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Massive parallel analysis of the binding specificity of histone-like protein HU to single- and double-stranded DNA with generic oligodeoxyribonucleotide microchips

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ABSTRACT

A generic hexadeoxyribonucleotide microchip has been applied to test the DNA-binding properties of HU histone-like bacterial protein, which is known to have a low sequence specificity. All 4096 hexamers flanked within 8mers by degenerate bases at both the 3′- and 5′-ends were immobilized within the 100 × 100 × 20 mm polyacrylamide gel pads of the microchip. Single-stranded immobilized oligonucleotides were converted in some experiments to the double-stranded form by hybridization with a specified mixture of 8mers. The DNA interaction with HU was characterized by three type of measurements: (i) binding of FITC-labeled HU to microchip oligonucleotides; (ii) melting curves of complexes of labeled HU with single-stranded microchip oligonucleotides; (iii) the effect of HU binding on melting curves of microchip double-stranded DNA labeled with another fluorescent dye, Texas Red. Large numbers of measurements of these parameters were carried out in parallel for all or many generic microchip elements in real time with a multi-wavelength fluorescence microscope. Statistical analysis of these data suggests some preference for HU binding to G/C-rich single-stranded oligonucleotides. HU complexes with double-stranded microchip 8mers can be divided into two groups in which HU binding either increased the melting temperature (T_m) of duplexes or decreased it. The stabilized duplexes showed some preference for presence of the sequence motifs AAG, AGA and AAGA. In the second type of complex, enriched with A/T base pairs, the destabilization effect was higher for longer stretches of A/T duplexes. Binding of HU to labeled duplexes in the second type of complex caused some decrease in fluorescence. This decrease also correlates with the higher A/T content and lower T_m. The results demonstrate that generic microchips could be an efficient approach in analysis of sequence specificity of proteins.

INTRODUCTION

Nucleic acid–protein interactions play an important role in cells and can be investigated by many different methods. Binding of small ligands to DNA has also been studied by several well-characterized techniques, such as protection of nucleic acids in a complex against chemical modification (1), nuclease footprinting assay (2,3), separation of complexes by electrophoresis (4), dialysis and optical methods (5). Oligonucleotides immobilized on filters or glass surfaces also provide means to assay protein–DNA interactions (6–10). All these methods are usually applied to discriminate significant specific binding from non-specific binding. For example, binding of the Cro repressor to its specific site is 10^6 times stronger than non-specific binding (11) and the binding constant of Hoechst 33258 to AT-rich sequences is 10^3 times higher than that to GC-rich sequences (5). It is more difficult to study specificities when the binding constants of a protein or small ligands to all sequences are of the same order of magnitude.

One typical protein known to bind non-specifically to double-stranded (ds)DNA is the bacterial histone-like protein HU. It is an abundant (30 000 dimers/cell), small (18 kDa), basic and heat stable protein associated with the bacterial nucleoid in *Escherichia coli* (12,13). HU protein is composed of two homologous polypeptides and the heterodimeric form, HUαβ, is predominant during stationary phase (14–16). This protein has the capacity to introduce *in vitro* negative supercoils in relaxed circular DNA in the presence of topoisomerase 1 and to condense DNA (17). HU binds to both dsDNA and single-stranded (ss)DNA (12) and to some other structures like nicks, gaps and cruciforms (15,18,19). Binding of HU protein to dsDNA seems to be non-specific and the specificity of binding to ssDNA has not yet been described.

In a previous study we investigated binding of Hoechst 33258 dye to dsDNA sequences using a generic microchip. The chip contains 4096 immobilized octadeoxyrbonucleotides

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(8mers) in which all possible 6mers are flanked at both ends by an equimolar mixture of the four nucleotides (20). A selected set of 1024 microchip 8mers was converted to the double-stranded form by hybridization with a mixture of fluorescently labeled complementary 8mers. The gel pad microchips containing immobilized oligonucleotides provide significant advantages over microchips based on glass or filters. Such gel pad microchips can be considered as arrays of picoliter test tubes in which specific processes are carried out in parallel in each of them. They have a higher capacity and provide a more homogeneous environment for different interactions and enzymatic reactions: a direct correlation has been found between thermodynamic parameters for DNA in solution and in the microchip gel pads (21).

