Production of DNA minicircles less than 250 base pairs through a novel concentrated DNA circularization assay enabling minicircle design with NF-κB inhibition activity

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ABSTRACT

Double-stranded DNA minicircles of less than 1000 bp in length have great interest in both fundamental research and therapeutic applications. Although minicircles have shown promising activity in gene therapy thanks to their good biostability and better intracellular trafficking, minicircles down to 250 bp in size have not yet been investigated from the test tube to the cell for lack of an efficient production method. Herein, we report a novel versatile plasmid-free method for the production of DNA minicircles comprising fewer than 250 bp. We designed a linear nicked DNA double-stranded oligonucleotide blunt-ended substrate for efficient minicircle production in a ligase-mediated and bending protein-assisted circularization reaction at high DNA concentration of 2 μM. This one pot multi-step reaction based-method yields hundreds of micrograms of minicircle with sequences of any base composition and position and containing or not a variety of site-specifically chemical modifications or physiological supercoiling. Biochemical and cellular studies were then conducted to design a 95 bp minicircle capable of binding in vitro two NF-κB transcription factors per minicircle and to efficiently inhibiting NF-κB-dependent transcriptional activity in human cells. Therefore, our production method could pave the way for the design of minicircles as new decoy nucleic acids.

INTRODUCTION

Synthetic double-stranded DNA minicircles (dsMCs) are attractive nano-objects that received attention for their use in therapeutic applications. dsMCs from 250 to 1000 bp in length recently entered the field of gene therapy as minivectors with a better delivery efficiency than conventional exogenous plasmid-based vector used as nucleic acid therapeutics to express small interfering RNA (1,2). Reducing the size of conventional plasmids to small dsMCs offers several advantages such as increased resistance to the shearing forces associated with nucleic acids delivery system (3), significantly better biostability in human serum and in the living cell (1) and an improvement in DNA intracellular delivery and trafficking (4,5). To our knowledge, the potential of small dsMCs of sizes down to 250 bp as nucleic acid therapeutics has not yet been investigated because their quantitative production is a limiting step for biological assays. In more fundamental studies, dsMCs less than 200 bp in length have been used for several purposes. In the field of DNA nanotechnology, small dsMCs were employed as building objects to assemble nanoarchitectures after the incorporation of G-quadruplexes, RNA hairpins or chemically functionalized oligodeoxynucleotides leading to the possibility of creating molecular devices with a broad range of functions (6–8). dsMCs in the range of 100 bp in length also offer the unique opportunity to mimic short DNA loops containing permanent bending and topological constrains formed during essential DNA-dependent transactions such as transcription and recombination (9–12). Producing minicircles with customized DNA sequences, chemical functionalization and supercoiling remains a hurdle for further applications including the study of interactions between minicircle and other molecules such as proteins and drugs. Therefore, despite a growing interest for dsMCs less than 250 bp
in length, no versatile method currently exists for the ratio-
nale, design and production of small minicircles to carry out
more straightforward experiments by exploiting the unique
structure of this DNA nanomaterial in studies from the test
tube to the cell.

