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# Preferential Binding to DNA Sequences of Peptides Related to a Novel XPRK Motif

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**Abstract**—Two dodecapeptide amides: $(WPRK)_3NH_2$ [WR-12] and $(YPRK)_3NH_2$ [YR-12], and a 30-mer polypeptide amide (SP-30) were synthesized by solid-phase peptide methodology. DNase I footprinting studies on a 117-mer DNA showed that WR-12 and YR-12 bind selectively to DNA sequences in a manner similar to SP-30 which has a repeating SPK(R)K sequence. The most distinctive blockages seen with all three peptides occur at positions 26–30, 21–24 and 38–45 around sequences 5′-GAATT-3′, 5′-TAAT-3′ and 5′-AAAACGAC-3′, respectively. However, it appears that YR-12 is better able to extend its recognition site to include CG pairs than is SP-30. At low concentrations YR-12 was able to induce enhanced rates of DNase I cleavage at regions surrounding some of its binding sites. To obtain further quantitative data supplementary to the footprinting work, equilibrium binding experiments were performed in which the binding of the two peptides to six decanucleotide duplexes was compared. Scatchard analyses indicated that WR-12 may be more selective for oligomers containing runs of consecutive purines or pyrimidines. On the other hand, YR-12 binds better to d(CTTAGACGTC)- d(GACGTCTAAG) than to the other oligomer duplexes, denoting selectivity for evenly distributed C/G and A/T sequences.


## Introduction[1]

Owing to the growing importance of understanding the regulation of gene expression as well as the biochemical function of specific genes, we are interested in the search for novel peptides that have the ability to bind to DNA in a sequence-specific manner. Many transcription factors, repressors and promoters which have very high DNA binding and sequence-recognition abilities utilize characteristic DNA binding domains such as the helix-turn-helix, zinc finger and leucine zipper motifs which enable tight interaction with the major groove of the double helix.[2–5] Another well known DNA binding motif which locates ligands in the minor groove is based on the repeating sequences SPKK, SPRR or SPRK which are found in histones, steroid hormone receptors, various segmentation gene products and some oncogene products.[6–8] The SPXX motif has been reported to assume a β turn stabilized by two hydrogen bonds, and the side chains of the two basic residues engage in salt bridges with the DNA phosphate groups.[7,8] An octapeptide SPRKSPRK (S2) and a 49-mer polypeptide (S6) containing six SPRK, SPQK or SPRR repeats have been shown to bind to DNA and to compete with the minor groove binding drug Hoechst 33258. Both S2 and S6 peptides were demonstrated by hydroxyl radical footprinting to bind to A/T-rich sites.[6]

In an ongoing search for peptides endowed with sequence specific DNA-binding properties, we have chosen to vary the composition of the SPXX sequence, envisaging that the repeating sequence XPRK might afford a new DNA binding motif. Thus, we carried out the synthesis of two peptide amides: $(WPRK)_3NH_2$ (WR-12) and $(YPRK)_3NH_2$ (YR-12). Tryptophan and tyrosine residues were incorporated because both residues often serve as fluorescent chromophores and the Trp indole

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side chain is known to be capable of intercalating into DNA.[9,10] Agarose gel electrophoresis revealed that both peptides do indeed significantly reduce the gel mobility of supercoiled DNA (results not shown). This prompted the use of quantitative DNase I footprinting titration to study the sequence selectivity of DNA binding by the new peptides. A 30-mer polypeptide with sequence corresponding to a fragment of histone H1 containing six repeats of the SPRK, SPKK or SPRR motif was also synthesized to serve as a reference compound for DNase I footprinting studies. To obtain further quantitative data relating to sequence-specific DNA binding we carried out Scatchard analyses of WR-12 and YR-12 binding to six decanucleotide duplexes employing fluorescence quenching methodology.

## Materials and Methods

### Chemicals

All the protected amino acid derivatives were purchased from Bachem California (Torrance, CA, USA) and AnaSpec, Inc. (San Jose, CA, USA) or synthesized in our own laboratory according to published procedures. All other analytical reagents were purchased from Acros, Tedia or Sigma.

### Chemical methods

Melting points were determined on a Mel-Temp apparatus (Cambridge, MA, USA) and are uncorrected. Optical rotations were determined on a Rudolph Autopol II instrument. Semi-preparative and analytical HPLC (Vydac reversed-phase columns, TP201; column 1, 1×25 cm; column 2, 0.4×25 cm) were performed using a Hitachi L-7100 pump equipped with a gradient elution device and a Soma S-3702 variable wavelength UV detector connected to a PC computer equipped with Ezchrom analytical software. Mass spectra were determined with a Finnigan/Thermo Quest MAT 95XL instrument operating in the electrospray ionization (ESI) mode in Chung-Hsing University. Fluorescence spectra were measured with a Hitachi F-4500 instrument. Decanucleotide duplexes were purchased from BioBasic Inc., Toronto, Canada.

### Biochemicals

Radiolabelled nucleoside triphosphates [α-$^{32}$P]dATP were obtained from NEN Life Science Products at a specific activity of 6000 Ci/mmol. *Taq* polymerase (Promega), T4 polynucleotide kinase (Promega), AMV reverse transcriptase (Promega) and the restriction endonucleases *Eco*RI (Roche) and *Pvu*II (New England Biolabs) were used according to the supplier's recommended protocol in the activity buffer provided. All other chemicals were analytical grade reagents, and all solutions were prepared using doubly deionized, Millipore filtered water.

