4726–4733 Nucleic Acids Research, 1993, Vol. 21, No. 20

Cpf1 protein induced bending of yeast centromere DNA element I

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Received July 15, 1993; Revised and Accepted September 9, 1993

ABSTRACT

The centromere complex is a multicomponent structure essential for faithful chromosome transmission. Here we show that the *S.cerevisiae* centromere protein Cpf1 bends centromere DNA element I (CDEI) with the bend angle ranging from 66° to 71°. CDEI DNA sequences that carry point mutations which lead to reduced Cpf1 binding affinity and *in vivo* centromere activity are still able to show bending. The Cpf1 induced bend is directed towards the major groove with the bend centre located in CDEI. An intrinsic bend cannot replace the Cpf1 induced DNA bend for *in vivo* centromere function. An *in vivo* phasing experiment suggests that both the distance and the correct spatial arrangement of the CDEI/Cpf1 complex to CDEII and CDEIII are important for optimal centromere function.

INTRODUCTION

Chromosome segregation in eukaryotic organisms is a very precise process. In the yeast chromosome loss occurs only once in 100000 mitotic cell divisions. An essential component involved in chromosome segregation is the centromere, which is made up of centromere DNA (*CEN* DNA) and centromere proteins (for reviews, see ref. 1 and 2). This DNA/protein complex interacts with the spindle apparatus to ensure equal distribution of duplicated chromosomes.

In budding yeast centromere DNA consists of an approximately 120 bp long DNA fragment comprising three centromere DNA elements named CDEs (2, 3).

CDEIII located at the right boundary of the *CEN* DNA plus part of CDEII are essential for basal centromere function (4, 5). CDEI at the left end of the centromere DNA is required for high fidelity centromere function (4, 5, 6). CDEII is extremely AT rich while the DNA sequences of CDEI and CDEIII are highly conserved in all 15 out of the 16 yeast centromeres analyzed (for review, see ref. 2). Mutational analysis of these elements from *CEN3* (6, 7) and *CEN6* (8, 9, 10) has shown the importance of the conserved bps for centromere function.

Proteins interacting with *CEN* DNA have been identified for CDEI and CDEIII elements (11, 12, 13, 14, 15, 16). CDEI is 8bps long and bound by a homodimer of Cpf1 protein, which is involved in both centromere and promoter function. Disruption of the *CPF1* gene results among other phenotypes in a 20 to 50 fold increase in mitotic chromosome loss (11, 12, 13, 17). The DNA bound form of Cpf1 appears to be required for centromere function. Certain amino acid changes in Cpf1 abolish binding to DNA *in vitro* and result in reduced centromere function (14).

Mutational analysis of *CEN* DNA sequences indicates a possible interaction between the CDEI and CDEIII DNA/protein complexes (9). This is supported by the observation that all *S.cerevisiae* centromere DNAs studied thus far are protected against nucleases due to the formation of a specific chromatin structure. They all show a characteristic 160 bps protected region that includes CDEI, CDEII and CDEIII sequences (18, 19, 20).

The types of interaction responsible for assembly and activation of the centromere complex are not understood. They might involve DNA/protein contacts as well as protein/protein contacts. One way to facilitate interaction between DNA binding proteins bound at distant sites is protein induced DNA bending. Bending contributes to the overall structure of higher order complexes in several recombination-, transcription- and replication systems in both prokaryotes and eukaryotes (for review, see ref. 21). Several proteins that induce bending have been identified such as the bacterial regulatory proteins CAP/CRP and integration host factor (IHF) (22, 23, 24, 25). The importance of these DNA distortions has been shown for the integration host factor (IHF), where binding of IHF stimulates site specific cleavage and recombination *in vivo* (26, 27). The IHF induced bend could be replaced by an intrinsic DNA bend that showed a similar *in vivo* activity (28).

In this work we studied the interaction of CDEI with its binding protein Cpf1. Permutation as well as phasing analysis showed that binding of Cpf1 induces a directed bend in CDEI. *In vivo* experiments indicated that the Cpf1 induced bend cannot be substituted by an intrinsic bend. All CDEI point mutants showed bending similar to wildtype CDEI. Bending might therefore be an inherent property of Cpf1 bound to CDEI.

MATERIALS AND METHODS

Yeast strains and media

For permutation and phasing analysis crude protein extracts were prepared from protease-deficient yeast strain ABSY60 (*a pra1 prb1 prc1 cps1 ade his*). Chromosome fragment loss rates were

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determined as described (9, 10) using YJH6 (*a/α ura3-52/ura3-52 lys2 801^{amber}/lys2 801^{amber} ade2-101^{ochre}/ade2-101^{ochre} trp1-Δ63/trp1-Δ63 leu2-Δ1/leu2-Δ1 CYH2^{R}/CYH2^{R}*) and YJH13 (*α ura3-52 lys2 801^{amber} ade2-101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1 CYH2^{R}*). The different nonselective (YPD) and selective (SD) media for the yeast strains were as described previously (8).

Preparation of crude yeast protein extracts

Cells were grown in 50−200 ml YPD to 1 OD at 600 nm, collected by centrifugation and washed twice with 20 ml of buffer A100 [20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) NaOH (pH 7.5), 5 mM $MgCl_2$, 10 mM mercapthoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM EDTA, 15% glycerol, 100 mM NaCl]. Cells were lysed in 1ml A100 using glass beads, the mixture was centrifuged and 700 μl of the supernatant were mixed with 300 μl 5 M NaCl. This solution was incubated on ice for 10 min and then centrifuged at 10,000 rpm for 30 min at 4°C. The resultant supernatant was stored at −20°C.

Plasmids

Plasmid pBend2 used for the permutation analysis of the three CDEIs was a gift from Sankar Adhya (29). DNA Probes for the phasing analysis were cloned in pBlueskript II SK+ (Stratagene). For measuring mitotic chromosome fragment loss rates all centromere constructs were cloned into pKE5 (9).

