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The *Schizosaccharomyces pombe* Mating-type Gene *mat*-Mc Encodes a Sequence-specific DNA-binding High Mobility Group Box Protein*

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The *Schizosaccharomyces pombe* gene *mat*-Mc plays a determinative role in the sexual differentiation of the fission yeast. The mat-Mc protein has been suggested to belong to a novel family of so-called high mobility group (HMG) box proteins, characterized by homology to high mobility group-1 and -2 proteins. Several HMG box proteins, including the mammalian sex-determining gene product SRY and the lymphoid transcription factors TCF-1 and LEF-1, have been shown to bind to DNA in a sequence-specific fashion. To analyze possible DNA-binding properties of mat-Mc, we have cloned and expressed its putative HMG box in *Escherichia coli*. Gel retardation analysis revealed that the mat-Mc HMG box recognizes the AACAAAG heptamer in a sequence-specific fashion. Combined T→C and A→I substitutions on both strands of the AACAAAG heptamer, which change the surface of the major groove while leaving the minor groove intact, did not interfere with sequence-specific binding of mat-Mc. Methylation interference analysis confirmed that the mat-Mc HMG box contacts adenine residues in the minor groove. By using a circular permutation assay, the mat-Mc HMG box was observed to bend DNA. These results indicate that mat-Mc is indeed a member of the HMG box family with DNA-binding characteristics assigned earlier to other members of this novel transcription factor family.

In recent years a number of structural protein motifs have been identified that mediate binding to DNA. Among these are the helix-loop-helix (1), the helix-turn-helix (2), the leucine zipper/bZip (3), the zinc finger (4), and the homeodomain (5). By characterization of the RNA polymerase 1 transcription factor UBF1, a new DNA binding motif was recently identified as a region of homology to the high mobility group (HMG)[1]–1 protein (6). This so called HMG box is defined by a loose consensus sequence of approximately 80 amino acids (7). A number of HMG box proteins have been identified since, including the *Schizosaccharomyces pombe* ste11 gene product (8), the products of the human sex-determining gene SRY (9, 10), the SRY-like *Sox* genes (9), the lymphoid-specific transcription factors TCF-1α/Lef-1 and TCF-1 (11–14), as well as the mitochondrial transcription factor mtTF1 (15). An evolutionary study of the HMG box family has revealed that two major subfamilies can be discrimated (16). One of these subfamilies contains proteins with a single HMG box, that bind to variants of the consensus motif (A/T)(A/T)CAAAG. Members in this subfamily are the TCF/*Lef* genes, the SRY/*Sox* genes, and the *S. pombe* gene *ste11*. DNA-binding occurs in the minor groove, as shown for TCF-1, TCF-1α/Lef-1, and SRY by methylation- and diethylpyrocarbonate carboxylation interference footprinting and T→C/A→I nucleotide substitutions (13, 17, 18), and is accompanied by the induction of a strong bend in the DNA helix (18, 19). The second subfamily includes proteins with multiple HMG boxes with a rather nonspecific affinity for DNA, such as the HMG-1 and -2 proteins, UBF, and mtTF1. Database searches have predicted that the product of the *S. pombe* mating-type gene *mat*-Mc should also be considered a member of the HMG box family (9–13). By homology, mat-Mc contains a single HMG box most closely related to that of ste11 and SRY, and might therefore mediate sequence-specific DNA-binding (Fig. 1).

The life cycle of the yeast *S. pombe* consists of haploid and diploid stages. Haploid cells express one of two mutually exclusive mating types, h^+ and h^- (20, 21). These haploid cells can fuse to undergo sexual differentiation and form a diploid h^+/h^- zygote upon nutrient limitation or nitrogen starvation. Diploids undergo meiosis, forming an ascus which consists of four haploid spores (20). Full meiotic competence and expression of the h^+ and h^- mating types is conferred by the mating-type region, which consists of three loci: *mat1*, *mat2*, and *mat3* (22–24). *mat2*-P (plus) and *mat3*-M (minus) are not transcribed, but their information is transposed via a cassette mechanism to the mat1 locus during a switch of mating type, giving rise to an h^+ or h^- phenotype (25, 26). Both donor loci, *mat2*-P and *mat3*-M, contain two open reading frames. These encode the P-specific polypeptides Pc and Pi and the M-specific polypeptides Mc and Mi, respectively. The Pc and Mc products appear to be necessary and sufficient to confer the full h^+ and h^- mating phenotypes, respectively, whereas all four mat genes are necessary for full meiotic competence (27).