Here we have used a generic microchip for large-scale parallel analysis of HU binding to different 8mer duplexes containing variable 6mer cores. This type of microarray enables one to study in parallel up to 4096 melting curves of DNA complexes with HU protein and a statistical analysis can be applied to find those motifs which are preferred for binding. The statistical analysis could uncover the low level specificity of HU protein–DNA binding.

HU protein was found to have a low specificity for some motifs in dsDNA and GC-rich ssDNA. These results demonstrate that gel-based generic microchips can be a convenient and efficient approach to study nucleic acid–protein interactions.

MATERIALS AND METHODS

Chemicals

The 4096 octadeoxyribonucleotides used to manufacture the generic microchips, each containing a 6mer core and 3′-terminal amino linker, were purchased from CyberSyn (USA). All other oligonucleotides were synthesized with an Applied Biosystems 394 DNA/RNA synthesizer using standard phosphoramidite chemistry and the 3′-C(7) amino modifier CPG (Glen Research, USA). The 8mer mixture containing 3′-NH_2 groups was fluorescently labeled with Texas Red sulphonyl chloride dye (Molecular Probes, Eugene, OR) according to the manufacturer's protocol.

Generic microchips

The generic microchips were manufactured in two steps. First, the arrays of 4200 (60 × 70) 5% polyacrylamide gel pads fixed on a hydrophobic glass slide (100 × 100 × 20 μm spaced at 200 μm) were prepared by photopolymerization (22). Then, 1 nl droplets of 1 mM solutions of oligonucleotides in water were applied to each gel pad (6) and the oligonucleotides were immobilized by reductive coupling of their amino groups with the aldehyde groups of the gel (23).

HU protein

Native HUαβ protein was purified from *E.coli* strain JRY1 as previously described (16), with a modification aimed at removing nuclease activity (19). The protein concentration was determined by absorbance at 230 nm, where A_{230} = 2.3 corresponds to 1 mg/ml HU protein.

For the experiments with ssDNA HU protein was labeled with FITC (30 μg/mg protein), in accordance with the standard protocol (24), in 0.15 M NaCl and 0.2 M sodium carbonate buffer, pH 9.0, at room temperature for 1.5 h. FITC was removed from the labeled protein by gel filtration on Sephadex G-25.

Hybridization of HU with ssDNA

Complexes of labeled HU with single-stranded 8mers on the generic microchip were produced by incubation of the protein (0.5 mg/ml) with the microchip in a 200 μl hybridization chamber at 0°C for 12 h in 20 mM Tris–HCl pH 7.2, 20 mM NaCl, 5 mM EDTA and 0.1% Tween-20. The melting curve measurements for the HU complexes with single-stranded microchip oligonucleotides were carried out as described below for the complexes with dsDNA.

HU hybrydization with dsDNA

To convert some 8mers of the generic microchip from the single-stranded to the double-stranded form it was hybridized with a mixture of fluorescently labeled 8mers [5′-MM(A/C)MM(A/C)MM-3′-TR, where M is an equimolar mixture of the four nucleotides] in a 200 μl hybridization chamber at 0°C for 24 h. The hybridization solution contained 200 μM oligonucleotides, 100 mM NaCl, 20 mM Tris–HCl pH 7.2, 5 mM EDTA and 0.1% Tween-20. After hybridization the solution was replaced with the same buffer without oligonucleotides. The hybridization chamber with the microchip was then placed on the thermotable of a fluorescence microscope and non-equilibrium melting curves were recorded for all elements of the microchip. The temperature was increased from –2 to + 50°C at a rate of 2°C/h in 1°C steps. After measuring the melting curves of the duplexes in the absence of HU protein the fluorescently labeled oligonucleotides were washed off the microchip with water. A second round of hybridization and melting experiments was repeated under the same conditions, but in this case the solution after hybridization was replaced with buffer containing HU protein (0.5 mg/ml) and the probe was incubated for 12 h at 0°C. Then the same melting procedure was performed.

