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Label free analysis of transcription factors using microcantilever arrays

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Abstract

We report the measurement of protein interaction with double-stranded DNA oligonucleotides using cantilever microarray technology. We investigated two different DNA-binding proteins, the transcription factors SP1 and NF-κB, using cantilever arrays as they allow label-free measurement of different biomolecular interactions in parallel. Double-stranded DNA oligonucleotides containing a specific binding site for a transcription factor were sensitized on gold-coated cantilevers. The binding of the transcription factor creates a surface stress, resulting in a bending of the cantilevers. Both transcription factors could be detected independently at concentrations of 80–100 nM. A concentration dependence of the bending signal was measured using concentrations from 100 to 400 nM of NF-κB. The experiments show that the recognition sequence of one transcription factor can serve as a reference for the other, highlighting the sequence specificity of transcription factor binding.

Keywords: Transcription factor; SP1; NF-κB; Cantilever sensor; Proteomics; DNA binding protein

1. Introduction

Cells respond to changes in their environment by regulating gene expression. In recent years, several groups have developed technologies, such as DNA microarrays, 2D electrophoresis of proteins or enzyme-linked immunosorbent assays (ELISAs) to analyze gene expression at the genomic and proteomic level (Honoré et al., 2004). Here, we propose to investigate gene regulation not only at the mRNA or protein level but also at the level of transcription factors using microcantilever array technology. While DNA microarrays have also been used very recently to analyze DNA binding proteins (Mukherjee et al., 2004) they have the disadvantage that labeled DNA binding proteins are required. In the case of microarrays, the proteins have to be cloned and expressed with an epitope tag, which is then recognized by a fluorescently labeled antibody. Especially the cloning steps make it difficult to analyze a total set of transcription factors from a cell. Surface plasmon resonance (SPR) is another method used to investigate DNA binding proteins (Linnell et al., 2004); but SPR can not be scaled up in number of simultaneous channels that easily. In contrast microcantilevers do not suffer from these problems, because they do not require cloning or labeling and can be easily scaled up. Recently microcantilever arrays (Fig. 1A) were used to investigate DNA hybridization (Fritz et al., 2000; McKendry et al., 2002) and antibody–antigen interaction (Wu et al., 2001; Arntz et al., 2003). For this purpose, microfabricated silicon cantilevers are coated on one side with gold to allow sensitization of the surface with thiol-modified receptor molecules, i.e. DNA oligonucleotides or antibodies. A surface stress is generated upon biomolecular recognition of a ligand in solution, resulting in a bending of the microcantilever due to steric hindrance and electrostatic effects (McKendry et al., 2002). The bending is detected by laser beam deflection (Fig. 1B).

Transcription factors, such as NF-κB or SP1, play an important role in either the initiation or regulation of gene expression by binding to sequence-specific sites in the promoter region of genes. Mutations in these binding sites have

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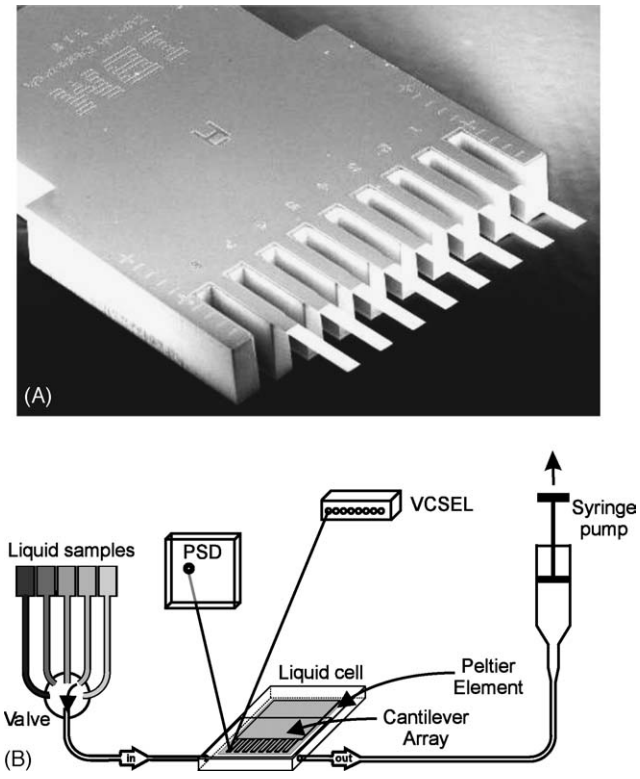