In the budding yeast *Saccharomyces cerevisiae*, the α1 and α2 products of the *MAT* genes are DNA-binding proteins that directly interact with their target genes (28–30). Because the *S. pombe* mating-type genes *Pi* and *Mc* are predicted to encode a homeobox (27) and an HMG box protein, respectively, a similar DNA-binding regulatory mechanism offers an attractive mechanism of action. To determine whether the mat-Mc protein indeed binds DNA in a sequence-specific fashion and should be classified as an HMG box protein, we have now expressed the putative HMG box region from mat-Mc in *Escherichia coli* and tested the DNA-binding characteristics of the recombinant protein.

MATERIALS AND METHODS

Cloning of the mat-Mc HMG Box Using Polymerase Chain Reaction—Isolation of the HMG box fragment (amino acids 94–181 (27)) of the mat-Mc protein was achieved by polymerase chain reaction-aided cloning. 100 ng of total genomic DNA of *S. pombe* were used in a polymerase

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X07642.
‡ Supported by a Pionier Grant from the Dutch Organization for Scientific Research. To whom correspondence should be addressed.
[1] The abbreviation used is: HMG, high mobility group.

chain reaction together with oligonucleotides 5′-GGGGAATTCCG-GAAAGACACTACGT-3′ and 5′-GGGAAGCTTATCTTTTAACTTTATT-3′. Reaction volume was 50 µl containing 1 unit of Taq polymerase used according to the manufacturer's instructions (Promega). Cycle conditions were: 60 s at 92 °C, 60 s at 55 °C, 120 s at 72 °C with a final extension at 72 °C after 35 cycles. Reaction products were cut with *Eco*RI and *Hin*dIII and ligated into the *Eco*RI/*Hin*dIII-digested pIH902 plasmid (New England Biolabs), to yield pIH-HMGMc. After transformation into bacterial strain DH5α, bacteria were grown on LB agar plates. Colonies were picked, and plasmids were screened for a correct mat-Mc HMG box insert using sequence analysis.

Production and Purification of the mat-Mc Protein—pIH-HMGMc was transformed into TB1 bacteria. Cells were grown in LB containing 100 µg/ml ampicilin. Upon reaching mid-log phase, the bacteria were induced for 4 h by inositol 1,4,5-trisphosphate to a final concentration of 0.3 mM to produce a malE/HMGMc fusion protein. Bacteria were harvested by centrifugation (10 min, 4000 × *g*, 4 °C) and resuspended in ice-cold lysis buffer (10 mM $NaPO_4$, 15 mM NaCl and 0.05% Tween 20, pH 7.4). Cells were sonificated (three times for 3 min each), after which the cellular debris was removed by centrifugation (10 min, 15,000 × *g*, 4 °C). The malE/HMGMc fusion protein was purified in a single affinity chromatography step with amylose-coated resin beads (Biolabs) according to the manufacturer's instructions. Purification of the fusion protein was tested by SDS-polyacrylamide gel electrophoresis on a PhastSystem (Pharmacia LKB Biotechnology Inc.), using pre-cast 8–25% gradient gels. Protein bands were visualized by silver staining.

The fusion protein was found to be approximately 90% pure, as analyzed by SDS-polyacrylamide gel electrophoresis (data not shown).

Gel Retardation Assay—Retardation assay experiments were carried out as described elsewhere (13). The oligonucleotides used were: MWε-1, GGGAGACTGAGAACAAAGCGCTCTCACAC annealed to CCCGTGTGAGAGCGCTTTGTTCTCAGTCT; MWε-1Sac, GGGAGAC-TGAGCCGCGGTCGCTCTCACAC annealed to CCCGTGTGAGAGC-GACCGCGGCTCAGTCT; Tα2, CCCAGAGCTTCAAAGGGTGCC-CTACTTG annealed to GGGCAAGTAGGGCACCCTTTGAAGCTCT; MWε-I12, ACTGAGIICAAAGCGCTCT annealed to AGAGCGCTTTGC-CCTCAGT; MWε-G12, ACTGAGGGCAAAGCGCTCT annealed to AGAGCGCTTTGCCCTCAGT; MWε-I456, ACTGAGAACIIIGCGCTCT annealed to AGAGCGCCCCGTTCTCAGT; MWε-G456, ACTGAGAAC-GGGGCGCTCT annealed to AGAGCGCCCCGTTCTCAGT. All oligonucleotides were synthesized on an Applied Biosystems Inc. 381A machine.