Probe construction for circular permutation analysis and determination of the bending angle

CDEI sequences for the permutation analysis were cloned as 28 bp long oligonucleotide duplexes carrying XhoI sites on either end (*CEN2*, 5'TCGAGTTAAATAAGT**CACATGATT**TATGTAGGAC3', 5'TCGAGTCCTACATAAA**TCATGTGA**CTTATTTAAC3'; *CEN3*, 5'TCGAGTCAAATATCAT**CATGTGAC**TTATTTGTAC3', 5'TCGAGTACAAATAA**GTCACATG**ATGATATTTGAC3'; *CEN6*, 5'TCGAGTATTTTTATAG**CACGTGATG**AAAAGAAAC3', 5'TCGAGTTTCTTTTC**ATCACGTG**CTATAAAAATAC3') (CDEI sequences are boldface). CDEI DNA fragments were cloned into the unique *SalI* site of pBend2 resulting in pRN162 (*CEN6*), pRN160 (*CEN2*) and pRN161 (*CEN3*). All inserts were sequenced after cloning into pBend2. The DNA fragments used for the permutation analysis were isolated from the plasmids pRN160−162 by cleavage with one of the restriction enzymes indicated in Fig. 1A and 3' end labeled using *Taq* DNA polymerase.

Bending angles were determined according to Thompson and Landy (25).

Probe construction for phasing analysis

Each construct was obtained with 3 successive PCR reactions using a set of 5 oligonucleotides. The final PCR products were cloned as *Hin*dIII/*Xba*I fragments into pBluescript II SK+. The PCR products carry the *CEN6*-CDEI sequence 5'TTGAAGACTATATTTCTTTTC**ATCACGTG**CTATAAAAA3' followed by one of the following spacer 5'GATATCC3' (pRN152), 5'GATATCCGC3' (pRN154), 5'GATATCCGTCC3' (pRN155), 5'GATATCCGTCGTC3' (pRN153), 5'GATATCCGTCGTCGC3' (pRN156) and 5'GATATCCGTCGTCGTCC3' (pRN157)and the intrinsic bend DNA sequence 5'TGGCCAAAAAACCGCAAAAAACGCCAAAAAAGG3'. The *CEN6*-CDEI-A-tract fragments were cloned as *Hin*dIII/*Stu*I fragments into pBluescript II SK+ *Hin*dIII/*Sma*I. The resulting plasmids pRN169−174 were cleaved with *Bss*HII to give the 232 bp to 242 bp long DNA fragments for phasing analysis.

Probe construction for bending analysis of *CEN6*-CDEI point mutations

CEN6-CDEI fragments carrying point mutations were cloned as 65 bp *Hin*dIII/*Xba*I DNA fragments into *Hin*dIII/*Xba*I cut pBluescript II SK+. The *Hin*dIII site is 27 bp left of CDEI, the *Xba*I 38 bp right of CDEI. The resulting plasmids pHP1[CDEI-wt], pHP3[CDEI (1-G)], pHP2[CDEI (1-T)], pHP4[CDEI (2-A)], pHP5[CDEI (2-C)], pHP6[CDEI (2-G)], pHP16[CDEI (9-A)], pHP17[CDEI (10-C)], pHP18[CDEI (1-G/9-A/10-C)], pHP8[CDEI (3-G)], pRH6[CDEI (5-T)], pRH7[CDEI (6-A)] and pRH2[CDEI (8-T)] were cleaved with *Bss*HII resulting in 199bp which were then 3' end-labelled.

Construction of *CEN6* mutations

Replacement of CDEI in *CEN6* by a DNA fragment with an intrinsic bend was done as follows: pRN152 to pRN157 (see above) were cleaved with *Eco*RV/*Hin*cII to delete CDEI and then religated. The *Acc*65I/*Ecl*136II DNA fragments from the resulting plasmids were cloned into *Acc*65I/*Swa*I cut plasmid pLA427/*CEN6* (47) giving plasmids pRN196 to pRN201. A CDEI deletion mutation was created in pLA427/*CEN6* by cleavage with *Acc*65I/*Swa*I, fill in and religation (pRN180). Mutant CDEI sequences were cloned into *BamHI*/*SalI* digested pKE5 giving pRN202−207 and pRN179 (CDEI deletion). An 108° intrinsic bend was introduced into *CEN6* by tandem ligation of 54° intrinsic bend inserts. To do this the pRN196 to pRN201 *Eag*I/*Stu*I fragments were cloned into the corresponding sites of pRN163−168 vectors. From the resulting vectors pRN208 to pRN213 the 108° intrinsic bends were cloned into pLA 427/*CEN6* thus obtaining pRN187−192. The mutant *CEN* DNA sequences were cloned as *BamHI*/*SalI* fragments into the *BamHI*/*SalI* digested pKE5 vector thus obtaining pRN181−186.

The insertions of spacer DNA into CDEII from *CEN6* were made by PCR using a mutagenic oligonucleotide located in CDEII and a second oligonucleotide left of CDEI. The mutagenic oligonucleotides are: 5'ATTAAAAAATTTAAATTAATATAATTATTTTTATAGCACGTGAT3' (CDEII+3bp), 5'ATTAAAAAATTTAAATATTAATATAATTATTTTTATAGCACGTGAT3' (CDEII+5bp), 5'ATTAAAAAATTTAAATTAATAATTATAATTATTTTTATAGCACGTGAT3' (CDEII+7bp), 5'ATTAAAAAATTTAAATATATAATTAATATAATTATTTTTATAGCACGTGAT3' (CDEII+10bp). The PCR products were cloned as *BamHI*/*Swa*I fragments replacing the wt sequence in *CEN6* in pBluescript II SK+ and from there as *BamHI*/*SalI* fragments into the chromosome fragmentation vector pKE5 yielding plasmids pMKE18, pMKE8, pMKE19 and pMKE9.

Gel retardation assays

DNA/protein binding reactions have been described (48). 2 to 5 fm of 3' labeled DNA probes were incubated with protein (amount as in figure legends) in 20 μl for 5 min at 23°C. Buffer used in the reaction was A100 plus 0.5 μg calf thymus DNA and 5 mM Pefabloc. DNA/protein complexes were analysed on nondenaturing polyacrylamide gels (7.76% acrylamide:0.24% bisacrylamide) in 25 mM Tris, 195 mM glycine (pH 8.9) buffer.

