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Sequence-Specific Minor Groove Binding Ligands as Potential Regulators of Gene Expression in Xenopus Laevis Oocytes

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Abstract

The mouse mammary tumor virus (MMTV) promoter is induced by glucocorticoid hormone. A robust hormone- and receptor-dependent gene activation could be reproduced in Xenopus laevis oocytes. The homogeneous response in this system allowed a detailed analysis of the DNA-protein interactions following hormone activation. The strategy of artificial regulating of gene activity by sequence-specific minor groove binding ligands is very attractive. We have synthesized and studied the interaction with DNA of bis-linked netropsin derivatives in which two monomers are attached *via* short linkers in head-to-head and tail-to-tail manners. We have found that cis-diammine-platinum bridged bis-netropsin added to Xenopus oocytes media penetrates cellular and nuclear membrane and binds selectively to the MMTV promoter at the DNA segment that partly overlaps with the site recognized by glucocorticoid receptor. DNase I footprinting studies demonstrate that there are more stronger binding sites for cis-diammine-platinum bridged bis-netropsin on the naked MMTV DNA which are found to be inaccessible for its binding in oocytes.

Introduction

In the past decade a considerable progress has been achieved in the design and synthesis of compounds which can bind to DNA at selected sites. Most of the newly synthesized sequence-specific DNA-binding ligands are derivatives of antiviral and antitumor antibiotics netropsin and distamycin A. X-ray (1-3) and NMR (4) studies show that netropsin and distamycin bind in the minor DNA groove at runs of four or five AT- base pairs, respectively. Their binding specificity derives from specific hydrogen bonding interactions between the amide NH groups of the antibiotic molecule and the thymine O2 and adenine N3 atoms, van der Waals contacts with the floor and both sides of the minor groove and electrostatic interactions.

In the present work, we have attempted to use bis-linked netropsin (**Nt**) derivatives with a final aim to affect the gene expression directed by mouse mammary tumor virus (MMTV) promoter. The MMTV promoter is induced by glucocorticoid hormone *via* glucocorticoid receptor (**GR**). The interaction of bis-linked netropsin derivatives with the DNA of the MMTV promoter in the context of the living cell was studied by intranuclear DNA injection in Xenopus oocytes. The oocyte system allows easy detection (due to high copy number of injected templates) of ligand-DNA interaction in the chromatin context. We have used bis-linked netropsin (**Nt**) and distamycin (**Dst**) derivatives, **←Nt-Pt(NH_3)$_2$-Nt→)** (or **Pt-bis-Nt** for simplicity), **←Dst-Pt(NH_3)$_2$-Dst→)** (or **Pt-bis-Dst**), and **-Nt→(CH_2)$_5$←Nt-** (or **Lys-bis-Nt** for simplicity) in which two monomers are linked in the tail-to-tail and head-to-head manners (Figure 1). They exhibit different sequence preferences on binding to DNA. In the present paper, we show that **Pt-bis-Nt** added directly to the oocyte media penetrates cellular and nuclear

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membrane and selectively binds to a site with the sequence 5′-TCTTAAAA-3′ on the MMTV promoter. This DNA region partly overlaps with the strong binding site for glucocorticoid receptor. DNase I footprinting studies demonstrate that there are more stronger binding sites for **Pt-bis-Nt** on the naked MMTV DNA which are found to be inaccessible for its binding in a living cell.

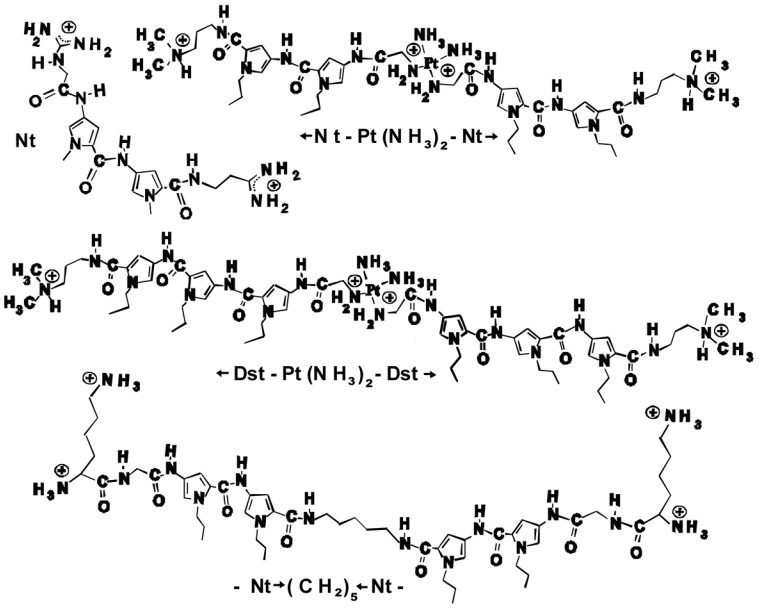

Figure 1: Chemical structures of netropsin (**Nt**) and its derivatives containing two monomers attached *via* short linkers in the head-to-head and tail-to-tail manners. The arrows indicate directions from the N-terminus to the C-terminus in each netropsin-like fragment.