The available production methods for small dsMCs
present several qualitative and quantitative limitations. A
minicircle-producer plasmid through integrase-dependent
DNA recombination in bacteria was unable to gener-
ate minicircles of less than 250 bp due to unpredictable
sequence- and length-dependent negative effects on the
recombination efficiency (2,13). In vitro, a recombinab-
based methodology involves time-consuming preparation
and purification of both plasmids and recombinase protein.
Furthermore, the minicircle recovery step is labor-intensive
and yields low amounts of material, in the microgram range
(14,15). Another approach consists in using a linear DNA
fragment the ends of which need to be in close proximity to
each other while leaving them accessible for covalent sealing
by enzymatic ligation. The main hurdle to such an approach
comes from the intrinsic stiffness of the DNA double helix
that makes the cyclization reaction very inefficient for DNA
lengths less than about 300 bp, as the DNA rigidity domi-
nates in the order of its persistent length (~150 bp) (16). In
reported ligase-mediated DNA circularization assays which
have been used extensively to study DNA bending through
minicircle formation in the presence or absence of protein-
induced bending (9), the DNA concentration was kept very
low in the nanomolar range or less. Such a weak DNA con-
centration is indeed necessary in order to favor the forma-
tion of minicircles arising from the intramolecular circular-
ization of flexible or bent linear DNA molecules and to min-
imize as much as possible linear DNA multimers formed
competitively through DNA concentration dependent in-
termolecular reaction. Because a very low DNA concentra-
tion renders the circularization reaction unfit for the quan-
titative production of dsMC, a key requirement for mini-
circle production is to design a more efficient circulariza-
tion reaction by increasing DNA bendability. Higher effi-
ciency of the circularization reaction has been achieved us-
ing specific sequences endowed with intrinsic bendability
(so called adenine tracts) but this approach precludes the
possibility of incorporating freely any DNA sequence of in-
terest (17). A smart strategy employed DNA substrates with
long cohesive ends, the flexibility of which was exploited to
produce a few tens of micrograms of ligatable minicircles
(18). This process requires labor-intensive prepara-
tion of plasmid-based linear DNA substrates. When using
oligodeoxynucleotides, a low minicircle yield is observed
due to the formation of byproducts, i.e. truncated DNA
strands and circular multimers (19). Therefore, a versatile
method for the production of minicircles having fewer than
250 bp in length with customized DNA sequences with or
without chemical functionalities is not yet available. The
production of sufficient quantities of material is another im-
portant criterion to be fulfilled with a view to design dsMCs
performing functions inside the cell.

Here, we report a cell-free method for the produc-
tion of dsMCs less than 250 bp in size. We designed a
nicked double-stranded DNA oligonucleotide blunt-ended
substrate for efficient minicircle production in a ligase-
mediated and bending protein-assisted circularization reac-
tion at high DNA concentration. Various minicircles can
be produced containing a variety of site-specific chemical
modifications or with controlled extent of physiological
supercoiling. As a proof of principle of our method, we de-
gined a minicircle endowed with the capacity to bind effi-
ciently the transcription factor NF-κB and to inhibit its
transcriptional activity in cellulo. This novel production
method might be exploited to help in the design of mini-
circles as therapeutic nucleic acids.

MATERIALS AND METHODS

Single-stranded oligodeoxynucleotides from Eurogentec
were synthesized with or without chemical modifications
as indicated in the text. For the oligodeoxynucleotide se-
quence composition incorporated into the minicircles de-
dsigned for NF-κB-dependent biochemical and biological
studies, we used the Jaspar data base (http://jaspar.binf.ku.
dk) to select minicircle sequence with the highest and low-
est score for specific NF-κB binding and non-specific se-
quences including the design of scrambled minicircles, re-
spectively. Proteinase K, T4 DNA ligase, T4 polynucleotide
kinase and Exonuclease III were purchased from Thermo
Fisher, T5 exonuclease and low molecular weight DNA
ladder were from New England Biolabs and CircLigase
from Epicentre. Full length rat NF-κB p50 homodimer and
bacterial formamidopyrimidine (Fpg) repair protein were
purchased from Promega and New England Biolabs, re-
spectively. Bal31 nuclease was from New England Biolabs.
Streptavidin and SYBR Green I nucleic acid gel stain were
purchased from Sigma-Aldrich. Tumor Necrosis Factor α
(TNFα) was from Miltenyi Biotec.

The pET-28a-derived vector containing the ARS binding
factor 2 protein (Abf2p) gene kindly provided by F. Culard
(CNRS-CBM, Orléans, France), was used for the trans-
formation of BL21 (DE3) pLysS (Novagen). The trans-
fomed strain was grown at 37°C to an OD600 of0.5 and
ducted with isopropyl-1-thio-β-D-galactopyranoside (Cal-
biochem) at a final concentration of 100 μM for 6 h. Af-
ter centrifugation, the cells were resuspended in a sonica-
tion buffer (50 mM Tris-HCl, 500 mM NaCl, 20 mM imi-
dazole, 5 mM β-mercaptoethanol pH 8) supplemented with
a protease inhibitors mixture (Sigma-Aldrich) and 100 mM
phenylmethylsulfonylfluoride (Pierce) and then sonicated.
All purification steps were performed at 4°C. The lysate ob-
tained after extract centrifugation was applied to a nickel
agarose column (Qiagen) and N-terminal histidine-tagged
Abf2p was eluted with buffer containing 100 mM imidazole.
Abf2p was concentrated to 3 mg/ml on an Amicon Ultra-4
10K (Millipore) and stored at −80°C in a buffer contain-
ing 25 mM Tris-HCl, 100 mM NaCl, 1 mM dithiothreitol,
5% glycerol pH 8 (protein purity > 95%). In addition to its
high production yield, His-tagged-Abf2p presents the ad-
vantage of supporting several cycles of thawing and freez-
ing and long storage time at −80°C for at least 2 years that
make this protein a robust biochemical DNA bender.
Production of double-stranded closed relaxed and supercoiled minicircles

