determination of the equilibrium dissociation constants were performed using InPlot, version 4 (GraphPad, San Diego, CA) with the one site binding (hyperbola) equation $Y = B_{\max} X / K_d + X$, where Y = specific binding, $B_{\max}$ = maximum binding, K_d = equilibrium dissociation constant and X = radioactive DNA probe concentration.

ICP-4 purification

ICP-4 was purified from HSV-1, type F, infected HeLa cells (8 h, 10 p.f.u./cell) using a protocol similar to that described by Kattar-Cooley and Wilcox which yields more than 90% pure ICP-4 protein (52). The following two modifications were made in the purification scheme; (i) Q-Sepharose (Pharmacia) was used in the anion-exchange chromatography step instead of DEAE-Trisacryl M; (ii) The affinity support was a 4 ml DNA-Sepharose column (ICP-4-binding site-Sepharose) containing 2 mg of covalently bound plasmid pCPC-1B. Plasmid pCPC-1B DNA was linearized with *EcoRI* and immobilized to cyanogen bromide-activated Sepharose as described (53). Both the ICP-4 protein and activity were monitored during the course of purification by immunoblot analysis (54) and DNA binding assays to ensure that the purified protein was active. The purified protein was stored in aliquots at -70°C.

Nitrocellulose DNA-binding assays

Binding reactions were performed under standard conditions and the mixture was applied to nitrocellulose filters equilibrated with binding buffer using a slot-blot apparatus (Minifold II, Schleicher and Schuell). Each slot was washed three times with 200 μ l of

binding buffer over a period of <3 min. Control experiments indicated that little DNA is retained on the filters in the absence of protein. The DNA-protein complexes were visualized by autoradiography or quantitated by scintillation counting of the radioactivity retained in each slot.

DNase I protection footprint analysis

The DNA probes used in the footprinting experiments were labeled on one strand by end-filling with the Klenow fragment of DNA polymerase I. The probe used in the DNase I footprint analysis of USF was derived from plasmid pUC-DD and labeled at the *Hind*III (*Dde*I) site, whereas the ICP-4 probe was a *Hind*III-*Eco*RI fragment from plasmid pIGA103 (38) labeled at the *Hind*III site.

DNA-cellulose competitive elution assay

Calf thymus DNA-cellulose (Sigma Chemical Co.) was treated as described (55). The DNA-cellulose suspension was aliquoted in tubes (7 μ g DNA/tube), pelleted by microcentrifugation for 10 min at top speed and TFE3 (0.5 μ g in 200 μ l) was added to the pellet and mixed for 60 min at 22°C. Protein elution took place in the presence of increasing amounts of the synthetic, double-stranded oligonucleotide poly(dI-dC) (Pharmacia Biotech. Inc.) in the absence or presence of 1 mM spermidine, as described (55). The amount of the TFE3 that eluted from the column was determined using an ELISA assay and rabbit polyclonal anti-TFE3 antibodies.

Glutaraldehyde crosslinking of TFE3

One microgram of purified, bacterially expressed TFE3 was crosslinked for 3 min at 22°C with 0.1% glutaraldehyde in 20 mM Hepes-KOH, pH 7.9, 0.2 mM EDTA, 0.1 M NaCl, 20% (v/v) glycerol and 0.5 mM DTT, as described (56). The reaction volume was 25 μ l and crosslinking was terminated by addition of an equal volume of 2 $\times$ SDS-PAGE loading buffer. The complexes were analyzed by SDS-PAGE on 10–13% linear gradient gels and immunoblot analysis was performed using a rabbit polyclonal anti-TFE3 antiserum.

RESULTS

Polyamines enhance ICP-4 binding to its cognate site

ICP-4, an immediate early protein encoded by herpes simplex virus type 1, is a sequence-specific DNA binding protein. It recognizes and binds as a dimer to a complex motif that contains the sequence ATCGTC in its core (57–59). ICP-4 binding sites are found in promoters of many HSV-1 genes, and in other regions scattered throughout the viral genome (58). One of the best characterized binding sites exists at the cap site of the IE-3 (ICP-4) gene (60). Binding of ICP-4 to this region results in autoregulation (61,62).

