TBP binding to the TATA box induces a specific downstream unwinding site that is targeted by pluramycin

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Background: The TATA-binding protein (TBP) is one of the major components of the human TFIID multiprotein complex. It is important in directing the initiation of RNA transcription at a site immediately downstream of the TATA sequence (TATA box) found in many eukaryotic promoters. The crystal structure of TBP complexed with an oligonucleotide containing the TATA box revealed a protein with an approximate two-fold symmetry which apparently has symmetrical interactions with DNA. It is not known how an asymmetric effect involving downstream activation can be produced by an apparent symmetric complex. We set out to examine the state of DNA in the TBP–DNA complex using pluramycin, a small molecular weight probe of DNA accessibility.

Results: Binding of TBP to the TATA box facilitates intercalation of pluramycin at a defined site immediately downstream of the TATA sequence through an apparent transient unwinding of the DNA. Pluramycin adducts are detected by the production of DNA strand breakage products upon heating. Incubation of pluramycin with the TBP–DNA complex facilitates the trapping of the specific complex by intercalation. Gel mobility shift and circularization assays reveal that the binding of pluramycin on the 3'-side of the TATA box region considerably stabilizes the TBP–DNA complex.

Conclusions: We propose that the TBP–DNA–pluramycin ternary complex is a 'specific' binding mode in which TBP and pluramycin make compensatory alterations in DNA, accounting for the improved stability of the ternary complex. We also propose a model of the ternary complex that explains the observed asymmetric effect of TBP binding to the TATA box.

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Introduction

TATA-binding protein (TBP) is a major component of the transcription factor IID (TFIID) found in all mammalian cells [1,2]. The association of TFIID with the TATA box is the first step in the formation of a transcription complex that can initiate the synthesis of mRNA [3–7]. Recent studies suggest that recruitment of TBP to the promoter can be a rate-limiting step for transcription *in vivo* and that interaction of TBP with the activation domains of other transcription factors may stabilize the binding of TBP to the promoter [8,9]. TBP is highly conserved in eukaryotic organisms and is one of the most intensively studied proteins involved in transcription. The shape of the carboxy-terminal portion of TBP resembles a saddle consisting of two roughly symmetric halves [10]. The inner surface of the saddle interacts with the minor groove of the TATA sequence, causing striking distortions in DNA [11–13]. The minor groove of the TATA box is widened and flattened and interacts with the whole concave underside of the molecular saddle through a variety of hydrophilic and hydrophobic contacts [2,10]. As a consequence of this unprecedented mode of interaction between a DNA-binding protein and DNA, the entire TATA box is severely bent through an angle of about 80° toward the major groove, with an associated unwinding of the TATA box of about 100° [11–13].

Despite the detailed structural analysis of TBP and the TBP–TATA box complex, several important features remain to be examined. Among these are the question of how TBP binding affects the structure of the regions that flank the TATA box and the structural origin of the asymmetry that follows initiation of transcription at the 3'-side of the TATA box, as a result of binding of an almost symmetric protein. We wished to explore the possibility that the asymmetric effect of TBP binding to the TATA sequence might be due to a transient unwinding occurring preferentially to the downstream side of the TATA box. We therefore designed experiments with pluramycin, which should preferentially intercalate in unwound DNA sites. Thus, dynamic effects propagated preferentially to one side or the other of the TATA box as a consequence of the TBP-induced unwinding of the TATA site can be monitored. Pluramycin (Fig. 1a) is a member of the family of anthraquinone-derived antitumor antibiotics, which are broadly characterized as DNA-threading intercalation agents (Fig. 1b) [14–18]. In addition to their intercalation properties, these drugs also form covalent adducts with N7 of guanine, which upon heat treatment give rise to DNA strand breakage products [15], thus revealing their site of intercalation (Fig. 1c).

Results

Specific and nonspecific binding of hTBP to DNA containing a TATA box

A DNase I protection assay was carried out initially to monitor the kinetics of formation of the human TBP (hTBP)–TATA box complex using a 64-base-pair (bp),

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double-stranded oligonucleotide (64M) (see Fig. 2) containing the muscle-specific promoter of the human myoglobin gene [19,20]. As illustrated in Figure 3a (lanes 2 and 10), the TATA box (5'-TATAAAAA) was completely protected from DNase I digestion in the absence of competitor DNA, and a region exterior to the TATA box was also weakly protected by hTBP (see brackets to the right of lane 10 in Figure 3a, denoting specific and nonspecific hTBP binding). To quantitate the affinity of hTBP for the flanking region of the TATA box (nonspecific binding) and for the TATA box region (specific binding), unlabeled template DNA (40 molar excess) was added to each reaction, and the dissociation of the hTBP–DNA complex was analyzed by DNase I footprinting after various intervals. The autoradiogram in Figure 3a was scanned by an LBK laser scanner, and the fraction of the specific and nonspecific hTBP–DNA complexes was calculated to determine the dissociation rates of the hTBP–DNA complexes in solution. By plotting the natural log of the fraction of probe DNA bound versus time, the first-order dissociation rate constants were calculated (Fig. 3b). As expected, the nonspecific complex between hTBP and the flanking regions of the TATA box was much less stable, with a half-life of 10 min, while the specific complex between hTBP and the TATA box region was much more stable, with a half-life of 120 min. The dissociation rate constants for the specific and nonspecific hTBP–DNA complexes in solution were calculated to be 8.9×10^{-5} s^{-1} and 2.6×10^{-3} s^{-1}, respectively. The ~30-fold difference in dissociation rates for the two types of hTBP binding sites provides evidence for both a class of weak, nonspecific binding sites between hTBP and the TATA binding region and a class of stronger, specific binding sites that have also been found with yeast TBP (yTBP) [21]. A similar pattern of protection was also observed with yTBP (unpublished results), but in this case, a region exterior to the TATA box was more weakly protected by yTBP compared to hTBP. Furthermore, the nonspecific complex between yTBP and

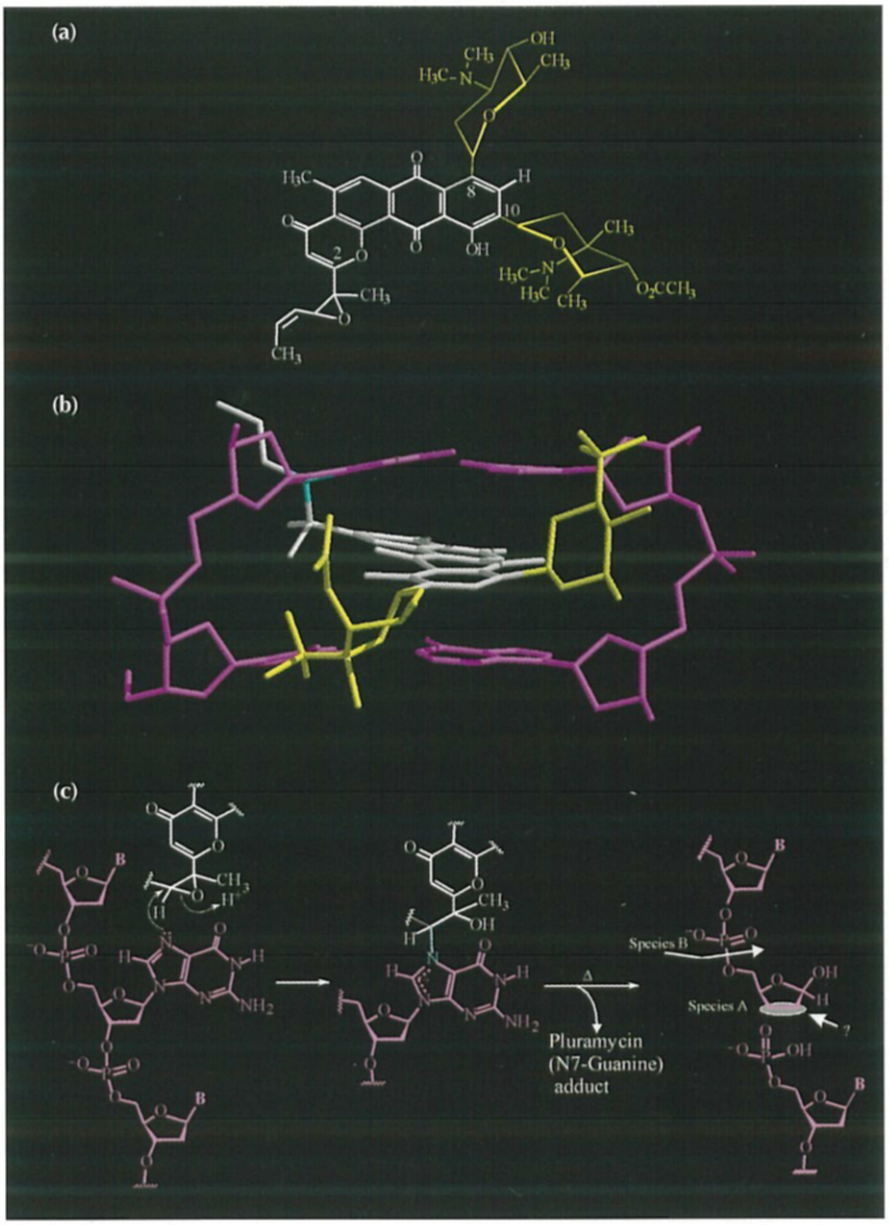


Fig. 1. Pluramycin as a probe of DNA accessibility. **(a)** Structure of pluramycin. **(b)** Solution conformation of the pluramycin–(N7-guanine) DNA adduct showing the intercalated moiety side chain at C2 (white), which undergoes reaction with N7 of guanine (cyan), and the sugars at C8 and C10 (yellow), which interact within the minor groove of DNA [18]. **(c)** Mechanisms for the covalent reaction of pluramycin with DNA and the heat-induced strand breakage of the pluramycin–(N7-guanine) DNA adduct [15]. The multiplicity of bands in Figs 3, 4, and 6 for each alkylation site are due to incomplete chemical degradation, which gives rise to both species **A** and **B**. Species **A** can be converted to species **B** with piperidine treatment.