Electrophoresis was performed at 6°C with a field strength of 7 V/cm for the time given in the figure legends.

Expression of Cpf1 protein in *E.coli*

Cpf1 DNA sequences were cloned into expression vector pHK255 giving plasmid pHK255/*CPF1* (R. Lyck and J. H. H., in preparation). XL-1-Blue *E.coli* cells transformed with pHK255-I/*CPF1* were grown to OD600 0.3. Cpf1-expression was induced by addition of 1 mM IPTG (final concentration 1 mM) for 2 to 3 hours. Cells were collected by centrifugation, washed, resuspended in 500 µl A100 and sonified. The lysed cells were centrifuged and the cleared supernatant stored at −20°C.

RESULTS

The Cpf1 protein induces bending at centromere DNA element I

We investigated the effects of Cpf1 binding to the CDEI sequence from chromosome VI (*CEN6*). Binding of yeast Cpf1 protein to DNA fragments carrying CDEI sequences can be detected by gel retardation assays (Fig. 1A, lane 2). A protein extract from a *CPF1* deletion strain does not show binding to CDEI sequences (Fig. 1A, lane 3) (13). Cpf1 protein expressed in *Escherichia coli* can also bind CDEI DNA sequences (Fig. 1A, lanes 4 and 5). The CDEI DNA structure in the CDEI/Cpf1 complex was analyzed by circular permutation analysis, which allows the detection of intrinsic as well as protein induced DNA bends (22). Bent DNAs (or bent DNA/protein complexes) show a slower than expected relative electrophoretic mobility. The degree of aberrant migration depends on the bending angle as well as on the position of the bend with respect to the ends of the DNA molecules. Maximal reduction of migration is observed when the bend is located at the centre of a DNA fragment. The 34 bp oligonucleotide duplex containing the *CEN6*-CDEI sequence was cloned into the unique SalI site of pBend2 (29, Fig.1B). This allowed cleavage with different restriction enzymes, each of which generated a DNA fragment of 153 bps length. These DNA fragments carry CDEI at different positions relative to the ends of the molecules. The end-labelled DNA fragments were incubated with wildtype yeast extracts.

DNA fragments that carried CDEI centrally yielded the slowest migrating DNA/protein complexes (Fig. 1C, lanes 3, 4 and 5). The CDEI binding site present at either end of the DNA fragment led to faster migrating complexes (Fig. 1C, lanes 1 and 7). Only slight differences in the mobilities of the free DNA probes were observed (Fig. 1C). The pattern of different mobilities for the CDEI/Cpf1 complexes strongly indicated that binding of Cpf1 induces a bend in the DNA (22). Furthermore bacterially produced Cpf1 protein bends wild type CDEI DNA as does the Cpf1 present in crude yeast extracts (Fig. 1 D).

The bend angle induced by Cpf1 was determined by an empirical relationship, which has been established for the relative electrophoretic mobility retardation caused by bending and the bending angle (25). Using the relative mobilities of the fastest (B, Fig.1C, lane 7) and slowest (P, Fig.1C, lane 4) migrating complexes and correcting for the slight variations found for the free DNA probes a bend angle of 69 degrees was obtained. The relative mobilities were plotted as a function of the localization of the protein binding site on the DNA fragment. This allowed us to localize the overall bend centre in the Cpf1 binding site of CDEI (Fig. 1. E).

Cpf1 induced DNA bending is a general feature of centromeric CDEI/Cpf1 complexes

To determine if the Cpf1 induced DNA bend is a common feature of all centromeric CDEI/Cpf1 complexes, the CDEI elements from *CEN2* and *CEN3* were assayed with crude protein extract. In Fig. 1F (P) the 153bp DNA fragment used carried CDEI centrally, while the other two fragments carried CDEI at either end (called M and B, Fig. 1F). All CDEI elements showed bending upon Cpf1 binding (Fig. 1F). The bend angles for *CEN2*-CDEI and *CEN3*-CDEI were calculated to be 71 and 66 degrees respectively. These results show that Cpf1 induced DNA bending is a feature of all centromeric CDEI sequences.

Cpf1 induced DNA bending at CDEI is directed to the major groove

To discriminate between a Cpf1 induced directed DNA bend or a higher DNA-flexibility (for review, see ref, 30) we used phasing analysis. This technique is a specific method for the identification and analysis of DNA bends. It is based on the phase-dependent interaction between a protein-induced bend and an intrinsic DNA bend localized on the same DNA fragment (31, 32). If no directed bend is induced by protein binding, then no difference in complex mobility is observed, when the distance between the intrinsic and induced bend is changed. If both the protein induced bend and the intrinsic bend are directed towards the minor groove (31) and have a spacer length of n helical turns between them then a slower migrating complex will be observed. If the induced bend is directed towards the major groove and the spacer length is n helical turns, the two bends will counteract each other and lead to a faster migrating complex (Fig. 2A). To analyze Cpf1 induced bending, DNA fragments were used which carried the CDEI sequence separated by a spacer from an intrinsic DNA bend. The intrinsic DNA bend has an angle of 54° and is made up of three phased A6 tracts (31). The length of the spacer was varied in steps of 2 bps over one helical turn from 37 to 47 bps (Fig. 2A), thus rotating the intrinsic bend once around the DNA axis relative to the CDEI site. The fastest migrating complex consisting of the DNA probe and Cpf1 protein has a spacer length of 41 bps (Fig. 2B, lane 3). The mobilities of the free probes vary slightly due to a second weak intrinsic bend located in the polylinker of the vector (Fig. 2B). To determine the relative orientation of the Cpf1 induced bend, the mobilities of the complexes were plotted against spacer length (Fig. 2 C). As the intrinsic bend is directed towards the minor groove at the centre of the A-tract (31) the migration behavior seen in Fig. 2 B is in accordance with a Cpf1 induced bend towards the major groove.

