A non-curved chicken lysozyme 5' matrix attachment site is 3' followed by a strongly curved DNA sequence

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ABSTRACT
Matrix attachment regions (MARs) partition the genome into functional and structural loop-domains. Here, we determined the relative matrix affinity of cloned fragments of the chicken lysozyme 5' MAR. We show that this region contains a non-curved high-affinity binding site, which is 3' followed by a strongly curved DNA sequence that exhibits weak matrix binding. DNA curvature is not a physical property required for strong matrix binding. Possible biological functions of this sequence arrangement, particularly of the strongly curved DNA, are discussed.

INTRODUCTION
Sequence-directed DNA curvature has been recognized as a structural motif common to many sites of regulatory relevance, for instance origins of replication in plasmids, prokaryotes and eukaryotes (1—5), termination sites of transcription and replication (6, 7), and eukaryotic promoters (8). Other functional important sequences such as centromers also contain sequences determining intrinsic DNA curvature (5). A novel group of sequences of significant structural and functional relevance are matrix (scaffold) attachment regions (MARs; SARs) (for review see 9, 10). MARs are mostly found upstream and downstream of regulatory transcription units (9—12) and frequently near transcriptionally cis-acting elements (9—13). The MAR located 5' of the chicken lysozyme gene has been recently shown to mediate elevated, position-less dependent expression of genes stably transfected into chicken or heterologous cells (14, 15). This suggests that MARs are important for gene expression as well as for DNA packaging. Four MARs (three within mouse and rabbit immunoglobulin genes and one between the Drosophila hsp70 heat-shock genes at locus 87A7) show sequence characteristics of intrinsically curved DNA (5). Analyzing several scaffold attachment regions of Drosophila genes, Homberger (16) found curved DNA in or near all of these SARs. These studies, however, do not bear on the issue of whether the presence of DNA curvature is a prerequisite of the matrix binding sites themselves.

We analyzed the chicken lysozyme 5' MAR and found that this matrix attachment region contains a non-curved high-affinity matrix binding site well separated (by more than 300 bp) from a strongly curved DNA sequence. Analyzing cloned DNA fragments of synthetic origin we could show that strongly curved DNA of different sequences as well as a straight control DNA bind only weakly or not at all to matrix. Thus DNA curvature seems not to be a physical property required for strong matrix binding. Yet the strong DNA curvature that we found well separated from the high-affinity binding site might have the function to guide the DNA away from the matrix, to participate in elevating gene expression of a near-by gene (14, 15) or to function as a preferred site for nucleosome formation or DNA topoisomerase I reaction. Other MARs have to be studied in similar detail to check whether this sequence arrangement (strong DNA curvature clearly separated from a weakly or non-curved binding site) is of more general nature.

MATERIALS AND METHODS
The matrix-DNA fragment binding assay was performed as described previously (11). Briefly, nuclear matrices prepared from hen oviducts were incubated for 90 min at 23 °C in binding buffer (50 mM NaCl, 10 mM Tris-HCl, 2 mM EDTA, 0.25 M sucrose, 0.25 mg/ml bovine serum albumin, pH 7.5) with 32P-labeled DNA fragments (at 20 ng/ml) and unlabeled E. coli competitor DNA (at 300—500 μg/ml). Following incubation, matrices were pelleted and washed. Purified matrix-bound fragments were then electrophoretically resolved on agarose gels and Southern-blotted onto nitrocellulose filters for autoradiography.

DNA samples (appr. 0.5 μg) were electrophoresed in three gel systems: (i) on 1.1% agarose gels in TPE (36 mM Tris, 30 mM NaH₂PO₄, 1 mM EDTA, pH 8.0), (ii) on prerun 6% acrylamide-methylenebisacrylamide (40:1) gels in TBE (45 mM Tris-borate, 1.25 mM EDTA, pH 8.6) at 6°C and about 7 V/cm

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for 14 h, and (iii) on 6% acrylamide-methylenebisacrylamide (40:1) gels in TBE at room temperature and about 9 V/cm for 9 h in the presence of 1 \( \mu \)g/ml ethidium bromide in the gel, the sample buffer and the running buffer. After electrophoresis, gels were stained with ethidium bromide and photographed under UV light. Electrophoretic mobilities were compared to those of multimers of a 123 bp fragment carrying a segment of the rat prolactin gene (BRL) (17).

Restriction enzyme digestion and subcloning into the pUC19 vector were performed as described (18). For nucleotide sequencing plasmids were digested on each side of the insert with a pair of restriction enzymes generating 3' and 5' overhanging strands, respectively. Serial external deletions were then constructed with exonuclease III and S1 nuclease, and sequences were determined by the Sanger dideoxynucleotide chain-termination method (19) using denatured plasmid DNA (20).

Plasmid pAT71 contains the homopolymer sequence \( \text{dA}_7 \text{dT}_7 \) cloned into the pJW300 vector and was sequenced following the Maxam and Gilbert sequencing protocol (21). Plasmids pK1A108 and pK4A108 contain the sequences \( \text{d(GACAGGACTC)} \) (no \( \text{dA} \) dinucleotide, straight) and \( \text{d(GACAAAACTC)} \) (same purine-pyrimidine sequence, block of \( \text{dA}_8 \), strongly curved), respectively (22). Plasmid APO (a gift from Dr. M. Gartenberg) contains the sequence \( \text{d(GCAAAGAATTGCAAGATG)(A/G)G}_2 \) (alternating blocks of \( \text{dA}_8 \) and \( \text{dA}_9 \)) cloned into the pGEM-2 vector. The \textit{Drosophila melanogaster} repetitive sequence (23) is a 1142 bp Sall-Sall fragment with an AT-content of 60\% cloned into the pGEM-1 vector. All plasmids were purified in CsCl-gradients in the presence of ethidium bromide.