DNA-bending Assay—The oligonucleotides TCGAGAACAAAGCG and CTAGCGCTTTGTTC (containing the cognate motif AACAAAG) were kinased, annealed, and inserted between the *Xba*I and *Sal*I sites of pBEND2 (31). Using the enzymes *Mlu*I, *Bgl*II, *Nhe*I, *Spe*I, *Xho*I, and *Bam*HI individually, fragments of constant length were excised from the resulting plasmid pBEND-HMG, effectively yielding probes with variant positions of the TCF-1 binding site. The fragments were dephosphorylated, kinased with [γ-^{32}P]ATP, and purified over acrylamide. A standard gel retardation analysis was subsequently performed.

Methylation Interference Footprinting—Probes were labeled either at the positive or negative strand with [γ-^{32}P]ATP using T4-polynucleotide kinase. Probes were purified on a sequencing gel. After annealing, the probes were purified on a non-denaturing acrylamide gel. The labeled probes were partially methylated using dimethylsulfate according to Siebenlist and Gilbert (32). 100,000 cpm of methylated probe was used in a 5-fold scale-up of the gel retardation reaction. After fractionation by gel retardation assay the gel was subjected to autoradiography. Bound and free probes were cut out and recovered by electroelution. After cleavage at the G and A residues using NaOH, the reaction products were analyzed on a 12.5% polyacrylamide, 8 M urea sequencing gel.

RESULTS AND DISCUSSION

Several HMG box proteins bind the AACAAAG and TTCAAAG heptamer sequences, as occurring in the CD3-ε and TCR-α gene enhancers (8, 11, 12, 13, 33–36). To determine if the putative HMG box of mat-Mc could bind DNA, we made use of these two heptamer motifs. Gel retardation experiments were performed using probes representing the relevant regions from the CD3-ε enhancer (probe MW-ε1) and from the TCR-α enhancer (probe Tα2). Sequences of these probes are given in Fig. 2*A*. As is shown in Fig. 2*B*, the malE/HMGMc fusion protein was capable to bind the MW-ε1 probe (*lane 2*), but did not bind the Tα2 probe (*lane 4*). The fusion protein did not bind to the negative control probe MW-ε1Sac (*lane 3*), nor did purified malE protein itself generate a retarded band with the MW-ε1 probe (*lane 1*).

For a number of HMG box proteins, notably SRY, TCF-1, and TCF-1α/LEF (17, 18), DNA binding has been shown to occur in the minor groove. The most direct line of evidence supporting this conclusion derives from A→I/T→C substitution experiments as first described by Star and Hawley (37). Changing the adenine residues in the binding motif for inosine residues, and altering the thymines for cytosines, will leave the surface of the minor groove intact while the major groove surface changes dramatically. This is illustrated in Fig. 2*C*. DNA binding still occurred when adenine residues on position 1 and 2 (Fig. 2*D*, *lane 1*) or on positions 4, 5, and 6 (*lane 3*) of the AACAAAG motif were substituted by inosine residues. Of note, A→I substitutions on positions 1 and 2 resulted in a significantly lower signal than similar substitutions on positions 4, 5, and 6. A similar phenomenon was observed with TCF-1 (17) and might indicate that determinants in the major groove contribute to binding affinity. Nevertheless, when guanine substitutions were introduced on positions 1, 2 (*lane 2*) or 4, 5, and 6 (*lane 4*) of the motif, DNA binding was completely abolished (*lanes 2* and *4*). These results indicated that DNA binding was principally dictated by determinants within the minor groove of the DNA helix.