Fig. 2. Sequences of oligonucleotides used in these studies. 64M is the sequence of the muscle-specific promoter of the human myoglobin gene. In oligonucleotide 64MI, the highlighted nucleotides flanking the TATA box are altered. The TATA sequence is boxed.

the flanking regions of the TATA box was much less stable than the nonspecific complex formed by hTBP, in agreement with the observations made by Hawley and colleagues [21]. We speculate that the amino terminus of hTBP may be important in modulation of nonspecific complex formation, since the carboxyl terminus is highly conserved between yTBP and hTBP [10–13], while the amino-terminal region varies considerably in length and in primary structure in different species [22]. To eliminate the nonspecific binding complex between hTBP and DNA in subsequent experiments, an excess of unlabeled competitor DNA was added to the preformed hTBP–DNA complex, and the reactions were incubated for 10 min before further experimentation.

Effect of specific binding of hTBP to the myoglobin TATA box on pluramycin modification of 64M

Pluramycin was used as a probe to identify possible unwinding sites generated by hTBP binding to the TATA box. The effect of hTBP binding to the TATA box on both the DNase I cleavage and pluramycin modification patterns is shown in Figure 4. In the absence of hTBP binding to the TATA box, pluramycin produces highly reactive bonding sites on both sides of the TATA box (see lanes 4 and 5 in Fig. 4a and arrows, which correspond to alkylated guanines). However, in the presence of hTBP, the pluramycin alkylation site immediately downstream of the TATA box shows enhanced cleavage, while alkylation of sites adjacent to the 5'-side of the TATA box is severely inhibited (compare lanes 4 and 6 and lanes 5 and 7 in Fig. 4a). The pluramycin modification pattern in the absence and presence of hTBP on the other strand is shown in Figure 4b (lanes 4 and 5, respectively). In contrast to the top strand, significantly enhanced pluramycin alkylation sites are not evident, and alkylation of the guanine immediately adjacent to the downstream site of the TATA box is not significantly inhibited. The unique reactivity of this downstream site is clearly related to the binding of TBP to

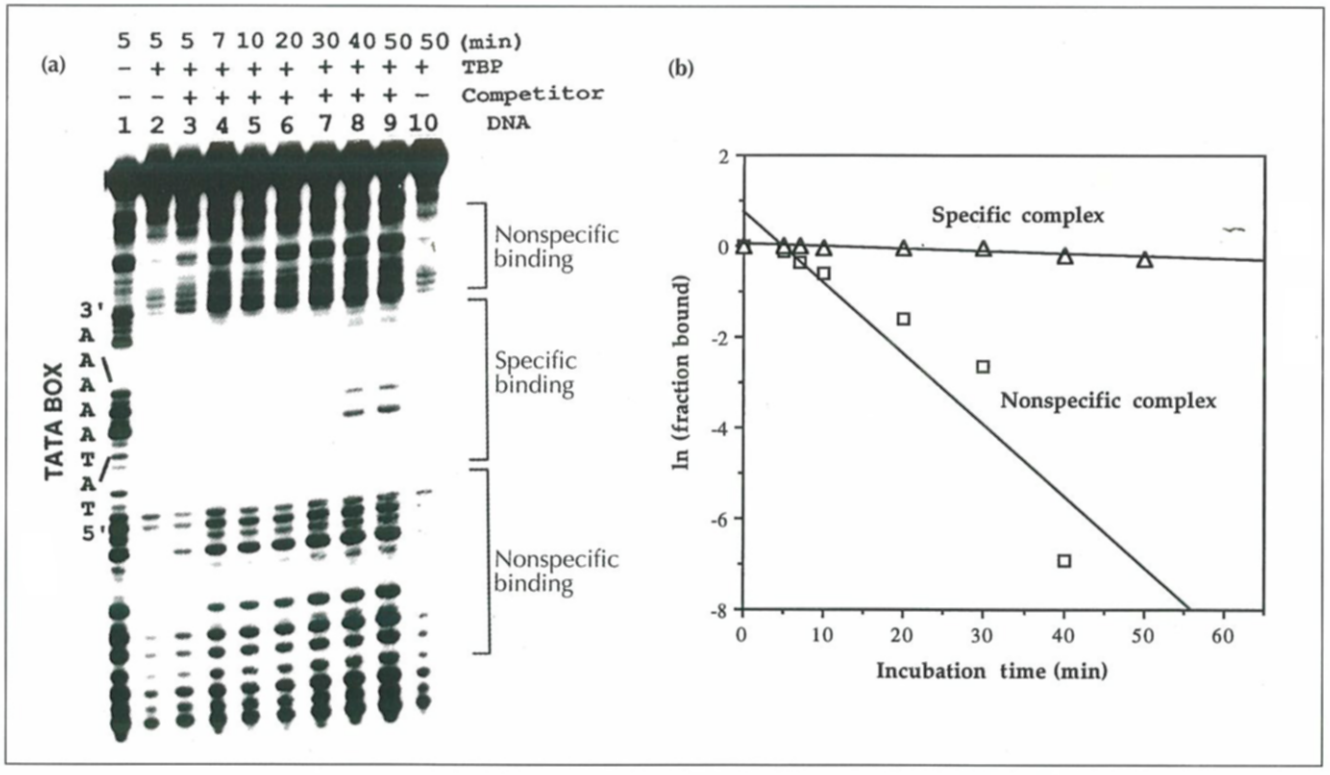


Fig. 3. Formation of specific and nonspecific complexes of hTBP with 64M. **(a)** DNase I footprinting. hTBP–DNA complexes were formed as described in Materials and methods and incubated for various periods of time (shown in the figure heading) in the absence (lanes 2 and 10) or presence (lanes 3–9) of unlabeled competitor DNA. For the footprinting analysis, 20 μl of the sample was removed, added to DNase I, and incubated an additional one min at 37 °C. The DNase I reactions were quenched with alkaline dye and processed for analysis on a sequencing gel. Brackets on the right-hand side of the gel show regions of specific and nonspecific hTBP binding. **(b)** Dissociation rates of the specific (–Δ–) and nonspecific (–❑–) complexes in solution. The natural log (ln) of the fraction of the DNA bound to hTBP is plotted against time (min). The first-order dissociation rate constants, calculated as the negative of the slopes, were determined to be 8.9×10^{-5} s^{-1} and 2.6×10^{-3} s^{-1} for the specific and nonspecific complexes, respectively.