Melting measurements

All measurements of HU binding and of melting curves were performed using an automated 3.5 × 3.5 mm field epifluorescence microscope with mercury lamp excitation and a filter for Texas Red dye (LOMO, Russia). The second filter on the same device was used to measure the fluorescence of FITC-labeled protein. The microscope was equipped with a CDD camera (Princeton Instruments, USA), a Peltier thermotable with a temperature controller (Melcor, USA) and a computer supplied with a data acquisition board (National Instruments, USA) (20–22). The fluorescence intensity was measured at each temperature by scanning the generic microchip by field, each containing 100 gel pads. It takes 2 s to acquire an image of 100 pads. The scanning system consisted of a two-coordinate table, stepped motors and a controller (Newport, USA). Special software was designed for experimental control and data processing using C^{++} or the LabVIEW virtual instrument interface (National Instruments, USA) (20–22).

RESULTS

Massive parallel measurements of HU protein–oligodeoxyribonucleotide interactions on a generic microchip

The generic 6mer microchip contains all possible 4096 single-stranded hexadeoxyribonucleotides NNNNNN (where N is one of the four bases A, G, T or C). These core 6mers are flanked within 8mers of general structure gel–5′-MNNNNNNM-3′ at both the 3′- and 5′-ends with a 1:1:1:1 mixture of the four bases M (20). This extension of 6mers to 8mers stabilizes the duplexes and increases their length covered with HU protein. HU is known to interact with 8–9 bases on DNA (19). The resulting 8mers are immobilized within gel pads; each gel pad contains only one core 6mer.

HU protein is known to bind ssDNA and dsDNA but no significant sequence specificity was detected. We decided, however, to re-examine the specificity of HU protein–DNA complexes by massive statistical analysis of the large volume of data obtained using a generic 6mer microchip.

Four techniques have been applied for these studies. The first and second are based on direct binding of fluorescently labeled HU to all single-stranded oligonucleotides on the microchip and on measuring the melting curves of HU complexes. The massive amount of data obtained consists of the fluorescence intensities for all the 4096 hexamers as well as the melting temperatures (T_m) of protein–ssDNA complexes. The affinity of the protein for each oligonucleotide correlates both with the intensity of fluorescence of the corresponding gel pad and with the T_m value. The third technique is based on indirect measurement of protein binding to dsDNA. The large amount of data obtained consists of a set of ΔT_m values, i.e. shifts in melting temperatures for all double-stranded oligonucleotides caused by HU binding. A positive (or negative) ΔT_m indicates that protein binding increases (or decreases) the stability of a particular duplex. In this method the protein is not fluorescently labeled but one strand of the duplex is labeled with Texas Red. The fourth technique is additional to the third and includes analysis of the fluorescence intensity of labeled DNA duplexes in the absence and the presence of unlabeled HU protein.

Measurements of T_m for HU–DNA complexes and for dsDNA alone and in complex with HU are well reproduced and suitable for use in quantitative analysis. All measurements were repeated on the same microchip at least twice and twice on different generic microchips. The T_m values were reproducible with an accuracy of ±1.5°C on the same microchip; on two different microchips the reproducibility was ±2°C. However, it was observed that changes in T_m during measurement in repeated experiments have the same tendency to increase or decrease for all gel pads to the same extent, therefore, the reproducibility in ΔT_m values is close to ±1°C.

Binding of HU protein to single-stranded microchip octamers

HU protein is known to bind to ssDNA (19,25,26). In a recent paper by Bonnefoy and Rouviere-Yaniv (26) ssDNA fragments of 20–40 bp or more were used to measure the binding constant. Oligonucleotides of this length are forced by HU to adopt some secondary structure. In our experiments binding of HU protein to ssDNA was measured for gel-immobilized short octamers lacking any secondary structure.