To extend these results, methylation interference footprinting was performed. In this assay, dimethyl sulfate is used to methylate a double-stranded end-labeled DNA probe. Dimethyl sulfate methylates guanine residues at N-7 which projects into the major groove. Adenine residues are methylated by dimethyl sulfate at N-3 which occupies the minor groove. The end-labeled, methylated MW-ε1 probe was subjected to a standard gel retardation experiment, after which the free and the retarded probes were eluted and cleaved at methylated purine residues.

```
hSRY     QDRVKKPMNAFIVWSRDQRRKMALENPRMRNSEISKQLGYQWKMLTEAEKWPFFQEAQKLQAMHREKYPNYKYR | PRRKAKMLPK
SOX-4     GHIKRPMNAFMVWSQIERRKIMEQSPDMHNAEISKRLGKRWKLLKDSDKIPFIQEAERLRLKHMADYPDYKYR | PRKKVKSGNA
SOX-5     PHIKRPMNAFMVWAKDERRKILQAFPDMHNSNISKILGSRWKAMTNLEKQPYYEEQARLSKQHLEKYPDYKYK | PRPKRTCLVD
TCF-1    KPTIKKPLNAFMLYMKEMRAKVIAECTLKESAAINQILGRRWHALSREEQAKYYELARKERQLHMQLYPGWSAR | DNYGKKKRRS
LEF-1    RPHIKKPLNAFMLYMKEMRANVVAECTLKESAAINQILGRRWHALSREEQAKYYELARKERQLHMQLYPGWSAR | DNYGKKKKRK
ste11    KSSVKRPLNSFMLYRRDRQA        EIPTSNHQSISRIIGQLWRNESAQVKKYYSDLSALERQKHMLENPEYKYT | PKKRSTVRRP

cons        iK P NaFm       r k   e p       is ilG rW  l    ek  y    a   r   Hm   yP ykyr | p   k

mat-Mc   TERTPRPPNAFILYRKEKHATLLKSNPSINNSQVSKLVGEMWRNESKEVRMRYFKMSEFYKAQHQKMYPGYKYQ | RKNKVKR
```

FIG. 1. **Comparison of the amino acid sequences of the HMG box regions of known sequence-specific HMG box proteins.** Ste11 (8); SRY (9, 10); *Sox*-4 and *Sox*-5 (9, 36); TCF-1 (13), and LEF-1 (12). Positions of identity are depicted in *capitals* in the consensus sequence (*cons*). Residues occurring at identical positions in at least four out of six sequences are given in *lowercase letters*. *Below*, the postulated sequence-specific HMG box of mat-Mc is given. Sequences *within the box* are highly basic (see "Discussion"), a feature shared between all sequence-specific HMG box proteins.