**Trp-Pro-Arg-Lys-Trp-Pro-Arg-Lys-Trp-Pro-Arg-Lys-$NH_2$ (WR-12).** This peptide was synthesized using solid-phase methodology by manual operation of a Protein Technology PS3 peptide synthesizer. The first Fmoc-protected amino acid was coupled to the Nova Rink amide AM resin using PyBOP/NMM in DMF. All of the $N_\alpha$-Fmoc-protected amino acids (in 4 equivalent ratio excess to the resin) were coupled in a stepwise fashion using PyBOP/NMM in DMF after deprotection of the *N*-Fmoc group by piperidine. The side chains of Arg, Lys and Tyr are protected by the Pmc, Boc and Trt groups, respectively. *Trp-Pro-Arg(Pmc)-Lys(Boc)-Trp-Pro-Arg(Pmc)-Lys(Boc)-Trp-Pro-Arg(Pmc)-Lys(Boc)*-Resin was treated with the cleavage reagent (0.75 g phenol, 10 mL TFA, 0.5 mL thioanisole, 0.25 mL EDT) for 1.5 h, and then lyophilized. The resin was washed with dry ether (2×30 mL), filtered, and then washed with 5% acetic acid (200 mL). The combined filtrate was lyophilized and the product was purified by semi-preparative reversed-phase HPLC (column 1) using gradient elution. Eluent A: 5% MeCN, 95% $H_2O$, 0.1% TFA; Eluent B, 95% MeCN, 5% $H_2O$, 0.1% TFA. A linear gradient was achieved by increasing the MeCN content from eluent A to eluent B in 30 min. $R_t$ (column 2), 12.4 min. Mp 165–167 °C, $[\alpha]_D^{28}$ −60.1 (*c* 0.0208, $H_2O$); ESIMS requires: 1720.08, found: 1721.

**Tyr-Pro-Arg-Lys-Tyr-Pro-Arg-Lys-Tyr-Pro-Arg-Lys-$NH_2$ (YR-12).** This peptide was synthesized using a similar procedure as for WR-12. The crude product was purified by semi-preparative reversed-phase HPLC (column 1) using gradient elution as described for the purification of WR-12. $R_t$ (column 2), 10.20 min. Mp 131–133 °C, $[\alpha]_D^{28}$ −84.88 (*c* 0.0324, $H_2O$); ESIMS requires: 1937.17, found: 1937.

**Ser-Pro-Gln-Lys-Arg-Ala-Ala-Ser-Pro-Arg-Lys-Ser-Pro-Arg-Lys-Ser-Pro-Lys-Lys-Ser-Pro-Arg-Lys-Ala-Ser-Ala-Ser-Pro-Arg-Arg-$NH_2$ (SP-30).** This peptide was synthesized using a similar procedure as for WR-12. The crude product was purified by semi-preparative reverse-phase HPLC (column 1) using gradient elution as described for the purification of WR-12. $R_t$ (column 2), 9.468 min. Mp 158–160 °C, $[\alpha]_D^{28}$ −9.09 (*c* 0.033, MeOH/$CH_2Cl_2$, 1:1); ESIMS requires: 3328.85, found: 3329.

### DNase I footprinting

A 117-mer fragment was isolated from the plasmid pBS by double digestion with *Eco*RI/*Pvu*II. It was labelled as previously described.[11,12] Reactions were conducted in a total volume of 8 μL. Radiolabelled DNA (2 μL) was mixed with varying concentrations of peptide (4 μL) dissolved in various buffers (1×TN buffer: 10 mM Tris–HCl, 10 mM NaCl, pH 7.4; 5 mM sodium cacodylate buffer, pH 6.0, or 7.0) and equilibrated at room temperature for 60 min. DNase I (2 μL) was added and the reaction allowed to proceed for 8 min. The DNase I solution (in 20 mM NaCl, 2 mM $MgCl_2$, 2 mM $MnCl_2$) was adjusted to yield a final concentration of 0.009 unit/mL so as to limit the digestion to less than 30% of the starting material in order to minimize the incidence of multiple cleavages in any strand. The digestion was stopped by adding stop solution (3 μL) containing 80% formamide, 10 mM EDTA, 0.1% bromophenol blue,

and 0.1% xylene cyanol. Samples were heated at 90 °C for 4 min and chilled in ice for 4 min prior to electrophoresis.

### Electrophoresis and autoradiography

The products of DNA cleavage were resolved by polyacrylamide gel electrophoresis under denaturing conditions (0.3 mm thick, 8% acrylamide containing 8M urea). G + A tracks were generated by treatment of the radiolabeled DNA with 80% formamide. Briefly, to 2 μL of the treated DNA were added 20 μL of water and 5 μL of an 80% formamide solution containing tracking dyes. The sample was then heated at 90 °C for 45 min with the Eppendorf tube cap opened, cooled on ice for 4 min and then loaded on to the gel. After electrophoresis (about 1.45 h at 70 Watts, 2500 V in TBE buffer, BRL sequencer model S2), gels were soaked in 10% acetic acid/10% methanol for 15 min, transferred to Whatman 3MM paper, dried under vacuum at 80 °C for 45 min, and placed against a Molecular Dynamics 425E PhosphorImager storage screen overnight. The electrophoretic band areas were analyzed by a PC computer installed with Viber Lourmat BIO-ID software (Marne La Valle, Cedex 1, France). Footprinting data are presented in the form in ($f_a/f_c$) representing the differential cleavage at each site relative to that in the control ($f_a$ is the fractional cleavage at any bond in the presence of the peptide and $f_c$ is the fractional cleavage in the control, i.e., absence of peptide). The results are displayed on a natural logarithmic scale. Positive values indicate enhanced cleavage whereas negative values indicate blockage.

### Binding of peptides to duplex oligonucleotides

Equilibrium titration studies employing six decanucleotide duplexes with YR-12 or WR-12 were carried out in Tris–HCl buffer (pH 7.3) at 37 °C. Briefly, 20-μL aliquots of oligonucleotide (30 μM) were added to the peptide solution (19.6 μM) and the mixture was allowed to equilibrate for 5 min. Excitation was provided by irradiation at the wavelength maximum of 275 and 305 nm for YR-12 or WR-12, respectively; and emission was measured at 290 and 350 nm. Lines were fitted by the method of least-squares to the linear regions of the data points using the Scatchard equation $r_b/m = K_a(n - r_b)$ where $r_b$ is the ratio of bound peptide to nucleotides, $n$ is the saturation binding density (number of binding sites per nucleotide), $K_a$ is the apparent association constant and $m$ is the free peptide concentration.