Fig. 1. (A) SEM image of a microfabricated cantilever array. A cantilever is 500 μm long, 100 μm wide and 500 nm thick. The pitch distance from the middle of one cantilever to the middle of the next is 250 μm. The cantilevers are coated with 2 nm of Ti and 20 nm of Au on top. The Au layer serves as a substrate to functionalize the cantilevers with thiol-modified oligonucleotides on one side. (B) The cantilever array is placed inside a liquid filled cell having a volume of 50 μl. Different samples can be injected using a syringe pump and a five-fold valve. A Peltier element placed below the liquid cell serves to assess the thermal and mechanical properties of the cantilevers. Cantilever bending is detected by laser beam deflection using a time-multiplexed vertical-cavity surface-emitting laser (VCSEL) array and a position-sensitive detector (PSD).

been shown to contribute to diseases, for example, in the case of the SP1 binding site in the collagen1A gene to changes in bone mass and subsequent osteoporotic fracture (Mirandola et al., 2002). Within the NF-κB binding site of the tumor necrosis factor (TNF) gene, mutations may possibly affect inflammatory responses to viral infections (Udalova et al., 2000). The activity of transcription factors is regulated through covalent modifications, like phosphorylation, or mutations in regulatory sites of the factor (Day et al., 2002) and therefore can also contribute to disease (Nakshatri et al., 1997; Wang et al., 1999; Scaife et al., 2002). Sometimes transcription factor binding sites overlap (Ponticos et al., 2004), so that a disruption of the site can result in the failure of multiple transcription factors to bind and hence affect gene regulation.

Conventional methods to analyze transcription factor binding require radioactive labeling of probes as in electromobility shift assays (EMSA) and are time consuming. More recently developed methods like DNA microarrays (Mukherjee et al., 2004) require cloning and labeling, or SPR (Linnell et al., 2004) which is difficult to scale up. Using an array of silicon microcantilevers sensitized with fragments of different promoter regions allows faster, label-free interaction measurements of transcription factors, and does not require cloning. Furthermore scaling up the number of microcantilevers on an array is relatively easy. Arrays with 4096 cantilevers have already been fabricated using standard silicon micromachining techniques (Pantazi et al., 2004). Moreover, multiple transcription factors can be analyzed in a single experiment. We chose NF-κB and SP1 in our study because of their important role in controlling inflammatory and immune responses as well as cell proliferation and development. For transcription factors to control cell behavior they get activated by complex signaling pathways in the cytosol. The signaling pathways themselves are triggered by, e.g. hormones which bind to receptors on the cell surface. The signal is then transferred to the transcription factor by a complex cascade of proteins and only then is the transcription factor activated and able to migrate from the cytosol to the nucleus to bind to its recognition site. NF-κB is a member of a large family of transcription factors known as the NF-κB/rel family proteins. The family consists among others of the proteins p50 and p65 (Hayden and Ghosh, 2004) and is regulated by IκB (Baldwin, 1996). They contain a 300-amino-acid-long domain at the N-terminus known as the rel homology domain (RHD), which is involved in dimerization and DNA binding (Ghosh et al., 1998). The two proteins can form p50/p50 homodimers as well as p50/p65 heterodimers. In our study, we investigated the p50/p50 homodimer, which recognizes the sequence GGGRNNYYCC (Sif and Gilmore, 1993), where G stands for guanine, C for cytosine, R for either adenine or guanine, Y for either thymine or cytosine, and N for any of the four bases. The second transcription factor, SP1, is a member of the zinc-finger family of transcription factors (Kriwacki et al., 1992). Unlike NF-κB, SP1 is in general present in all cells (Black et al., 2001) and recognizes the sequence KRGGCGKRRY (Briggs et al., 1986), where K stands for either guanine or thymine. This ambiguity in the recognition sequence can result in different recognition sites which all bind the same transcription factor albeit with a different affinity (Villard, 2004). It has been shown that these alterations in a binding site can result in different expression levels of mRNA of a subunit of the high-affinity IgE receptor (Nishiyama et al., 2004) which is involved in IgE-mediated allergic reactions.