The binding of ICP-4 to the IE-3 cap site was studied by electrophoretic mobility shift analysis (EMSA) of protein-DNA complexes on native, low-ionic strength polyacrylamide gels. Under these conditions a unique ICP-4-containing complex is detected in infected nuclear extracts but not in extracts from mock-infected cells (Fig. 1). The presence of ICP-4 in the complex was verified by supershift analysis using an ICP-4-

Figure 1. Spermidine enhances ICP-4 binding to its cognate site. Electrophoretic mobility shift analysis (EMSA) of DNA-protein complexes formed after incubation of a DNA probe with nuclear extracts from HeLa cells infected with HSV-1, in the presence of the indicated spermidine concentrations, was performed as described in Materials and Methods. The nonspecific competitor is sonicated salmon sperm DNA and the probe is the *Bam*HI-*Ava*I fragment from the IE-3 promoter cap-site. The EMSA profile of a mock-infected nuclear extract is included for comparison.

specific monoclonal antibody (data not shown). The effect of spermidine on ICP-4 binding was measured after the addition of the polyamine to the binding reactions in concentrations ranging from 10^{-6} to 10^{-2} M. Physiological concentrations of spermidine (10^{-3} M) enhanced ICP-4 complex formation ~8-fold (Fig. 1). Addition of an anti-ICP-4 monoclonal antibody in the binding reactions completely supershifted this complex (data not shown), demonstrating that it was the ICP-4 binding activity that was enhanced by spermidine. Increased amounts of non-specific competitor did not alter the concentration range required to produce the effect, whereas increased non-specific competitor lowered the amount of the ICP-4-containing complex (Fig. 1).

In addition to ICP-4, nuclear extracts from infected cells contain other proteins capable of binding the DNA probe used. Spermidine affects the resulting complexes (I, II and III; Fig. 1) to different extents, varying from no effect for complex I, to enhancement at high concentrations (10 mM) for complex II, and inhibition of binding by physiological concentrations for complex III (Fig. 1). When nuclear extracts from mock-infected HeLa cells were tested, the presence of a novel DNA-protein complex was revealed in the presence of a narrow range of spermidine concentrations centered around 1 mM (Fig. 2). The data presented in Figure 2 demonstrate that the formation of complex II, or a complex with similar electrophoretic mobility, is stimulated by 10 mM spermidine even when nuclear extracts from mock-infected cells are used. These results indicate that spermidine differentially affects formation of DNA-protein complexes.

When the three naturally occurring polyamines were examined for their effects on ICP-4 binding activity, we noted that the enhancement of complex formation correlated with increasing charge. Putrescine²⁺ had only a minor effect, even at concentra-

Figure 2. Spermidine affects the binding of HeLa nuclear factors to the IE-3 promoter cap site. Binding reactions between the IE-3 probe and HeLa nuclear extracts were performed in the presence of the indicated concentrations of spermidine. A 6 h HSV-1-infected HeLa nuclear extract is included for comparison. The positions of a complex containing ICP-4 and of complexes I, II and III are indicated together with that of a prominent, slow migrating, polyamine-dependent complex (PA).

tions as high as 10 mM (Fig. 3), whereas spermine⁴⁺ was the most potent, in terms of amount of complex formed and its ability to elicit the effects at lower concentrations (Fig. 3).

Trivalent but not mono- or divalent-cations can enhance ICP-4 binding

To determine if the observed effects on ICP-4 binding are polyamine-specific or result from their polycationic nature we tested the effect of the inert trivalent cation cobalt(III)hexamine³⁺. This cation elicited a concentration-dependent enhancement of ICP-4 complex formation that was very similar to that of spermine (Fig. 4). In contrast, the divalent cations Mg²⁺ and Ca²⁺ inhibited ICP-4 binding activity at concentrations of 1 mM or higher (data not shown). A similar inhibitory effect was produced by the monovalent cation Na⁺ at concentrations above 50 mM.

Polyamines might enhance ICP-4 binding by dissociating ICP-4 from inactive complexes with other proteins or by inducing the activity of an ICP-4 modifying enzyme present in the nuclear extracts. To explore these possibilities, we studied the effects of spermidine, spermine and cobalt(III)hexamine³⁺ on the binding activity of affinity-purified ICP-4 (Fig. 5). These polyvalent cations were all effective in increasing the amount of ICP-4-DNA complexes at concentrations similar to those required to enhance ICP-4 DNA-binding activity from nuclear extracts prepared from HSV-1-infected cells.