## Results and Discussion

Initial experiments using DNase I digestion showed that incubation of the peptides with the 117-mer DNA in sodium cacodylate or TN buffer gave better footprints than reaction mixtures prepared using phosphate buffer for incubation. Figure 1 shows typical digestion patterns for the 117-base-pair fragment in the presence of low concentrations of YR-12. Each gel lane contains about 70 bands that are well resolved and the derived differential cleavage plots are illustrated in Figure 4. Incubation of 200 nM YR-12 with DNA in sodium cacodylate buffer at pH 6.0 and pH 7.0 does not produce distinctive cleavage patterns indicative of simple sequence selectivity. However, incubation at pH 6.0 suggests some preferential effect on the sequence 5′-AAACGACG-3′. Incubation of 75 nM YR-12 with the same DNA in TN buffer, pH 7.4 produced better DNase I footprints and confirmed the existence of a selective effect at positions 37–44 and 20–30 comprising the sequences 5′-AAACGACG-3′ and 5′-GAATTG TAATA-3′, respectively. The most distinctive blockages include the 5′-AAA-3′, 5′-AATT-3′ and 5′-AATA-3′ sequences (Fig. 4c). Other regions of moderately diminished cleavage occur at sequences down to position 30 including the sequence 5′-AGTG-3′; at position 64–68 around the sequence 5′-GGTTT-3′; position 51–55 surrounding the sequence 5′-ACGAC-3′; and position 73–77 corresponding to the sequence 5′-TAACG-3′.

Inspection of the regions protected by 200 nM WR-12 incubated with DNA in sodium cacodylate buffer at pH 6.0 and pH 7.0 reveals more decisive blockages at positions 38–44 and 26–30 around the sequences 5′-AAAC GAC-3′ and 5′-GAATT-3′, respectively (Fig. 2 and 4b). The intensity of the blockages appear stronger than that produced by YR-12 under identical incubation conditions (Fig. 4b). When 75 nM WR-12 is incubated with DNA in TN buffer at pH 7.4, once again it produces significant blockages at positions 26–30 and 21–24 around sequences 5′-GAATT-3′and 5′-TAAT-3′, respectively (Figs 3 and 4c). Another notable blockage is identified at positions 38–44 comprising the sequence 5′-AAACGAC-3′.

The footprinting pattern of the reference compound SP-30 is illustrated in Figure 3. The chief difference between SP-30 and the other two peptides is that it does not contain any Trp or Tyr residues. SP-30 has twice as many XPK(R)K motifs as the other two peptides as a result of which it binds to DNA more tightly. Incubation of 7.5 nM SP-30 with DNA in TN buffer, pH 7.4 again reveals major blockages around positions 27–30, 21–24 and 38–45 around sequences 5′-GAAT-3′, 5′-TAAT-3′ and 5′-AAAACGAC-3′, respectively. Other blockages can be identified at positions 50–54 for the sequence 5′-ACGAC-3′; 65–69 for the sequence 5′-GGGTT-3′; and possibly position 58-6-1 comprising the sequence 5′-CCAG-3′ (Fig. 4c).

Comparison of the differential cleavage plots produced by the three peptides under varying conditions (Fig. 4) yields consensus preferential binding sites at positions 26–30, 21–24 and 38–44, showing that it is true that peptides containing the XPK(R)K motif prefer to bind near a nucleotide sequence containing consecutive adenines (A/T-rich sites). Close examination of the plots also reveals that there are tangible differences in the DNase I digestion patterns that occur in the presence of YR-12 and SP-30. YR-12 produces some footprints at regions where little blockage is induced by SP-30, for example between positions 60 and 64 corresponding to the sequence 5′-TTCCC-3′ and at position 33–37