Materials and Methods

Chemicals and Synthesis

←Nt-Pt(NH$_3$)$_2$-Nt→, **←Dst-Pt(NH$_3$)$_2$-Dst→**, and **(-Nt→(CH$_2$)$_5$←Nt-)** were synthesized and purified as described previously (5, 6). The concentrations of bis-netropsins were determined spectrophotometrically, using the molar extinction coefficient at 297 nm of 42,000 M^{-1} cm^{-1}. The concentration of **←Dst-Pt(NH$_3$)$_2$-Dst→** was determined, using the molar extinction coefficient at 303 nm of 60,000M^{-1} cm^{-1}.

DNA Fragments and DNase I Footprinting Studies In Vitro

End-labeled DNA fragment was obtained by cleaving of a modified plasmid pGEM7(f+) (Promega) containing inserts of synthetic oligonucleotides (7) within the polylinker containing the recognition sites for endonucleases NcoI and ApaI. The radioactive label was introduced into 3′-end of the DNA fragment, using [α-^{33}P]dATP (Isotop, Moscow), unlabeled dNTP and Klenow fragment of *Escherichia coli* DNA polymerase I (Boehringer Mannheim). The DNA fragment was isolated by PAGE in 5% PAG (8). To prepare a ligand-DNA complex, a solution (10 μl) of the DNA fragment (about 10^4 Bq) in 10 mM Tris-HCl (pH 6.0), containing 0.25 M NaCl and ligand solutions in the same buffer (10 μl, ligand concentrations are given in the figure captions) were mixed and cooled to 0° C. A solution (20 μl) of DNase I (~1 μg/ml) (Sigma) in 10 mM Tris-HCl (pH 6.0), containing 0.25 M NaCl and 5 mM $MnCl_2$ was added, and the mixture was kept for 3 min at 0° C. The reaction was quenched by a solution (85 μl), containing 0.15 M NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 10 μg /ml tRNA. After phenol extraction, the DNA was precipitated with ethanol, washed with 70% ethanol, dried, and dissolved in 95% formamide (1 μl), containing 15 mM EDTA (pH 8.0), 0.05% Bromphenol Blue, and 0.05% Xylene Cyanol FF. The mixture was heated for 1 min at 90° C, rapidly cooled, and loaded on a 6% denaturing PAG (40 cm long) with a gradient thickness of 0.15-0.45 mm (9). The PAGE was performed for 55 min at 100 W (2.3 kV). Prior to superimposition, the gel was fixed in 10% acetic acid and dried on a glass plate preliminarily treated with γ-methacrylpropyloxysilane (LKB, Sweden).

Xenopus laevis oocytes were prepared and injected as described previously (10, 11). Construction of the MMTV reporter and the plasmid designed for *in vivo* transcription of rat **GR** mRNA was described previously (11). **Pt-bis-Nt** and **Lys-bis-Nt** were added directly to the oocyte media to a final concentration of 250 μM.

DNase I and Dimethylsulfate (DMS) Footprinting In Situ*, RNA Analysis*

Ten injected oocytes were collected and homogenized in 300 μl of 10 mM Tris-HCl (7.5), 50 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$, and 5% glycerol by pipetting up and down. The homogenate was divided into three tubes and DNase I (Roch Applied Science) was added (XXX units per tube). Following incubation at 25° C for 3 min, the reaction was stopped by addition of SDS (to 1%) and EDTA (to 10 mM). DNA was purified by proteinase K treatment, phenol/chloroform extractions, and ethanol precipitations. The DNase I pattern was visualized by primer extension as described previously (10, 12). [^{32}P]-radioactivity scans were done with a Fuji Bio-Imaging analyzer BAS-2500, using the Image Gauge V3.3 software.

RNA Analysis and Dimethylsulfate (DMS) Footprinting In Situ

RNA analysis and DMS *in vivo* footprinting were done as described previously (11, 12).

Results and Discussion

Figure 2 shows a reporter DNA construct, pMTV:M13, used for injection in *Xenopus laevis* oocytes. Position of the primer used for primer extension analysis of DNase I digestion pattern is shown as a solid black arrow. Indicated are the hexanucleotide elements recognized by glucocorticoid receptor (GR) as well as interaction sites for transcription factor 1 (**NF1**), octamer-binding protein (**OCT**), and box TATA-binding protein (TATA). The insert shows the DNA sequence around the strong interaction site for **GR**. The hexanucleotide elements recognized by **GR** are boxed. **Pt-bis-Nt** recognizing sequence is shown as a light grey box.

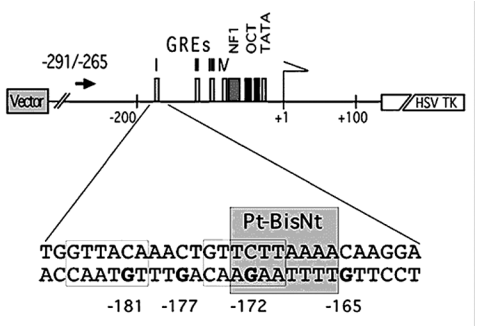

Figure 2: A reporter DNA construct containing MMTV promoter.