2. Materials and methods

2.1. Reagents

Recombinant human NF-κB (rhNF-κB) and recombinant human SP1 (rhSP1) were purchased from Promega/Catalyse (Wallisellen, Switzerland). MicroSpin™G-25 columns were from Amersham Biosciences (Otelfingen, Switzerland).

HPLC-grade water was purchased from Fluka (Buchs, Switzerland). Oligonucleotides 5′-functionalized by a hexyl spacer with a thiol group were obtained from Microsynth GmbH (Balgach, Switzerland). They had the following sequences for NF-κB binding (consensus binding sequence is underlined): 5′-GAC AGT TGA GGG GAC TTT CCC AGG CAA AAA GCC TGG GAA AGT CCC CTC AAC TGT C-3′; SP1 binding oligonucleotide: 5′-GAC ATT CGA TCG GGG CGG GGC GAG CAA AAA GCT CGC CCC GCC CCG ATC GAA TGT C-3′. Oligonucleotides were delivered at a concentration of 100 μM in water containing 100 μM DTT (dithiothreitol) to protect their thiol group.

2.2. Cantilever preparation

A microfabricated array of eight identical silicon cantilevers (Fig. 1A) with 250 μm pitch, a length of 500 μm, a thickness of 500 nm and a spring constant of 0.0025 N/m were provided by the Micro-and Nanomechanics group at the IBM Zurich Research Laboratory. The cantilevers were prepared as described in detail elsewhere (Fritz et al., 2000; McKendry et al., 2002), except Fritz et al. (2000) and McKendry et al. (2002) used 1 μm thick cantilevers in their experiments with a spring constant of 0.02 N/m. In our experiments we use thinner cantilevers for increased sensitivity. Briefly, oligonucleotides were annealed to obtain the hairpin loop structure by heating them to 95 °C and letting them cool slowly to 30 °C. Then they were extracted twice with ethyl acetate (SDS, Peypin, France) to remove DTT, dried and redissolved at a concentration of 40 μM in 50 mM TEAA buffer (triethyl ammonium acetate, Fluka, Buchs, Switzerland). Eight microcapillaries (o.d. 250 μm; i.d. 150 μm; from Garner Glass, Claremont, CA) were filled with oligonucleotide solution in an alternating pattern, thereby functionalizing the cantilevers either with a sensing layer or a reference layer of double-stranded oligonucleotides. Afterwards the cantilever array was washed in 50 mM TEAA.

2.3. Instrument

The functionalized cantilever array is inserted into a liquid chamber (volume: 50 μl) and mounted at an angle of 11° with respect to the incident laser beam (time-multiplexed vertical-cavity surface-emitting laser (VCSEL); wavelength 760 nm, Avalon Photonics, Zurich, Switzerland). The laser beam is redirected by a mirror to a PSD (position-sensitive detector (PSD), SiTek, Partille, Sweden). Data are acquired using a multifunctional data-acquisition board (National Instruments, Austin, TX) driven by LabView software. The software also controls the liquid-handling system of the setup, the syringe pump (GENIE, Kent Scientific Corp, Torrington, CT), and a 10-position valve system (Rheodyne, Rohnert Park, CA). The entire setup is placed inside a temperature-controlled box (Intertronic; Interdiscount, Switzerland), which is temperature-equilibrated through a fuzzy logic controller by the LabView software at ± 0.02 °C accuracy.

2.4. Binding experiment

First, we had to remove the DTT from preparations of rhSP1 and rhNF-κB, because of competition with the thiol-modified oligonucleotides for binding to the gold surface of the cantilevers. For this purpose, we used three Microspin G-25 columns (Amersham Biosciences) to extract the protein preparations sequentially and remove the DTT. The columns were washed three times with binding buffer either for SP1 (20 mM HEPES pH 7.5, 0.1% Tween 20, 5 μM $ZnSO_4$, 6 mM $MgCl_2$, 50 mM KCl, 50 mM NaCl) or NF-κB (10 mM HEPES pH 7.9, 50 mM KCl, 0.05% NP40, 10% Glycerol) and stored overnight at 4 °C. Twelve hours later proteins were diluted accordingly in binding buffer and passed three times through the pre-equilibrated columns as described by the manufacturer, to remove the DTT. The volume of the eluate was adjusted to 200 μl with the appropriate binding buffer. Binding experiments were conducted at 23 °C; for this purpose the environmental box was equilibrated for 2 h. After that the cantilever array was washed three times at a flow rate of 25 μl/min with a total volume of 600 μl binding buffer or until the baseline was stable. Then 200 μl of protein at the desired concentration was injected at 25 μl/min.