The next experiments were designed to ask whether the bend induced at CDEI contributes to centromere function.

Point mutations in CDEI do not alter Cpf1 induced bending

Once the properties of the Cpf1 induced bend were established, it was of interest to test if these properties would change when non wildtype CDEI sequences were used. Mutation analysis of *CEN6*-CDEI had revealed that certain single base pair changes in CDEI increase the mitotic chromosome loss rate up to 30 fold (9). We therefore asked whether a correlation existed between the strength of the CDEI/Cpf1 bend and the *in vivo* activity of the mutant CDEI sequences. We selected CDEI point mutants

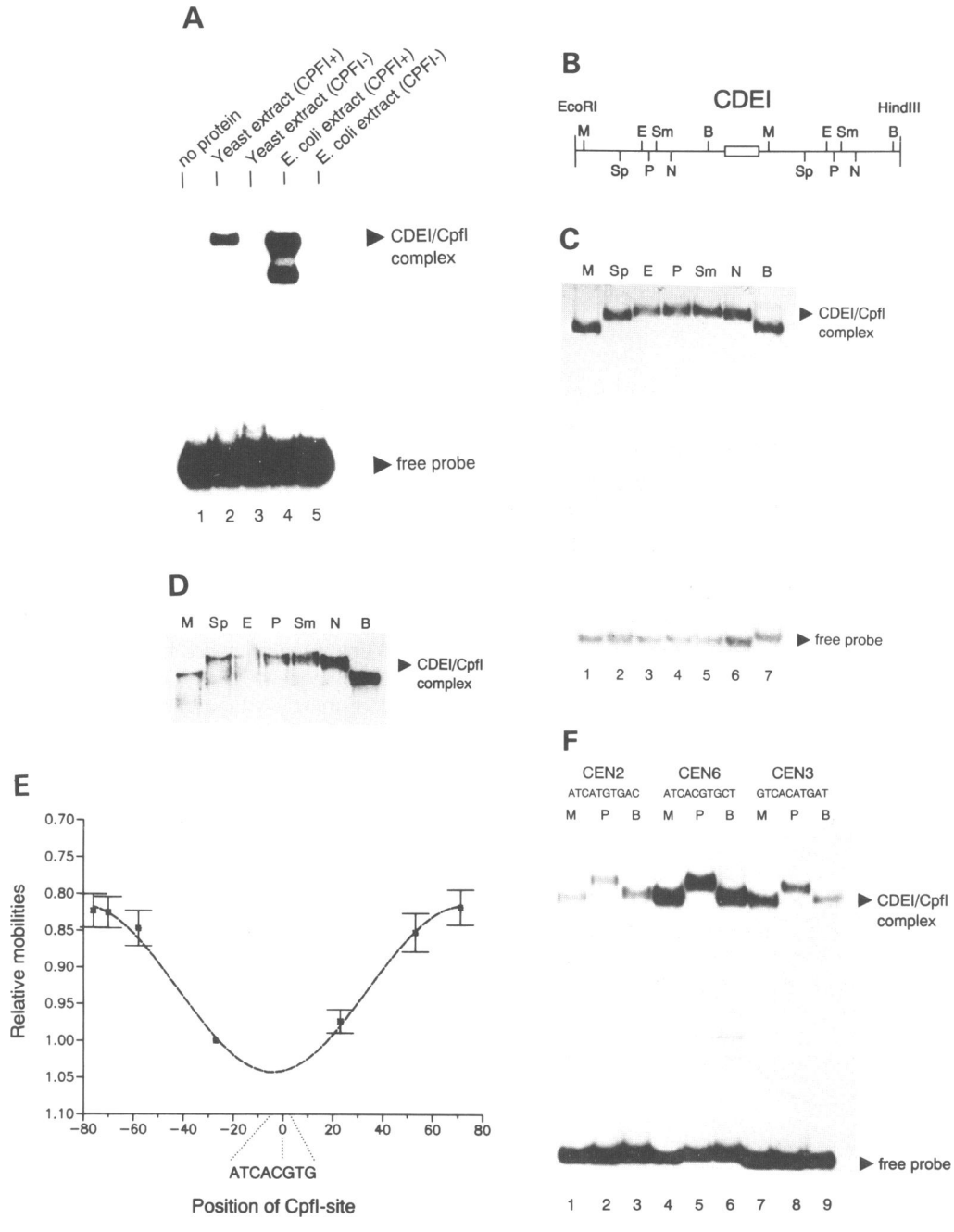

Figure 1. Circular permutation analysis of DNA bending induced by binding of Cpf1 protein. **(A)** DNA-binding activity of Cpf1 protein. Gel retardation analysis was carried out using a 199 bp DNA fragment (5 fmole/sample) carrying the *CEN6*-CDEI wildtype sequence. Lane 1: no protein added; lane 2: 1 μl (10 μg) crude yeast protein extract (*CPF1*+ strain); lane 3: 1 μl (10 μg) crude yeast protein extract (*cpf1*-strain); lane 4: 1 μl (0.2 μg) crude *E.coli* protein extract (expressing Cpf1, the second faster migrating band corresponds to a Cpf1 degradation product); lane 5: 1 μl (0.2 μg) crude *E.coli* protein extract (no Cpf1 expression). **(B)** DNA fragments used for bending analysis. The 153 bp probes used for the circular permutation analysis were generated from pBend2 (27), which carried in its polylinker the 28 bp long *CEN6*-CDEI DNA fragment (open box). The restriction sites used to create the various probes are indicated (M=*Mlu*I; Sp=*Spe*I; E=*Eco*RV; P=*Pvu*II; Sm=*Sma*I; N=*Nru*I; B=*Bam*HI). **(C)** Electrophoretic mobility shift analysis of Cpf1 protein bound to circulary permutated probes. The end-labelled 153 bp long probes (5 fmole/sample) were incubated with 1 μl (10 μg) of crude yeast protein extract (experimental procedures) and the complexes analyzed by PAGE. The probes are designated according to the restriction enzyme used for their preparation (see Fig. 1B). **(D)** Electrophoretic mobility shift analysis of *E.coli* expressed Cpf1 protein bound to circulary permutated probes as described in Fig. 1B. Only the DNA/protein complexes are shown. **(E)** Mobility plot of CDEI/Cpf1 complexes. The relative mobilities of DNA/Cpf1 complexes were corrected for the slight variations in probe mobility and normalized to the fastest migrating complex. These mobilities were plotted as a function of the position of the CDEI sequence (center of the CACGTG palindrome) relative to the ends of the probes. The CDEI sequence is indicated. The numbers on the X-axis indicate the distance in base pairs from the center of the CDEI palindrome to the ends of the DNA fragment. Data are based on seven independent experiments. Standard deviations are shown as vertical bars. As the fastest migrating