Preparation of nicked DNA templates was performed by mixing equimolar quantities of complementary single-stranded oligonucleotides in buffer containing 10 mM Tris-Cl and 25 mM NaCl, pH 7.5 at a concentration of 40 μM. Hybridization was carried out by slowly cooling the oligonucleotides mixture from 80°C to 15°C. A standard circularization reaction was carried out by adding one of the overlapping nicked duplexes shown in Supplementary Figure S1 at a final concentration of 2 μM in a buffer containing 40 mM Tris-Cl, 10 mM MgCl2, 0.5 mM ATP and 5% glycerol, pH 7.8. A leading strand of 95 nucleotides (nt) or with both complementary strands was ligated using T4 DNA ligase and exonuclease T5 (200 units, 30 min at 37°C) to obtain covalently closed minicircles after carrying out a final incubation with proteinase K. The same protocol was followed to generate supercoiled minicircle in the presence of increasing concentrations of Ethidium Bromide (Sigma-Aldrich) as indicated in Figure 2A except that the presence of increasing concentration of Ethidium Bromide was characterized by a decrease in linking number (Lk) (underwound molecules with the possibility of negating supercoiling formation). The first 95 bp minicircle topoisomer shown in Figure 3 with a value of ΔLk = -1 is characterized by a superhelical density (σ) equal to 0.11 as deduced from the formula ΔLk/Lk0 where Lk0 is the number of helical turns in the original linear molecule. Minicircles of various sizes generated in the circularization reaction were analyzed on 8% denaturing gel (19:1, acrylamide:bisacrylamide; 8 M urea; TBE); DNA samples in formamide were heat denatured and electrophoresed at 18 W for 90 min.

Different binding assays were used for interaction studies of the linear DNA substrate and dsMCs according to the DNA interacting proteins. NF-κB p50/p50 protein was incubated with the 95 bp minicircle (10 ng) as a function of the protein concentration in 10 mM Tris-Cl, 50 mM NaCl, 3 mM EDTA, 0.2 mM DTT, 2% glycerol, 50 μg/ml acetylated BSA, pH 7.5 for 30 min at 4°C. The protein concentration was used, 5, 20, 40, 100, 120 nM and 2, 5, 20, 40, 60, 180 nM in the presence of minicircle containing 1 and 2 κB binding sites, respectively. The DNA binding activity of Ab2p and NF-κB was analyzed by electrophoretic mobility shift assay (EMSA). The reaction mixture was loaded on a native 5% polyacrylamide gel (19:1, acrylamide:bisacrylamide (w/w); TBE) and then electrophoresed for 1 h. After staining and quantification of band intensity, the fraction of DNA bound to protein for each retarded band was calculated according to the following equation: fraction minicircle bound = [protein-minicircle complex/total minicircle], NF-κB binding data obtained with minicircle containing a single κB binding site were fitted to a square hyperbola using non-linear least squares analysis with the Origin 9.0 software (Microcal). When the minicircle contained two κB binding sites, the binding curves corresponding to unbound, singly bound and doubly bound forms were fitted as previously described (20) where the dissociation constant for each binding site was assumed to be identical since these two binding sites are identical. The cooperativity parameter was determined by measuring the maximum value of the singly bound form using equation numbered 16 (20).

For the gel-based methodology, gel staining was carried out by using SYBR Green before exposition to a Typhoon phosphorimager to determine bands intensity using ImageQuant 5.1 software. The DNA was visualized by fluorescence and thus the molar ratio of the starting linear duplex to 150 ng of 95 bp minicircle produced as previously described (20) where the dissociation constant for each binding site was assumed to be identical since these two binding sites are identical. The cooperativity parameter was determined by measuring the maximum value of the singly bound form using equation numbered 16 (20).