2.5. Data analysis

Bimetallic response and mechanical properties of the cantilevers were assessed by applying 2 V to a Peltier element situated directly below the chamber for 30 s (thermal cycle). This resulted in a 1 °C pulse for deflection calibration. The cantilever with the highest response was used to normalize the response of the others provided the responses did not differ by more than 10% of magnitude. Normalized data from reference cantilevers were subtracted from the data of the sensing cantilevers to obtain a differential signal. A baseline correction, needed because the drift behavior of different cantilevers varies slightly, was applied, using a linear fit of data in the time interval between the start of the experiment and the injection of the transcription factor. The slope of the linear fit then was subtracted from all differential signals.

3. Results and discussion

Transcription factors recognize binding sites on double-stranded DNA (dsDNA). Therefore, we sensitized the cantilever surface with thiol-modified 55-mer oligonucleotides forming hairpin loops. The structure of the hairpin loop provides a 25-base-pair-long dsDNA stem containing the recognition site for the transcription factor. Furthermore the two self-complementary strands are connected by a 5-base-long loop (A_5), which prevents loss of the second strand due to dehybridization (Fig. 2). We measure differential signals

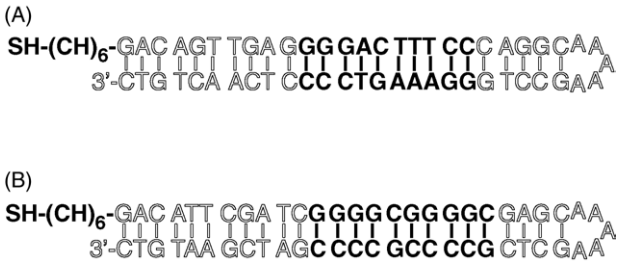

Fig. 2. Schematic representation of the oligonucleotides containing the recognition sites for the transcription factors NF-κB (A) and SP1 (B). Shown in black are the thiol groups with their $(CH)_6$ linkers and the binding sites recognized by the transcription factors. The lines between the two strands indicate base pairing. DNA sequences that do not serve transcription factor binding and the A_5 loop at the end are shown as letters with contours.

to cancel out signals that occur because of changes in the refractive index and nonspecific interactions with the cantilevers. For this purpose we use the DNA-binding sequence of one transcription factor, e.g. of NF-κB, as reference for the other transcription factor, SP1, and vice versa. Binding conditions can vary significantly, depending on which transcription factor is analyzed and which method is used. For example, various additives, such as ions, BSA or detergents, are added to the binding buffer to reduce nonspecific adsorption or prevent denaturation of the proteins investigated. Furthermore, SP1 requires Zn^{2+}, which could affect the cantilevers (Xu et al., 2002). Therefore, we had to investigate the binding buffer used during the experiments (Fig. 3A and B). One important part was to eliminate DTT from the binding buffers. DTT is a common compound used in protein chemistry to prevent aggregation of proteins due to cross-linking of free thiol groups by oxidation (Cleland, 1964). Because DTT contains two thiol groups itself, it could interfere with the Au layer on the cantilever surface, thereby disrupting the functionalization with the thiol-modified oligonucleotides. Moreover, essential ions, such as Zn^{2+} in the case of the zinc-finger-containing-transcription factor SP1, have to be considered, as these ions could influence the cantilevers. Several investigations indicate that metal ions can influence gold thin films: Gold coated cantilevers can be used to measure the presence of metal ions (Xu et al., 2002); adsorption of metals and metal ions can change the surface resistance of gold coated electrochemical sensors (Glück et al., 1999; Tucceri, 2004). Therefore, initial experiments were conducted with a basic HEPES pH 7.5 buffer as shown in the grey curve of Fig. 3A. But this buffer did not allow binding of SP1 to its target sequence. In subsequent experiments buffer conditions were changed until a buffer was found that allowed SP1 to bind. For SP1, it turns out that the optimal conditions are 20 mM HEPES pH 7.5, 0.1% Tween 20, 5 μM $ZnSO_4$, 6 mM $MgCl_2$, 50 mM KCl, 50 mM NaCl as indicated by the black curve in Fig. 3A. Initial SP1 buffer was also used to investigate NF-κB binding, because in future experiments where multiple transcription factors are analyzed in one experiment, it would be feasible to have a common buffer for all factors. But the