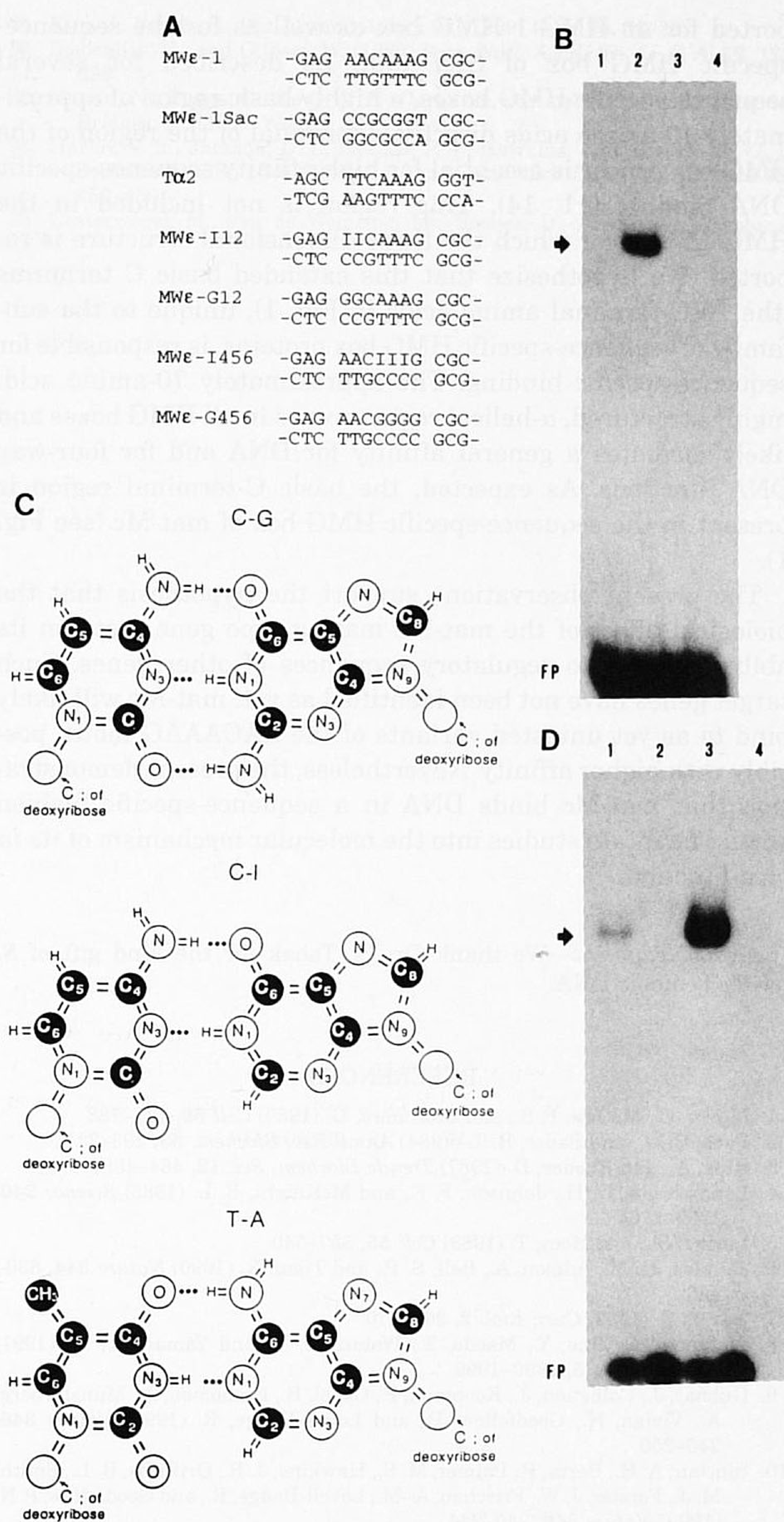

FIG. 2. **Sequence-specific minor groove binding by the HMG box of mat-Mc.** *A*, Sequences of the probes. The sequences of the heptamer binding motifs in the probes used are given with three flanking base pairs on each side. *B*, Gel retardation analysis. The malE/HMGMc fusion protein binds only to the MWε-1 probe (*lane 2*). The fusion protein does not bind to probes MWε-1Sac and Tα2 (*lanes 3* and *4*). As a control, the malE protein alone does not bind to the MWε-1 probe (*lane 1*). FP indicates the free probe. *C*, Graphic depiction of C-G, C-I, and T-A pairs. The upper side of the base pairs is exposed to the major groove, whereas the lower parts of the base pairs protrude into the minor groove. The parts of C-G and C-I pairs that form the major groove surfaces are identical, whereas C-I and T-A pairs have identical minor groove surfaces. *D*, inosine substitution experiments. The recombinant mat-Mc HMG box shows binding to the MWε-I12 probe (*lane 1*), albeit with reduced affinity, and to probe MWε-I456 (*lane 3*). Guanine substitutions on positions 1, 2, 4, 5, and 6 are not tolerated as the malE/HMGMc fusion protein does not bind to probes MWε-G12 and MWε-G456 (*lanes 2* and *4*). The *arrow* indicates the position of the malE/HMGMc retarded band.

As shown in Fig. 3, N-3 methylation of Ade-1, Ade-2, Ade-4, Ade-5, and Ade-6 on the upper strand interfered strongly with DNA binding. Methylation of Gua-3 and Gua-7 at N-7 did not significantly affect DNA binding. These results were very similar to those reported previously for the well studied HMG box genes *Lef*-1 (18), *TCF*-1, and *SRY* (17) and confirmed the notion