Cryo-electron microscopy

A 3 μl drop of 95 bp minicircle solution at 248 μg/ml concentration in buffer containing 10 mM Tris-Cl, 1 mM EDTA, pH 8 was deposited onto a glow-discharged carbon-filmed R2/2 grid (Jena) and vitrified in liquid ethane using a cryo-fixation device (UMR 8502 CNRS-LPS, Orsay, France). The cryo-specimens were transferred to a Gatan 626 cryo-holder (Gatan Inc.) and examined at −180°C with a JEM 2011 cryo-transmission electron microscopy (Jeol)
using an acceleration voltage of 200 kV and a 50 000-fold magnification. Images were recorded with 1 to 2.5 μm of de-focus under low-electron-dose micrograph conditions on a Kodak SO163. The calculated diameter of minicircle was derived from the formula $P/\pi$ where $P$ is the perimeter as determined by multiplying the bp number of the linear DNA with the bp width equal to 0.34 nm. The longest diameters measurement was performed from analysis of 32 imaged minicircles using Image J software. The histogram shown in Figure 2D was obtained by splitting the range of diameter values into 5 equal-sized classes as derived from Scott’s normal reference rule. The distribution was fitted with a Gaussian function using the origin 9.0 software (Microcal) providing a mean value of the diameter as indicated further. It was also verified whether the diameter values measured in the sample were normally distributed by using the Shapiro–Wilks normality test. As we failed to reject the null hypothesis with a $P$-value of 0.35, the diameter values were normally distributed. We then performed a parametric test to determine whether the mean value of diameters was statistically different from the diameter value calculated for a perfect round-shaped minicircle. The one-sample $t$-test with null hypothesis that the difference between measured diameters and calculated diameter values is zero, failed to reject the null hypothesis with a large $P$-value of 0.39 indicating no statistical difference between the mean diameter value and the calculated diameter value. Statistical analysis was derived from NIST/SEMATECH e-Handbook of statistical methods at (http://www.itl.nist.gov/div898/handbook/index.htm).

**RESULTS AND DISCUSSION**

**DNA circularization reaction at high DNA concentration**

We were first interested in revisiting the ligase-mediated multimerization–circularization reaction at high DNA concentration (2 μM) in the absence or presence of a bending protein. The electrophoresis experiment shown in Figure 1A indicates that a blunt-ended 95 bp duplex with phosphorylated 5'-ends that co-migrates expectedly at similar distance to the molecular weight marker of 100 bp (lanes 1 and 2), formed mainly linear dimers and also higher molecular weight multimers in ligase-mediated reaction in the absence of bending protein (lane 3); no monomer circular species was formed as deduced from the absence of any ligation product migrating at the expected position as indicated (Figure 1A, right side) (24). This result shows that circularization of linear DNA smaller than the persistence length is inefficient as expected (25) and indicates that T4 DNA ligase efficiently joined blunt-ended DNA duplexes (26). Next, we studied the high DNA concentration circularization reaction in the presence of a bending protein to tentatively enable an intramolecular circularization reaction. For that purpose, we used the well-known bending protein Abf2p that has two conserved boxes named box A and box B like the high-mobility-group (HMG) box domain binding protein HMG1 (27). Abf2p was found to induce DNA circularization as the major ligation reaction in the low DNA nanomolar concentration range (Supplementary Figure S2), in agreement with the circularization efficiency of other bending proteins including HMG1 (A+B) (28). In contrast, when the circularization reaction is carried out at high DNA concentration of 2 μM in the presence of Abf2p (Figure 1A, lane 4), 95 bp monomer minicircle species represents only 30 ± 6% of total ligation products (Figure 1B). Therefore, the high DNA concentration promotes the contaminating intermolecular reaction over the cyclization intramolecular reaction the efficiency of which is thus not sufficient to generate a high amount of minicircle species.