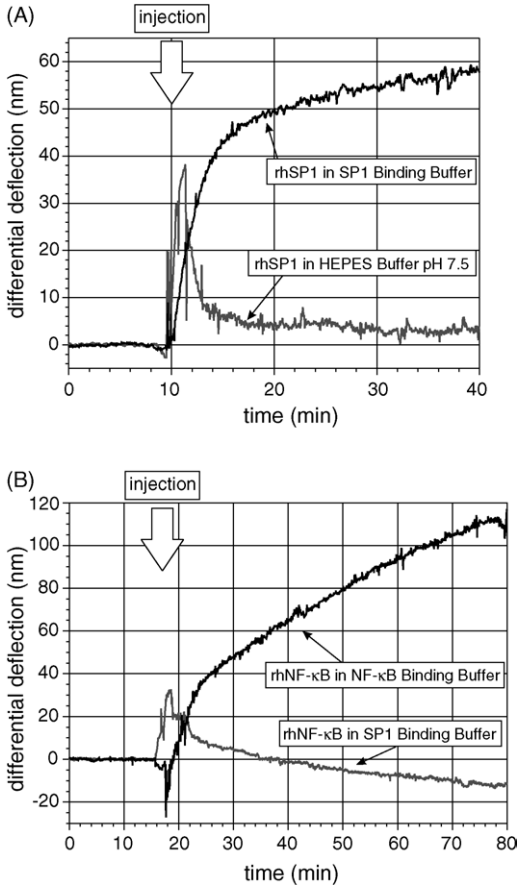

Fig. 3. Investigation of buffer conditions for binding the transcription factor rhSP1. (A) 200 μl 80 nM of rhSP1 was injected. Initially very simple buffer conditions were chosen (20 mM HEPES, pH 7.5). In subsequent experiments, we found that optimized conditions promote binding of rhSP1 (SP1-binding buffer: 20 mM HEPES pH 7.5, 0.1% Tween 20, 5 μM $ZnSO_4$, 6 mM $MgCl_2$, 50 mM KCl, 50 mM NaCl). Optimizing binding conditions for rhNF-κB. (B) 200 μl 400 nM rhNF-κB was injected. rhNF-κB required a different buffer (NF-κB-binding buffer: 10 mM HEPES pH 7.9, 50 mM KCl, 0.05% NP40, 10% glycerol).

SP1 buffer conditions turned out to be unsuitable for NF-κB binding as revealed in the grey curve of Fig. 3B. NF-κB requires different conditions, as shown in the black curve of Fig. 3B (10 mM HEPES pH 7.9, 50 mM KCl, 0.05% NP40, 10% Glycerol). The addition of detergent may well stabilize the proteins and reduce the nonspecific adsorption to the cantilevers (Wilkins-Stevens et al., 1995). The microcantilever method is an excellent tool to optimize buffer conditions for binding processes on surfaces.

Fig. 4A shows signals from the injection of 100 nM rhSP1. A deflection of 300–450 nm is observed for all cantilevers, even for the reference cantilevers possibly because of changes in the refractive index. Only the differential signals (Fig. 4B) indicate an actual bending of 54.0 ± 10.4 nm due to transcription factor binding. This demonstrates the importance of the differential signal in cantilever measurements, as also pointed out in previous work (Arntz et al., 2003; McKendry et al., 2002). The positive differential signal means by conven-