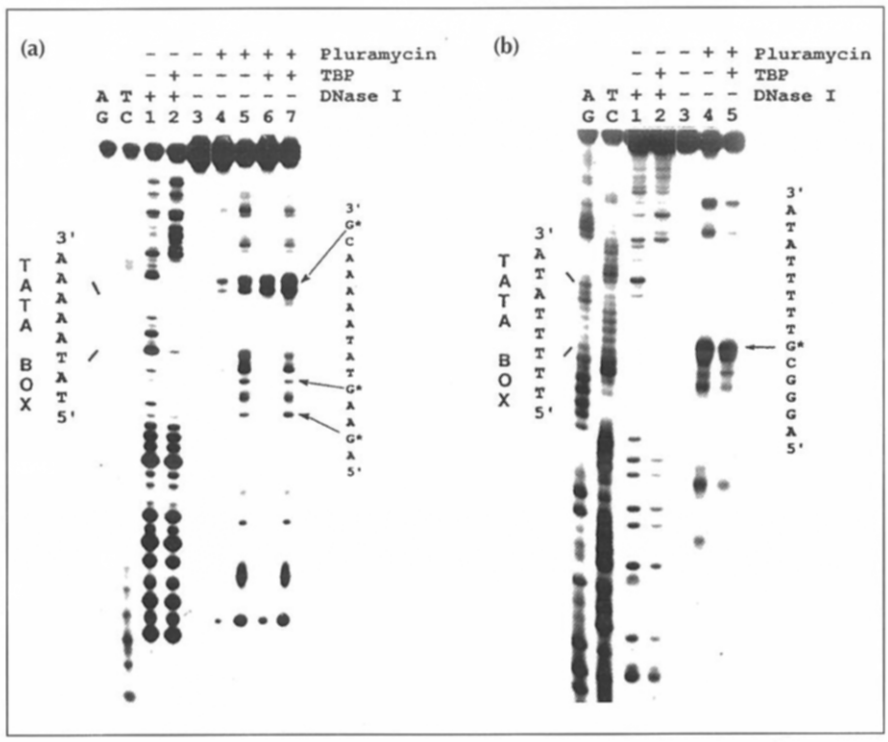


Fig. 4. Effect of specific hTBP binding to the TATA box on the pattern of pluramycin modification of 64M on the (+) and (–) strands. The specific hTBP–DNA complex was formed as described in Materials and methods and modified with pluramycin for 5 min. Drug modification was terminated by adding 100 μl of double-distilled water (DDW) containing 10 μg of calf thymus DNA followed by heating the reactions at 95 °C for 15 min to induce DNA strand breakage at the drug modification sites [15]. Lanes 1 and 2 of (a) [(+) strand] and (b) [(–) strand] are DNase I footprinting without and with hTBP, respectively. For (a), lanes 4 and 6 contained 100 nM of pluramycin (final concentration) and lanes 5 and 7 contained 400 nM of pluramycin. Drug modification was carried out for a period of 5 min. For (b), lanes 4 and 5 contained 400 nM of pluramycin.

the TATA box, as is the dampened reactivity of the other sites on both sides of the TATA box. To determine if sequence context could change the pattern of enhanced and diminished reactivity, a second sequence (64MI), in which the flanking sequences to the TATA box were different, was also tested for this asymmetric pattern of pluramycin modification in the presence of hTBP.

Effect of multiple pluramycin intercalation sites on both sides of the TATA box

Although the natural myoglobin muscle promoter TATA sequence was useful in that it provided a result suggestive of a downstream side-specific effect of TBP binding on pluramycin intercalation, it suffered from three potential drawbacks. First, a previous study on the sequence selectivity of pluramycin [16] has revealed that pluramycin prefers the 5'-CG★ sequence to the 5'-AG★, 5'-TG★, and 5'-GG★ sequences (★ indicates the pluramycin modification site). Therefore, we could not exclude the possibility that the absence of 5'-CG★ sequences on the upstream side of the TATA box might affect the overall efficiency of pluramycin modification in that region. Second, the enhanced pluramycin reactivity site might be a function of a special sequence context in proximity to the binding of hTBP to the TATA box. Third, this natural sequence did not reveal whether the effect was also specific for a site at a predetermined distance from the TATA box, in addition to being selective for the downstream side. Therefore, a new DNA template was designed in which repetitive 5'-CG★ sequences were included on both strands of the upstream and downstream sides of the TATA box in order to map precisely the hTBP-induced intercalative high-reactivity sites for pluramycin (see 64MI in Fig. 5d). Figure 5a,b shows an experiment similar to that shown in Figure 4 using the new DNA template. Pluramycin showed enhanced intercalation/alkylation reactivity with only a single site (c★ in Fig. 5c), which is located on the downstream side of the TATA box. Other sites (d, e, f, i, j and k) on the downstream side showed diminished reactivity or, in one case (h), similar reactivity. Significantly, site 'h' is located on the other side of the pluramycin intercalation site, which shows enhanced reactivity on the other strand (site c, Fig. 5c). Sites on the upstream side (a, b, and g) all showed reduced reactivity (see Fig. 5c). Therefore, the results using the natural myoglobin muscle enhancer sequence (Fig. 4) and those using the artificial sequence containing multiple CG★ sites for pluramycin modification are entirely consistent and reveal that binding of hTBP to the TATA box results in enhancement of pluramycin intercalation/reactivity that is specific for the downstream side of the TATA box and for a site immediately to the 3'-side of the TATA box (both results are summarized in Fig. 5d). In addition, a common site on the opposite strand, which is located on the other side of the pluramycin intercalation site in both 64M and 64MI, shows negligible changes in alkylation reactivity. Therefore, these results suggest that it is unlikely that these common sites of enhanced or similar pluramycin reactivity in 64M and 64MI are due to either a structural consequence of a specific sequence context or the inherent sequence selectivity of pluramycin, but they are in accord with a common unwinding site at this 5'-CG sequence, resulting from TBP binding to the adjacent sequence.

Effect of pluramycin modification on conversion of nonspecific to specific hTBP–TATA-box complexes

Since hTBP binding to the TATA box creates a high reactivity intercalation site for pluramycin, it was important to

determine how pluramycin would affect conversion of nonspecific to specific hTBP–DNA complexes. A gel mobility shift assay was used to monitor the conversion of nonspecific to specific hTBP complexes at 37 °C by adding a 40-molar excess of unlabeled competitor DNA and drawing aliquots at various times, which were then immediately loaded onto a native gel at 4 °C. Three parallel incubations were carried out without drug (control) or with pluramycin or (+)-CC-1065 (a minor-groove adenine N3 alkylating agent) added at the same time as competitor DNA. The results in Figure 6a (graphically represented in Fig. 6b), show that in the presence of pluramycin the amount of conversion of the nonspecific to the specific complex was significantly increased compared to a control experiment without pluramycin (compare left and middle panels). This is in sharp contrast to an experiment in which the conversion to the specific complex was almost completely inhibited in the presence of (+)-CC-1065 [23],