complex was used for normalization, no standard deviations were calculated for it. The points are connected by the calculated best fit of a cosine function to the set of data. The curve maxima points to the overall bend center in CDEI. **(F)** Comparative permutation analysis of CDEI from centromeres 2, 3 and 6. For the different CDEI elements DNA fragments were generated as described (Fig. 1A and 1B). For each CDEI clone three DNA fragments were generated, which carried CDEI at either the left end (*Mlu*I = M), right end (*Bam*HI = B) or in the centre of the fragment (*Pvu*II = P). Gel retardation analysis was done as in Fig. 1C and the bend angles for *CEN2*-CDEI and *CEN3*-CDEI determined as described in Fig. 1E.

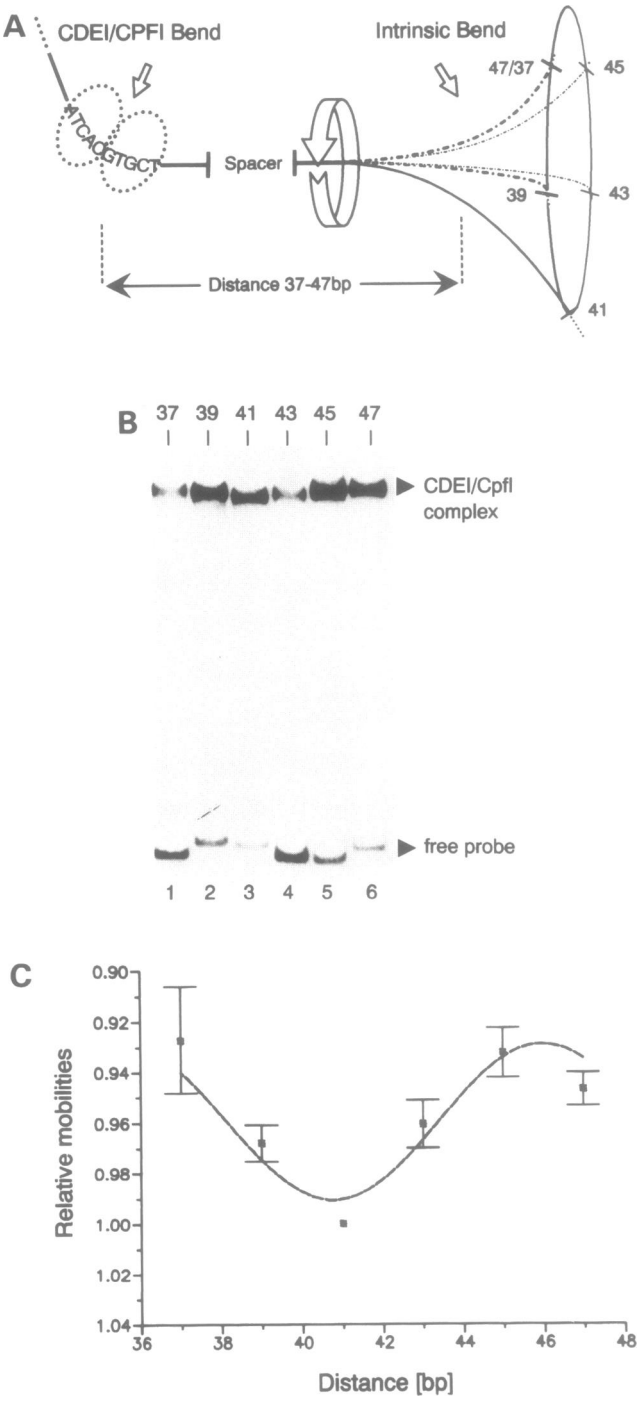

Figure 2. Phasing analysis of the Cpf1 induced bend. **(A)** Diagramatic representation of the Cpf1 induced DNA bend and an intrinsic DNA bend. The induced bend is shown to be directed towards the major groove. The scheme illustrates the result of a rotation (circular arrow) of the intrinsic bend relative to the induced bend by insertion of a spacer. The spacer insertions change the distance from the center of the CDEI palindrome (short arrow) to the center of the intrinsic DNA bend (short arrow) from 37 to 47bp. All probes vary in spacer length only. **(B)** Electrophoretic analysis of Cpf1 bound to the CDEI 'phasing' variants. Probes for phasing analysis were 228−238 bp. The intrinsic DNA bend had an estimated bend angle of 54° (25). The probes are named by the bp distance (Fig. 2A). **(C)** Mobility plot of phasing analysis. The corrected (as in Fig. 1D) relative mobilities of the DNA/protein complexes were plotted as a function of the distance from the center of CDEI to the center of the intrinsic bend (Fig. 2A). The diagram represents data from three independent experiments. Standard errors are shown as vertical bars except for the fastest migrating complex, which was used for normalization. The points are connected by the calculated best fit of a cosine function to the set of data.

which showed a 2 to 30 fold decrease in mitotic centromere function. CDEI DNA sequences carrying point mutations at positions flanking the central palindrome CACGTG, which affect the function at most 2 fold, were tested using yeast crude extract (Fig. 3A). Mutations in the central CDEI palindrome which have a strong effect on centromere function [e.g. CDEI(3-G)] did not appear to bind to Cpf1 protein from crude yeast extracts (Fig. 3B). Only when large amounts of bacterially produced Cpf1 protein were added to these mutant CDEI DNA sequences could binding be observed (Fig.3C). Electrophoretic analysis of the mutant CDEI-Cpf1 complexes revealed no significant variation in their mobility relative to wild type complexes (Fig. 3 A and C). These data suggest that the bending properties of the CDEI/Cpf1 complex do not change when mutated CDEI sequences are used.