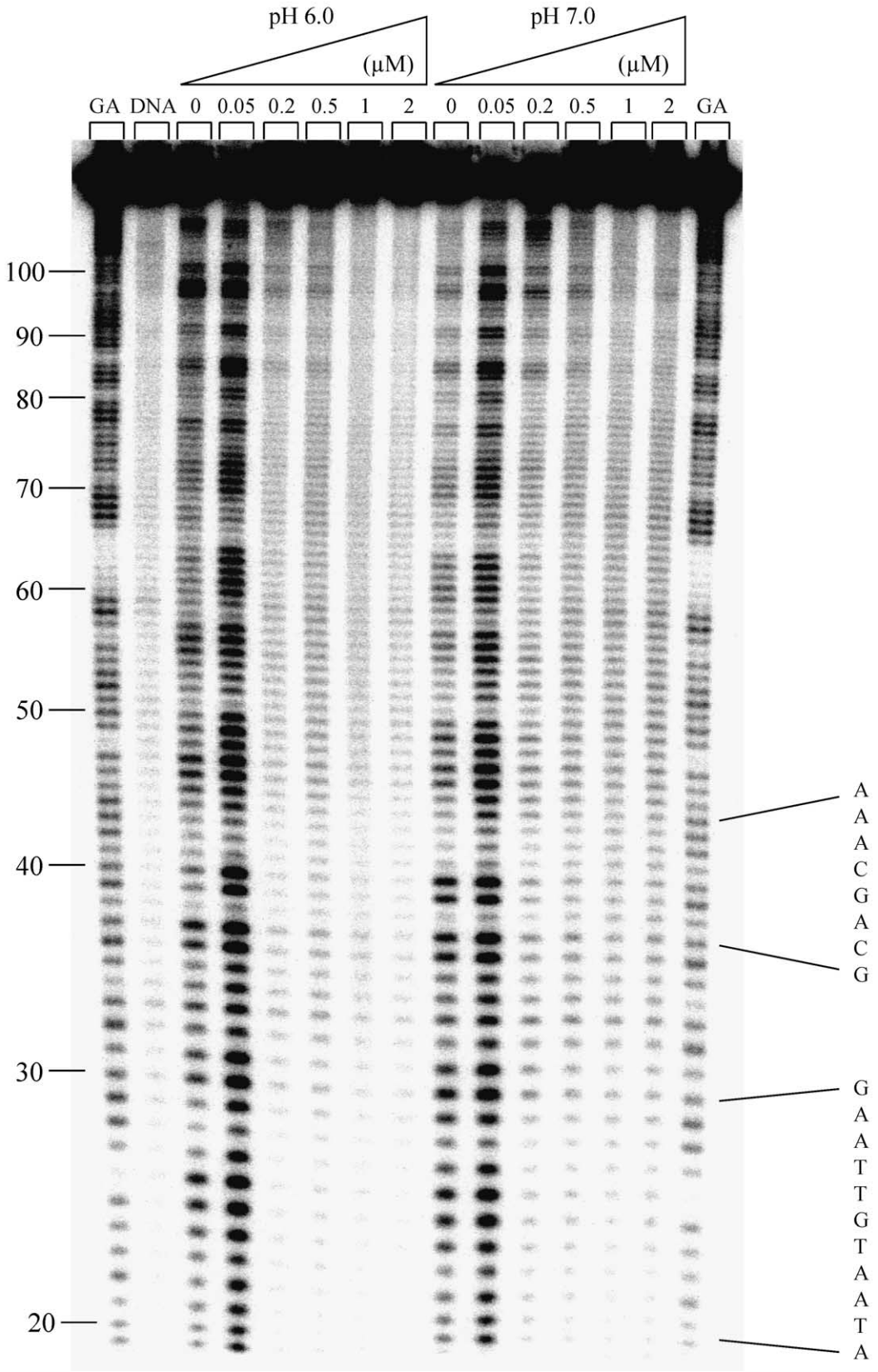


**Figure 1.** DNase I footprinting of YR-12 bound to the 117 bp duplex DNA fragment labelled with [α-$^{32}$P]dATP on the 3′-end in the presence of AMV reverse transcriptase. YR-12 was incubated with the DNA in 5 mM sodium cacodylate buffer (pH 6.0 and pH 7.0) at room temperature for 60 min before DNase I cleavage. GA represents a Maxam-Gilbert purine sequencing track.

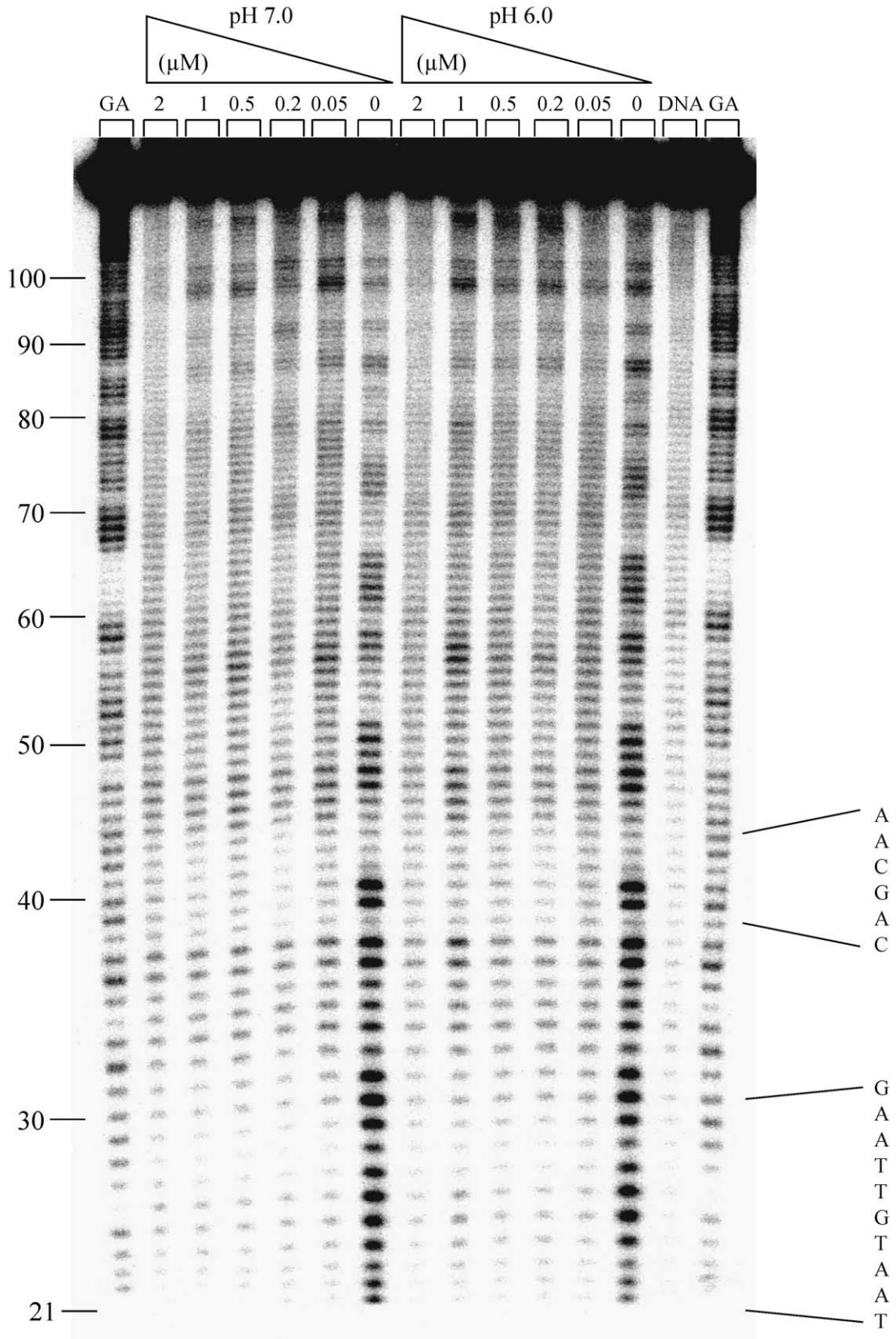


**Figure 2.** DNase I footprinting of WR-12 bound to the 117 bp duplex DNA fragment labelled with [α-$^{32}$P]dATP on the 3′-end in the presence of AMV reverse transcriptase. Reaction conditions as indicated in the legend to Figure 1.