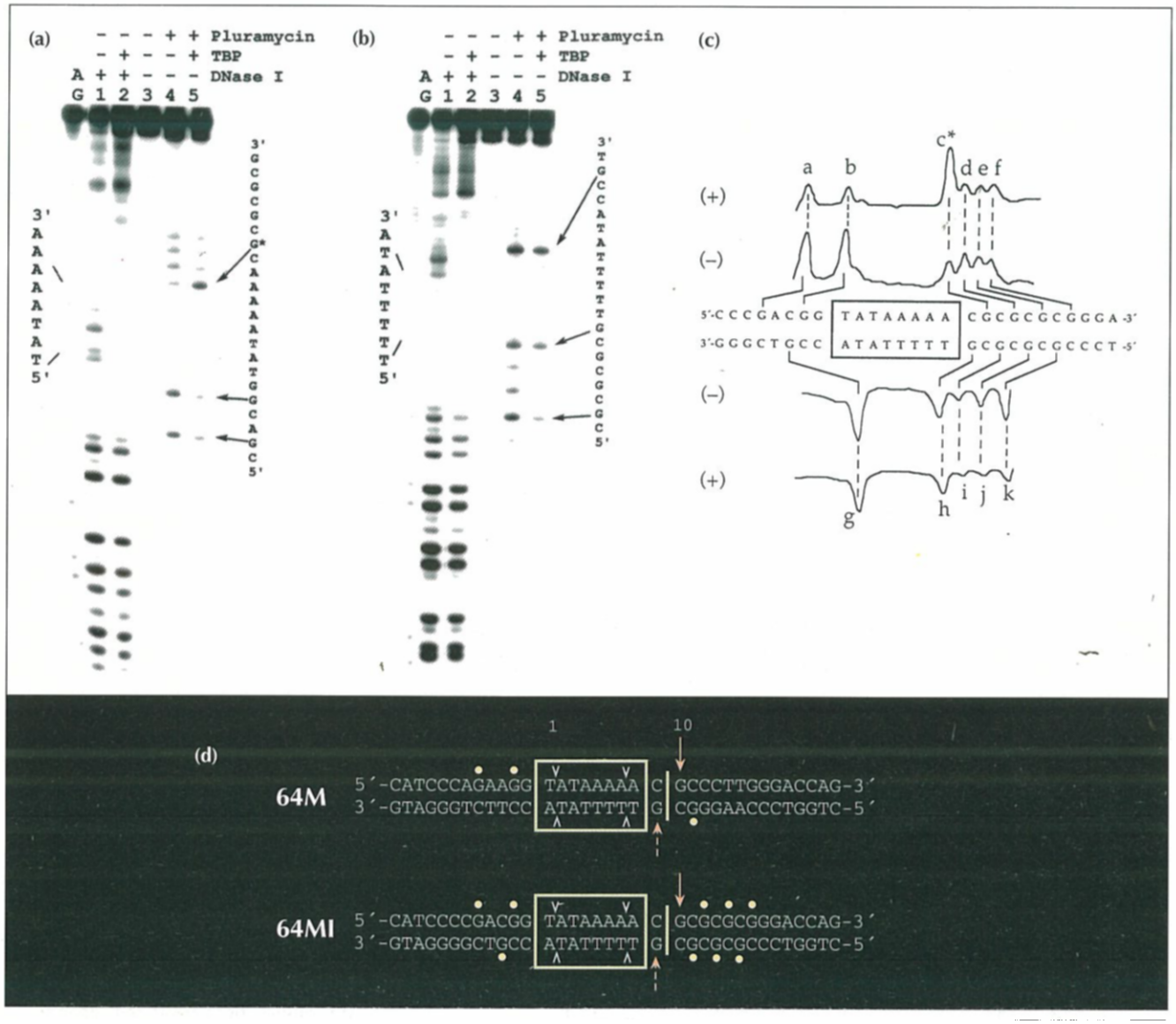


Fig. 5. Effect of specific hTBP binding to the TATA box on the pattern of pluramycin modification of the 64MI DNA template on the (+) and (–) strands. The specific hTBP–DNA complex was formed as described in Materials and methods, and 200 nM of pluramycin (final concentration) was incubated with the reaction mixture for 5 min. Pluramycin modification was stopped by adding 100 μl of DDW containing 10 μg of calf thymus DNA followed by phenol/chloroform extraction and ethanol precipitation. DNA pellets were dissolved in 20 μl of 1M piperidine solution, and samples were heated at 95 °C for 15 min to induce strand breakage at drug modification sites, as described in Materials and methods. The pattern of pluramycin protection on the (+) strand **(a)** and (–) strand **(b)** of oligomer DNA 64MI. The sequences to the left and right of (a) and (b) show the sequence of the TATA element and pluramycin alkylation sites on both sides of the TATA box, respectively. **(c)** Pairs of densitometer scans of lanes 4 and 5 from (a) and (b) of this figure. Inside scans are in the absence (–) and outside scans are in the presence (+) of TBP. Sites 'a' through 'k' are pluramycin alkylation sites (the asterisk shows the pluramycin-enhanced site). The TATA box is enclosed in a rectangle. **(d)** Schematic representation of pluramycin enhancement, no change, and protection sites on a portion of oligomer DNAs 64M and 64MI. The yellow dots indicate sites of inhibition, solid red arrows indicate sites of enhancement, dotted red arrows indicate no change, and the blue arrows indicate sites of phenylalanine insertion. The dinucleotide numbering system used later in the paper is noted above the sequence.

presumably due to the selective modification of the minor groove of the TATA box by (+)-CC-1065 (see right panel; our unpublished data).

Subsequently DNA was isolated from both the unbound and the hTBP-bound samples (I and II in Fig. 7a), and the thermal strand-breakage assay was then used to determine the extent and the site selectivity of pluramycin modification in both samples of DNA on the (+) and (–) strands (Fig. 7b). In accord with previous experiments, this assay reveals that pluramycin modification is specifically enriched at the two adjacent guanines on opposite strands immediately to the downstream side of the TATA box in the specific hTBP-bound DNA (compare lanes B and F in Figure 7b for both the (+) and (–) strands).

To obtain further evidence for the increased conversion of nonspecific to specific complexes in the presence of pluramycin, the effect of increasing quantities of drug in the incubation mixture on the amount of the specific hTBP–DNA complexes was analyzed by the gel mobility shift assay. The results shown in Figure 8 indicate that as the concentration of pluramycin is increased, the amount of the hTBP–DNA complex also increases. In a parallel experiment, the pluramycin-modified DNA was recovered from the hTBP–DNA complexes, and the thermal strand-breakage assay was then used to monitor the extent of pluramycin modification. This result also shows a positive correlation between the extent of pluramycin modification at the downstream sites and the amount of conversion of nonspecific to specific hTBP–DNA complexes (data not shown).

Pluramycin modification of downstream sites increases the stability of the hTBP–DNA complex

A direct comparison of the stability of the hTBP–DNA complex in the absence and presence of pluramycin specifically bound at the downstream enhancement sites was made by determining the dissociation rate of the respective specific hTBP–DNA complexes (left and right panels of Fig. 9, respectively); both experiments were carried out in the presence of excess nonlabeled competitor DNA. The results in the right panel show that in the presence of pluramycin the specific hTBP–TATA box complex is quite stable, at least up to 120 min. In contrast, about a 50 % dissociation of the complex occurred in the absence of pluramycin during the same period of time, based upon the densitometric measurements of the specific complex (unpublished results). Therefore, intercalation of pluramycin into the region immediately downstream of the TATA box considerably stabilizes the specific hTBP–DNA complex.

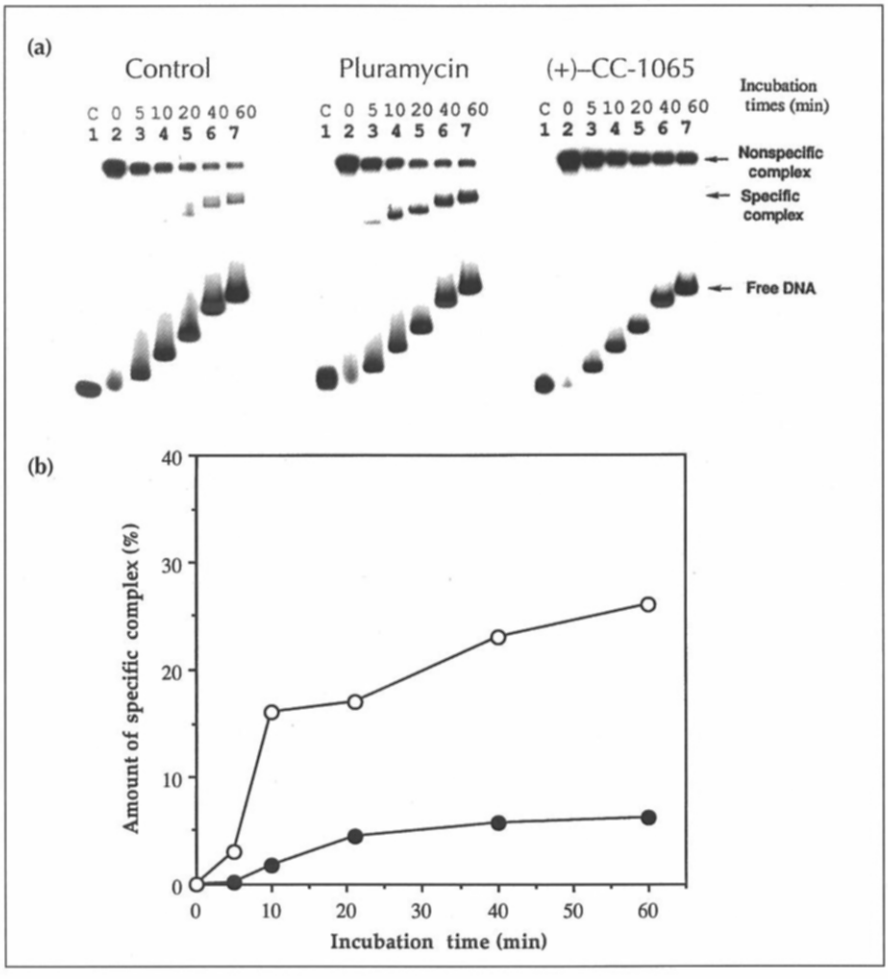


Fig. 6. Effect of pluramycin and (+)-CC-1065 modification of 64M on the conversion of the nonspecific to the specific binding complex between hTBP and the TATA box. The hTBP–DNA complexes were formed at 37 °C in a final volume of 100 μl, as described in Materials and methods. **(a)** A 20-fold excess of cold-template DNA was added to reactions without (left panel) or together with 400 nM of pluramycin (center panel) or 200 nM of (+)-CC-1065 (right panel). At the indicated times (min), the extent of hTBP–DNA complex formation was determined by a band shift analysis. A sample (5 μl) of the reaction was loaded onto the gel and separated by electrophoresis at 4 °C. Lane 1 shows a control reaction without hTBP, lane 2 shows an hTBP–DNA complex in the absence of competitor DNA, and lanes 3–7 are incubation with cold-template DNA for the indicated times before loading on the gel. **(b)** The left and center autoradiograms shown in (a) were scanned with a densitometer and the percentage of specific complex formed was plotted against time (–●–, control; –○–, incubated with pluramycin).

