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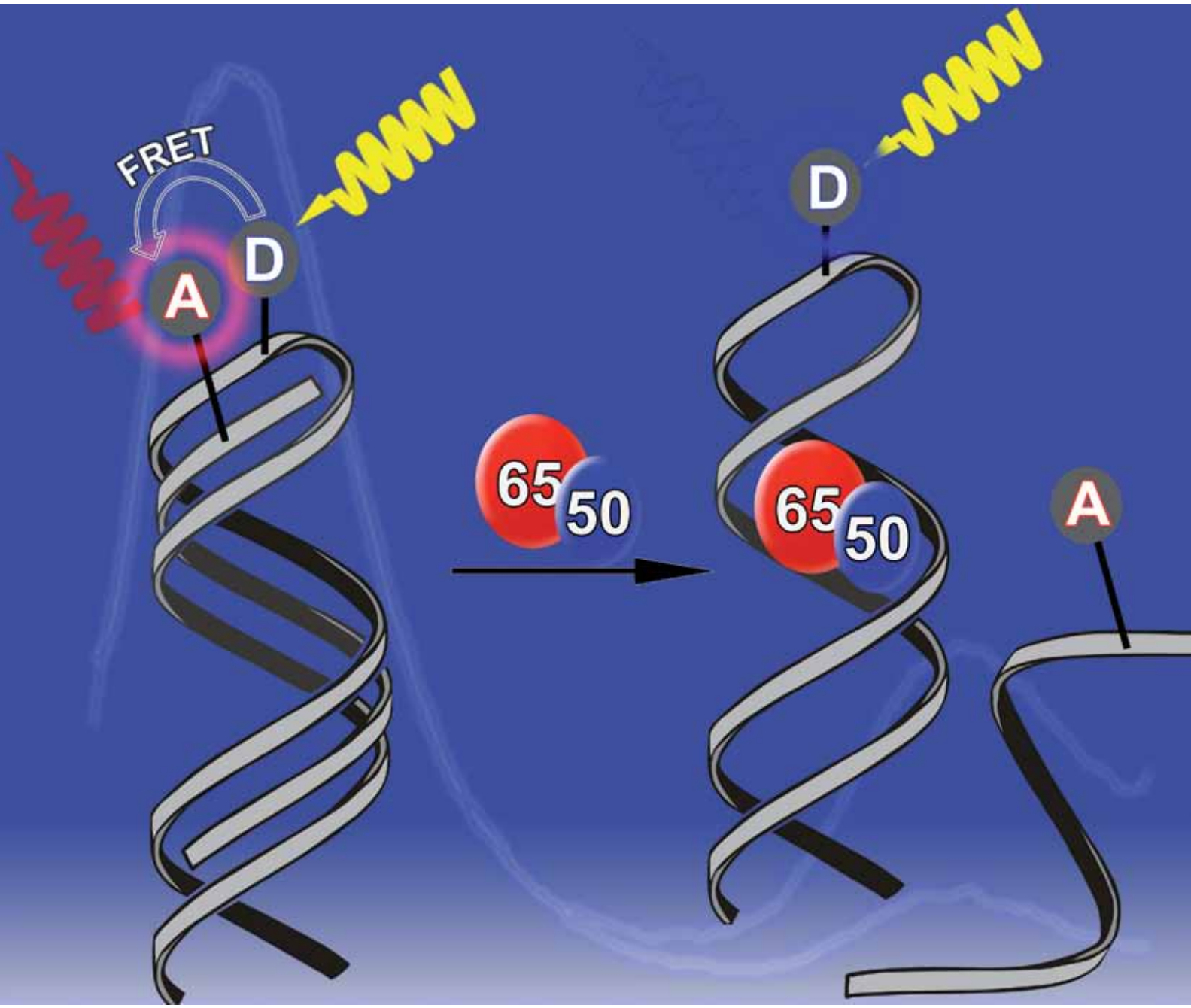

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FULL PAPER

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A new assay format for NF-κB based on a DNA triple helix and a fluorescence resonance energy transfer

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Herein we report a feasibility study for a new concept to detect DNA binding protein NF-κB based on a DNA triple helix formation in combination with a fluorescence resonance energy transfer (FRET). The new principle avoids expensive antibodies and radioactivity and might have implications for assays of other DNA binding proteins.