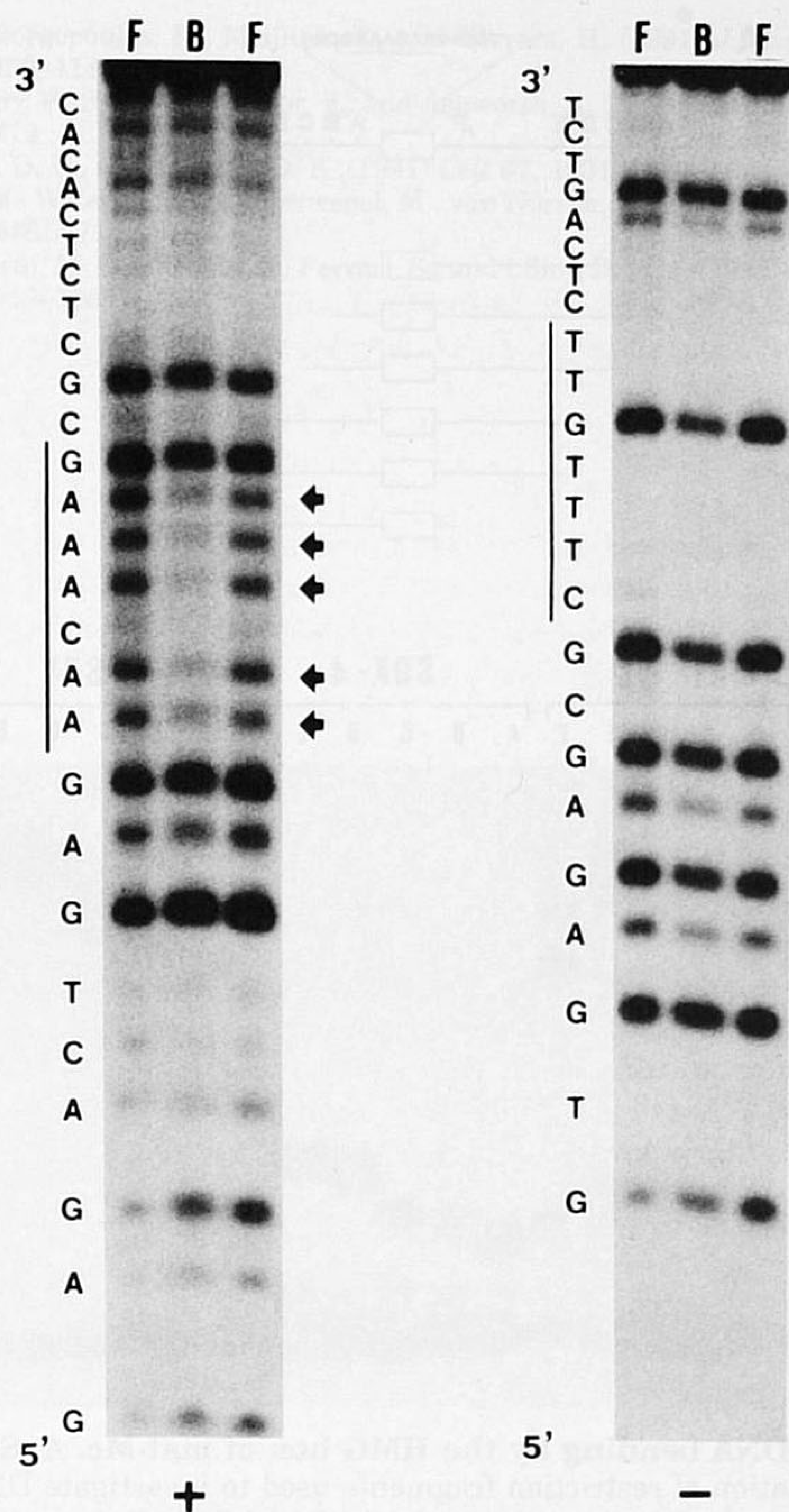

FIG. 3. **Methylation interference footprinting of malE/HMGMc on the AACAAAG motif.** The *left panel* represents interference analysis of the positive (+) strand; the *right panel* that of the negative (−) strand. *F*, cleavage products of the free probe after gel retardation analysis; *B*, cleavage products of the bound probe after gel retardation analysis. The *bar* indicates the binding motif of malE/HMGMc. The *arrows* point to methylated residues that interfere with binding. Interference is only seen with N_3 methyl groups of adenine residues on the positive strand.

that the mat-Mc HMG box binds the AACAAAG motif in the minor groove of the double helix.