Figure 3. Effects of polyamines on ICP-4 binding activity. The effects of putrescine, spermidine and spermine on ICP-4 binding to its cognate site were tested by performing the binding in the presence of the indicated polyamine concentrations and analyzing the complexes by EMSA. The ICP-4-DNA complexes were visualized by autoradiography and the polyamine effects were found to be reproducible after repeating each experiment at least three times for each polyamine. A representative gel was quantitated using a Betascope 603 (Betagen), as described in Materials and Methods and the results are given above. The amount of ICP-4-DNA complex measured in the absence of polyamines is set to one and values obtained in their presence are given as multiples of this value.

Figure 4. Cobalt(III)hexamine³⁺ enhances ICP-4 DNA binding. HSV-1-infected (6 h) HeLa nuclear extracts were the source of ICP-4 and its binding was tested in the absence or presence of the indicated concentrations of cobalt(III)hexamine³⁺ by EMSA. The position of the ICP-4-containing complexes is indicated.

Polyamines stimulate the formation of ICP-4-DNA complexes without affecting their dissociation

The stimulatory effect of polyamines on ICP-4 binding might result from either an increased rate of complex formation or stabilization of preformed complexes by these polycations. Since

Figure 5. Spermidine, spermine and cobalt(III)hexamine³⁺ effects on the binding activity of purified ICP-4. Affinity purified ICP-4 was incubated with the IE-3 probe and the DNA-protein complexes were measured by a nitrocellulose filter binding assay as described in Materials and Methods. The experiment was repeated three times and the results of a representative experiment are given. The amount of the ICP-4-DNA complexes formed in the absence of polyamines is set to one and results are expressed as multiples of this value.

the dissociation of ICP-4-DNA complexes is a slow process (52) it was unlikely that the stimulatory effect of polyamines resulted from stabilization of these complexes. To experimentally eliminate this possibility we measured the effect of 1 mM spermine, which produces the maximal effects on ICP-4 binding, on the dissociation of preformed complexes. A 500-fold molar excess of homologous cold competitor DNA was added to the reaction mixtures at the end of the binding reactions and the amounts of the labeled complexes that remained over a period of time were measured. The polyamine had no measurable effect on the dissociation of protein-DNA complexes (Fig. 6).

To determine if polyamines affected the association rate (k_{on}), the kinetics of complex formation were examined. At each time point a 500-fold excess of unlabeled, homologous, competitor DNA was added and the complexes were immediately loaded on a running native, low-ionic strength gel. Spermine addition both accelerated the rate (k_{on}) of ICP-4 complex formation, and increased the amount of complex. In the presence of spermine ICP-4 complex formation was complete in less than 2 min (Fig. 7). In contrast, complex formation in the absence of the polyamine continued to increase over 30 min. The effect is due to the polycationic nature of polyamines because it can be reproduced by cobalt(III)hexamine³⁺ (Fig. 7).

Polyamines do not alter the DNA site occupancy of ICP-4

Changes in DNA and protein conformation induced by polyamines may result in altered contacts between a protein and its DNA binding site. For example the site occupancy of the *E. coli* SSB protein changes from 35 nt (SSB₃₅) in the absence of polyamines to 56 nt (SSB₅₆) in their presence (63,64). Alterations in the ICP-4 DNA site occupancy should be detected by DNase I protection footprint analysis as changes in the DNA region that

Figure 6. Effect of spermine on the stability of ICP-4-DNA complexes. The effect of spermine (1 mM) on the stability of ICP-4-containing complexes was tested by adding a 500-fold excess of unlabeled probe to the binding reactions after complex formation. At the indicated times samples were withdrawn and loaded on a running native polyacrylamide gel for EMSA analysis of the remaining complexes.