around the sequence 5′-GGCCA-3′. In addition, within the nucleotide sequence comprising the major binding site at position 37–44, YR-12 and WR-12 seem to produce greater blockage affecting nucleotides 37–39 (5′-ACG-3′). It thus appears that YR-12 has greater capability to extend its recognition sites to encompass CG pairs than can SP-30.

It is of interest that lower concentrations of YR-12 appear to enhance the rate of nuclease cleavage at many regions of the DNA, including some positions close to binding sites (relatively less enhancement is observed for WR-12 and SP-30). Incubation of 117-mer DNA with 50 nM YR-12 in sodium cacodylate buffer at pH 6.0 seems to promote cutting even at positions 38 and 39

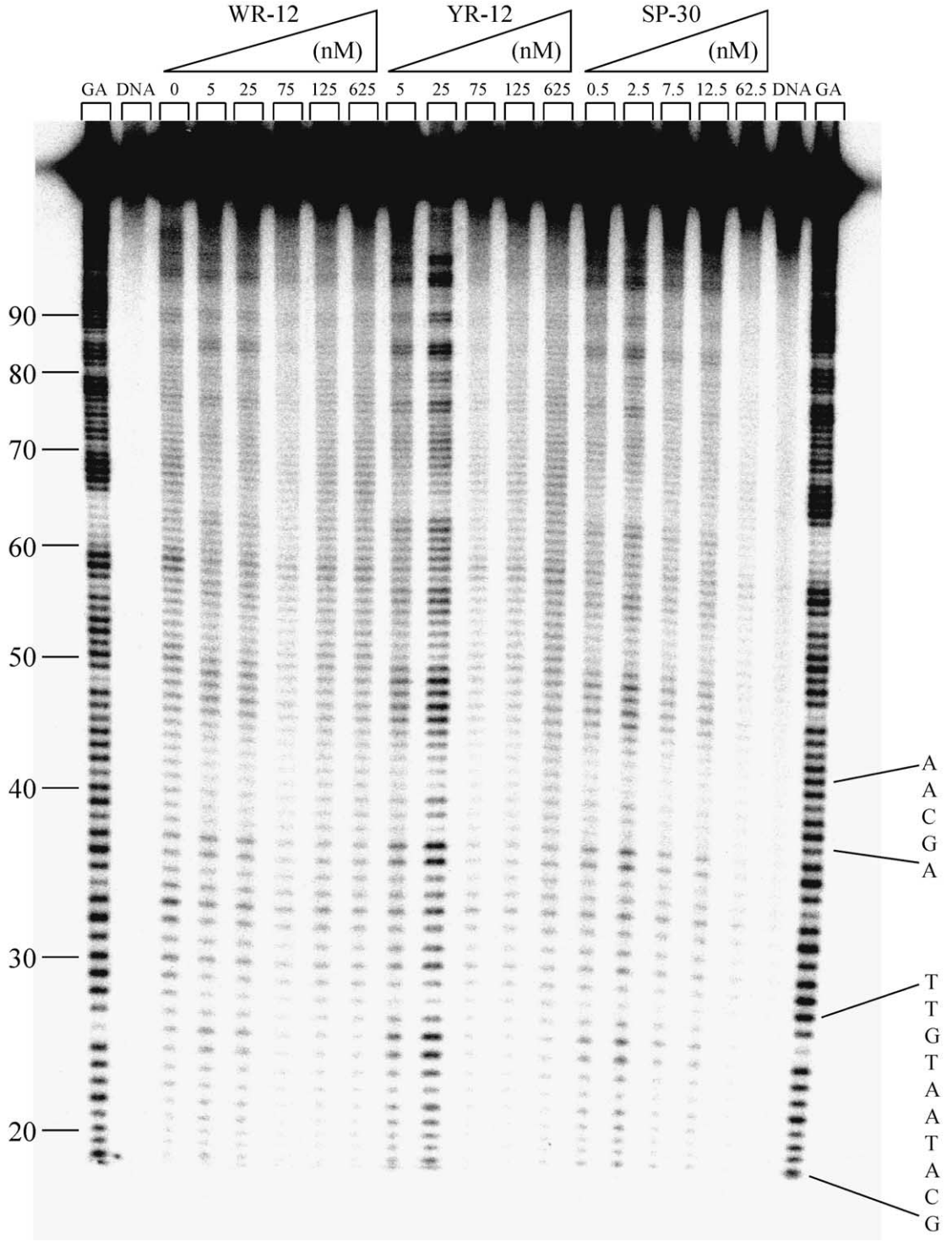


**Figure 3.** Comparative DNase I footprinting of WR-12, YR-12 and SP-30 bound to the 117 bp duplex DNA fragment labelled with [α-$^{32}$P]dATP on the 3′-end. Each peptide was incubated with DNA in 1×TN buffer (pH 7.4) at room temperature for 60 min before DNase I cleavage.