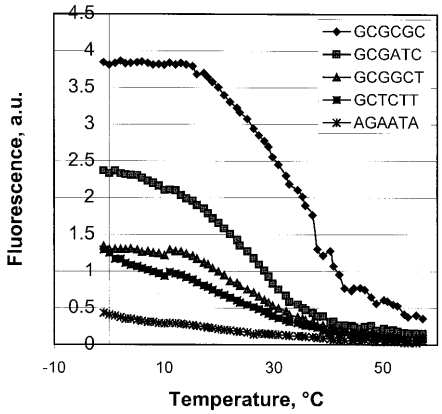


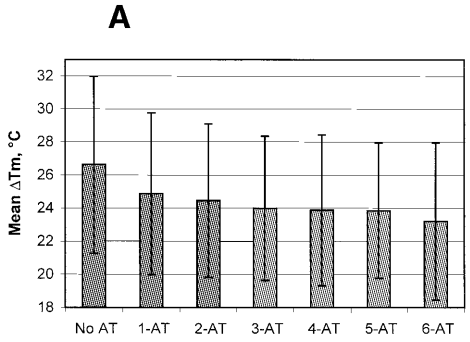
Figure 1. Non-equilibrium melting curves for complexes of FITC-labeled HU protein with five microchip-immobilized octamers containing specific 6mer cores.

HU was found in our experiments to have a much lower affinity for ssDNA as compared with dsDNA. We detected only negligible binding of HU to single-stranded microchip 8mers under the conditions of efficient binding to 8mer duplexes in 100 mM NaCl. Therefore, the protein–ssDNA interaction measurements were carried out in 20 mM NaCl. The affinity of HU for ssDNA of different compositions was measured by comparing their temperature-dependent dissociation. HU complexes with microchip 8mers were formed at 0°C, then the temperature of the microchip was gradually increased and complex dissociation was monitored by fluorescence of FITC-labeled HU protein in each gel pad. Nearly 4000 melting curves of HU–ssDNA complexes were obtained. Some typical dissociation curves are presented in Figure 1. The dissociation curves of these complexes are not cooperative, suggesting that one HU heterodimer forms a complex with one immobilized octamer. The T_m values for 4000 melting curves for HU–ssDNA complexes were approximated by the least squares method using the following equation:

$$f(T) = A + \{B/[1 + (T/T_m)^N]\} \quad \mathbf{1}$$

where T is the temperature (K), $f(T)$ is the measured signal, T_m is the melting temperature, $A + B$ is the the initial signal, B is the the final signal and N is the the cooperativity factor. When the approximation was done we obtained close to 4000 T_m values for HU complexes with all the single-stranded hexamers.

We undertook statistical analysis of these T_m values to obtain the preferential motifs for HU binding to hexamers. The results presented in Figure 2A show that the T_m values of the complexes drop from 27 to 23°C when the G/C content of the oligonucleotide core decreases from 6 to 0 bp. Figure 2B shows a similar dependence of the fluorescence intensity on G/C composition of 8mers in complexes with labeled HU protein: fluorescence increases with an increase in G/C content of the 6mer core of the gel-immobilized oligonucleotides. Analysis of the 4 base motifs demonstrates that GCGC is clearly the strongest sequence for HU binding to ssDNA (data not shown). However, the deviations in T_m and HU binding are



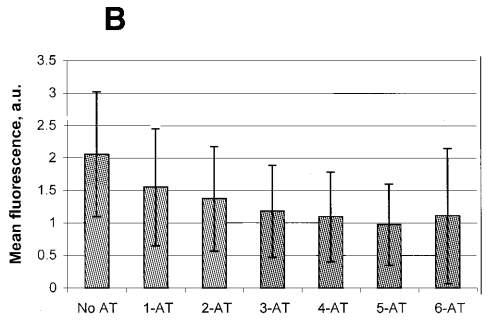


Figure 2. Mean melting temperatures (**A**) and labeled HU protein binding (**B**) for oligonucleotides with different numbers of A/T bases in the hexamer core. HU binding is estimated by measuring the fluorescence of labeled HU located in each gel pad of the generic microchip. The standard values and deviations are indicated by columns and bars, respectively.