Figure 7. Effect of spermine or cobalt(III)hexamine³⁺ on the association of ICP-4-DNA complexes. The effect of spermine (1 mM) or cobalt(III)hexamine³⁺ (1 mM) on the association of ICP-4 complexes was tested by allowing the binding reaction to take place for the indicated time, then stopping the reaction by adding a 500-fold excess of unlabeled probe and loading the reaction mixture on a running EMSA gel.

is protected from nucleolytic cleavage. Therefore, we used DNase I footprinting to ask if polyamines altered the DNA sequence that was protected from cleavage. Spermidine (1 mM) was used in this experiment because it enhances the ICP-4 DNA-binding activity without affecting the activity or specificity of DNase I (Fig. 8), in contrast to spermine which decreases the activity of this enzyme (data not shown). The DNA regions protected from DNase I cleavage by ICP-4 were identical in the absence or presence of spermidine (Fig. 8), demonstrating that the region of DNA protected by this protein does not change in the presence of the polyamine.

Figure 8. DNase I footprint protection analysis of (A) ICP-4-DNA and (B) USF-DNA complexes formed in the presence or absence of polyamines. The DNA fragments were incubated for 20 min, in the presence or absence of 1 mM spermidine (SPD), with or without the appropriate purified proteins and then treated with DNase I, as described in Materials and Methods. The deproteinized digestion products were analyzed on 6% sequencing gels, dried and autoradiographed. The protected DNA sequences are indicated along the side of each footprint and the nucleotides encompassing the core binding sites for ICP-4 and USF are marked with stars. The marker lanes (M) are a G-ladder for the ICP-4 footprint and end-labeled *MspI*-digested plasmid pBR322.

Effect of polyamines on the DNA-binding activities of other proteins

The DNA-binding activities of nuclear proteins other than ICP-4 were also affected by polyamines (Figs 1 and 2). The identities of these proteins are unknown but they all bound the same DNA fragment. To extend our observations, we studied the effect of polyamines on the binding activities of other known DNA binding proteins (or DNA binding protein domains). Included in our study were proteins, or domains of proteins, with the following structural motifs: basic-helix-loop-helix/leucine zipper (bHLH-ZIP; USF and TFE3) (65–67), basic/leucine zipper (bZIP; Ig/EBP and NF-IL6) (44), zinc finger (YY1) (68) and helix-turn-helix (Oct-1 and λ repressor) (69).

The binding activities of the bHLH-ZIP proteins tested (USF and TFE3) were stimulated by spermidine (Fig. 9), although both the magnitude and the concentration-dependence of the response differed. USF and TFE3 recognize and bind to identical DNA sequences and were originally cloned based on their interaction with similar binding sites. USF was characterized based on its interaction with a site in the adenovirus major late promoter (MLP) (65), whereas TFE3 was cloned by virtue of its interaction with the μ E3 site in the immunoglobulin heavy chain (IgH) gene enhancer (66). This allowed us to test whether the differential effects of spermidine on their binding resulted from interactions with a specific sequence, i.e. the USF site of the MLP or the μ E3

Figure 9. Spermidine effect on the binding of (A) recombinant USF and TFE3 proteins and (B) Ig/EBP and NF-IL6 proteins to their cognate DNA-binding sites. Binding reactions were performed in the presence of the indicated concentrations of spermidine and the DNA-protein complexes were analyzed by EMSA. Protein-DNA complexes were visualized by autoradiography and the polyamine effects were found to be reproducible after repeating each experiment at least three times. Representative gels were quantitated using a Betascope 603 (Betagen). The amount of protein-DNA complexes, for each protein, measured in the absence of spermidine is set to one and values obtained in its presence are given as multiples of this value.

site of the IgH enhancer, or if they were unique for a given protein-DNA interaction. Because swapping probes did not change the spermidine effect (data not shown), we conclude that the effectiveness of spermidine does not depend on the DNA sequence context in which the binding site is embedded, but on the nature of the particular protein-DNA interaction.

The possibility that polyamines affect non-specific rather than sequence specific DNA-protein interactions is unlikely because the binding reactions were performed in the presence of a vast excess of non-specific competitor DNA. To experimentally eliminate this possibility, however, we performed a DNA-cellulose competitive elution assay (55) using bacterially expressed TFE3 as a model protein. TFE3 was bound to DNA-cellulose and the effect of 1 mM spermidine on the elution of the protein from the column by increasing concentrations of non-specific competitor DNA poly(dI-dC) was measured. The presence of spermidine did not increase the ability of the non-specific competitor DNA to elute the DNA-bound TFE3 (data not shown), proving that, at least for TFE3, polyamines primarily affect sequence-specific rather than non-specific protein-DNA interactions.