Introduction

The transcription factor nuclear factor κB (NF-κB) is a dimeric protein whose monomers belong to the Rel/NF-κB protein family. So far, five mammalian NF-κB proteins named p65 (RelA), cRel, RelB, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100) have been described. The heterodimer p65/p50 is the most common one. In resting cells, NF-κB heterodimers are retained in the cytoplasm by IκBs, specific inhibitors that mask their nuclear localisation sequences and prevent nuclear transport and thereby DNA binding. Stimulation by cytokines, such as IL-1β or TNF-α, viral or bacterial pathogens leads to phosphorylation-induced ubiquitination of the IκB proteins which are then degraded by the 26S proteasome.[1] The liberated NF-κB dimers translocate to the nucleus where they are involved in transcriptional activation of more than 300 genes including those encoding cytokines, adhesion molecules, acute phase proteins, regulators of apoptosis and inducible effector enzymes.[2] These multiple target genes underline the pivotal role of NF-κB in the innate and adaptive human immune system. Consequently, its abnormal activation and deregulation leads to various diseases including those related to inflammation and enhanced cellular proliferation.[2,3] Additionally, an important role of NF-κB in induction of inducible chemoresistence is discussed.[4] Therefore, NF-κB has become a prime target for intense drug discovery and drug development.[5] Hence, there is a need for robust methods allowing for the rapid, cheap and quantitative detection of NF-κB DNA-binding activities.

The most common assay is the electrophoretic mobility shift assay (EMSA) detecting slower migration of protein-DNA complexes relative to free DNA molecules.[6] This assay is very sensitive, but rather time consuming. Furthermore, quantifications are difficult and radioactive material is needed. Renard *et al.* established a DNA binding assay on the basis of a modified enzyme-linked immunosorbent assay (ELISA) in which the NF-κB-dependent gene expression is studied using either luciferase or β-galactosidase as reporters.[7,8] Recently, a fluorescence resonance energy transfer (FRET) was used to study protein-DNA interactions.[9–12] The pertinent assay combines an exonuclease III (ExoIII) protection strategy with FRET detection or the SYBR® Green I staining method.[13] In this assay the NF-κB protein protects the probe from ExoIII digestion, which results in a high FRET signal. In another assay the activated NF-κB was detected by FRET following a restriction endonuclease digestion.[14] These FRET assays can be adapted to high-throughput screening formats, but are based on the expensive SYBR® Green I or an additional enzyme, respectively.

One of the DNA sequences to which NF-κB has a high binding affinity is the gene for the inducible nitric oxide synthase (iNOS).[15] In this gene the binding site for NF-κB (bold) overlaps with a 14 mer homopyrimidine sequence (italic), as outlined in Fig. 1. Homopyrimidine sequences are prone to Hoogsteen type triple helix hybridizations leading either to TAT or C^+GC motifs, respectively (Fig. 2).[16]

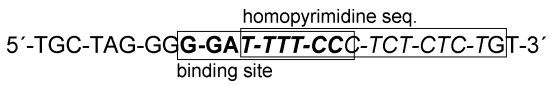

Fig. 1 Relevant stretch of the iNOS DNA sequence.

Our idea for a new NF-κB assay format is depicted in Fig. 3. A backfolding DNA sequence containing the double-stranded binding site for NF-κB and the homopyrimidine sequence for the triple helix formation is equipped with a FRET donor, whereas the third oligonucleotide involved in Hoogsteen pairing to the described backfolding DNA carries a FRET acceptor. In this arrangement, the donor and the acceptor chromophore are in close proximity which should yield in an intense FRET. Binding of NF-κB will replace the third oligonucleotide strand resulting in a decreased FRET.

As suitable donor and acceptor entities we had envisaged the carbostyril **1** and the Ru(II)bathophenanthroline complex **2**, respectively (Fig. 4). We have already successfully used both dyes in the past to establish efficient FRET systems in DNA.[17]

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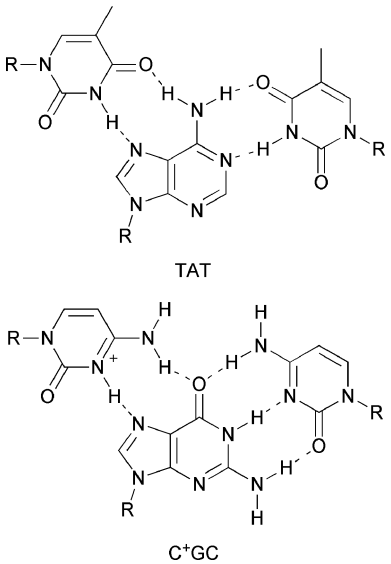

Fig. 2 (a) Hoogsteen TAT triad. (b) Hoogsteen C$^+$GC triad.