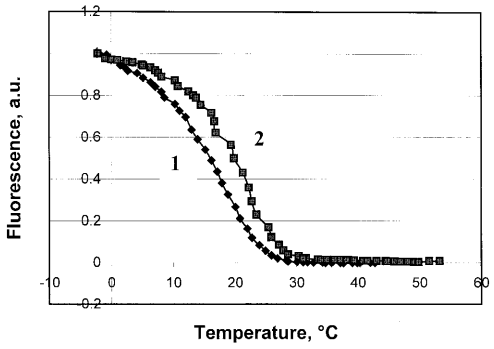


Figure 3. Non-equilibrium melting curves for a microchip duplex measured in the absence (1) and presence (2) of HU protein. A duplex was formed by hybridization of the microchip oligonucleotide gel–5′-MAGTCTGM-3′ with the fluorescently labeled oligonucleotides 3′-MTCAGACM-5′-TR from the hybridization mixture.

significant and some 6mers with a high and low G/C content overlap each other within the values of these parameters. This indicates a relatively low specificity of HU protein binding to ssDNA. Such a specificity was detected by statistical analysis of a large set of data for all hexamers.

Analysis of the T_m of HU–dsDNA complexes

The third technique is based on the conversion of single-stranded oligonucleotides on a generic chip to the double-stranded form by hybridization with a mixture of fluorescently

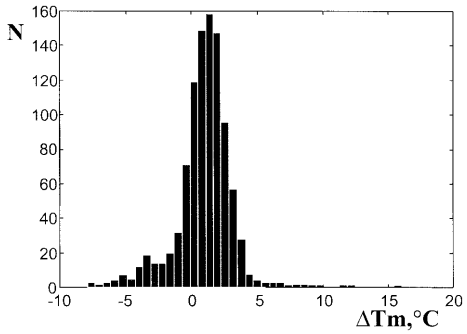
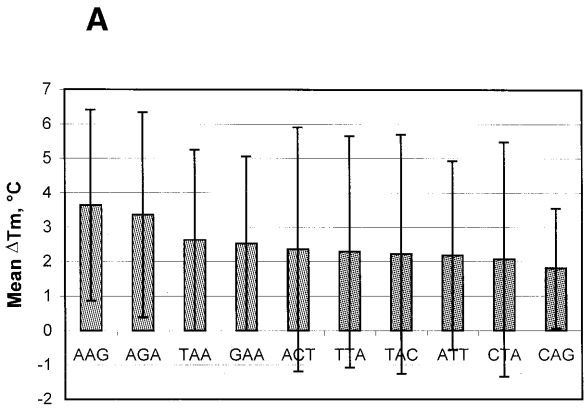


Figure 4. Distribution of the number of oligonucleotides (*N*) characterized by different ΔT_m. $\Delta T_m = T_m(+HU) - T_m(-HU)$.



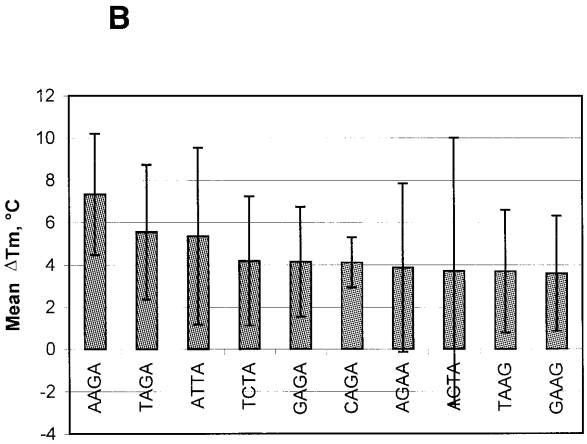


Figure 5. Average shifts of T_m (ΔT_m) for the 10 best motifs with 3 base long (**A**) and 4 base long (**B**) motifs. The standard mean values and deviations of ΔT_m are indicated.

labeled 8mers. To avoid hybridization of the competitor oligonucleotides with each other in solution instead of with immobilized oligonucleotides the mixture contained 1024 different non-complementary oligonucleotides of structure 5′-MM(A/C)MM(A/C)MM-3′-NH_2-TR.