To implement DNA circularization efficiency in a sequence-independent manner, we introduced a DNA...
structural distortion to stimulate the protein-assisted circularization reaction with a catalyst-like activity. A DNA nick which is known to induce flexible bending (17,29,30), is specifically recognized by HMGB1 in a structure-specific manner (31) indicating that a DNA nick could increase the bending protein assisted-circularization reaction efficiency. In addition, DNA nick is efficiently sealed by T4 DNA ligase (32) that fulfills our requirement to generate a final minicircle product devoid of nicks. We therefore designed a nicked double-stranded 95 bp blunt-ended duplex containing one internal nick per strand, the base sequence being identical to the non-nicked duplex used above. This nicked duplex possesses a centrally placed overlapping region of 15 bp as schematically drawn in Figure 1A, indicating the nucleotide length of DNA strands used to build it. In the absence of bending protein, both nicked and non-nicked duplexes, which have identical electrophoretic mobility, yielded only linear multimers arising from the high DNA concentrated circularization reaction (Figure 1A, lanes 3,6). In contrast to the non-nicked duplex (lane 4), the circularization reaction of the nicked duplex in the presence of Abf2p generated a huge amount of circular species with a faint amount of linear multimers and almost complete consumption of the starting duplex (lane 7). As shown in Figure 1B, minicircle species represent near 90% of all ligation products when circularization reaction is carried out with the nicked DNA substrate. Thus, our assay named Concentrated Nicked DNA Circularization Assay (CNDCA) gave a high yield of DNA circular species that has never been reported previously. This circularization efficiency can be explained by several key physical-chemical parameters specific to our CNDCA. Abf2p exhibits the same order of binding activity with the starting linear nicked DNA substrate and the minicircle end product (Figure 1C); this could be due to the fact that Abf2p has no C-terminal acidic tail (27) that was previously shown to be responsible for the well-known preferential recognition of minicircles by an HMG-box domain protein (33). This Abf2p binding activity is different from previous data that reported that bending protein exhibited a large binding preference for dsMC over linear DNA by at least one order of magnitude, resulting in a weak circularization efficiency (34). Thus, Abf2p is likely not significantly displaced from the nicked DNA substrate in the course of minicircle formation, contributing to CNDCA efficiency. We also determined whether the yield of circularization reaction was dependent on nicks position. For that purpose, the centrally positioned overlapping region containing nicks was displaced close to one or other 95 bp duplex end. Circularization reaction was found to be decreased near 5-fold with nicks situated close to DNA ends as compared to nicks in the central position of DNA (Supplementary Figure S3). CNDCA is thus effective when nicks are in central position of the linear DNA substrate that strongly suggests that DNA bending induced by Abf2p occurs in the center of DNA bringing its two ends into close proximity.

Methodology for one pot CNDCA-based DNA production of DNA minicircles

We then designed a CNDCA-based strategy allowing one pot preparation of DNA minicircles. The production of minicircles of increasing size was achieved by using starting blunt-ended nicked duplexes containing one or several overlapping regions to avoid the use of long oligodeoxynucleotides, since full length oligodeoxynucleotide synthesis by the solid-phase phosphoramidite method enables high yield and purity in the range of less than 65 nucleotides in length. Therefore, the presence of nicks within the DNA substrates is relevant not only for high circularization efficiency but also for producing minicircles of increasing size without the need to use long oligodeoxynucleotides. As shown in Figure 2A, the starting linear nicked duplex having phosphorylated 5'-ends except at one internal nick posi-
Figure 2. CNDCA-based production of minicircles. (A) Scheme illustrating the method of DNA circularization through CNDCA and the derived pathways used to prepare relaxed, supercoiled dsMC and nicked minicircles. The filled space in grey within the central segment of the starting linear duplex refers to the overlapped region with nick indicated by an arrow. P indicates the presence of a phosphate group at DNA 5′ end. The length of the starting linear DNA has an integral number of helical turns to prepare the relaxed covalently closed DNA minicircle. (B) Image of stained denaturing polyacrylamide gel showing the 95 bp double-stranded minicircle (lane 1) and the nicked DNA minicircle (lane 2) respectively generated according to pathways 1 and 3. The denaturing conditions enabled strand separation of the single nicked duplex showing that the covalently closed minicircle is free of strand nick as confirmed by the position of the migration of control DNA samples indicated on the right side. (C) Cryo-electron microscopy micrograph showing 95 bp closed minicircles produced according to pathway 1; scale bar indicates 20 nm. (D) Histogram of the longest diameters measured from cryo-electron micrograph of 95 bp minicircles (n = 32). The Gaussian fit provides a mean diameter value of 10.1 ± 1.2 nm. (E) Image of stained denaturing polyacrylamide gel showing DNA minicircles of various sizes as generated according to pathway 1; the sequence of input linear nicked DNA duplexes with integer multiples of the helical repeat is shown in Supplementary Figure S1.