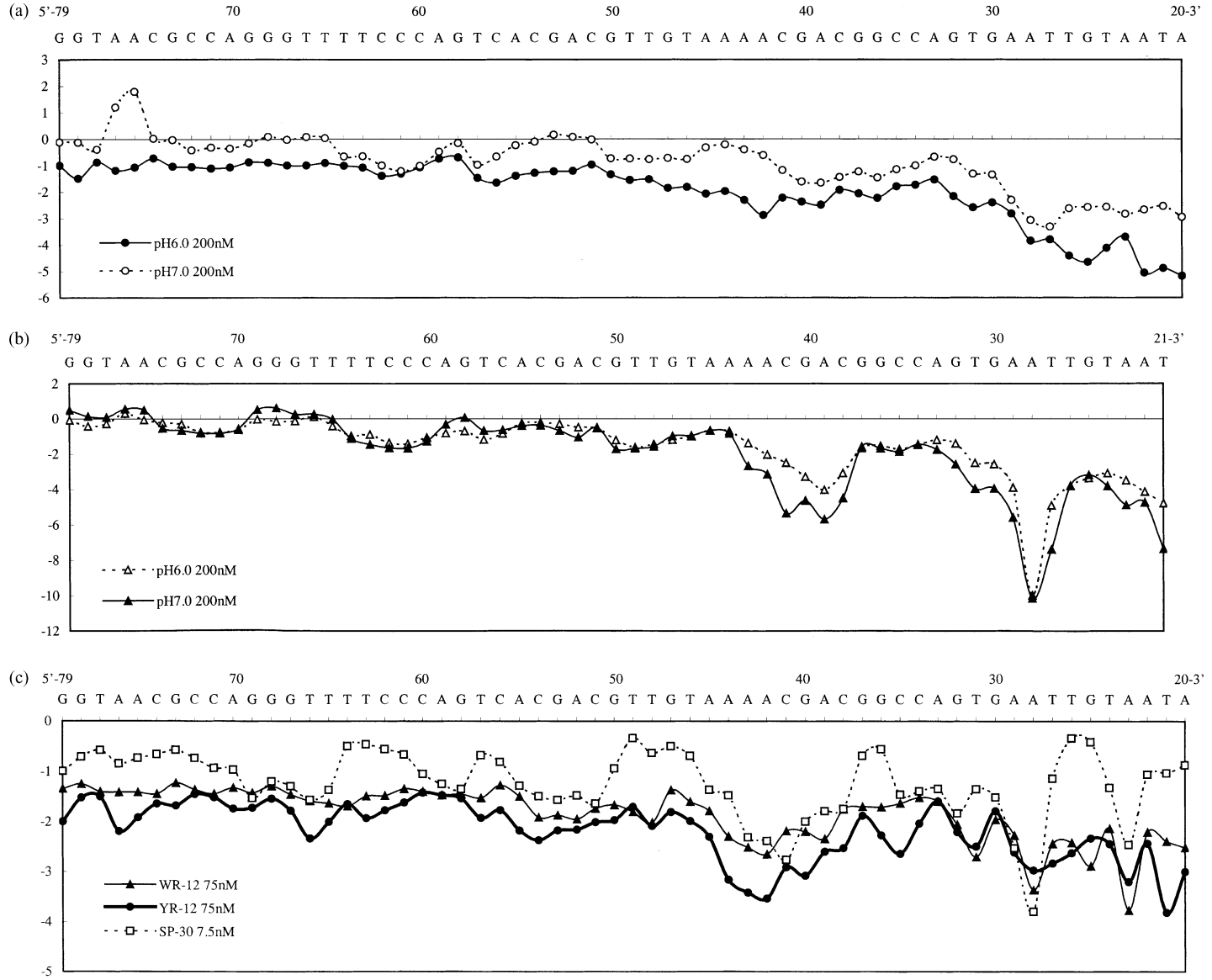


**Figure 4.** Differential cleavage plots for the susceptibility of the 117 bp duplex DNA fragment to DNase I after incubation with 200 nM YR-12 (panel a) or 200 nM WR-12 (panel b) in sodium cacodylate buffer at pH 6.0 and pH 7.0. Panel c shows the differential cleavage of DNA by 75 nM WR-12, 75 nM YR-12 and 7.5 nM SP-30 after incubation with DNA in 1×TN buffer (pH 7.4) at room temperature for 60 min prior to DNase I cleavage. The ordinate scales are the dimensionless difference ($\ln f_a - \ln f_c$) calculated as previously described.[14]