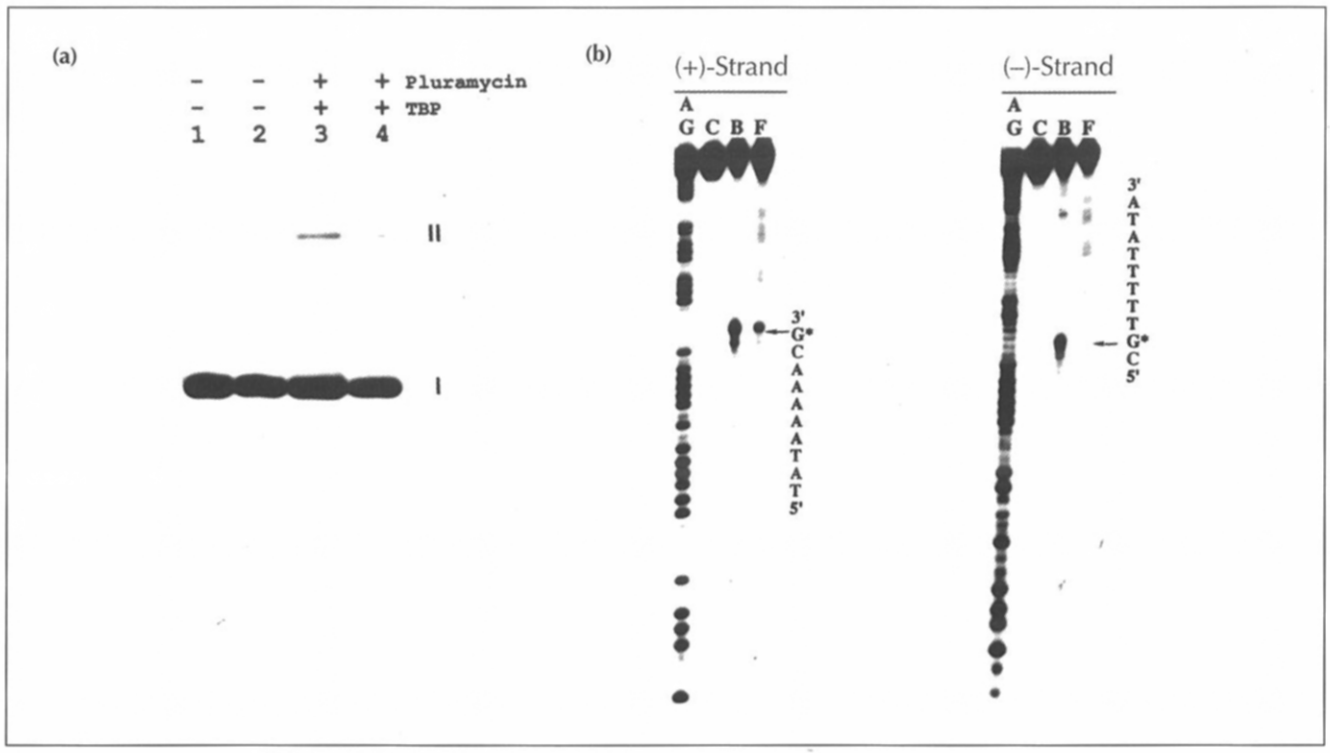


Fig. 7. Determination of the extent and sites of pluramycin modification in unbound and TBP-bound oligonucleotides. **(a)** Gel electrophoretic isolation of the pluramycin–modified specific hTBP–64M complex after modification with pluramycin. The specific hTBP–DNA complex was formed as described in Materials and methods. After addition of 20 molar excess of unlabeled competitor DNA, the reaction was incubated for a further 10 min, modified with 200 nM of pluramycin for 5 min, and loaded onto a 5 % polyacrylamide gel to separate the hTBP–64M complex from the free 64M. Lanes 1 and 3 correspond to (+) strand-labeled template DNA and lanes 2 and 4 correspond to (–) strand-labeled template DNA (I and II refer to the free 64M and hTBP–64M complexes, respectively). **(b)** Determination of the site of pluramycin modification on the (+) and (–) strands of 64M recovered from the specific hTBP–DNA complex. DNA molecules corresponding to bands I and II in (a) from the (+) and (–) strands were recovered from the gel (see Materials and methods) and subjected to the thermal strand-breakage assay [15]. Lane headings 'F' and 'B' refer to the free 64M and hTBP–64M complexes, respectively, obtained from lanes 3 [(+) strand] and 4 [(–) strand]. The sequence to the right of the (+) and (–) strands shows the pluramycin alkylation sites (denoted with an asterisk) on the downstream side.

Pluramycin modification stabilizes the hTBP-induced bending and unwinding of DNA

In previous studies, the ability of TBP to bend DNA has been demonstrated by means of a gel mobility shift assay and crystallographic observation [11–13,24]. In this study, the stability of the hTBP-induced DNA bending and unwinding was examined by studying the efficiency of cyclization of multimers of a 53-bp oligomer DNA fragment containing the TATA box, carried out in the presence of T4 DNA ligase. Significantly, no net change in the linking number occurs because the unwinding is offset by changes in writhing [25]. As shown in Figure 10a, in the presence of T4 DNA ligase, the 53-bp DNA fragment formed a set of linear dimers, trimers, and tetramers (L2, L3 and L4) and closed circular trimers (C3) but was not converted into covalently closed circular dimers (C2). However, upon the addition of hTBP at a protein to DNA ratio of 3 to 1, circular dimers (106 bp) appeared among the ligation products. In subsequent experiments, the effect of pluramycin modification on the efficiency of the formation of circular dimers was examined. Pluramycin modification of DNA (Fig. 10c, lanes 5 and 6) increased the number of circular dimers; only one third of the amount of hTBP was required to produce the same amount of C2. These results support the idea that pluramycin modification of guanine residues at the 3'-side of the TATA box stabilizes a specific binding mode of hTBP to DNA by forming a stably unwound form of DNA adjacent to the hTBP binding site. This result also suggests that the specific binding mode of the hTBP–TATA box complex that is stabilized by pluramycin has no net change in bending unless additional writhing occurs to compensate for this.

Discussion

The main purpose of this study was to use pluramycin as a molecular probe to determine the effects of hTBP binding on local DNA structure and the dynamics of the event in the vicinity of the TATA box. Pluramycin belongs to the anthraquinone-derived antitumor antibiotic family, which also includes the altromycins, hedamycin, and rubiflavin (for review, see [14]). In a recent study, Beerman and colleagues [26] used hedamycin as a DNA interactive agent to examine how drug modification of DNA affects TBP binding. Their study shows inhibition of TBP binding to the adenovirus major late promoter. This finding is not in conflict with

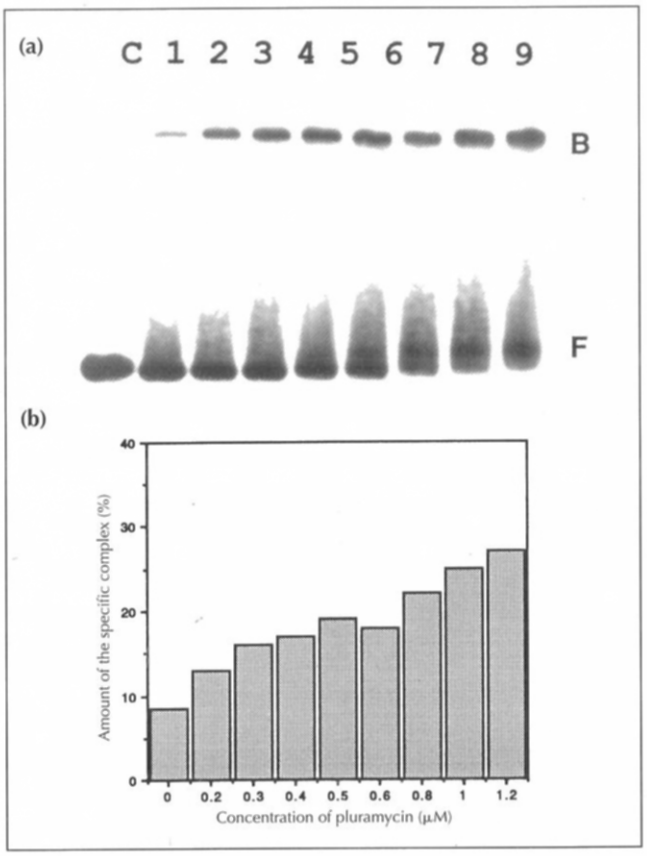


Fig. 8. Determination of the effect of increasing concentrations of pluramycin on the formation of the specific hTBP–DNA complexes using a gel shift assay. **(a)** hTBP–DNA complexes were formed as described in Materials and methods, and a predetermined amount of pluramycin was added to each of the incubations. After incubation for 30 min, the amounts of the specific hTBP–DNA complexes were determined by densitometer scans of individual lanes. Lane C, control (in the absence of hTBP). Lanes 1–9 contained hTBP plus 0, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0 and 1.2 mM, respectively, of pluramycin. **(b)** Histogram of the percentage of the specific complex plotted against the concentration of pluramycin determined from (a).