Intrinsically bend DNA cannot substitute for Cpf1/CDEI in centromere function

We next asked whether the Cpf1 induced bend can be replaced functionally by a DNA sequence-directed bend as has been shown for some other systems (28). A series of centromere mutants was created, in which CDEI was replaced by an 108° intrinsic bend (Fig. 4 and Materials and Methods). A spacer (Sp) of 2, 4, 6, 8 or 10 bps was introduced between intrinsic bend and the rest of the *CEN* sequence thus rotating the intrinsic bend relative to the rest of the *CEN* DNA. This rotation is intimated in Fig. 4 by the term RBD (RBD, relative bend direction). These centromere mutants were placed on artificial chromosomes and the mitotic loss rate of the various constructs was analyzed as described (9) (Fig. 4). Determination of mitotic loss rates using the R/S system is highly reproducible exhibiting standard deviations between 10% and 20% only (9, 10). Deletion of CDEI and part of CDEII (strain YRN325, Fig. 4) showed an 87 fold increase in mitotic chromosome loss rate compared to wild type (strain YRN401, Fig. 4). Introduction of the 108° intrinsic DNA bend did not improve the mitotic loss rate regardless of how the new bend was placed relative to CDEII and CDEIII sequences (strains YRN407 to YRN411, Fig. 4). Similar results were obtained with a 54° intrinsic DNA bend (data not shown). Therefore an intrinsic bend cannot substitute for the CDEI/Cpf1 complex in centromere function.

In vivo phasing analysis of the centromeric CDEI/Cpf1 complex

Previous experiments had suggested that the DNA/protein complexes located at the CDEI and CDEII/CDEIII sites might interact with each other *in vivo* (9). As Cpf1 induces a directed bend at CDEI, we asked if *in vivo* function of the centromere required a specific position of the CDEI/Cpf1 complex with respect to the DNA/protein complexes located at CDEII and CDEIII. A/T rich spacers of 3, 5, 7 and 10 bps length were introduced into CDEII DNA at a position 17 bps right of CDEI. This rotated the CDEI/Cpf1 complex around the axis relative to CDEII and CDEIII (Fig. 5). These constructions were placed on artifical chromosomes and the mitotic loss rate was determined as described above. The mitotic centromere activity of these centromere mutants showed that insertions of 3 or 5 bps (up to half a helical turn) increased the chromosome loss rate 1.9 fold and 3.5 fold respectively (strains YMS618 and YMS608, Fig. 5). In contrast insertions of 7 or 10 bps (up to a full helical turn) increased the loss rate about 2.5 fold (strains YMS619 and YMS609, Fig. 5). Interestingly insertions of 3 to 10 bps do not