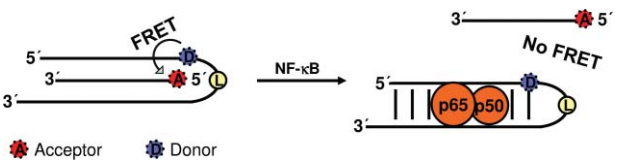

Fig. 3 Principle of FRET Assay for NF-κB. D: FRET-donor, A: FRET-acceptor, L: Hexaethyleneglycol spacer, p65/p50: NF-κB heterodimer.

Results and discussion

Oligonucleotide synthesis

In order to achieve the replacement of the third oligonucleotide by binding of NF-κB the stability of the triple helix had to be tuned appropriately.

Since the stability of triple helix formations is dependent on several factors like pH, length and salt concentrations, we have carried out a careful evaluation of these parameters.[18–21] Based on the iNOS sequence we have synthesized the backfolding DNA (bfDNA) sequences **3** and **4** as well as the oligonucleotides **5–7** (Fig. 5). All sequences were purified by RP-HPLC and their purity was checked by PAGE.

In order to increase the stability of the double helix, the two iNOS-derived strands were connected *via* a hexaethyleneglycol spacer to form a backfolding DNA which allows for optimal hybridization[22] and also leads to an increase of the stability of the triple helix at the same time.[23] Since C$^+$GC-base pairs of the Hoogsteen type require slightly acidic conditions to protonate the cytosine, the pertinent melting experiments of triple helices **8–13** were carried out at pH 6. The obtained melting curves are depicted in Fig. 6.

The results revealed the expected dependency of the melting of the third strand on its chain length. Melting temperatures slightly above 40 °C were obtained with both backfolding sequences and the 14 mer sequence **5**. Hence, it can be assumed that such triple helixes should be sufficiently stable at r.t. to establish the new assay principle. In a further set of studies we investigated the dependency of the most stable triplexes on the salt concentrations.

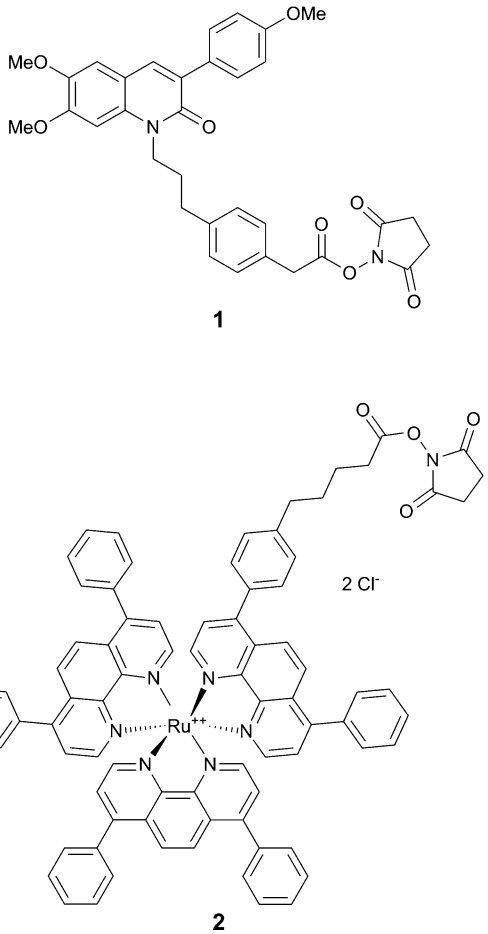

Fig. 4 Envisaged dyes for the FRET system (activated as hydroxysuccinimide esters).

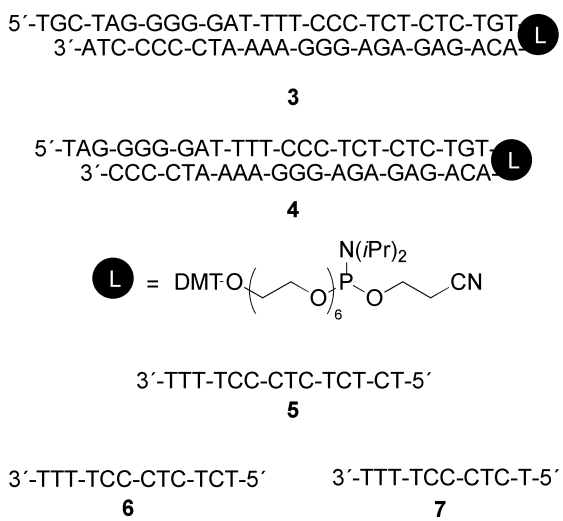

Fig. 5 Synthesized oligonucleotides for the evaluation of the melting curves.