After hybridization with fluorescently labeled 8mers and washing, the non-equilibrium melting curves for all duplexes formed on the microchip were recorded at increasing temperature.

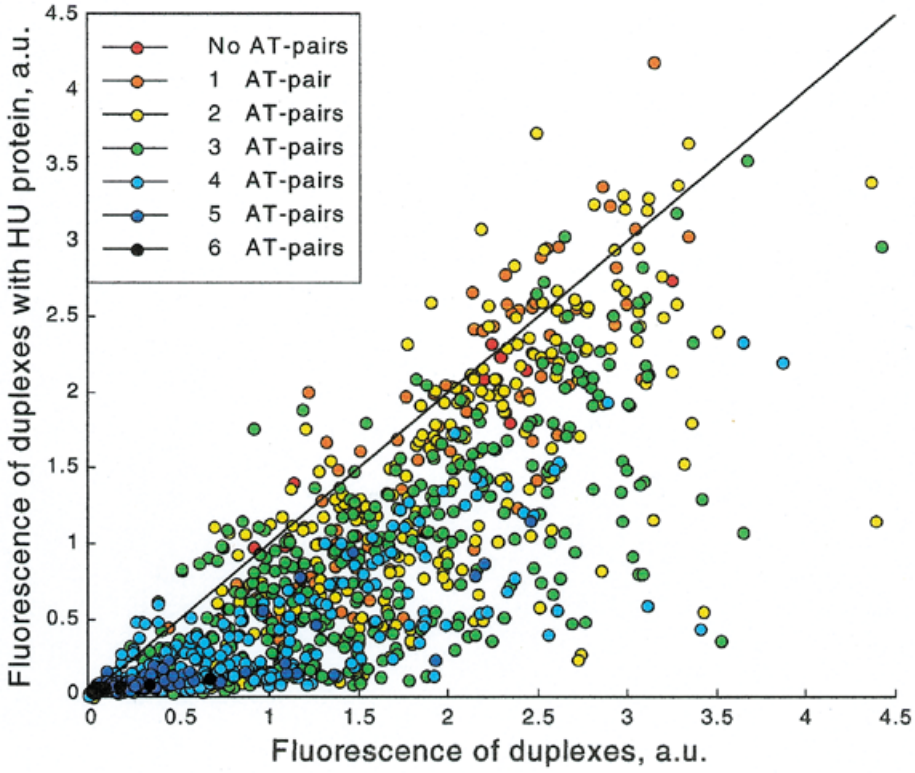


Figure 6. The effect of HU protein binding on fluorescence intensity of labeled microchip dsDNA with different GC contents.

For the second stage of the experiment the hybridization and melting curve measurements were repeated on the same microchip under the same conditions, however, in this case incubation was performed in the presence of HU protein to allow formation of protein–oligonucleotide complexes. The melting curves were obtained in the same way as in the absence of HU protein.

Figure 3 demonstrates two such melting curves obtained for the same oligonucleotide, AGTCTG. All 1024 melting curves were approximated by the least squares method using equation **1**. When the approximation was done we obtained 1024 pairs of T_m values for melting curves in the absence and presence of HU protein. As a result, the overall $\Delta T_m = T_m(+\text{protein}) - T_m(-\text{protein})$ for all duplexes was obtained. Fourteen oligonucleotides were excluded from consideration owing to a weak hybridization signal. A total of 1010 ΔT_m values were subjected to statistical analysis.

The ΔT_m values were arranged as a histogram, presented in Figure 4. This histogram clearly demonstrates the existence of two classes of complexes formed by HU protein and double-stranded oligonucleotides. The first, major class of ~850 complexes has a positive ΔT_m shift of approximately +3°C. The second class of weak complexes, comprising nearly 150 examples, shows a negative ΔT_m shift of approximately –3°C.