tion yielded an intermediate nicked dsMC used in turn for the production of either relaxed (pathway 1), constrained (pathway 2) dsMCs or controlled nicked minicircles from purified single-stranded minicircles (pathway 3). Final minicircle products arising from pathways 1 and 3 using an overlapping 95 bp duplex with an integer number of helical repeats as starting linear DNA substrate were analyzed by denaturing PAGE (Figure 2B). dsMC obtained from pathway 1 migrated as one band indicating that it is entirely covalently closed (lane 1) while the single nicked minicircle assembled according to pathway 3 expectedly yielded two bands arising from strand dissociation under the denaturing conditions. Migration of the 95 nt single-stranded minicircle control and starting linear oligodeoxynucleotides (lanes 3 and 4) confirmed the double- and single-stranded nature of the minicircles generated from pathways 1 and 3, respectively. Next, the 95 bp dsMC generated from pathway 1 was directly visualized by cryo-electron microscopy (cryo-EM) in similar conditions to those previously used (35). Our purpose is to know whether this minicircle is close to perfect circle and thus without constrain which should be the case as DNA used here has an integer number of helical repeats. As can be seen on the micrograph shown in Figure 2C, 95 bp dsMCs have an apparently regular circular shape. Next the longest diameters were measured from minicircles in the sample (n = 32) and histogram of measured diameters is shown in Figure 2D. The measured diameters distribution was then fitted with a Gaussian function providing a mean diameter value of 10.1 ± 1.2 nm. Conducting a one-sample-t test (see Material and Methods), we next verified that there was no statistical difference between this mean diameter value and the calculated diameter for per-
formed from out of phase nicked DNA duplexes of 90 and 100 bp in size. When the circularization reaction was carried out with non-nicked linear DNA of 90 and 100 bp, circular species formation was nearly completely inhibited (Supplementary Figure S4). This differential circularization efficiency indicates that linear nicked DNA substrate makes circularization reaction still effective for minicircles production from out of phase DNA duplexes.

Minicircles functionalized with chemical modifications or containing physiological supercoiling

Commercially available synthetic DNA oligonucleotides functionalized with various base analogs and chemical modifications were used to generate functionalized minicircle for various applications as biochemical tools and potential therapeutics. Firstly, we were interested in the production of 95 bp minicircle containing a base modification such as 8-oxoguanine residues that is formed naturally when DNA is subjected to oxidative conditions or ionizing radiations. Such lesion is substrate for the well known bacterial-derived formamidopyrimidine (Fpg) repair protein that catalyzes the excision of 8-oxoguanine yielding a strand nick (37), the formation of which can be easily exploited to directly reveal the presence and the location of such base residue within functionalized minicircle. The production yield of single and double 8-oxoguanine-modified minicircles was insensitive to the presence of base modification within starting overlapping modified duplex (Supplementary Figure S1). When the minicircle containing a single or double base modification was incubated in the presence of Fpg, we observed a pattern of DNA fragments on denaturing PAGE that corresponds to cleavage of DNA strand at expected position of 8-oxoguanine present within minicircles allowing to validate our strategy for production of minicircles with site specifically base modification (Supplementary Figure S5A). Secondly, dsMCs with site specifically placed labels were produced. After completion of our protocol using input nicked duplex containing one biotin residue linked by a spacer to the C5-atom of pyrimidine ring, we obtained a pure closed dsMC with similar yield to that obtained for unmodified minicircle production. The biotine-labeled minicircle was able to bind to streptavidin as shown by the slower migration of complex (Supplementary Figure S5B). Biotinated minicircles could be used for further biochemical applications based on the use of streptavidin coated surfaces.