Table 1 Melting points for Hoogsteen base pairs of triple helix **8** and **11** were recorded in a sodium acetate buffer (100 mM) at pH 6 with the following salt concentrations:

Salt concentration (mM)	*Mp of* **8** *(°C)*	*Mp of* **11** *(°C)*
No salt	*40.1*	*36.3*
150	*40.6*	*37.5*
450	*43.6*	*40.5*
1000	*48.5*	*45.1*

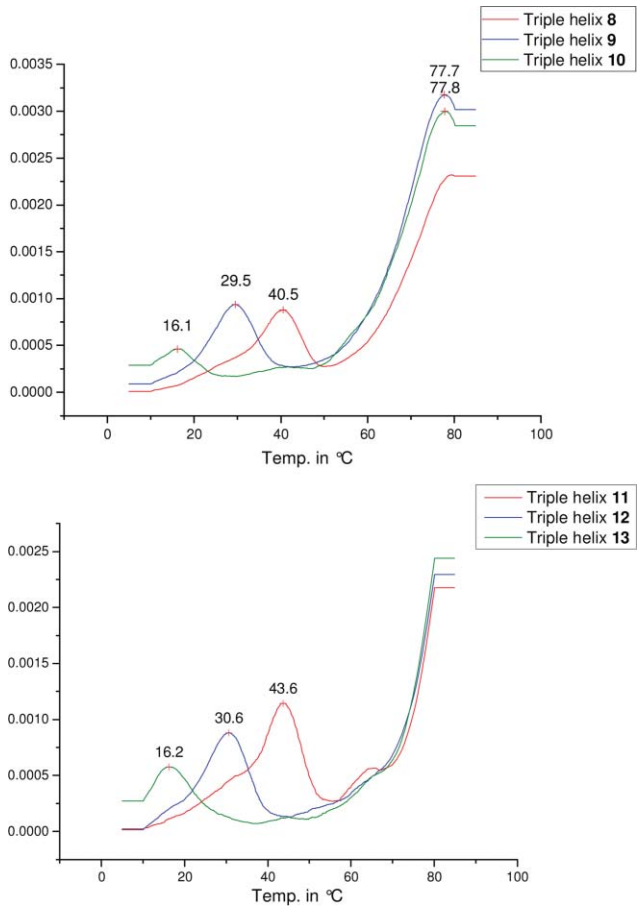

Fig. 6 Melting curves of the triple helices **8**–**13**. Conditions: Triple helix **8**: bfDNA **3** with homopyrimidine sequence **5**; triple helix **9**: bfDNA **3** with homopyrimidine sequence **6**; triple helix **10**: bfDNA **3** with homopyrimidine sequence **7**; triple helix **11**: bfDNA **4** with homopyrimidine sequence **5**; triple helix **12**: bfDNA **4** with homopyrimidine sequence **6**; triple helix **13**: bfDNA **4** with homopyrimidine sequence **7**; NaCl concentration: 450 mM, D: 1st derivative of absorption; measured in a sodium acetate buffer (100 mM) at pH 6.

These results indicated that the modification of the salt concentrations offers a further possibility to manipulate the stability of the triple helix.

Concurrent with the melting experiments, we performed binding studies of NF-κB with the bfDNAs **3** and **4** using Jurkat T cells, an acute T-cell leukaemia cell line. Cells were stimulated with TNF-α for one hour. Total protein extracts were prepared and analyzed for NF-κB DNA binding in an EMSA. Stimulation with TNF-α induced one novel DNA binding activity. Competition assays identified this complex as an NF-κB complex, which was more pronounced with bfDNA **4** than with **3** (Fig. 7). Position of the