Statistical analysis of these two types of complexes showed a higher A/T content for the minor class and a lower one for the major class. The probability of the presence of one, two or more A/T pairs in each class of duplex was calculated (data not shown). The minor class was found to contain mainly four, five and sometimes six A/T base pairs, whereas less then four A/T base pairs were present in the major class.

The specificity of HU protein binding to dsDNA was studied. This complex was believed to be non-specific (25,26). The use of the generic microchip provides additional possibilities for finding the motifs in DNA sequences which may be preferential for protein binding. All measured ΔT_m values were used for a statistical analysis of specificity. Figure 5 shows average ΔT_m values for 8mers containing different trinucleotide and tetranucleotide motifs as well as deviations from the average values for different 6mer cores. First, we found that the 3 bp motifs **AAG** and **AGA** have the strongest shift in T_m in the major class of complexes (Fig. 5A). A 4 bp motif **AAG**A, which is a combination of **AAG** and **AGA**, has the highest ΔT_m. The deviations in ΔT_m are significant, thus indicating that the specificity of protein binding to dsDNA is rather low and only a statistical analysis of a large data set could reveal the preferential motifs. No motifs were identified for the minor class of HU complexes with double-stranded oligonucleotides, although the ΔT_m values were usually lower for duplexes with long A/T sequences. A similar method has already been used to analyze binding of Hoechst 33258 to DNA (20).

Analysis of the fluorescence intensity of HU–dsDNA complexes

The fourth technique used was to analyze the influence of HU protein on the fluorescence intensity of duplexes. The first observation is that the fluorescence signal of some duplexes formed on the generic microchip decreased markedly after HU binding. Figure 6 shows a plot of the fluorescence of microchip duplexes in the presence of HU protein against the signals obtained in its absence. It appears that HU protein only decreased fluorescence of A/T-rich duplexes, which are

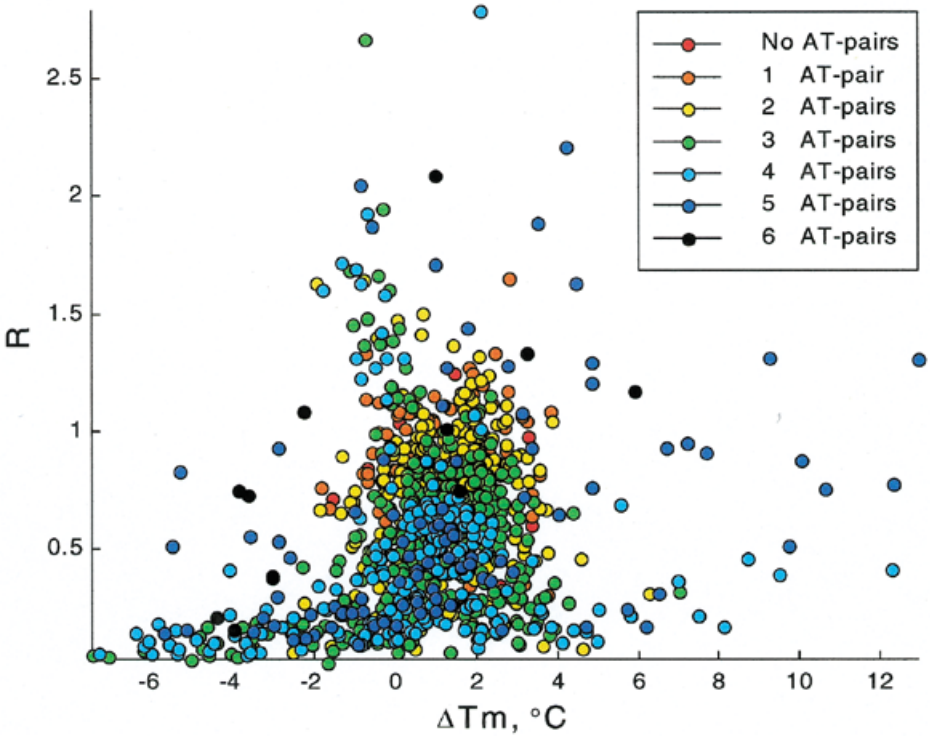


Figure 7. The effect of HU protein on fluorescence of the microchip dsDNA with different ΔT_m. R = fluorescence(+HU)/fluorescence(–HU).

presented in the left shoulder of the ΔT_m histogram in Figure 4, where ΔT_m is negative.