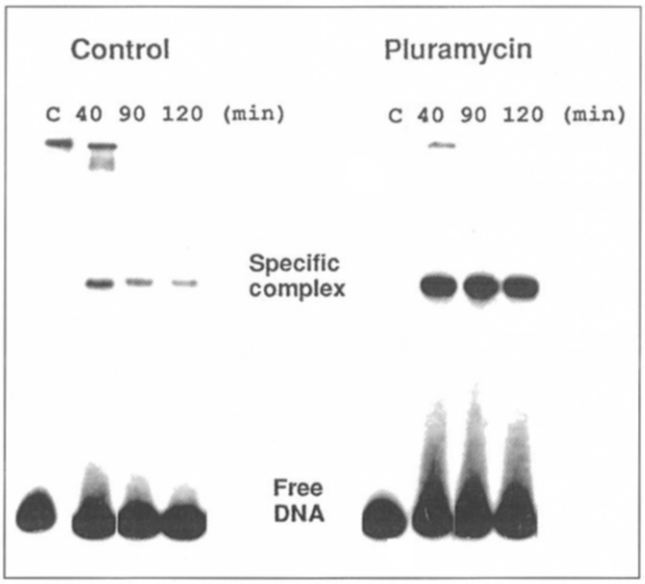


Fig. 9. Dissociation kinetics of the specific hTBP–DNA complex in the presence (right panel) or absence (left panel) of pluramycin, determined by a gel mobility shift assay. Using the pluramycin-modified DNA (right) or unmodified DNA (left), hTBP–DNA complexes were formed as described in Materials and methods. To determine the dissociation rate of the hTBP–DNA complex, a 40-molar excess of unlabeled competitor DNA was added to the reactions, which was then incubated for the indicated periods of time shown in the lane headings. The residual amount of the specific hTBP–DNA complex at various time intervals was analyzed by gel mobility shift assay (see text).

the results reported here, because binding of hedamycin to the guanine (G*) adjacent to the 3'-side of the TATA box in this promoter (5'-TATAAAAG*GG) blocks the insertion of the amino-terminal pair of phenylalanine residues into the ApG dinucleotide, presumably resulting in the prevention of TBP–DNA complex formation.

We have recently shown that these antibiotics interact with DNA in a threading intercalating mode, but they also alkylate N7 of guanine with a sequence specificity that lies to the 5'-side of the covalently modified guanine [14–18]. As a requirement for pluramycin reaction with DNA, the helix must be unwound and the minor groove must be accessible to the sugar moieties of pluramycin (see Fig. 1b). Pluramycin has a critical advantage over other, better known unwinding probes such as MPE·Fe(II) or Cu–phenanthroline as a molecular probe for protein-induced unwinding of DNA, because it forms a stable covalent attachment at the intercalation site. The other agents hit and run, leaving strand breaks at the intercalation site. Although in previous studies using DNase I, MPE·Fe(II) and Cu–phenanthroline, the area protected by TBP extended beyond the base pairs normally considered part of the TATA box consensus sequence [27–29], these footprinting methods generally did not reveal the relative importance of individual bases in the interaction with the protein or altered DNA structure upon TBP binding to the TATA box. Moreover, the demonstrated compatibility of pluramycin intercalation with hTBP binding to the TATA box and the increased stability of the ternary complex could not have been determined with these strand breakage reagents.

In this study we demonstrate that hTBP binding to the TATA box creates an enhanced pluramycin intercalation site immediately downstream of the TATA sequence. This results in increased covalent reactivity with one of the guanines flanking this intercalation site (see Figs 4, 5d). This result is surprising, since in the crystal structures of TBP bound to DNA solved by both Burley and colleagues [12] and Sigler and colleagues [11], there is no indication of a deformation of DNA at, or adjacent to, the enhanced pluramycin reactivity/intercalation site. In fact, at the 8–9 and 9–10 dinucleotides (pluramycin intercalates between base pairs 9 and 10, numbered with the first T of TATA as nucleotide 1; see Fig. 5d), the co-crystals show B-form DNA. Therefore, we propose that pluramycin is detecting a transient unwinding effect

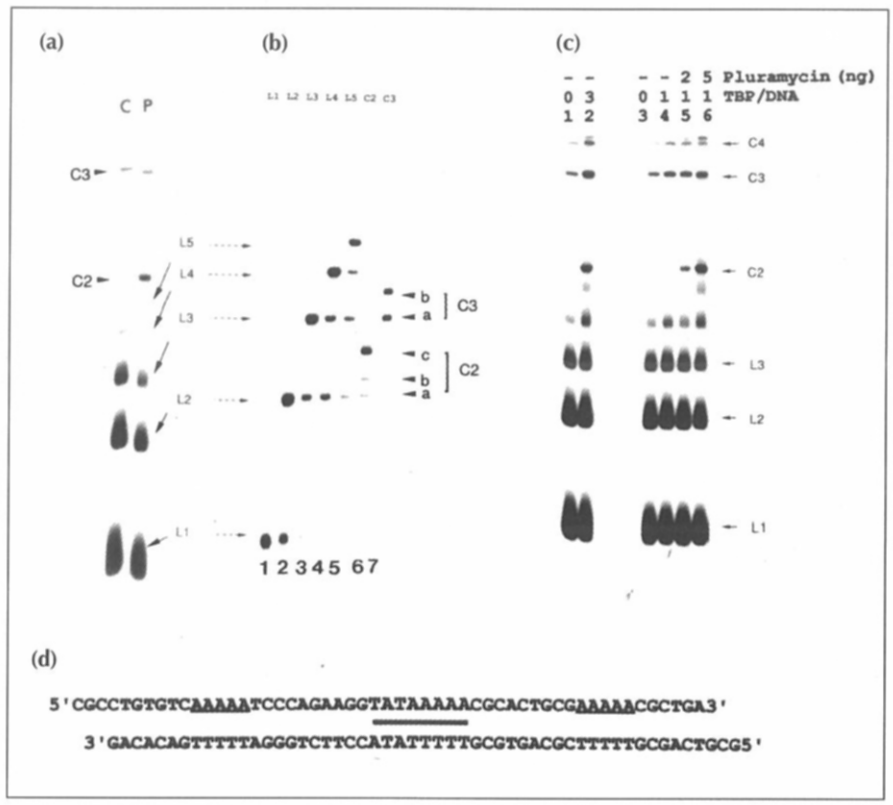


Fig. 10. **(a)** Effect of incubation with hTBP on the T4 ligase-catalyzed conversion of a 53-bp oligonucleotide to dimer 106-bp circles. L1–L5 indicate linear monomer to linear pentamer, and C2 and C3 indicate circular dimer and circular trimer DNA. Lanes C and P represent the ligation products without and with three molar excess of hTBP in the reaction. **(b)** Denaturing gel analysis of the DNA species (L1–L5 and C2–C3) isolated from the gel shown in (a). For DNA molecules designated as C2 and C3, bands a, b and c represent linear, circular single-stranded and undenatured DNA species, respectively [36]. **(c)** Effect of pluramycin modification on the efficiency of hTBP-induced cyclization. Lanes 1 and 3 show control reactions without hTBP and lanes 2 and 4 represent ligation with three- and one-molar excesses of hTBP, respectively. Lanes 5 and 6 contain 2 and 5 ng of pluramycin, respectively, in addition to hTBP at a one-molar excess. **(d)** Sequence of the 53-bp oligonucleotide used in the cyclization experiments. The bar indicates the TATA box consensus sequence, and the flanking A5 tracts are underlined.

rather than a stable structural change in this region, and this is associated with hTBP binding to the adjacent TATA box. It is important to differentiate between the possibility that enhanced reactivity of pluramycin at this site is a direct effect of a stable change in minor groove geometry at the upstream base pair, providing an increased proximity effect at the alkylation site, and the possibility that the indirect unwinding effect is transient and related to the adjacent binding of hTBP to DNA. We believe the former explanation, involving a favorable minor-groove geometry change, is unlikely because this would be revealed in the X-ray studies. It would also be sequence dependent, and therefore should result in sites of enhancement that would vary with the different flanking sequences (such as in 64M and 64MI). This is clearly not the case, since the site of pluramycin-enhanced reactivity occurs at just one defined position relative to the TATA box, irrespective of sequence context. On the contrary, this observation argues for a source that is common to both the 64M and 64MI sequences (that is, the TBP–5'-TATAAAAA sequence complex) and is independent of sequence context.

We propose that the enhanced pluramycin reactivity is due to a transient unwinding effect occurring specifically at the 9–10 dinucleotide step that stabilizes pluramycin intercalation at this site. This proposal is consistent with the enhanced reactivity at site 'c' and no change in reactivity at site 'h' (Fig. 5c) and the corresponding pluramycin reactivities shown in Figure 4. Furthermore, pluramycin intercalation adjacent to the hTBP–TATA-box complex appears to be associated with a 'specific' binding mode that stabilizes a ternary complex in which compensatory alterations occur in DNA induced by both ligands. This conclusion is further supported by competition experiments showing the enrichment of DNA specifically modified by pluramycin at the enhancement site in specific hTBP–TATA box complexes and by the increased efficiency of circularization of the C2 species in the presence of pluramycin demonstrated in the ligation experiments. Last, in order to determine directly the effect of flexibility of adjacent base pairs to pluramycin alkylation, we have determined the relative reactivity of a site having a one-base bulge to the 5'-side of the intercalation/guanine alkylation site for pluramycin. Such one-base bulges dramatically increase the covalent reactivity of the adjacent guanine to pluramycin (10 to 100 times greater reactivity) (S.-J. Lee, M. Hansen and L.H.H., unpublished results). This is consistent with the proposal presented here that the adjacent binding of hTBP produces an unwinding of the CG dinucleotide and, thus, enhanced reactivity of pluramycin.