We have used DNase I footprinting to monitor the binding of **Pt-bis-Nt** to its target site on the MMTV promoter (Figure 3). In order to accomplish this, a pool of oocytes was injected with a double strand MMTV construct, the pool was split into three groups to which mock solution, **Lys-bis-Nt** or **Pt-bis-Nt** was added. Ligands were added directly to oocyte media immediately after injection. After 5 hours of incubation oocytes were harvested and treated with increasing amounts of DNase I, DNA was isolated and the digestion pattern was visualized by primer extension, using ^{32}P-labeled primer; naked ds DNA was DNase I digested and assayed the same way as a control. Strong protection is observed between nucleotides in positions -173 to -165. Interesting, no protection in this DNA region is observed on adding **Lys-bis-Nt** to the oocyte media.

With mock solution (no ligand) added we observed digestion pattern different from that seen on adding DNase I to solution containing the naked MMTV DNA (compare lanes 1-3 with lane 11 in Figure 3). This reflects chromatinization of the injected ds DNA.

Pt-bis-Nt binds both in the extended conformation and parallel-stranded hairpin form to a DNA segment, harboring the sequence 5′-TCTTAAAA-3′

Physicochemical studies show that cis-diammine Pt(**II**)-bridged bis-netropsin forms two types of complexes with poly[d(AT)]·poly[d(AT)] and DNA oligomers harboring sequences 5′-CC$(TA)_n$CC-3′, where n = 4 or 5 (7, 13, 14). The first type corresponds to the binding of ←**Nt-Pt(**NH_3**)**$_2$**-Nt**→ in the extended conformation

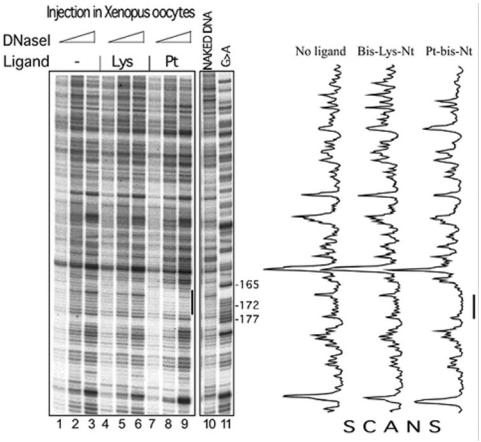

Figure 3: DNase I *in situ* footprinting reveals **Pt-bis-Nt** binding to its target site that partly overlaps with the strongest affinity site for glucocorticoid receptor (GRE I). No ligand (-), **Lys-bis-Nt** and **Pt-bis-Nt** (Pt) were added to injected oocytes to a final concentration 250 μM. After 5 hours of incubation oocytes in groups of 10 were treated with DNase I, see ***Materials and Methods***. Digestion pattern was developed by primer (-291/-265) extension. Naked DNA control (Naked DNA) and sequence marker (G>A) are shown. Black bar designates region protected from the DNase I cleavage. Corresponding Gs around the protected region are indicated. Radioactivity scans of lanes corresponding to the highest DNase I concentration are shown to the right.

and is characterized by the saturation ratio of one bis-netropsin molecule bound to approximately 8 successive AT-base pairs. The second type of the complex reflects binding of ←**Nt-Pt(NH$_3$)$_2$-Nt**→ in the hairpin form which is built on the basis of a parallel side-by-side motif. The binding approaches saturation when one bis-netropsin molecule is bound to approximately four or five AT-base pairs. In the extended conformation ←**Nt-Pt(NH$_3$)$_2$-Nt**→ binds with the strongest affinity to a DNA site with the sequence 5′-TTTTAAAA-3′. It exhibits approximately four times lower affinity for interaction with a DNA region with the sequence 5′-AAAATTTT-3′. In contrast, **-Nt→(CH$_2$)$_5$←Nt** exhibits an opposite order of preferences binding more strongly to 5′-AAAATTTT-3′, than to 5′-TTTTAAAA-3′. After X-ray irradiation of complexes between ←**Nt-Pt(NH$_3$)$_2$-Nt**→ and DNA X-ray induced DNA cleavage is observed at DNA regions with sequences 5′-TXT-TAAYA-3′, where X and Y are predominantly A or T (15). The cleavage was also detected in a DNA region with the sequence 5′-TCTTAAAA-3′, although **Pt-Bis-Nt** exhibits lower affinity for this site, as compared with binding to the strongest affinity site harboring the sequence 5′-TTTTAAAA-3′.