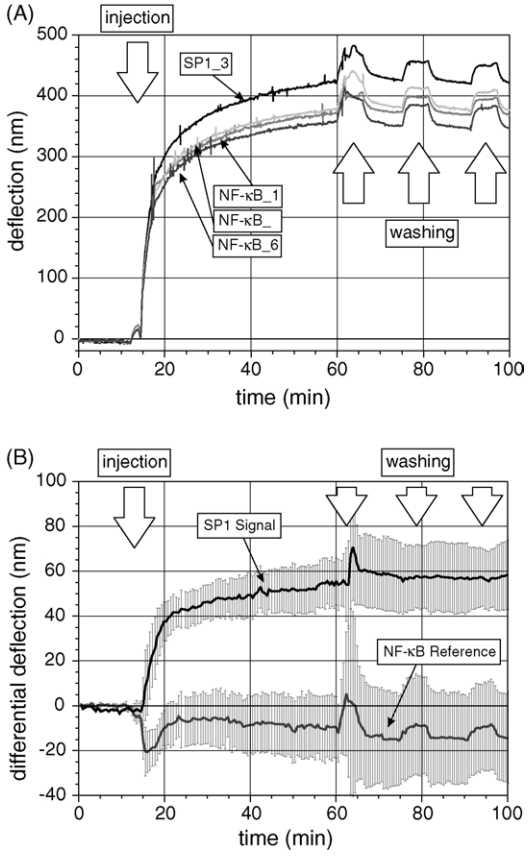

Fig. 4. Representation of raw data from an experiment in which 80 nM of rhSP1 is injected. (A) The bending curves of cantilever 3 (SP1_3), functionalized with SP1-binding oligonucleotides and three reference cantilevers (NF-κB_1, NF-κB_4, and NF-κB_6) functionalized with NF-κB-binding oligonucleotides. For clarity only four representative curves are displayed. Other cantilevers of the same array exhibited the same behavior. Comparison of averages and standard deviations of 3 SP1 signals with 3 NF-κB references. (B) Average differential SP1 signal obtained from differences in the responses of cantilever 3 and of cantilever 1 (SP1_3–NF-κB_1), cantilever 4 (SP1_3–NF-κB_4), and cantilever 6 (SP1_3–NF-κB_6). Average differential NF-κB reference signal calculated from differences in the responses of cantilever 4 and of cantilever 1 (NF-κB_4-NF-κB_1), cantilever 6 (NF-κB_4-NF-κB_6), cantilevers 6 and 1 (NF-κB_6-NF-κB_1).

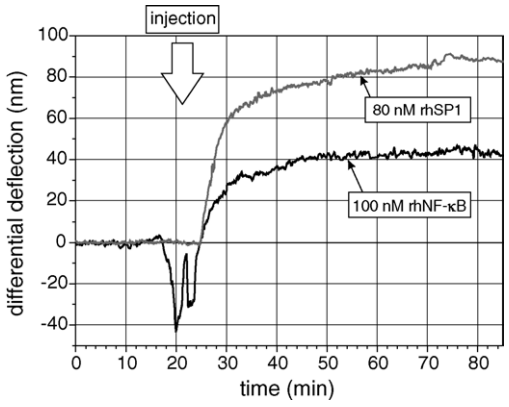

Fig. 5. Comparison of rhNF-κB with rhSP1. Differential signal of experiments conducted by injection of 100 nM rhNF-κB or 80 nM rhSP1 to two different cantilever arrays. For rhNF-κB, cantilevers functionalized with SP1-binding oligonucleotides were used as reference. Cantilevers functionalized with the rhNF-κB-binding oligonucleotides served as references when rhSP1 was injected.

tion compressive stress. Peaks during injection of the protein originate from liquid flow over the cantilevers. In Fig. 5, we demonstrate that the binding site for SP1 can serve as reference for NF-κB and vice versa. In these experiments, the differential responses shown, for example for NF-κB binding, were obtained by subtracting the signal of a reference cantilever functionalized with the SP1-binding oligonucleotide from the signal of the NF-κB oligonucleotide-functionalized cantilever. This evidences the specificity of transcription factor binding. Furthermore the experiments indicate that the surface stress created by the binding of the transcription factors is of more complex origin than a steric model (Lang et al., 2002; McKendry et al., 2002) suggests. In this model, mainly steric hindrance is the cause of the compressive stress that is bending the cantilever. First the molecular weight of the rhNF-κB is about 105 kD, whereas the molecular weight of rhSP1 is about 85 kD. If we assume a similar density of the two proteins (Quillin and Matthews, 2000) they will have a comparable size. The two proteins have to penetrate the double-stranded oligonucleotide layer to bind to their recognition sequences and should thereby exert a similar steric hindrance. This should result in a similar surface stress for NF-κB and for SP1 at the same protein concentration, but not a two fold difference. SP1 exhibits a differential bending signal of 90 nm, whereas NF-κB has one of 45 nm. Second, McKendry et al. (2002) use oligonucleotides in their studies which are chemically much simpler than complex proteins which consist of many different amino acids. One explanation for the two fold discrepancy in the differential bending signal could be that NF-κB and SP1 have a different influence on the electrostatic charges of the phosphate backbone. It was reported (Strauss and Maher, 1994) that the shielding of the electrostatic charges of the phosphate backbone by DNA-binding proteins can result in the bending of DNA which would produce an additional strain. Considering the neutralizing effects DNA-binding proteins have on electrical charges of the phosphate backbone, we can assume that the electric repulsion between the DNA molecules on the cantilever is reduced. However, reducing the electrostatic repulsion on the surface of the cantilever is equivalent to a tensile stress, whereas the binding of proteins due to steric effects creates a compressive stress. Therefore, the shielding of the electrostatic charges of the phosphate backbone can reduce the bending signal of the cantilever that would result from steric effects. From these observations it seems that NF-κB is able to screen the electrical charges of the phosphate backbone more efficiently than SP1 can.