The activities of another pair of structurally related proteins belonging to the bZIP family, Ig/EBP and NF-IL6, were also differentially affected by spermidine. NF-IL6 was stimulated more than Ig/EBP by spermidine, although the concentration of polyamine required to stimulate Ig/EBP binding was lower (Fig.

Figure 10. Effect of spermidine on the binding activities of recombinant YY1 and Oct-1 proteins. The binding reactions were performed in the presence of the indicated spermidine concentrations and the products were analyzed by EMSA. The positions of the GST-YY1- or Oct-1-DNA complexes, as well as those of the free, unbound probes, are indicated. Note that different probes were used in the two experiments (see Materials and Methods).

9B). Thus it is not possible to predict the nature or magnitude of the polyamine effect for a given DNA-binding protein based only on the structural taxonomy of its DNA-binding domain.

Not every DNA-binding protein tested was stimulated by polyamines. The activity of the DNA-binding domain of Oct-1, a helix-turn-helix protein, was actually inhibited by spermidine concentrations higher than 1 mM (Fig. 10) whereas the DNA-binding activity of λ repressor, another helix-turn-helix protein, was unaffected by polyamines (data not shown). The DNA-binding activity of the GST-YY1 fusion protein was only modestly stimulated by spermidine (Fig. 10).

In each instance, spermine and cobalt(III)hexamine³⁺ could substitute for spermidine, although the effective concentrations and the magnitude of binding increase differed (data not shown). This indicates that, as with ICP-4, the polyamine effects on the rest of the proteins included in this study result from their multivalent cationic nature.

The effect of spermidine on USF and TFE3 DNA-binding activities did not result from alterations in the lifetime of protein-DNA complexes as their dissociation rates remained unaffected (data not shown). In contrast, their association rates (k_{on}) were increased (data not shown), as observed with ICP-4. Saturation binding experiments with USF indicated that this resulted from increased affinity of the protein for its cognate DNA binding site in the presence of 1 mM spermidine (Fig. 11). The same was true for TFE3 (data not shown). DNase I footprinting of USF-DNA complexes formed in the absence or presence of 1 mM spermidine (Fig. 8B) revealed that the spermidine-induced conformational change, which accelerates protein-DNA complex formation, does not affect the DNA-site occupancy by USF.

Despite their well established effects on DNA conformation polyamines might also affect the conformation and/or the assembly state of individual proteins. Because several of the proteins used in this study can oligomerize [e.g. ICP-4 (57, 59), USF (51), TFE3 (70), Ig/EBP (71) and NF/IL-6 (71)] glutaraldehyde crosslinking was used to examine the effects of polyamines on the assembly of one of them (TFE3). TFE3 binds DNA as a dimer (70) although trimeric and tetrameric forms have been described (56). Increased assembly of a particular oligomeric

Figure 11. Saturation analysis of USF binding in the presence of the spermidine. A constant amount of recombinant USF was incubated for 20 min with increasing concentrations of a labeled DNA probe containing a USF binding site in the presence or absence of 1 mM spermidine. The products of the binding reactions were analyzed by EMSA on a native 6% acrylamide gel and the amounts of free and protein-bound DNA probe were quantitated using a Betascope 603 (Betagen). The calculations of the dissociation constants were performed as described in Materials and Methods.

form in the presence of spermidine can be detected by crosslinking and Western blotting following gel electrophoresis of the products. Concentrations of spermidine (1 mM) and spermine (0.1 mM) that increased TFE3 binding to DNA did not produce any significant differences in the crosslinking pattern of the protein, which migrated predominantly as a dimer (Fig. 12). The general inhibition of crosslinking observed in the presence of increasing concentrations of spermidine can be attributed to the presence of the excess reactive amino groups, provided by the polyamine, in the reaction. It can be reproduced even by putrescine which has no effect on the interaction of TFE3 with DNA. The slightly decreased mobility of TFE3 monomers and dimers in the polyamine-containing crosslinking reactions can be attributed to polyamine crosslinking to the protein and was found to occur even when the experiment was repeated with glutathione S-transferase (GST) (data not shown). The absence of a spermidine-specific effect on the equilibrium of the various multimeric forms of TFE3 *in vitro* does not exclude the possibility of other conformational changes induced by this polyamine.