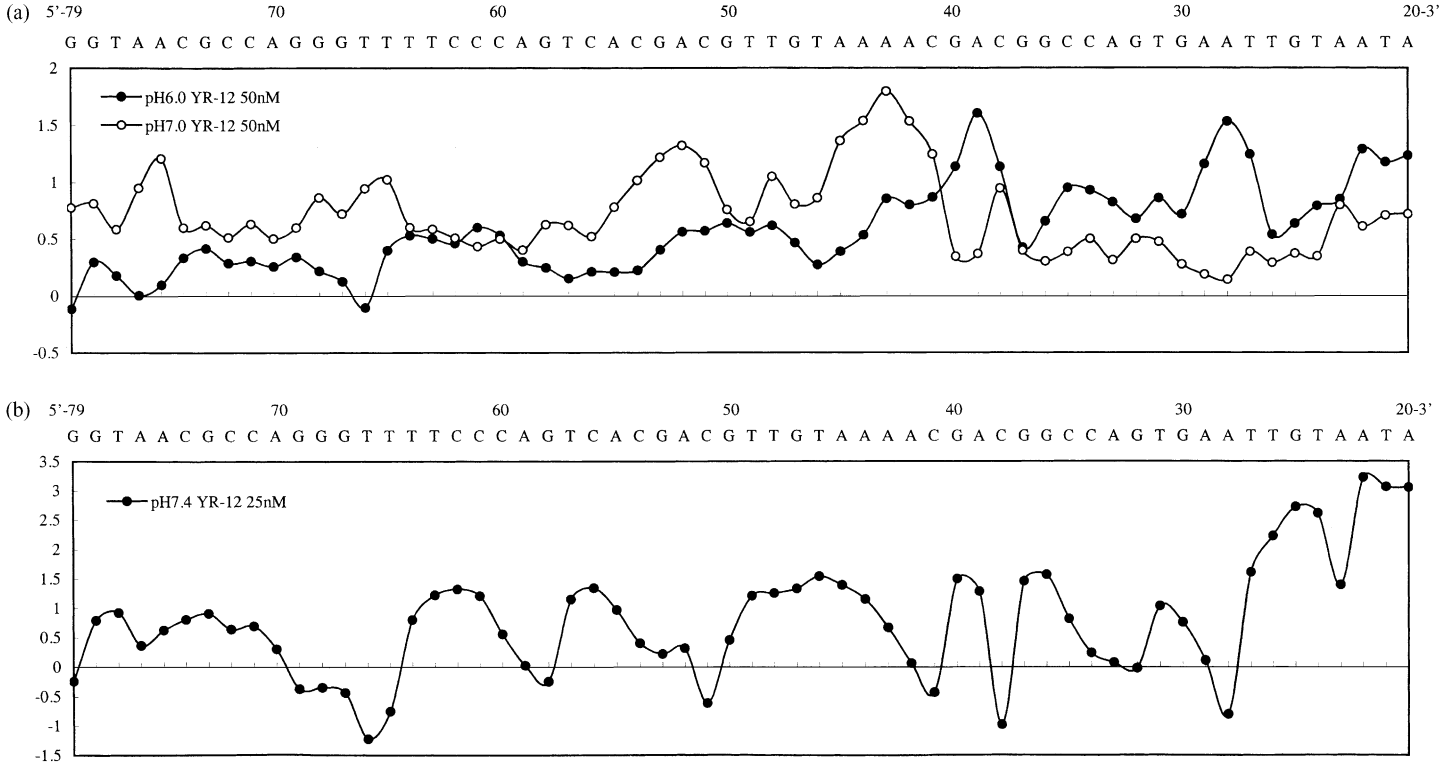


**Figure 5.** Differential cleavage plots showing enhancement of DNase I cleavage after incubation of 50 nM YR-12 with the 117 bp duplex DNA fragment in sodium cacodylate buffer at pH 6.0 and pH 7.0 (panel a), or after incubation with 25 nM YR-12 in 1×TN buffer at pH 7.4 (panel b) at room temperature for 60 min.

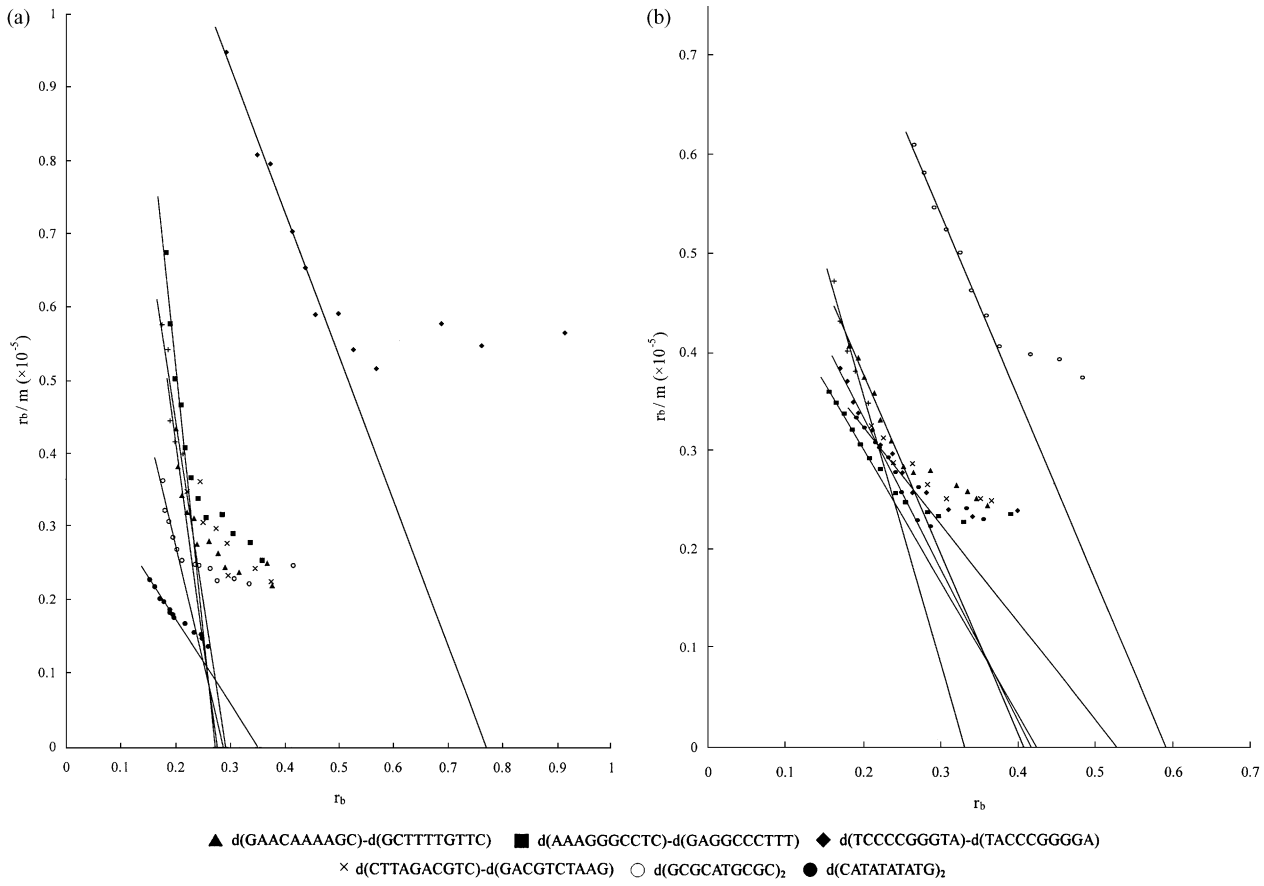


**Figure 6.** Comparative Scatchard plots for the interaction between WR-12 (panel a) or YR-12 (panel b) and six decanucleotide duplexes in Tris–HCl buffer, (pH 7.3) at 37 °C.