The binding of **Pt-Bis-Nt** in the extended conformation and hairpin form can be easily discriminated by means of CD spectroscopy. Figure 4 displays the difference CD spectra for complexes between ←**Nt-Pt(NH$_3$)$_2$-Nt**→ and DNA oligomers with sequences 5′-CCTTTTAAAACC-3′ and 5′-CCTCTTAAAACC-3′. The difference CD spectra were calculated by substraction of the CD spectrum of nucleic acid alone from the CD spectra of complexes between ←**Nt-Pt(NH$_3$)$_2$-Nt**→ and corresponding DNA oligomer at different ligand to DNA oligomer molar ratios (C/O). Here C is the concentration of the ligand. O is the molar concentration of the DNA oligomer. The CD difference spectra obtained for complexes of ←**Nt-Pt(NH$_3$)$_2$-Nt**→ with duplex 5′-CCTTTTAAAACC-3′ exhibits a positive CD band with a peak at 315 nm and negative CD band at 270 nm which are characteristic of bis-netropsin binding in the extended conformation (Figure 4). The CD spectral profiles obtained for complexes between ←**Nt-Pt(NH$_3$)$_2$-Nt**→ and DNA oligomer, harboring the sequence 5′-CCTCTTAAAACC-3′, look quite different (Figure 4). They exhibit two positive CD bands at 290 and 328 nm which are characteristic of bis-netropsin binding in the parallel-stranded hairpin form (12).

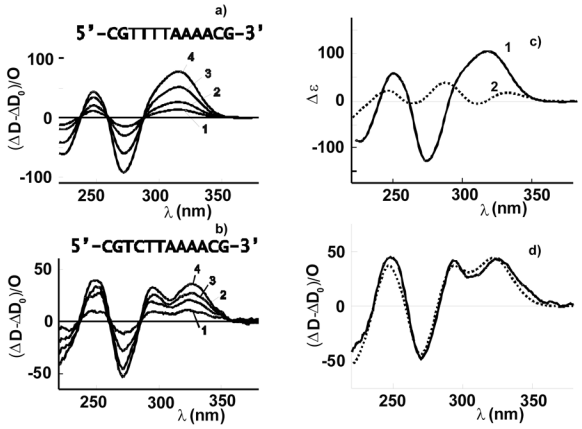

Figure 4: The CD difference spectra obtained for complexes of **Pt-bis-Nt** with DNA oligomers 5′-CCTTTTAAAACC-3′ (**a**) and 5′-CCTCTTAAAACC-3′ (**b**) at different ligand/DNA oligomer molar ratios (curves 1-4). The C/O values were equal to 0.18, 0.36, 0.73, and 0.91 in the system, containing duplex 5′-CCTTTTAAAACC-3′ (26.2 μM), and equal to 1.4, 2.3, 6.8, and 9.1 in the system, containing duplex with the sequence 5′-CCTCTTAAAACC-3′ (15 μM). ΔD and ΔD$_0$ are the measured CD amplitudes in 1 cm cell for ligand-DNA complex and naked DNA, respectively. O is the molar concentration of the DNA oligomer. Experiments were done at 20° C in 0.01 M sodium cacodylate buffer (pH 7.0) in the presence of 0.1 M NaCl. The reference CD spectral profiles for binding of **Pt-bis-Nt** in the extended conformation and hairpin form (panel *c*, curves 1 and 2, respectively). Decomposition of difference CD spectral profile for a complex of **Pt-bis-Nt** with the dodecamer of the sequence 5′-CCTCTTAAAA-3′ into constituents, corresponding to the binding of bis-netropsin in the extended conformation and hairpin form (panel *d*). The experimental and calculated CD spectral profiles are shown by solid and dotted lines, respectively. The concentration of the DNA oligomer was 12.5 μM, C/O=2.7.

The CD and NMR spectroscopy studies show that ←**Nt-Pt(NH_3)$_2$-Nt**→ forms 1:1 complex with the nanomer duplex 5′-CCTATATCC-3′ and binds most probably exclusively in the parallel-stranded hairpin form (14). The difference CD spectrum obtained for this complex was taken as a reference CD spectral profile characteristic of bis-netropsin binding in the hairpin form (14). Decomposition of the difference CD spectral profiles for complexes of ←**Nt-Pt(NH_3)$_2$-Nt**→ with dodecamer 5′-CCTCTTAAAACC-3′ into components, corresponding to different binding modes of **Pt-bis-Nt**, was achieved by least square fitting of the experimental and calculated CD contours, using the reference CD spectral profiles for binding of the bis-netropsin in the extended conformation and hairpin form. Decomposed CD spectral profiles obtained at different molar ratios of ligand to the DNA oligomer can be used for quantitation of different bound ligand species in the system under study. A decomposed CD spectral profile obtained at C/O = 2.7 is shown in Figure 5. The best fit between the experimental and theoretically calculated curves is observed when the occupancies of the dodecamer by **Pt-bis-Nt**, binding in the extended conformation and hairpin form are equal to 0.34 ± 0.01 and 0.65 ± 0.02, respectively.