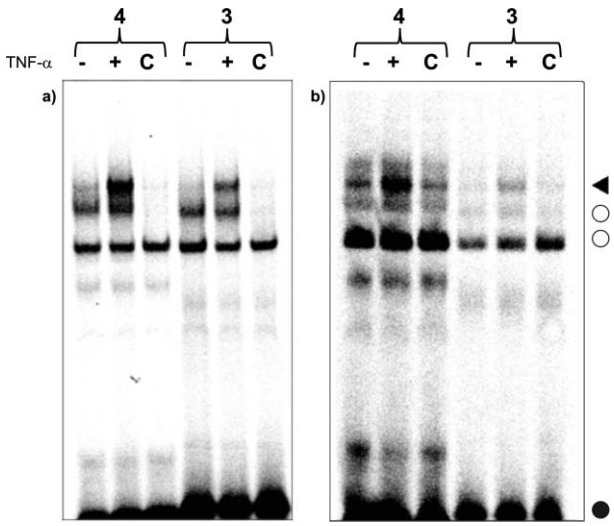

Fig. 7 NF-κB binding to backfolding DNA (**3** and **4**) in Jurkat cells; (a) at pH 7 (b) at pH 6. Lanes 1 and 4 show unstimulated control cells; lane 2 and 5, cells treated with 200 U/ml TNF-α for 1 h using either bfDNA **3** or **4**. Equal amounts of protein from cell extracts were analyzed for NF-κB activity by EMSA. Results from the competition assay are shown in lanes 3 and 6. ◀ indicates the position of NF-κB DNA complexes. ○ denotes a non-specific activity binding to the probe and ● shows unbound oligonucleotide.

NF-κB DNA complex was further verified by the addition of recombinant p50/p65 (data not shown).

The promising outcome of the binding studies of NF-κB to the backfolding DNA **4** and the melting experiments prompted us to synthesize the oligonucleotides **14** and **15** carrying the FRET donor and the FRET acceptor, respectively. In sequence **15** the Ru-complex as acceptor is placed at the 5′-end to minimize interference with the hybridization. In the bfDNA **14** the carbostyril donor is positioned at the 3′-end of the homopyrimidine stretch so that a close proximity to the acceptor upon triple helix formation is guaranteed (Fig. 8).

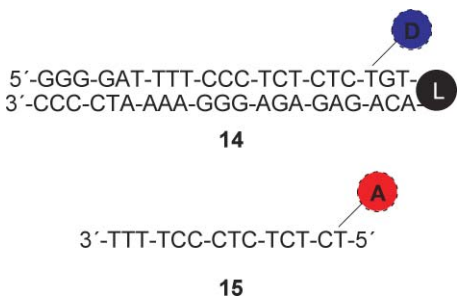

Fig. 8 Labelled oligomers for assay.

Both dyes were incorporated by post-synthetic labelling. The specific labelling in **15** was achieved by incorporation of a 5′-amino-5′-deoxy-thymidine as last building block.[24] After deprotection and removal from the solid support this resulted in a primary amino-function at the 5′-end to which **2** was coupled selectively. For the preparation of the donor-modified bfDNA **14** a 5-aminopropargyl-2′-deoxy uridine building unit was incorporated in the appropriate position. Again this yielded in a primary amino function after deprotection and cleavage from the solid

support, to which **1** was coupled.[17] Purification of the labelled oligonucleotides was performed by preparative RP-HPLC and their purity was checked by analytical PAGE (Fig. 9).

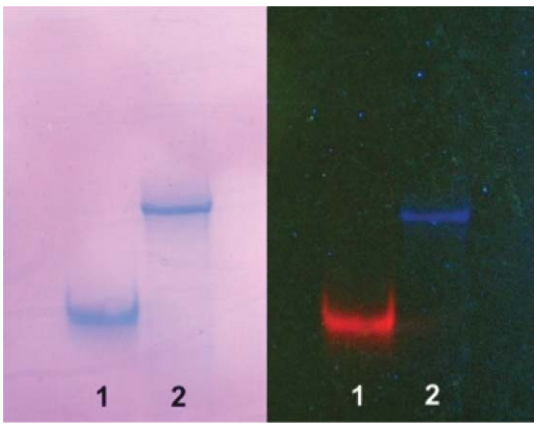

Fig. 9 Analytical PAGE of oligonucleotide **15** (lane 1) and bfDNA **14** (lane 2). Left: stained bands at vis; right: emission after exposure at 366 nm.

FRET measurements

When mixing the two oligonucleotides **14** and **15** at pH 6 the results were not clear cut but by lowering the pH to 5.5 we could indeed observe an emission at 620 nm after excitation at 360 nm which is indicative for the FRET and to be expected after formation of the triple helix. Addition of a pure heterodimer p65/p50 in small excess led to a substantial decrease of the emission at 620 nm and hence, of the FRET (Fig. 10a). The reduction occurred almost spontaneously after the addition of NF-κB and remained stable as was followed in a time course experiment (Fig. 10b). The still observable emission at 620 nm can be attributed to direct excitation of the Ru-complex.

To demonstrate a correlation between the NF-κB concentration and intensity of the FRET acceptor emission, different concentrations of the heterodimer of NF-κB were added to the triple helix solution and the corresponding emission spectra were recorded after an incubation time of 5 min. The results are depicted in Fig. 11.