LEF-1, SRY, and TCF-1 reportedly cause a flexure in the DNA upon binding (18, 19). To investigate if DNA bending is an intrinsic property of the HMG box of mat-Mc, a circular permutation assay was performed as described previously (19). The AACAAAG binding motif was subcloned into the bending vector pBEND2 (31), yielding pBEND2-HMG. This vector bears the motif flanked by two directly repeated sequences containing a number of endonuclease sites. Digestion with six individual enzymes and end-labeling of the released fragments yielded a set of probes of constant length (128 bp) but with the AACAAAG motif located at variant positions. Linear DNA displays anomalous migration behavior in acrylamide upon bending. This effect, observed as a decrease in migration velocity, is strongest when the bend occurs in the middle of the DNA probe.

As demonstrated in the *left section* of Fig. 4*B*, anomalous migration behavior was observed upon complexation of the malE/HMGMc fusion protein with the various probes excised from pBEND2-HMG. In the *Mlu*I probe used in *lane A*, the motif is located at the 3′ end of the DNA. From *lanes B* to *E*, the motif is placed progressively more to the centre of the probe. The *Bam*HI probe used in *lane F* contains the motif at its 5′ end. The protein/probe complex migrated increasingly slower when the motif was shifted from the 3′ end to the centre of the

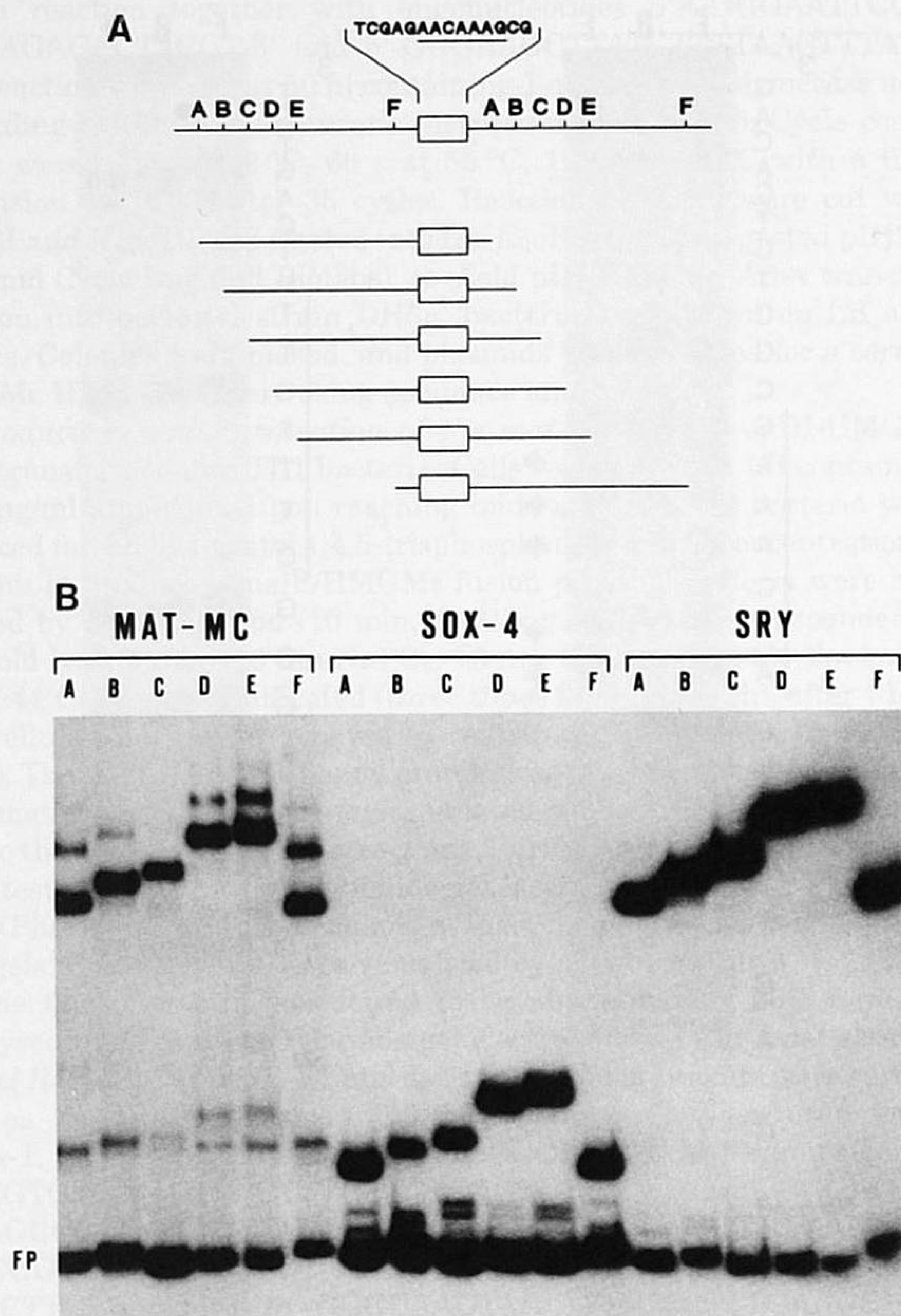

FIG. 4. **DNA bending by the HMG box of mat-Mc.** *A*, Schematic representation of restriction fragments used to investigate DNA bending. Oligonucleotides containing the AACAAAG binding motif were inserted into the pBEND2 vector (28), yielding pBEND-HMG. Two direct repeats containing endonuclease sites allow the generation of fragments of identical length on which the position of the binding motif varies. Enzymes used: *A*, *Mlu*I; *B*, *Bgl*II; *C*, *Nhe*I; *D*, *Spe*I; *E*, *Xho*I; *F*, *Bam*HI. *B*, DNA bending as observed in a circular permutation assay. DNA bending patterns of the malE/HMGMc fusion protein (*MAT-MC*), the SOX-4 HMG box (*SOX-4*), and the SRY HMG box expressed as a malE fusion-protein (*SRY*). HMG box induced bending was observed in all three cases.

probe (*lanes A–E*). As expected, placing the motif at either end of the probe resulted in protein/DNA complexes with identical mobilities (*lanes A* and *F*). This experiment demonstrated that binding of the mat-Mc HMG box to its cognate motif introduced a bend in the DNA helix. Similar migration patterns were obtained for the HMG box of Sox-4, an HMG box protein cloned recently in our laboratory (38) (Fig. 4*B*, *middle section*), and for a fusion protein of malE with the HMG box of human SRY (17) (*right section*). The rate of anomalous migration velocity observed for mat-Mc and SRY appeared very similar, indicating comparable angles of bending. Notably, SRY has been calculated to induce a bend of 85° in the DNA helix (18).