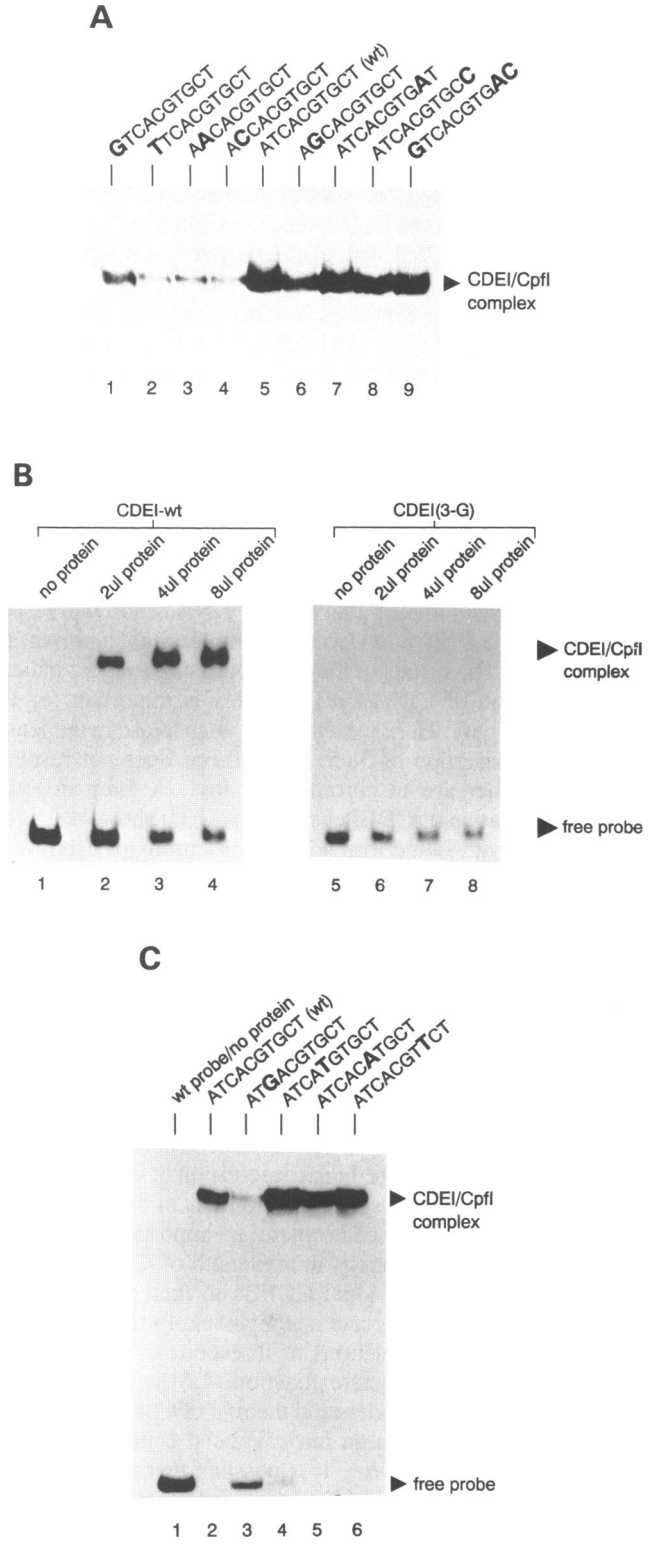

Figure 3. Complex mobilities of wt and point mutated CDEI/Cpf1. **(A)** The 199 bp *CEN6*-CDEI DNA fragments carrying point mutations in CDEI (5 fmole/sample) were incubated with 1 μl (10 μg) of crude yeast protein extract and analyzed by PAGE. The mutations are indicated by bold letters. **(B)** Increasing amounts of crude yeast protein extract (2 μl, 4 μl, 8 μl) were used in gel retardation assays with either wt-CDEI (lanes 2 to 4) or mutant CDEI(3-G) (lanes 6 to 8) (9). **(C)** Gel retardation assay of DNA fragments carrying *CEN6*-CDEI point mutations in the CDEI palindrome CACGTG using bacterially expressed Cpf1.

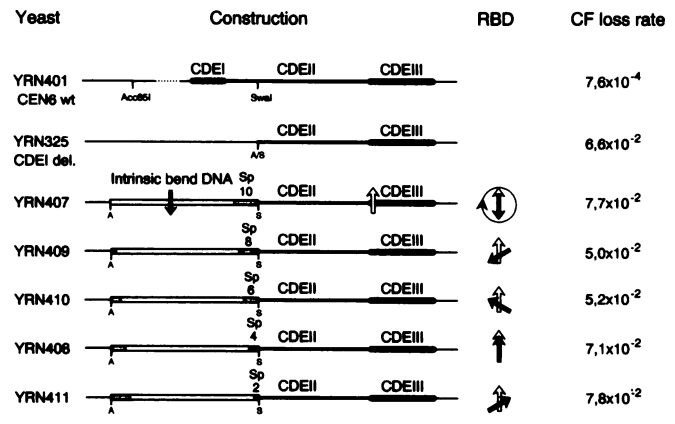

Figure 4. Diagramatic representation of wt *CEN6* and various constructs carrying intrinsic DNA bends instead of CDEI. The 108° intrinsic bend was rotated around the DNA axis relative to the rest of the CEN DNA by insertion of spacer DNA (Sp) of 2, 4, 6, 8 or 10 bps length (shaded boxes). Arrows mark the relative orientation of the intrinsic bend (black arrow) compared to CDEII and CDEIII (white arrow). This relative orientation is indicated as RBD (Relative Bend Direction). The mitotic loss rates of chromosome fragments (CF) carrying these *CEN6* constructs are given.

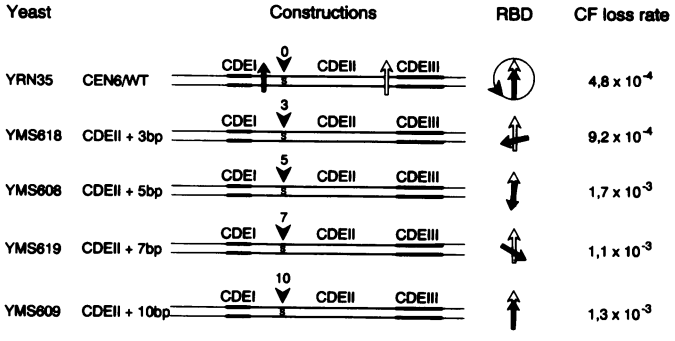

Figure 5. Diagramic representation of *in vivo* phasing analysis of the CDEI/Cpf1 complex relative to CDEII and CDEIII in CEN6. The CDEI/Cpf1 complex was rotated around the DNA axis relative to the rest of the centromere by insertion of spacer DNA (3, 5, 7 and 10 bps) within CDEII of CEN6 (indicated by short, black arrows). Long arrows indicate the relative orientation of the CDEI/Cpf1 complex (black arrow) compared to the rest of the centromere (open arrow) and are presented as RBD.

lead to a systematic increase in chromosome fragment loss rate. This suggests that both the distance and the correct spatial arrangement (phase dependence) of the CDEI/Cpf1 complex to CDEII and CDEIII are important for optimal centromere function. The effects of both parameters are probably additive thus leading to the observed pattern in loss rate (Fig. 5).

DISCUSSION

Here we show that Cpf1 upon binding to CDEI induces a bend directed towards the major groove of the DNA. Our results demonstrate for the first time protein induced DNA bending of the centromere nucleo-protein complex. Thus far DNA bending has only been demonstrated to be involved in the regulation of gene expression, DNA replication and site specific DNA recombination (21).

Cpf1 protein induces bending of centromere DNA element I (CDEI)

The bend angle in the CDEI/Cpf1 complex has been determined for three different yeast centromere DNAs (*CEN2*, *CEN3* and *CEN6*) and these are 71°, 66° and 69° respectively. The bend angles are similar to the ones induced by the proteins encoded by the proto-oncogenes *c-fos* and *c-jun* (79−94°) (32) and testis determining factor SRY (83°) (33). Cpf1 belongs to the group of basic helix-loop-helix proteins which recognize the conserved sequence CACGTG including the oncoproteins Myc and Max, the mammalian transcription factors USF, TFE3 and TFEB (34, 35, 36, 37, 38). It has been shown recently that Max homodimers, c-Myc/Max heterodimers as well as USF, TFE3 and TFEB induce a bend with an estimated angle of 53° to 80° (39, 40). Interestingly the bend induced by these proteins is directed towards the minor groove, while Cpf1 bends its target sequence towards the major groove.

Cpf1 and many other helix-loop-helix proteins recognize the consensus sequence CACGTG, which carry two CA dinucleotide steps. Such CA elements are also found in the binding site for the prokaryotic CRP protein (22). The crystallographic structure of CRP complexed with its site shows that most of the bending is brought about by kinks at the two CA dinucleotide elements (23). Such kinks are discussed to possess unusual DNA structures possibly involving partially unstacked bases (41). It is open at present, whether such kinks are present in the CDEI/Cpf1 complex.