Conclusions

In summary, we have presented a feasibility study for a new principle for DNA-binding proteins. The new concept was exemplified for the transcription factor NF-κB heterodimer p50/p65 and a DNA-stretch derived from the inducible nitric oxide synthetase (iNOS) gene. Binding of NF-κB to the DNA leads to a replacement of the third strand bound by Hoogsteen pairing. Since this third strand is equipped with a FRET acceptor the replacement resulted in a reduced emission of the luminescence at 620 nm due to the diminished FRET. Hence, the assay avoids the need for radioactivity as in EMSAs and of antibodies as in alternative assays. The new principle has therefore also implications for assays of other DNA-binding proteins as well. Future experiments will be focused on other heterodimeric combinations of NF-κB and on precise quantification experiments with the new format.

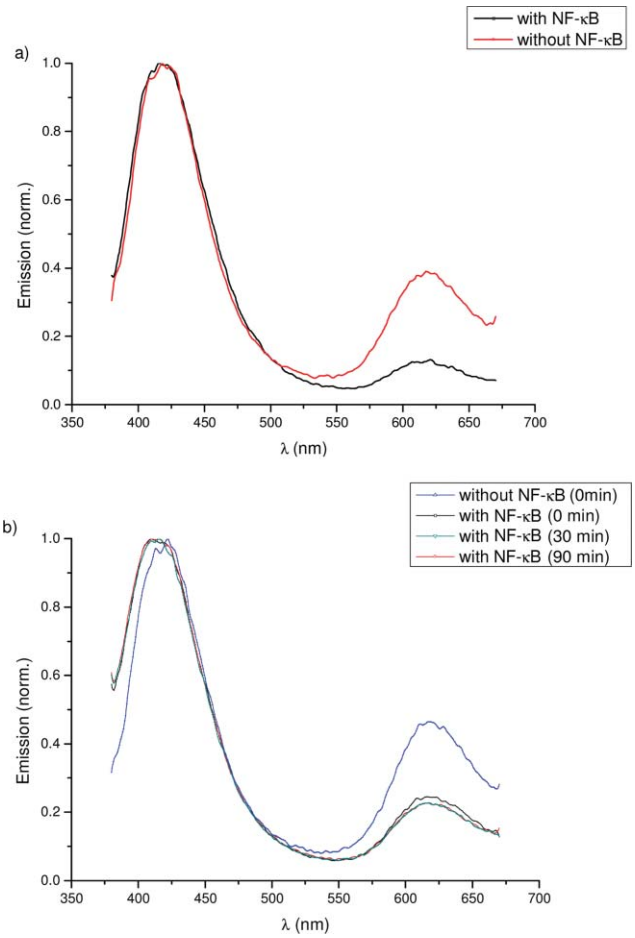

Fig. 10 (a) FRET of triple helix before and after addition of NF-κB. (b) Time course after addition of NF-κB.

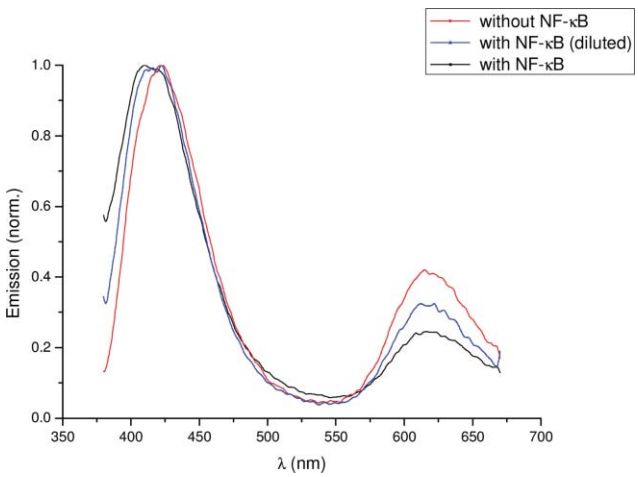

Fig. 11 FRET of triple helix with different concentrations of heterodimers of NF-κB.