which lie towards the 3′-end of the main site at position 40–44 (Figs 1, 3 and 5a). However, at pH 7.0 the enhancement shifts to the higher 40's with another possible peak around position 51–55. On the other hand, incubation with 25 nM YR-12 in TN buffer at pH 7.4 induces several different enhancements as shown in Figures 3 and 5b. These enhancements in nuclease cleavage may account for the apparent observation that the footprinting patterns are not closely related to concentration of the peptides tested. Comparable enhancement effects have been reported with actinomycin D, echinomycin, and mithramycin binding to DNA.[13–15] Enhanced susceptibility to DNase I cleavage has been interpreted as resulting from the induction of a locally wider helical minor groove by neighouring ligands, restoring it to a more 'average' B-DNA-like structure. At higher concentrations of YR-12, blockage of the cleavage action of DNase I is restored since any conformational transition is swamped by the footprinting action of bound peptide blocking access of the nuclease to its binding sites.

To obtain additional quantitative data supplementary to the DNA footprinting of YR-12 and WR-12 we performed Scatchard analyses on six decanucleotide duplexes. The sequences of these oligonucleotides were tentatively chosen from the known sequences of two fragments: EcoRI-PvuII of pBS (265-mer) and fragment EcoRI-BsrBI of PBR-322 (156-mer). The methodology used was modified from that of Chen's work on the equilibrium binding of actinomycin D to various oligonucleotide duplexes.[16–18]

Representative Scatchard plots[19] resulting from fluorescence quenching titrations are illustrated in Figure 6 and the various binding parameters are collated in Table 1. It is apparent that these six oligomers display varying binding affinities for YR-12 and WR-12. For example, WR-12 binds to d(AAAGGGCCTC)-d(GAGGCCCTTT), d(GAACAAAAGC)-d(GCTTTTCTTC) and d(CTTAGACGTC)-d(GACGTCTAAG) with apparent binding constants 6, 5, and 4 times higher than for the A/T-rich self-complementary oligomer d(CATATATATG)$_2$, respectively. Likewise, the binding constant of YR-12 on d(CATATATATG)$_2$ is lower than for the same three oligomers having less A/T rich sequences, though the differences are less marked (up to 3-fold). The tightest binding of WR-12 is seen with the oligomers d(AAAGGGCCTC)-d(GAGGCCCTTT) and d(GAACAAAAGC)-d(GCTTTTCTTC), which might suggest some selectivity for runs of consecutive purines or pyrimidines. On the other hand, YR-12 binds best to the oligomer d(CTTAGACGTC)- d(GACGTCTAAG), perhaps denoting selectivity for evenly distributed C/G and A/T sequences. The Scatchard plots also indicate that the two oligopeptides bearing the XPRK motif may bind to more than one site in some of the decanucleotide duplexes, witness the varying values of $n$, pointing

**Table 1.** Binding parameters of peptides to decanucleotide duplexes

| Peptide | Oligomer | $K_a$ ($M^{-1}\times10^5$) | $n$ |
|---|---|---|---|
| WR-12 | d(AAAGGGCCTC)-d(GAGGCCCTTT) | 7.14 | 0.273 |
| WR-12 | d(TCCCCGGGTA)-d(TACCCGGGGA) | 1.97 | 0.792 |
| WR-12 | d(CTTAGACGTC)-d(GACGTCTAAG) | 4.83 | 0.292 |
| WR-12 | d(GAACAAAAGC)-d(GCTTTTGTTC) | 5.50 | 0.276 |
| WR-12 | $d(CATATATATG)_2$ | 1.15 | 0.350 |
| WR-12 | $d(GCGCATGCGC)_2$ | 3.15 | 0.286 |
| YR-12 | d(AAAGGGCCTC)-d(GAGGCCCTTT) | 1.34 | 0.425 |
| YR-12 | d(TCCCCGGGTA)-d(TACCCGGGGA) | 1.54 | 0.418 |
| YR-12 | d(CTTAGACGTC)-d(GACGTCTAAG) | 2.72 | 0.331 |
| YR-12 | d(GAACAAAAGC)-d(GCTTTTGTTC) | 1.83 | 0.406 |
| YR-12 | $d(CATATATATG)_2$ | 1.0 | 0.528 |
| YR-12 | $d(GCGCATGCGC)_2$ | 1.87 | 0.593 |

The parameters are calculated from the Scatchard equation: $r_b/m = K_a(n-r_b)$ where $r_b$ is the ratio of bound drug to nucleotide concentration, $n$ is the saturation binding density (number of drug binding sites per nucleotide), $K_a$ is the apparent association constant and $m$ is the free peptide concentration.

to generally lower sequence selectivity than DNA binding proteins that bind specifically in the major groove.

Thus, both footprinting and Scatchard analyses indicate that YR-12 and WR-12 have different DNA binding properties from those of the SPXX peptides investigated previously, which are reported to show preference for A/T rich sequences.[6] The footprinting results also show that YR-12 and WR-12 can bind quite strongly to the 117-mer DNA at sub-micromolar concentrations. Since these small oligopeptides evidently have DNA sequence recognition properties that favor various regions of DNA, it is possible that on-going work on the XPRK motifs may generate potential agents for modulating gene expression as a focus for future research.

## Acknowledgements

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## References and Notes

1. Abbreviations generally follow the IUPAC-IUB recommendation as published in *J. Biol. Chem.* **1989**, *264*, 668. Other abbreviations: NMM, *N*-methylmorpholine; Fmoc, 9-Fluorenylmethyloxycarbonyl; Boc, *t*-butyloxycarbonyl; Trt, trityl; Pmc, pentamethylchroman-6-sulfonyl; TFA, trifluoroacetic acid; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium-hexafluorophosphonate.
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