Our studies with mutant CDEI sequences show that bending is an intrinsic property of Cpf1 binding, thus there seems to be no correlation between the *in vivo* activity and the bending properties of CDEI mutants. All mutant CDEI DNA/Cpf1 protein complexes exhibited an identical complex mobility indicating similar, if not identical bending properties. Even a mutation in the CA dinucleotide elements does not change the bend indicating that loss of one of the two dinucleotide steps is not critical for bending. This is interesting as some CDEI mutants differ in their affinity for Cpf1 protein 15-fold *in vitro* (Wilmen, Pick and Hegemann, in prep.) and *in vivo* show a *CEN* activity comparable to a CDEI deletion mutant (9). The main reason for the increase in chromosome loss observed for these CDEI mutants might therefore be the reduced affinity to Cpf1 protein rather than a change in their bending properties. Similar results have been obtained for the *E.coli* phage 434 repressor and two operator variants, where mutant binding sites exhibit decreased binding affinities but wildtype-like bending (42). However for mutations in the binding site of the cyclic AMP receptor protein (CRP) of *E.coli* it has been shown that binding affinity as well as bending angle were decreased (43).

While changes in the CDEI sequence do not change Cpf1 induced bending, deletion variants of Cpf1 protein have an effect on bending. Two different amino terminal deletions (deletions of 76 aa and 196 aa) of Cpf1 that leave the DNA-binding domain including the C-terminus intact show differences in the extent of bending or in the bending direction respectively (R.K.N. and J.H.H., unpublished data). This has also been reported for deletion variants of the bend-inducing proteins Fos and Jun and for the Flp recombinase (44, 45).

Role of the Cpf1 induced bend for *in vivo CEN* function?

We attempted to answer the question of whether the role of Cpf1 in mitotic function is the introduction of a bend in CDEI. For this the CDEI/Cpf1 complex was replaced by an intrinsic bend and these constructs were tested for mitotic centromere function. Our data show that the intrinsic bend cannot substitute for the CDEI/Cpf1 complex *in vivo*. It seems therefore likely that Cpf1 has properties necessary for precise chromosome segregation apart from its bending activity. Evidence for a purely structural role has been obtained for several prokaryotic DNA bending proteins by replacing the DNA/protein complex with an intrinsic bend (e.g. IHF, 28; CRP, 46). For eukaryotes such replacements have not been reported yet. At present we cannot rule out the possibility that the bend induced by Cpf1 is structurally different to intrinsically bend DNA. Cpf1 may bend mainly through kinks at the CA elements, while the intrinsic DNA carries bend centres over the whole DNA which is curved.

Our replacement studies suggest that Cpf1 has additional function(s) apart from inducing a DNA bend at CDEI. The CDEI/Cpf1 DNA/protein complex is needed for optimal centromere function in mitosis (13) and we have evidence that this complex interacts with other parts of the centromere complex (9). It was therefore of interest to know if the correct orientation of the CDEI/Cpf1 complex (and thus the DNA bend) with respect to the rest of the CEN DNA/protein complex was important for *CEN* function. The results of the *in vivo* phasing analysis indicate that the position of CDEI/Cpf1 complex is important for full centromere activity. Here we show that an increase in the length of CDEII by insertion of 3, 5, 7 or 10 bps does not result in a systematic increase in chromosome loss. A 5 bp insertion (relative orientation of CDEI changed by 180°) shows a 3.5 fold increase in chromosome loss whereas insertions of 7 bp or 10 bp lead to a 2.3 and 2.7 fold decrease in centromere activity respectively. This finding indicates that the correct spatial arrangement of CDE/protein complexes is important for centromere function and thus that the increase in chromosome loss rate is not simply due to an increase in the length of CDEII. It is likely that both the orientation of the CDEI/Cpf1 complex to CDEII and CDEIII as well as the length of CDEII contribute to the loss rate pattern observed in our in vivo phasing analysis. Consistent with this is the observation that the length of naturally occuring CDEII sequences in not very variable (83 bp to 86 bp, with one exception) (2). The high conservation in length of CDEII might suggest that optimal spatial arrangement of the CDE/protein complexes in the yeast centromere is important for proper function. The small variations in the length of CDEII might be adapted versions of individual CDEIIs to their corresponding CDEI and CDEIII DNA/protein complexes. Finally it has been shown previously that deletions or insertions in CDEII have a negative effect on centromere function (4,6).

At present we do not understand the role of Cpf1 induced bend in centromere function as an intrinsic bend cannot replace the CDEI/Cpf1 complex *in vivo*. It is possible that the bend DNA structure is enhancing local CDEI/Cpf1 interactions and/or that it is helpful for local unwinding of DNA such as CDEII which is extremely AT rich. Alternatively the CDEI/Cpf1 complex might facilitate interaction of the centromere with the nuclear scaffold. Another possibility could be the involvement of the CDEI/Cpf1 complex in organizing special chromatin structures at centromeres or promoters, which contain CDEI DNA sequences.

Preliminary data from our laboratory indicate that the bend induced by Cpf1 changes during the cell cycle. This might point to a role in the activation of the centromere in the mitotic cell cycle.

ACKNOWLEDGEMENTS

We are grateful to Dr U.Fleig for many helpful discussions during the preparation of this manuscript. We thank Dr M.Hollenhorst for assistance in preparing Fig. 1C and 2B, S.Heck for excellent technical assistance and synthesis of oligonucleotides, M.Bahr and D.Wegner for preparation of media, E.Gailhofer and C.Reitz for photographic work and Dr B.Boscheck for reading the manuscript. We are also grateful to R.Lyck for the bacterial Cpf1 protein and Dr S. Adhaya for a gift of pBend2. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 272, A1) to J.H.H. This research was conducted by R.K.N. and A.W. in partial fulfillment of the Ph.D. degree requirements.

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