As schematically shown in pathway 2 on Figure 2A, our method enabled also production of constrained dsMC based on religation of nicked dsMC in the presence of Ethidium Bromide (EtBr), an intercalating drug inducing a double helix linking number decrease by lowering the base pairs twist. Because biophysical characterization of small constrained 95 bp minicircle was not previously reported, we used native PAGE together with Bal31 nuclease assay that are well known technical approaches previously used to determine the formation of topoisomers and the presence of DNA distortions, respectively (13,15,18,36). A total of 95 bp dsMCs produced in the presence of EtBr exhibit an increase in electrophoretic mobility as a function of drug concentration, yielding two major bands corresponding to negatively supercooled topoisomers of decreased link-
Figure 4. Design of minicircles containing one and two κB binding sites efficiently bound by NF-κB p50/p50. (A and D) Sequence of 95 bp minicircles containing one and two κB binding sequences as indicated by the rectangular frame. (B and E) EMSA showing titration of minicircle by increased concentrations of NF-κB p50/p50 as indicated under Materials and Methods. (C and F) Plot of data from panels B and E, respectively; data were fitted to a square hyperbola using non-linear least squares analysis and according to previously reported equations (20) related to unbound (▲), singly bound (♦) and doubly bound (■) DNA.

ing number (ΔLk values of −1 and −2) (Figure 3, lanes 4, 6). This indicates that the overall compaction of minicircles is increased as a consequence to DNA writhing (13,15) as confirmed by magnesium dependent effect on the electrophoretic migration of topoisomers (Figure 3 and Supplementary Figure S6). The existence of writh structure was previously observed with a constrained 178 bp minicircle (15) but this is the first time to our knowledge that it is also observed with small 95 bp minicircles. We next used Bal31 nuclease to detect the presence of DNA distortions within our constrained minicircles. We found that both minicircle topoisomers are nuclease sensitive on the contrary to relaxed closed minicircle formed by religation in absence of EtBr (Figure 3 lanes 3,5,7) indicating the formation of a NF-κB/minicircle complex. Under these conditions, no gel-shifted complexes were detectable when the minicircle has no κB binding site revealing the specific nature of NF-κB interactions (Supplementary Figure S7). As deduced from the half-maximal binding equilibrium (Figure 4C), NF-κB exhibits a significant affinity for κB minicircle with a Kd value of 30 ± 3.6 nM that is similar to that reported with specific linear DNA substrates (39). Our data indicate that the pre-bent κB sequence did not substantially modulate NF-κB DNA binding activity that suggests that DNA bending is involved in indirect readout of κB sites by NF-κB. Our observation is in line with the fact that several linear κB sequences adopt a pre-bent conformation (40).

To exemplify the versatility of our method regarding the production of dsMCs with various sequence compositions and in order to design minicircles for potential therapeutic applications, we next produced relaxed minicircles containing one or several consensus sequences 5′-GGGACTTTCC/5′-GGAAAGTCCC of the immunoglobulin K-light-chain gene enhancer (κB). This sequence is part of numerous important nuclear factor K-light-chain enhancers of activated B cells (NF-κB) transcription factor-responsive genes (38). As anticipated from the molecular basis of our CNDCA-based minicircle production, the yield for minicircles containing one or several κB motifs was unchanged compared to minicircles with a random sequence composition and in particular was independent of the κB sequence position as described below. Our method for dsMC production thus provides the opportunity to study extensively the recognition mechanism of various DNA-binding proteins with pre-bent DNA as exemplified below with the transcription factor NF-κB using 95 bp dsMCs.

DNA minicircles containing one to several recognition sites for transcription factor binding activity

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specifically more than one NF-κB p50/p50 protein, we therefore prepared a 95 bp minicircle containing two κB sites (Figure 4D). The binding activity of NF-κB with this minicircle was carried out as described above. As shown in Figure 4E, two retarded bands are now present on the EMSA gel upon addition of the NF-κB protein, indicating the binding of two proteins per minicircle. To investigate the possible influence of the first bound protein on the specific binding of the second one, we used the previously described calculation that defines a protein binding cooperativity parameter (see Materials and Methods). This parameter was experimentally determined from the representation shown in Figure 4F where the fraction of each DNA species detected from EMSA was plotted as a function of the protein concentration. We found that the cooperativity parameter is 1.2, indicating that NF-κB binding to the first site did not notably distort the second κB binding site structure to modulate the protein affinity. When the second specific site was placed in a nearly opposite position to the first one, we still found that NF-κB bound with identical affinity to each binding site. However, when a third binding site was present, there was a significant decrease in binding to the minicircle that could partly be explained by protein hindrance and/or conformational change of the third binding site. Overall, this protein–DNA minicircle interaction study illustrates the potential of our methodology for the design of minicircles with various sequence compositions and shows for the first time that NF-κB efficiently binds pre-bent recognition sequences in a topologically closed DNA domain.