The dynamic effects of pluramycin modification of the region immediately downstream of the TATA box on the hTBP–DNA complex are also very informative. Pluramycin binding to this downstream region causes an increased conversion of nonspecific to specific complexes and a pronounced stabilization of the specific hTBP–DNA complex (in the absence of pluramycin, the half-life is 120 min, whereas in the presence of pluramycin, no significant dissociation was found during the same time period; see Fig. 9). Most likely, the enhanced stabilization (and circularization efficiency) of the hTBP–DNA complex by pluramycin intercalative binding is due to stabilization of a 'specific' binding

mode, resulting in compensatory alterations in DNA structure produced by both ligands.

Structure of the pluramycin–TBP–TATA-box

Based upon the X-ray structure of the TBP–TATA box complex [11–13], the NMR solution structure of the pluramycin–DNA complex [18], and the enhanced covalent reactivity site for pluramycin in the hTBP–DNA complex shown here, a molecular model for the ternary structure of the pluramycin–hTBP–DNA complex was constructed (Fig. 11). Docking of the covalently bound pluramycin in an unwound site at the 9–10 position, so that alkylation occurs at site 'c' (Fig. 5c), did not produce any steric clashes between pluramycin and TBP. After minimizing *in vacuo*, Lys97 forms an H-bond with a phosphate flanking the pluramycin binding site on the minor-groove side and the O-acetyl group of pluramycin, but this may be an artifact of the *in vacuo* minimization (Fig. 11a). It is intriguing that the major-groove side of the pluramycin intercalation site is wide open for interaction with other components of the transcriptional activation system, and if the 9–10 dinucleotide was unwound, it could be captured by an appropriately positioned aromatic moiety (Fig. 11b).

Implications of hTBP-induced pluramycin intercalation for nucleation of unwinding by hTBP

An important unanswered question related to the binding of TBP to the TATA box is how nucleation of unwinding is achieved in an asymmetric manner [30]. The TBP molecule has an approximate two-fold symmetry axis, and furthermore, its interactions with the TATA box are all matched in the two protein subdomains [11–13]. In contrast, the TATA binding sequence is asymmetric by virtue of indirect readout of sequence information, with the 5' half being more flexible, due to the alternating A's and T's, whereas the 3' half, consisting of an A4 tract, is much more rigid. As has been proposed by Sigler and colleagues [11], this would result in an increased amount of energy required for deformation of the 3' run of A's relative to that required for deformation of the TATA region. An apparent consequence of this sequence asymmetry in the TATA region is the induction of high-reactivity pluramycin alkylation sites specifically at a site on the downstream side of the TATA box upon binding of TBP.

How this induction of asymmetry from the TATA sequence to the downstream site identified by pluramycin is transmitted by TBP binding is still not clear. The results from this study suggest that a transient unwinding effect, rather than a direct sequence recognition mediated by a changed groove geometry, is responsible for the increased alkylation reactivity. Conceivably, this transient unwinding is due to a redistribution of torque energy initially trapped by TBP binding to the unwound TATA box. Unwinding of the TATA box as a result of TBP binding might take place in a two-step process (that is, first the 5'-TATA side, then the 3'-side A-tract) and during dissociation is then released stepwise in the reverse two-step mode. If this proposal is true, then at an intermediate step in the TBP dissociation process a favorable transition of torque energy and associated unwinding might be released specifically to the downstream side. This would create the unwound site intercepted by pluramycin intercalation. That this transient unwound state of the TBP–TATA-box complex is a specific binding mode is suggested by the stability studies on the ternary complex and the circularization efficiency experiments. Based upon these observations and the model of the pluramycin–TBP–TATA-box complex (Fig. 11), in which the unwound site is accessible via the major groove to other proteins involved in transcriptional activation, it is tempting to suggest that the intercalation site trapped by pluramycin might also be the nucleation site for the transcriptional start, which might be recognized by a planar aromatic moiety of a protein binding to this region.

A number of precedents exist for protein-induced distortion involving underwound regions of the helix. The case most relevant to the example described here is the transcriptional activation by the metal-responsive gene-regulatory protein (MerR) in the presence of nanomolar concentrations of mercuric ions [31]. The result of the addition of Hg is the conversion of the RNA polymerase complex from the closed conformation to the strand-separated, transcriptionally competent open complex. In parallel with the observations described here, an Hg–MerR-induced structural alteration in the promoter has been detected by the intercalation-sensitive reagents MPE and Cu–phenanthroline [31]. In this case, the origin of the asymmetry does not appear to be the MerR binding site, since this is palindromic, but may be induced by the asymmetric interaction of Hg with the MerR.

We would like to consider the biological implications of the immobilization of an intercalation agent like pluramycin at a site of protein-induced distortion of DNA. One of us has previously proposed that the biologically important lesions for DNA-reactive drugs like the pluramycins and CC-1065 result from special niches produced on DNA as a consequence of protein-induced distortion of DNA [32,33]. We have now documented two such examples, the one described here and the Sp1-induced bending sites that occur in the 21-bp repeat region of SV40 DNA, which maintains high-reactivity sites for CC-1065 between GC boxes III and IV and V and VI [34]. In the case described here, where hTBP binding to the TATA box results in an enhanced reactivity of pluramycin at a specific site downstream of the TATA box, which then immobilizes hTBP on DNA, one can propose at least two different biological consequences. First, the immobilization would lead to trapping of TBP and associated transcriptional factors at transcription start sites where pluramycin is covalently bound. These sites would presumably be unproductive transcriptional regions, leading to down regulation of

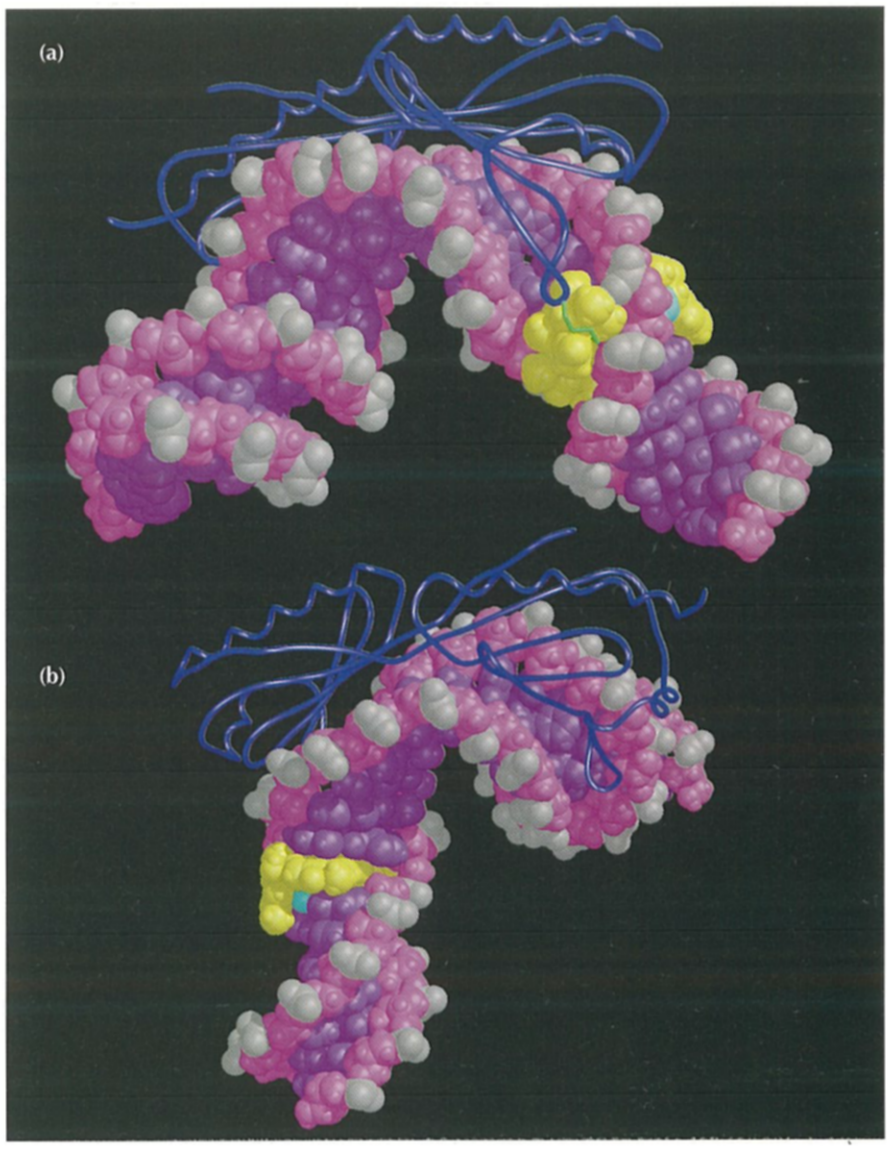
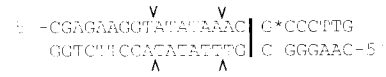