Experimental

General

Oligonucleotide synthesis was carried out on an ExpediteTM 8909 Nucleic Acid Synthesis system on a 1 μmol scale using

phosphoramidite chemistry. Standard phosphoramidites as well as nucleosides coupled to CPG solid support were obtained from Proligo/Sigma Aldrich. Cleavage of the synthesized oligonucleotides from the solid support and deprotection of the nucleobases was done by treating the oligo with a 25% NH_3 solution at r.t. over night. Oligonucleotide samples were desalted on NAP-10 columns. All oligonucleotides were analyzed by means of PAGE and HPLC. Polyacrylamide gels (20%) of 0.4 mm thickness were used. Pre-electrophoresis was performed for 2 h at 500 V with tris-borate running buffer. Oligonucleotide (1 µl, 0.1 OD units) and bromophenol blue/xylenecyanol solutions (2 µl) were heated to 90 °C for 2 min and rapidly cooled to 0 °C before being loaded on the gel. Electrophoretic separation was performed for 2 h at 500 V and 4 mA. Oligonucleotide bands were visualized at 366 nm or stained with a solution of 3,3′-diethyl-9-methyl-4,5,4′,5′,dibenzothiacarbocyanine bromide (Stains-All; Fluka). HPLC runs were performed on a Merck/Hitachi system; reversed phase: SP-250/10-Nucleosil-100-5-C18 or EC-125/4-Nucleosil-100-5-C18 columns, solvent A = 0.1 M $Et_3NH(OAc)$ buffer at pH 7.0, B = CH_3CN; ion exchange: 250/4 Dionex DNAPAc-PA-100 column, solvent A= 20 mM KH_2PO_4 in CH_3CN/H_2O 1:4 at pH 6.0, B = 1 M KCl, 20 mM KH_2PO_4 in CH_3CN/H_2O 1:4 at pH 6.0.

UV Spectra were recorded on a Perkin-Elmer-Lambda-35-UV/VIS spectrometer. Fluorescence spectra: Perkin-Elmer LS45 spectrometer.

Melting curves were recorded on a IA 9000 spectrometer of Electrothermal.

Column chromatography was performed on silica gel type 60 ACC35-70 µm, using an MPLC-system for chromatography of Büchi (Fraction Collector C660, Pump Module C605, Pump Manager C615, UV Photometer C635).

^1H, ^{13}C und ^{31}P-NMR spectra were measured on AC250, AM 400 und DRX 500 from Bruker as well as a Mercury VX 300 from Varian using $CDCl_3$ or d-DMSO as solvent. Coupling constants are in Hertz, all chemical shifts δ in ppm. Internal standard for ^1H-NMR was $CDCl_3$ (7.26 ppm), for ^{13}C-NMR-$CDCl_3$ (77.0 ppm).

Linker synthesis

(4′,4″-Dimethoxytrityl)-hexaethyleneglycol was prepared from hexaethyleneglycol and DMT-Cl according to reference.[25]

(4′,4″-Dimethoxytrityl)-hexaethyleneglycoyl-β-cyanethoxy-(diisopropylamino)-phosphine was prepared from (4′,4″-dimethoxytrityl)-hexaethyleneglycol according to reference.[25]

Oligonucleotide synthesis

DNA 3 and 4. The synthesis of **3** and **4** was carried out on a 1 µmol scale. Modified building block (4′,4″-dimethoxytrityl)-hexaethyleneglycoyl-β-cyanoethoxy-(diisopropylamino)-phosphine (0.1 M in CH_3CN) was incorporated automatically using an increased coupling time of 15 min. In the last cycle, the DMT-protecting group at the 5′-end was cleaved off. Cleavage from solid support was performed with 25% aq. NH_3 at r.t. over night. The crude oligos were purified by preparative PAGE and afterwards desalted on NAP-10 columns. After purification **3** and **4** were analyzed by analytical PAGE.

DNA 5–7. The synthesis of sequences **5–7** were carried out on a 1 µmol scale. In the last cycle, the DMT protecting group at the 5′-end was cleaved off. Cleavage from solid support was performed with 25% aq. NH_3 at r.t. over night. The crude oligos were purified by preparative PAGE and afterwards desalted on NAP-10 columns. After purification **5–7** were analyzed by analytical PAGE.