Transcription initiation normally requires the assembly of numerous auxiliary factors, together with the basic transcription machinery, on the promoters of genes (72; and references therein). This assembly might be subject to such structural constraints as steric interference or protein-induced DNA conformational changes. In the study of complex formation between USF, Ig/EBP and a DNA probe from the IgH enhancer (that contained binding sites for both proteins) we did not detect the formation of complexes containing both proteins bound to the same DNA fragment (Fig. 13). Instead, complex formation was inhibited, possibly as the result of interference of each protein on the scanning process that controls sequence recognition (73). Inclusion of spermidine, even at concentrations having no effect on the binding activities of the individual proteins, restored binding of each individual protein, and it also allowed visualization of a tripartite complex (Fig. 13). Thus addition of

polyamines permits enhanced detection of multiprotein–DNA complexes *in vitro*.

DISCUSSION

The consequences of polyamine binding on DNA structure have been the focus of many experimental, as well as theoretical, studies (15,16,18–22,24,26,29,74–78). These biogenic amines are among the most abundant intracellular cations and their structure combines special characteristics such as unique spacing of the positive charges, a hydrophobic backbone which could allow for secondary interactions, and structural flexibility. The results from numerous studies indicate that polyamine binding to DNA can induce such conformational changes as transitions from the B to A and Z forms (17–20), bending (15,29), and, at high polyamine concentrations, condensation in rod- or toroidal-shaped structures (21–26). Not surprisingly these conformational changes can have a bearing on the activities of DNA modifying enzymes such as restriction endonucleases (33,79) and topoisomerase II (80), as well as on Hin invertasome assembly (81).

The DNA-binding activities of transcription factors might also be expected to be influenced by polyamine-induced conformational changes of DNA structure. However, only the estrogen receptor has been examined so far (34). Its binding to the estrogen response element, increased in the presence of spermidine and spermine. However, the multivalent nature of polyamines was not necessary in this interaction as the divalent cations Mg^{2+} and Ca^{2+} had similar effects on the binding of this protein.

In the present work we describe a more extensive study of polyamine effects on the DNA-binding activities of several eucaryotic transcription factors and on the viral protein ICP-4, a major transcriptional regulator of HSV-1. Our primary interest is on how ICP-4, a sequence-specific DNA binding protein, regulates herpes simplex virus gene expression. The observation that both spermidine and spermine specifically associate with HSV virions (35,36), and that ICP-4 is present in purified virions (37), prompted us to study the polyamine effects on ICP-4 binding. The amount of spermine in purified virus is sufficient to neutralize close to 50% of the DNA phosphate (36) and induce significant structural alterations to the virus DNA.

We found that the binding activity of ICP-4, and other proteins such as USF, TFE3, Ig/EBP, NF-IL6 and YY1, is enhanced by spermidine and spermine, although the magnitude of the effect varies with each protein. The inert trivalent cation cobalt(III) hexamine³⁺, which, like polyamines, can neutralize the phosphate charges (24,25) as well as bend DNA (82), can generate similar effects. The possibility that proteins are also targets of the polyamine action can not be excluded. The absence of a spermidine effect on the equilibrium of the TFE3 oligomeric forms (Fig. 12) suggests that it does not affect its assembly but does not exclude the possibility that the assembly states of other proteins are affected, or that polyamine- or cation-induced conformational changes take place. The divalent cations Ca^{2+} and Mg^{2+} , which affect binding to the estrogen receptor (34), do not enhance the binding of the proteins studied in this work. When the polyamine effect was tested in complex protein mixtures such as nuclear extracts from HSV-1-infected HeLa only the ICP-4-containing complex and complex II increased in abundance (Fig. 1). The levels of other protein–DNA complexes were either unaffected (complex I) or decreased (complex III). Therefore, the observed polyamine effects do not represent artefacts due to

Figure 12. Effects of polyamines on TFE3 oligomerization. The basic HLH-ZIP domain of TFE3 was expressed in bacteria, purified and 1 μ g of purified protein was crosslinked with 0.1% glutaraldehyde (lanes 2–9) in the presence of the indicated spermidine (Spd), putrescine (Put) or spermine (Spm) concentrations as described in Materials and Methods. The products were analyzed by SDS–PAGE on a 10–13% gradient gel and detected by immunoblotting using a rabbit polyclonal anti-TFE3 antibody. Untreated TFE3 is shown in lane 1. The closed arrows on the left indicate the positions of protein molecular weight markers and the open arrows on the right the positions of the TFE3 oligomeric forms.