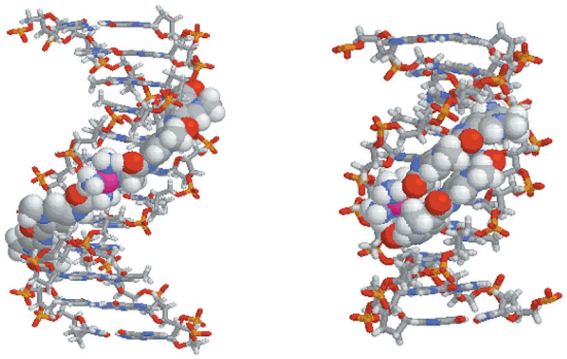

Figure 5: The proposed molecular models for binding of an analogue of Pt-bridged bis-netropsin (**←Nt-Pt(NH_3)$_2$-Nt→**) in the extended conformation and parallel-stranded hairpin form to the DNA dodecamer with the sequence 5′-CCTATATATACC-3′.

Figure 5 shows the proposed molecular models for binding of an analogue of Pt-bridged bis-netropsin (**←Nt-Pt(NH_3)$_2$-Nt→**) in the extended conformation and parallel-stranded hairpin form to the DNA dodecamer with the sequence 5′-CCTATATATACC-3′. The analogue is composed of N-methylpyrrole carboxamide units and contains no charged N-dimethylamino groups at two ends of the bis-netropsin molecule. Molecular modeling was carried out, using modified AMBER 4.1 force-field. For the electrostatic component of the empirical energy function the effect of solvent was approximated by a distance-dependent dielectric function and by reducing the net charge on the phosphate group to -0.46 e. A cis-Pt$(NH_3)_2^{2+}$ unit of the bis-netropsin molecule is bound to the nitrogen atoms of glycine residues in an approximately square planar coordination geometry. New atom types were created for Pt, nitrogens of glycine residues and the ammine N and H atoms. Force field parameters for these new atom types were developed by comparing force field parameters found in the literature with the structural features of published crystal structures. A set of fractional atomic charges of the Pt-bound glycine residues were modified from typical AMBER values to account for the expected electron withdrawal from groups coordinated to the positively charged Pt(II) (16, 17). In order to accommodate **Pt-bis-Nt** in the parallel-stranded hairpin form the width of the minor DNA groove should be increased by approximately 2 A. An increased width of the minor groove in the central part of the target site is also required for accommodation of cis-diammine-platinum group of the bis-netropsin molecule binding in the extended conformation to DNA. In the complex, platinated NH_3 groups are implicated in intramolecular hydrogen bonding to the glycine carbonyl oxygens, whereas the NH groups of each netropsin-like fragment are hydrogen bonded to the thymine O2 and adenine N3 atoms.

DNase I Footprinting Studies of Complexes Between bis-linked Netropsin Derivatives and Naked MMTV DNA

It is of interest to compare DNase I footprints generated on binding of **Pt-bis-Nt**, **Lys-bis-Nt**, and **Pt-bis-Dst** to the naked MMTV DNA with footprinting diagrams

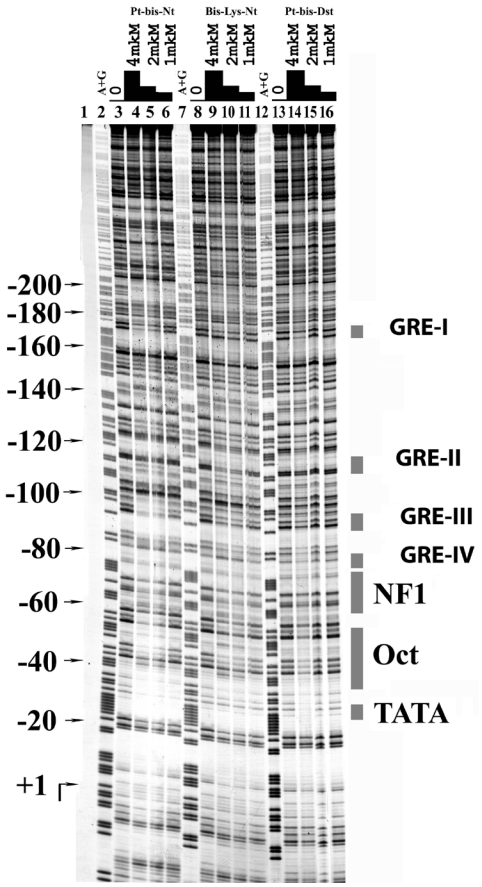

Figure 6: The patterns of DNase I cleavage of the DNA fragment, containing the insert of MMTV DNA. Lane 1, initial (untreated) fragment; lanes 2 ,7, and 12, chemical cleavage at purine residues; lanes 3, 8, and 13, DNase I cleavage of the free DNA fragment; lanes 4-6, 9-11, and 14-16, DNase I cleavage in the presence of 4, 2, and 1 μM **Pt-bis-Nt**, **Lys-bis-Nt**, and **Pt-bis-Dst**, respectively.