DNA 14. The synthesis of **14** was carried out on a 1 µmol scale. Modified building block (4′,4″-dimethoxytrityl)-hexaethyleneglycoyl-β-cyanoethoxy-(diisopropylamino)-phosphine (0.1 M in CH_3CN) was incorporated automatically using an increased coupling time of 15 min. Aminopropargyl-2′-deoxy uridine (68 mM in CH_3CN) was incorporated automatically at the specific site, by using standard coupling times. In the last cycle, the DMT-protecting group at the 5′-end was cleaved off. Cleavage from solid support was performed with 25% aq. NH_3 at r.t. over night. After replacement of NH_4^+ with K^+ the oligo was desalted with a NAP-10 column. Coupling of the donor was carried out in solution. To a soln. of the oligo (50 OD, 113 nmol) in DMF/1,4-dioxane/H_2O 1:1:1 (339 µl), iPr$_2$EtN (3.5 µl, 22 µmol) and the hydroxysuccinimide ester of donor **1** (1.5 mg, 2.6 µmol) were added. The mixture was incubated at 25°C in the dark for 24 h. After removal of the solvents, the residue was washed three times with cold EtOH (3 × 500 µl) to remove the excess of the donor. The crude **14** was purified by preparative reversed phase HPLC (EC-125/4-Nucleosil-100-5- C18 column, 0-60% B in 45 min; t_R = 16.28 min) and desalted on NAP-10 columns. After purification 7.9 OD of **14** (12.4 nmol) were obtained and analyzed by analytical PAGE.

DNA 15. The synthesis of **15** was carried out on a 1 µmol scale. Modified building block 5′-deoxy-5′-{[(4-methoxyphenyl)-diphenylmethyl]amino}thymidine-3′-[cyanoethoxy-diisopropyl-amino)-phosphine] (60 mg) was incorporated manually in the last cycle by using 0.7 ml of 0.3 M BMT as activation reagent. The (MeO)Tr-protecting group at the 5′-end was cleaved off. Cleavage from solid support was performed with 25% aq. NH_3 at r.t. overnight. Coupling of the acceptor was carried out in solution. To a soln. of the oligo (23 OD, 194 nmol) in DMF/1,4-dioxane/H_2O 1:1:1 (536 µl), iPr$_2$EtN (6.1 µl, 35.8 µmol) and (bathophenanthroline)ruthenium(II)-complex-hydroxysuccinimide ester **2** (6.1 mg, 4.5 µmol) were added. The mixture was incubated at 25°C in the dark for 24 h. After removal of the solvents, the residue was washed three times with cold EtOH (3 × 500 µl) to remove excess of acceptor. The crude **15** was purified by preparative reversed phase HPLC (EC-125/4-Nucleosil-100-5-C18 column, 0–80% B in 30 min; t_R = 20.58 min) and afterwards desalted by NAP-10 column. After purification 7.4 OD of 15 (37.5 nmol) were obtained and analyzed by analytical PAGE.

General procedure for the formation of triple helices

Equimolar amounts of the backfolding DNA (1.035 nmol) and the third oligonucleotide (1.035 nmol) were combined in an Eppendorf reaction tube. The solvent was removed under vacuum and the precipitate was dissolved in 1300 µl NaOAc buffer (450 mM, pH 5.5) to give a final concentration of 0.796 pmol/µl. The solution was heated to 90 °C for 2 min and afterwards slowly cooled to r.t.

General procedure for the NF-κB assay

14.7 μl of the triple helix solution (0.796 pmol/μl, 11.7 pmol) in NaOAc buffer (450 mM, pH 5.5) and 5.3 μl of pure NF-κB dimer (2.6 pmol/μl, 13.8 pmol) were combined in an Eppendorf reaction tube. The solution was immediately transferred into a fluorescence cuvette and the fluorescence spectrum was recorded. (Excitation at 360 nm. Emission recorded between 380–670 nm).

Cell Culture

Jurkat T cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL, Groningen, Netherlands).

Electrophoretic mobility shift assay

Protein extracts were prepared as described previously.[26] The protein content of the extract was determined and equal amounts of protein (10–20 μg) were added to a reaction mixture containing 20 μg bovine serum albumin (BSA) (Sigma), 2 μg of poly(dI-dC) (Roche Molecular Biochemicals), 2 μl of buffer D+ (20 mM Hepes, pH 7.9, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40, 2 mM DTT, 0.1% PMSF), 4 μl of buffer F (20% Ficoll 400, 100 mM Hepes, 300 mM KCl, 10 mM DTT, 0.1% PMSF), and 100,000 cpm (Cerenkov) of a P33-labelled oligonucleotide for NF-κB made up to a final volume of 20 μl with distilled water. The reaction mixture was either adjusted to pH 7 or 6 with HCl before added to the protein extract. Samples were incubated at room temperature for 25 min. Oligonucleotides **3** and **4** were labelled using [γ^{33}P]dATP (3000 Ci/mmol; Amersham Pharmacia Biotech) and a T4 polynucleotide kinase (New England Biolabs). The probes were resolved through nondenaturing 4% polyacrylamide gel electrophoresis.

Acknowledgements

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