Figure 7 shows the correlation between ΔT_m and HU fluorescence decrease. One can see that only some A/T-rich duplexes with negative ΔT_m values exhibit a decrease in fluorescence upon HU binding. Most G/C-rich duplexes are characterized by a positive ΔT_m and their fluorescence is unaffected by the protein. Similarly, the negative effect of HU on fluorescence is higher for microchip duplexes with a lower melting temperature (not shown).

The data in Figures 6 and 7 indicate a direct correlation between A/T content, ΔT_m and T_m and a decrease in dsDNA fluorescence in the complex with HU protein.

DISCUSSION

A massive amount of data describing the interaction of HU protein with ssDNA and dsDNA was obtained with the 6mer generic microchip. Use of the microchip was an efficient approach to study the protein–DNA interaction. A multi-wavelength, large field fluorescence microscope equipped with CCD camera provides a number of significant advantages. The protein–DNA interactions can be studied in the reaction chamber in real time for all microchip gel pads at increasing temperature. The DNA and the protein are labeled with two different fluorescent dyes and are monitored at different wavelengths.

Several parameters of protein–DNA interactions were studied for oligonucleotides of different structures. Quantitative assessment of the binding of labeled HU to the microchip single-stranded oligonucleotides directly evaluates their affinity. The affinity is increased for oligonucleotides with a higher GC content; the motif GCGC was found as slightly preferential for protein binding. The thermostability of HU complexes with ssDNA also seems to reflect the sequence specificity of the protein. This conclusion is supported by the direct correlation between HU binding and the melting temperatures of the protein–oligonucleotide complexes. T_m is also higher for GC-rich oligonucleotides.

The HU interaction with dsDNA occurs at a higher ionic strength and thus appears to be stronger than that with ssDNA. This interaction is a more complex process and a more indirect method was therefore applied for its study. The effect of HU binding on melting temperature of the microchip duplexes was measured. Two kinds of complexes were identified that are characterized either by an increase in T_m and duplex stabilization or by a decrease in T_m and destabilization of duplexes.

The destabilized complexes are characterized by a higher A/T content and longer A/T stretches. However, no specific binding motifs were identified. Among the stabilized duplexes the trinucleotides AGA and GAA and tetranucleotide AAGA are slightly preferential motifs, which was only revealed by statistical analysis of a large amount of melting data.

Studies of the HU interaction with labeled dsDNA showed that the protein caused a decrease in fluorescence of the microchip duplexes, probably due to their partial dissociation. The decrease was found only for A/T-rich and not for A/T-depleted duplexes and directly correlated with their higher A/T content and lower T_m. These measurements were carried out in 100 mM NaCl, in which the HU affinity for ssDNA is significantly lower than for dsDNA. Therefore, the decrease in fluorescence of DNA duplexes caused by HU binding could not be explained by their melting due to preferential binding of HU to ssDNA. This unusual behavior may be related to the affinity of HU for DNA cruciforms and gaps in duplexes (19),

where single- and double-stranded regions are adjacent to each other.

All the approaches described here can be applied to studies of other nucleic acid–protein interactions. In some cases the length of the immobilized oligonucleotides should be extended above the 8mers of the present generic microchip to obtain greater specificity in the interaction.

It should be recalled that during the first characteristics studies of HU protein it was observed that this protein associated with the *E.coli* nucleoid and could bind equally well to dsDNA and ssDNA (12). To document the HU–DNA interaction some studies of the effect of HU protein on thermal denaturation of λDNA have also been performed (27). These studies showed that melting of certain AT-rich portions of λDNA occurred first. It is very reassuring that the new and much more powerful technology of microchip analysis can confirm, and detail, these preliminary data performed a long time ago with more time consuming techniques.

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