obtained *in situ* when netropsin derivatives were added directly to the *Xenopus laevis* oocyte media (see Figure 3). In order to accomplish this, we have inserted into a polylinker region of a modified plasmid pGEM7(f+) (Promega) a fragment of MMTV DNA comprizing interaction sites for **GR** as well as sites recognized by nuclear factor I (**NF 1**), octamer-binding protein (**OCT**), and TATA box-binding protein (**TBP**). DNase I footprinting studies *in situ* reveal that **Pt-bis-Nt** added directly to the oocyte media penetrates cellular and nuclear membrane and selectively binds to a site with the sequence 5′-TCTTAAAA-3′ on the MMTV promoter. This site partly overlaps with the recognition site GRE I for **GR**. DNAse I footprinting diagrams obtained for complexes of **Pt-bis-Nt** with the naked MMTV DNA reveal protection zones at sequences 5′-TTATTATGATTA-3′ (nucleotides -246 to -235), 5′-TTTAAATAAGTTTAT-3′ (nucleotides -200 to -187), 5′-TTCTTAAAACA-3′ (nucleotides -176 to -164), 5′-GTGTTCATTTT-3′ (nucleotides -100 to -89), 5′-TTATGTAAAT-3′ (nucleotides -47 to -38) and 5′-ATAATATAAAAGAGTG-3′ (nucleotides -36 to -21) (Figure 7). The protected DNA regions between nucleotides -176 to -164 and -100 to -88 contain octamer sequences 5′-TCTTAAAA-3′ and 5′-TCATTTTG-3′ which may serve as the preferred sites for binding of **Pt-bis-Nt** in the extended conformation. These sites are partly overlapped with the hexanucleotide recognition elements in the binding sites GRE I and GRE III for **GR**. The protected zone between nucleotides -36 to -21 contains recognition site for the TATA-box binding protein (**TBP**) with sequence 5′-TATAAAAG-3′. It differs only in a single position (where A is replaced by G) from the consensus sequence for the preferred binding sites for **TBP**. Our CD studies show that **Pt-bis-Nt** exhibits a high affinity for binding to the DNA dodecamer 5′-CCTATAAAAACC-3′, containing recognition site for **TBP** in the core AT-rich sequence (data are not shown). In agreement with the results of CD studies, clear footprints are observed between nucleotides -36 to -21 on the naked MMTV DNA in the presence of as low as 0.5 μM **Pt-bis-Nt**. **Pt-bis-Dst** and **Lys-bis-Nt** also bind

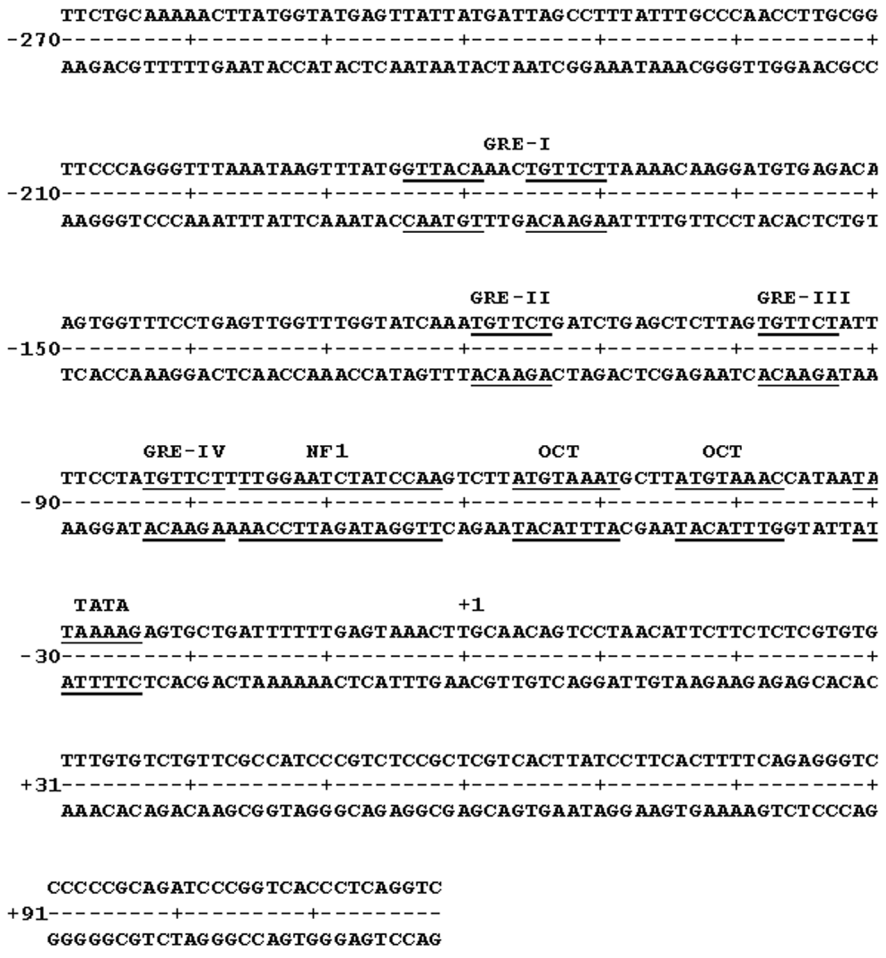

Figure 7: The nucleotide sequence of the insert of MMTV DNA. Indicated are the interaction sites for **GR**, nuclear factor 1 (**NF1**), octamer-binding protein (**Oct**), and TATA-box binding protein.