The present data indicate that the mat-Mc gene product should be classified as a sequence-specific HMG box protein. It displays all characteristics of previously characterized mammalian proteins in this HMG box subfamily: 1) it binds to the AACAAAG motif and produces a clear methylation interference footprint, 2) its sequence determinants are located predominantly in the minor groove of the DNA helix, and 3) DNA-binding is accompanied by the induction of a bend in the DNA.

A recent study describes the three-dimensional solution structure of the second HMG box of HMG-1 (HMG-1B (38)). It consists of three extended α-helices, that form an L-shaped structure. The authors suggest that this unusual shape is responsible for the curious affinity for four-way junctions reported for an HMG-1 HMG box as well as for the sequence-specific HMG box of SRY (39). As described for several sequence-specific HMG boxes, a highly basic region of approximately 10 amino acids directly C-terminal of the region of the HMG box proper is essential for high affinity sequence-specific DNA binding (11, 14). This region is not included in the HMG-1B box for which the three-dimensional structure is reported. We hypothesize that this extended basic C terminus (the 10 C-terminal amino acids in Fig. 1), unique to the subfamily of sequence-specific HMG box proteins, is responsible for sequence-specific binding. The approximately 70-amino acid, highly structured, α-helical region occurs in all HMG boxes and likely mediates a general affinity for DNA and for four-way DNA junctions. As expected, the basic C-terminal region is present in the sequence-specific HMG box of mat-Mc (see Fig. 1).

The present observations support the hypothesis that the biological effect of the mat-Mc mating type gene rests on its ability to bind to regulatory sequences of other genes. Such target genes have not been identified as yet. mat-Mc will likely bind to as yet untested variants of the AACAAAG motif, possibly with higher affinity. Nevertheless, the present demonstration that mat-Mc binds DNA in a sequence-specific fashion should facilitate studies into the molecular mechanism of its *in vivo* function.

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