Fig. 6 shows the dependence of the differential deflection on the injected rhNF-κB concentration with a deflection of 45 nm at 100 nM, of 70 nm at 200 nM, and of 130 nm at

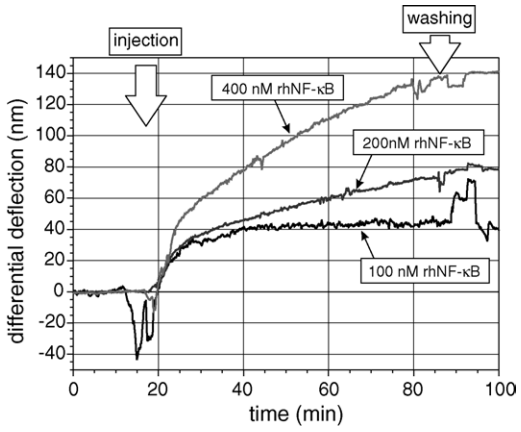

Fig. 6. Differential signals obtained by injection of three different rhNF-κB concentrations (100, 200 and 400 nM).

400 nM. The binding of NF-κB to its target sequence cannot be reversed by dilution with binding buffer. Even after a 20-fold exchange of the solution in the chamber (50 μl total volume), we were not able to wash off the bound protein, as indicated by a decrease in the differential signal. From Fig. 4B it can also be seen that SP1 cannot be washed off either. These observations indicate that the transcription factors have low dissociation constants. Indeed, the K_D of NF-κB (p50/p50 homodimer) as measured with surface plasmon resonance is 8×10^{-12} molar (Lumley et al., 2004) and that of SP1 is 3×10^{-10} molar, as measured in gel retardation assays. The high density of the oligonucleotides present on the cantilever surface and the low dissociation constants of the transcription factors are conclusive explanations for these observations.

4. Conclusions

This work demonstrates the feasibility of micromechanical cantilever sensors for investigating transcription factors. We were able to show that no labeling of the DNA probe is required, that different concentrations can be measured, and that in future applications many more than two transcription factors can be measured in parallel. Also, the integration with other applications, such as DNA sensing (Fritz et al., 2000; McKendry et al., 2002), investigation of antibody–antigen interactions (Arntz et al., 2003), and detection of bacterial growth (Gfeller et al., 2005) on a single cantilever array could result in a sensor that is capable of detecting multiple biomarkers simultaneously. This will allow us to investigate various disease stages that are characterized by the regulation and expression of different genes. The progression of cancer, for example, is characterized by the expression of different genes in early stages of the disease than in later stages (Diamandis, 2003). The ability to distinguish these stages characterized by different gene expression patterns originating at different levels of organism regulation with a combined device will help in the treatment of cancer. So far, we were only exploiting static mode measurements for transcription factor analysis, but in the future we are planning to use dynamic mode measurements. In the dynamic mode absolute mass changes can be measured due to changes in the eigenfrequency upon binding of proteins to the cantilever (Braun et al., 2005). Additionally, this should enable us to investigate quantitative aspects of transcription factors, like kinetic behavior during binding. Initial calculations indicate that about 100 pg of protein can be bound to the cantilever surface which is well in the sensitivity range of dynamic mode measurements. Either in static or in dynamic mode, micromechanical cantilevers will allow us in the future to investigate a multitude of biomarkers without amplification or labeling of the samples, which will reduce the artifacts introduced by these procedures and result in more accurate results.

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