to this DNA region, but exhibit lower affinities as compared to that of **Pt-bis-Nt**. Interesting, the TATA-box is found to be inaccessible for interaction with **Pt-bis-Nt** and **Lys-bis-Nt** in the MMTV minichromosomes, as revealed from DNase I footprinting studies *in situ,* whereas the octamer sequence 5′-TCTTAAAA-3′ (nucleotides -173 to -165) is fully protected by **Pt-bis-Nt**. However, the letter DNA segment serves as a weak interaction site for **Pt-bis-Nt** on the naked MMTV DNA (Figure 7). Evidently, access of DNA for interaction with bis-linked netropsin derivatives in MMTV minichromosomes depends on the rotational positioning of a target site relative to the histone octamer in a nucleosome, as observed earlier for binding of transcription factors (18).

Pt-Bis-Nt Binding is Independent on DNA Replication

Injection of ds DNA into the oocyte nuclei results in the formation of non-physiologically spaced, more open and less repressive chromatin structure (19). This structure could be more open for minor groove binding ligands as well. For this end, we decided to check whether the process of DNA replication could influence binding of **Pt-bis-Nt** to the MMTV promoter. Injection of ss DNA to Xenopus oocytes gives a unique possibility to mimic the process of DNA replication (19). So, a pool of oocytes were injected with ss MMTV reporter construct. Replication-coupled chromatin assembly generates more repressive chromatin than chromatin assembly on duplex DNA (19). Bis-linked netropsin derivatives were added to the oocyte media either immediately after injection (*i.e*., before DNA replication and chromatin assembly), or 6 hours after (*i.e*., after DNA replication and chromatin assembly). In both cases, DNase I digestion pattern after addition of **Pt-bis-Nt** revealed protected area adjacent to 3′-flank of GRE I. We concluded that **Pt-bis-Nt** binds to the same target site on the MMTV promoter irrespective on DNA replication.

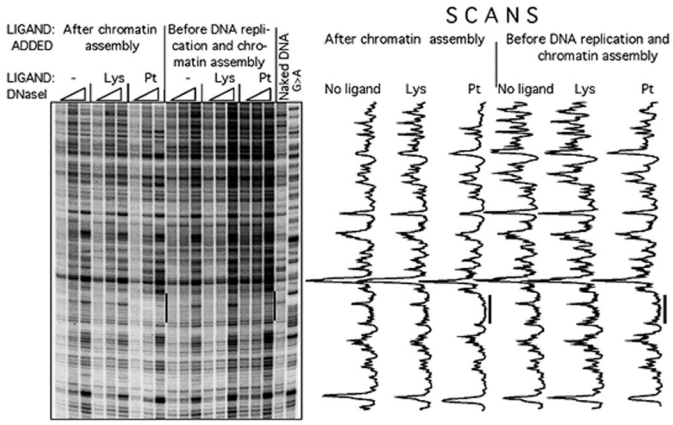

Figure 8: Pt-bis-Nt can bind its target site irrespective of DNA replication. No ligand ("-"), **Lys-bis-Nt** (Lys) or **Pt-bis-Nt** (Pt) were added either 5 hours after DNA injection ("After chromatin assembly") or immediately after DNA injection ("Before DNA replication and chromatin assembly"). See Figure 2 for further details.

Addition of Pt-bis-Nt has no Effect on Hormone-Induced Transcription.
***Pt-bis-Nt**-DNA Complex is "Transparent" for the Glucocorticoid Receptor*

To evaluate biological effect of bis-linked netropsin derivatives, we first checked their effect on the hormone-induced transcription of the MMTV promoter. For this end injected oocytes were treated (or mock treated) with bis-netropsin and glucocorticoid hormone triamcinolone acetonide was added to half of the oocytes at a final concentration of 1.0 μM. Oocytes were harvested and MMTV transcription was evaluated by the S1 assay (20). Addition of ligands has virtually no effect on transcription (Figure 4A). The fact that transcription was not changed after addition of ligand(s) strongly argues for a non-compromised binding of **GR** to the MMTV

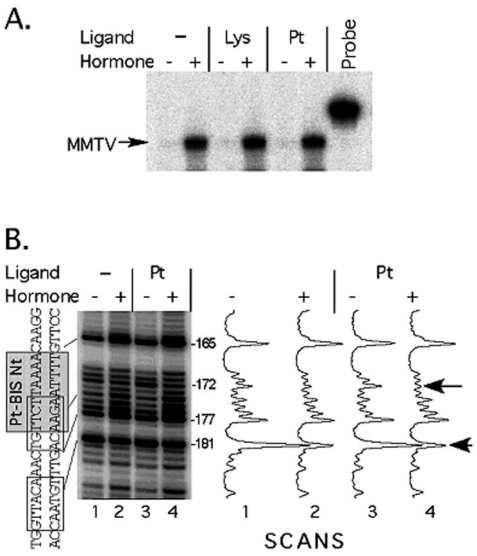

Figure 9: A. Transcription analysis by S1 nuclease protection of MMTV. Six oocytes in each case were harvested for S1 analysis and assayed; one oocyte equivalent per lane was loaded. **B**. DMS methylation protection over the GRE I segment. Oocytes in groups of five were treated with DMS, see ***Materials and Methods***. The methylation pattern was developed by primer (-291/-265) extension. Sequence around **Pt-Bis-Nt** binding site is given to the left. Corresponding Gs in the area are indicated to the right of the gel. Black arrows indicate protected guanosines upon hormone activation of the MMTV promoter. Radioactivity scans of lanes are shown to the right.