Figure 13. Spermidine stimulates the formation of tripartite complexes containing USF and Ig/EBP bound to DNA. USF and Ig/EBP were incubated, either separately or together, with the *DraI*–*DdeI* fragment of the IgH enhancer, which contains binding sites for both proteins, in the presence of the indicated spermidine concentrations. The products of the binding reactions were analyzed by EMSA and the positions of the DNA–protein complexes formed are indicated.

specific condensation and precipitation of the competitor DNA present in the binding reactions. If that was the case, then the binding activity of every DNA binding protein present in the nuclear extracts would be expected to increase. The increased binding results from higher affinities of the proteins for their cognate sites in the presence of polyamines and not from stabilization of the protein–DNA complexes after they were formed. Footprint analysis indicated that the DNase I protection

patterns of protein–DNA complexes formed in the presence or absence of polyamine were indistinguishable. Thus, polyamines appear only to increase the initial binding step without altering specific DNA–protein interactions. This differentiates the sequence-specific proteins studied in this work from a non-specific DNA-binding protein, the *E. coli* SSB, whose site occupancy changes from 35 nt (SSB₃₅) in the absence to 56 nt (SSB₅₆) in the presence of polyamines (63,64).

Not every DNA-binding activity is enhanced by polyamines. Spermidine, in millimolar concentrations, inhibited the DNA-binding activity of the DNA-binding domain of Oct-1. The activity of Ig/EBP, which is stimulated by spermidine, was inhibited by millimolar concentrations of spermine. At least a portion of the differential effects of polyamines on the activities of DNA-binding proteins may result from different effects on DNA conformation being produced by polyamines over the range of concentrations tested, e.g. micromolar concentrations are needed for B to Z transitions (17,18) whereas millimolar concentrations induce DNA bending (83). The conformation resulting from polyamine binding might, therefore, be favorable for the interaction of one protein with its binding site but not for another.

Expression of ornithine decarboxylase, the first enzyme in polyamine biosynthesis, is increased very rapidly upon stimulation of cell growth, paralleling that of the *c-myc* proto-oncogene (84). Moreover, constitutive over-expression of ornithine decarboxylase can lead to oncogenic cell transformation (85). These findings suggest that polyamines might have a role in modulating gene expression. The importance of polyamines for gene expression was realized more than two decades ago when a number of studies on polyamine-effects on transcription was performed (for reviews see 1,2,4,86). The findings from these studies are complicated because of the inhibition of contaminating nucleases by polyamines. Nevertheless, they demonstrate that polyamines stimulate the activities of all three eucaryotic RNA polymerases. Our findings complement these studies, which suggested that polyamines increase the rate of RNA polymerization, by implying that they might also regulate promoter-specific transcription initiation through their effects on the recruitment of transcription factors.

The present study also provides a new experimental tool for the *in vitro* study of protein–DNA interactions. By adding polyamines, at physiologically relevant concentrations, higher DNA-binding activities can be achieved for some proteins. A similar enhancement of DNA-binding activity, by the Epstein–Barr Virus BZLF1 protein, was observed when basic peptides were added to reaction mixtures (87). We also demonstrate that they can be used for the detection of protein–DNA complexes involving multiple proteins binding to the same DNA fragment. Polyamines can stimulate the formation of such tripartite complexes as shown using the binding of USF and Ig/EBP to a target fragment from the IgH enhancer as an example. Other potential applications include the use of polyamines in the sequence-specific DNA-affinity purification of factors that respond to them, and for screening expression libraries for polyamine-responsive DNA binding proteins. We have successfully used the latter method to screen a cDNA expression library from HeLa cells to identify HMG's Y and I as proteins that interact with the DNA sequence spanning the cap site of the IE-3 gene from HSV-1 (C. A. Panagiotidis and S. J. Silverstein, unpublished results). Therefore, we propose that the effects of polyamine addition should be

tested in the study of DNA-binding proteins as a means of achieving more physiologically relevant experimental conditions and possibly enhancing DNA-binding activities.

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