**Figure 5.** 95 bp κB minicircle delivered into cells inhibits NF-κB transcriptional activity. (A) Confocal microscopy images show that the minicircle is internalized in HEK293 cells; the red rectangular frame indicates the selected cell whose size is magnified 10 times as represented in the lower part (right: bright-field image of HEK293 cells; left: the fluorescence channel of fluorescein). (B) Responsiveness of the luciferase reporter gene driven by a NF-κB responsive promoter in the HEK293 cell line delivered with a minicircle containing two κB sequences (κB) as compared to the control non-specific minicircle (scramble). A specific decrease of luciferase activity is observed as a function of κB minicircle delivering concentration indicating κB minicircle-dependent NF-κB transcriptional inhibition. Data are means ± S.D. obtained from triplicate wells and are representative of six independent experiments (*P < 0.05).

**Inhibition of NF-κB cellular transcriptional activity by the κB minicircle**

The two κB sites minicircle produced by our CNDCA-based strategy was evaluated for its capacity to modulate in cellulo NF-κB-dependent transcription by its specific NF-κB binding activity. For that purpose, we next designed a new experiment based on the so-called decoy approach. The classical decoy strategy consists in the intracellular delivery of a short and linear DNA double-stranded oligonucleotide that contains one specific binding site able to trap a transcription factor of interest that in turn inhibits its activity (41). Here, our strategy consists in using a minicircle instead of linear DNA. We delivered either the κB minicircle or a control minicircle intracellularly using HEK293 cells that stably expressed a luciferase-reporter gene driven by a NF-κB responsive promoter.

In order to determine whether the DNA minicircle was properly internalized in HEK293 cells, a fluorescein-labeled 95 bp κB minicircle was delivered in cells thanks to His-IPEI, a transfection agent known to transfer DNA inside cells and to efficiently trigger its endosome escape into cytosol (42). The transfected cells exhibited a few fluorescent spots and diffuse fluorescence throughout the cytoplasm that likely correspond respectively to κB minicircles included in the cytoplasm and released minicircles that are located in both the cytosol and nucleus compartments (Figure 5A). Indeed, molecules comprising fewer than 100 bp are known to diffuse easily inside the cytosol and enter the nucleus (4). A similar observation was made with the non-specific minicircle (data not shown). Interestingly, the κB minicircle delivered intracellularly induced a specific and significant decrease in luciferase activity in a dose-dependent manner as shown in Figure 5B. The results show a 60% decrease in luminescence in cells
transfected with a 25 nM concentration of κB minicircle. For comparison, a linear anti-NF-κB decoy DNA oligonucleotide was previously shown to induce a similar decrease in luciferase activity at a 2-fold higher concentration than that used here with the κB minicircle in the same experimental conditions (22). Our data indicate that the κB minicircle was able to efficiently inhibit NF-κB-dependent transcriptional activity and hence acted as a ligand for cellular NF-κB. The CNDCA-based methodology opens the possibility of designing DNA minicircles as new decoy nucleic acids directed against NF-κB as shown here and also against other relevant transcription factors known as targets for the treatment of several human diseases (41).

CONCLUSION

Our novel method enables the quantitative production of a variety of DNA minicircles containing customized sequence and including the possibility of chemical functionalizations. The minicircles produced herein support the binding and the enzymatic activity of proteins such as transcription factor and DNA repair protein. The capacity of DNA minicircle bearing κB motifs to inhibit NF-κB transcriptional activity in human cells paves the way for the design of minicircles as new decoy nucleic acids. In this regard, minicircles containing various transcription factor binding sites could be developed for multitargeting as many promoters are known to be regulated by several transcription factors in a compensatory manner.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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