promoter. To check the occupancy of GREs *in vivo,* we used dimethylsulfate (DMS) methylation (21) and specific cleavage of DNA by alkali. Specific cleavage sites caused by DMS methylation and subsequent β-elimination were detected by primer extension. Addition of hormone resulted in a drastic reduction in DMS methylation over the GRE I (compare lanes 1 and 2, Figure 9B). Further addition of **Pt-bis-Nt** to the system did not result in any significant alteration of the protection pattern in either silent or hormone-activated MMTV promoter. This suggests that binding of **Pt-bis-Nt** to a site partly overlapped with GRE I did not interfere with GR-DNA interaction, *i.e*., complex of **Pt-bis-Nt** with DNA is "transparent" for **GR**.

Concluding Remarks

The eukaryotic genome is packaged into nucleosomes to form chromatin which regulates gene expression by controlling the accessibility of DNA for interaction with transcription factors. Many promoters, when packaged as chromatin, are transcriptionally repressed due to a reduced access of transcriprion factors to their target sites on DNA. The degree to which nucleosomes inhibit DNA-binding proteins from interacting with their target DNA sites is highly variable. Of particular significance are observations, showing that TATA-box binding protein (**TBP**) cannot associate with DNA packaged into a nucleosome (22). This may be attributed to the fact that binding of **TBP** to DNA is accompanied by significant DNA bending and widening of the minor groove (23). These structural changes are incompatible with the structure of DNA in the nucleosome (24). However, nucleosomes have only a modest inhibitory effect on the ability of a variety of activator proteins to binds their target sites (25-27). The inability of **TBP** to bind nucleosomal DNA indicates that entire Pol II transcriptional machinery is excluded from vast majority of promoters *in vivo* in the absence of transcription activators (27).

Nuclear receptors belong to a group of transcription activators that have the ability to interact with their target sites (response elements) in chromatin and recruit chromatin-modifying proteins and ATP-dependent chromatin remodeling complexes, resulting in transcriptional activation. ATP-dependent remodeling of the chromatin structure to allow binding of **TBP** and other transcription factors to DNA is the key step in regulation of gene activity in eukaryotes (27). Recent studies show that polyamides can be used to effectively block eucaryotic transcription factors from binding to their target DNA sites (28). Moreover, polyamides can inhibit transcription by RNA polymerase II both *in vitro* and in cell culture experiments (28). Recent X-ray studies show that DNA within nucleosome is accessible for interaction with polyamides, except for the target site located near the nucleosome dyad, which was completely blocked by interaction with the histone octamer (29, 30). Several laboratories have synthesized polyamide-dye conjugates to evaluate their nuclear localization in different cell lines in cultures (30-35). Dervan and coworkers have found that nuclear localization of polyamide-dye conjugates in different cell lines depends on polyamide composition, ring sequence, chemical structure of the dye, and dye-polyamide linker (30, 34). They have concluded that nuclear targeting in any particular cell line will require careful screening of candidate polyamides. Negative results in the targeted regulation of gene expression by polyamides can be explained by their failure to penetrate into a nucleus (34-36). Our present studies show that **Pt-Bis-Nt** can penetrate cellular and nuclear membrane of *Xenopus laevis* oocyte and bind selectively to a DNA segment, harboring the sequence 5′-TCTTAAAA-3′, which is partly overlapped with the recognition site for **GR**. Interesting, this site is occupied by **Pt-bis-Nt**, irrespective on whether **Pt-bis-Nt** was added to the oocyte media before or after MMTV DNA replication and chromatin assembly.

Considerable efforts were made to design DNA sequence-specific ligands or ligands conjugated to different effectors keeping in mind their possible use as therapeutic agents. Previous studies showed that **Pt-bis-bis-Nt** and **Lys-bis-Nt** exhibit lower cytotoxity in Vero cell culture than parent antibiotic netropsin (37). They

both strongly inhibit replication of herpes simplex virus of the first type in the Vero cell culture (37) with the selectivity indexes equal approximately to 60 and 150, respectively. Our present studies show that **Pt-bis-Nt** does not interfere with **GR** binding to DNA and has no marked effect on the transcription of a reporter gene from MMTV promoter. This enables us to suggest that **Pt-bis-Nt** can be considered as a DNA-binding "platform" for further design and synthesis of polyamide-peptide conjugates that could repress transcription of the MMTV promoter *via* interfering with **GR** binding or activate it in a non-hormone dependent fashion.

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