Fig. 11. Molecular models of the pluramycin–TBP–TATA-box complex. The structures were formed as described in Materials and methods. The ternary complex contains the following duplex DNA sequence:



where the arrows show phenylalanine insertions, the vertical line is the pluramycin intercalation site, and G* shows the alkylated guanine. **(a)** The minor-groove side of the pluramycin modification site on DNA. The sugar moieties of pluramycin (see Fig. 1) interact in the minor groove, and the vinyl epoxide flanks the left-hand side of the helix (pluramycin is shown in yellow). TBP is shown as a ribbon diagram (blue), except for Lys97 (green), which after minimization H-bonds with a phosphate and the O-acetyl group of pluramycin. The N7-alkylated guanine is shown in cyan. The DNA helix is shown in purple (bases), magenta (deoxyriboses), and gray (phosphates). **(b)** The major-groove side of the pluramycin modification guanine nucleotide 10 showing the clear accessibility of the pluramycin-trapped intercalation site from this side. Colors as for (a).

specific gene transcription and perhaps even general transcription because of the resulting depletion of transcriptional factors. Second, the 'fixation' of protein–DNA complexes is well known to have potent biological consequences, as, for example, in the case of the formation of cleavable complexes with topoisomerase I and II and certain anticancer drugs, such as camptothecin and VP16 [35].

Significance

The results of this study demonstrate that the specific binding of hTBP to the TATA box creates a unique unwinding site immediately downstream of the TATA box that is trapped by the intercalative drug pluramycin, which alkylates N7 of guanine at the flanking site. These results demonstrate the utility of pluramycin as a specific probe for protein-induced unwinding sites where intercalation may stabilize a specific binding mode of the protein–DNA complex. Based upon the compatibility of intercalation by pluramycin with hTBP binding to the TATA box and the proposed structure of the ternary complex, which shows clear accessibility via the major-groove side to an unwound site downstream of the TATA box, we propose that this site may be a nucleation site for unwinding of DNA recognized by a transcription factor. A mechanism involving an asymmetric spring loading and the release of torque associated with binding and dissociation of TBP from the TATA box is used to rationalize the asymmetric distribution of pluramycin intercalation sites. The consequences of the immobilization of TBP at TATA boxes by pluramycin modification of a downstream site may have potent effects on transcriptional control and lead to dramatic effects on cell survival.

Materials and methods

Materials

Human TBP and DNase I were obtained from Promega, and T4 DNA ligase and T4 polynucleotide kinase were from

United States Biochemical. Pluramycin was kindly provided by Abbott Laboratories (Chicago, IL) and used in this study without further purification. A stock solution was prepared by dissolving pluramycin (1 mg) in 1 ml of methanol and diluting it with double-distilled water (DDW) just before carrying out each experiment. The oligonucleotides (64M and 64MI in Fig. 2) were synthesized on an Applied Biosystems Model 380A oligonucleotide synthesizer and purified by polyacrylamide gel electrophoresis, as described before [15].

DNase I footprinting assay

To form hTBP–DNA complexes, 5 ng of the 5' end-labeled, 64-bp double-stranded oligonucleotide was added to hTBP (final concentration 20 nM) in 20 μl of a solution containing 25 mM Tris·HCl (pH 7.8), 50 mM KCl, 6.25 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM dithiothreitol (DTT), 0.01 % NP-40, and 5 % glycerol. After a 30-min incubation at 37 °C, 100 ng of unlabeled 64-bp oligonucleotide was added, and the reaction was incubated for a further 10 min. Following treatment for 1 min with 0.2 U of DNase I, the reaction was terminated by the addition of 40 μl of alkaline dye (80 % formamide, 10 mM NaOH) and then heated at 95 °C for 10 min. For measuring dissociation kinetics of the hTBP–DNA complex, 50 mg of DNA template was added to hTBP (final concentration 20 nM) in 200 μl of the same solution. After incubation for 30 min at 37 °C, 160 μl portions of the reaction were removed and added to 2 μg of the unlabeled template DNA. At the indicated times, 20 μl of the reaction were withdrawn, and the extent of hTBP–DNA complex formation was analyzed by DNase I footprinting. In all cases, kinetic experiments were repeated at least twice to ensure reproducibility.

Pluramycin protection experiments

The hTBP–TATA-box complex was formed in 20 μl of solution by incubating the reactions for 30 min at 37 °C, as described for the DNase I footprinting experiment. After the addition of 100 ng of unlabeled DNA, the reaction was incubated for a further 10 min, and the indicated amounts of pluramycin were added to the reactions. Reaction with drug was continued for 5 min and stopped by adding 100 μl of DDW containing 10 μg of calf thymus DNA, followed by heating the reactions at 95 °C for 15 min to induce DNA strand breakage at the drug modification sites [15]. In some experiments, free drug molecules were removed by phenol/chloroform extraction followed by ethanol precipitation. DNA pellets were dissolved in 20 μl of 1 M piperidine solution, and samples were heated at 95 °C for 15 min to induce strand breakage at drug modification sites. Samples were lyophilized, redissolved in 20 μl of alkaline dye (80 % formamide and 10 mM NaOH), and then separated by electrophoresis on a 10 % sequencing gel at room temperature at 1400 V for 2 h.

Formation of TBP–DNA complexes and the gel mobility shift assay

The gel mobility shift assay was performed by binding hTBP to 64M in a buffer containing 25 mM Tris·HCl (pH 7.8), 50 mM KCl, 6.25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 0.01 % NP-40, and 5 % glycerol. After incubation at 37 °C for 30 min, a 20-molar excess of unlabeled 64M was added to the reaction, and the reactions were further incubated for the indicated period of time. Free and hTBP-bound DNA were separated on a 5 % polyacrylamide gel (39:1 acrylamide/bisacrylamide) in Tris-borate-$MgCl_2$ (TBM) (50 mM Tris base, 40 mM boric acid, 5 mM $MgCl_2$) supplemented with 5 % glycerol. Gel electrophoresis was performed in TBM buffer at 4 °C for 4 h at 250 V.

Determination of the dissociation kinetics of the TBP–DNA complex

To prepare the 64-bp oligonucleotide modified with pluramycin at the 3'-side of the TATA box, 50 ng of 64M was incubated with hTBP (final 20 nM) in 200 μl of binding buffer at 37 °C for 30 min, and DNA was modified with pluramycin (400 nM) for 5 min in the presence of hTBP. DNA was re-isolated by phenol/chloroform extraction followed by ethanol precipitation. hTBP–DNA complexes were reformed using this pluramycin-modified DNA by the incubation of the reaction mixture at 37 °C for 10 min followed by further incubation for 120 min. During the incubation, a 40 molar excess of unlabeled DNA was added to the reaction for the indicated period of time, and the amount of the specific hTBP–DNA complex was analyzed using a gel mobility shift assay.

Cyclization experiments

A 53-bp double-stranded oligonucleotide containing the TATA box sequence was used as a DNA substrate for the cyclization experiment. This oligonucleotide was also designed to contain two A_5-tracts that are 16 bp away from the center of the TATA-box sequence to improve overall efficiency of cyclization (see Fig. 10d). The oligonucleotide (2 ng) was labeled with [γ-^{32}P]ATP and was incubated for 30 min at 37 °C with indicated amounts of hTBP in 20 μl of a ligase buffer. The ligation reaction was initiated by the addition of 200 U of T4 DNA ligase and incubated for 30 min. The effect of pluramycin modification on the efficiency of the formation of circular dimers was examined by adding pluramycin to the preformed hTBP–DNA complex prior to initiation of the ligation reaction. After ligation was complete, hTBP was removed from the DNA substrate by adding sodium dodecylsulfate to a 0.5 % concentration and heating at 65 °C for 15 min. Ligated reaction products were separated by electrophoresis in a 6 % polyacrylamide gel. Each band was then extracted from the gel, purified, and loaded onto a 4 % denaturing polyacrylamide gel to determine the size of the DNA, according to the method described previously [36].

Generation of molecular models

Molecular models were created by modifying the crystal structure of the TBP–DNA complex. Flanking sequences were mutated and elongated to closely match sequences used in the gel studies, and pluramycin A was docked on the molecule using the program MIDAS [37]. The molecular model was then minimized *in vacuo* using the molecular modeling package AMBER [38].

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