RESULTS AND DISCUSSION

One MAR subfragment shows very strong matrix binding affinity

The chicken lysozyme 5' MAR (11) was used to explore the potential relationship of MARs with DNA curvature. The chicken lysozyme 5' MAR was previously localized to a 2.95 kb BamHI-XbaI fragment and was found to contain multiple binding sites (11). We cut the BamHI-XbaI fragment by restriction enzyme digestion into halves (fragments B-1-P1 and P1-P2) and additionally subcloned six smaller fragments (see Fig. 1). The relative binding affinity of these fragments to nuclear matrices was determined by monitoring the binding of labeled restriction fragments to oviduct matrices in response to 300 to 500 \( \mu \)g of unlabeled \textit{E. coli} competitor DNA (nuclear matrix-DNA fragment binding assay) (11, 13). Fig. 2B shows that the halves of the lysozyme 5' MAR (B-1-P1 and P1-P2) exhibited nearly equal binding affinities confirming our previous results (11). Of the six subfragments (listed in Fig. 1) a 446 bp HindIII-HaelII fragment (H1-HaeII) showed a much higher binding affinity than that of the other subfragments and of the MAR halves (Fig. 2A and B).

Strong matrix binding is not solely due to AT-richness

Most MARs are AT-rich but AT-richness per se is not sufficient for matrix binding (10, 12). We used two AT-rich DNA fragments as controls, (i) a repetitive sequence from the \textit{Drosophila melanogaster} Y chromosome (60\% AT) (23) and (ii) a homopolymer \( \text{dA}_7 \text{dT}_7 \) stretch. The fragment containing the repetitive sequence (HR10/20) showed a weak affinity which, nevertheless, was significantly larger than that of vector DNA sequences (Fig. 2A). Similarly, the fragment containing a run of 71 bp of homopolymer \( \text{dA} \cdot \text{dT} \) (cloned in pJAT71) showed a weak affinity to oviduct matrices (Fig. 2C). The repetitive \textit{Drosophila} sequence HR10/20 and the \( \text{dA}_7 \text{dT}_7 \) homopolymer sequence (pJAT71) showed binding affinities similar to those of some other MAR subfragments analyzed here (e.g. H-1-H1 and HaeII-Sacl), however, a much weaker binding than the subfragment H1-HaeII (Fig. 2).

DNA fragments with different degrees of AT-richness (up to 100\% and different sequence organisation thus show binding to the nuclear matrix, however, to a much smaller extent than the subfragment H1-HaeII. The high binding affinity of this fragment is thus not solely due to its AT-richness.

The strongly binding fragment H1-HaeII is not curved

Measuring the relative mobility of DNA fragments allows to sensitively detect and to locate sequence-directed DNA curvature (24–26). Curved DNA fragments have a reduced mobility (are retarded) in polyacrylamide gels while they migrate normally in agarose gels. Here, we employed this method to determine whether portions of the chicken lysozyme 5' MAR contain sequences causing intrinsic DNA curvature. The fragments analyzed here were the same as those studied in the matrix-DNA fragment binding assay (see Fig. 1 and 2). These fragments migrated normally on 1.1\% agarose control gels (Fig. 3, left panel). On 6\% polyacrylamide gels the mobilities of selectively three subfragments (HaeII-Sacl, and two longer fragments, P1-P2 and E1-X1, that contain this subfragment) were significantly reduced (Fig. 3, middle panel, lanes 1, 7, and 8). Mobility reduction was quantified by the 'k-factor', which is defined as the ratio of the gel electrophoretic mobility of the fragment to that expected of a 'normal' DNA fragment of the same size (26). A 123 bp fragment carrying a segment of the rat prolactin gene and polymerized into multimers (BRL; 17) was used as a normal sequence (22). The k-factors of most fragments ranged between 0.94 and 1.2 (Table I). Notably, the k-factor of subfragment H1-HaeII that strongly binds to nuclear matrix was 0.99. Thus, the strongly matrix-binding fragment H1-HaeII is not or only weakly curved. In polyacrylamide gels non-curved molecules are not distinguished from weakly curved molecules (27).

Theoretical studies have indicated that DNA could be bent by the introduction of AA- TT wedges (28) and by the introduction of abrupt junctions between conformationally non-related sequence blocks (29). The wedge model of Ulanovsky and Trifonov (28) assumes that AA- TT includes a particular wedge angle while all other dinucleotides do not. By use of this model the path of the DNA in the matrix binding subfragment H1-HaeII in 3-dimensional space was calculated. Only a very weak curvature was detected (as is commonly found for normal DNA fragments; data not shown), in agreement with the experimental findings.
Fig. 2: Analysis of relative binding affinities to oviduct matrices. Chicken oviduct matrices were incubated in a binding assay with end-labeled cloned DNA fragments in the presence of 300–500 μg/ml of E. coli competitor DNA. The autoradiograms show the electrophoretically resolved DNA fragments purified from a 10% aliquot of the input sample and from the matrix-associated samples. Fragments are identified on the right handed side of the figures.

Fig. 3: Analysis of sequence-directed DNA curvature by gel electrophoresis. Cloned restriction fragments of the lysozyme 5' MAR were electrophoresed on a 1.1% agarose gel (left panel), a 6% polyacrylamide (PA) gel (middle panel), and a 6% polyacrylamide gel in the presence of 1 μg/ml of ethidium bromide (EthBr) (right panel). All gels contain identical samples. Lane 1, pUC-B-1-X1 cleaved with BamHI and PvuII; lane 2, pUC-B-1-H-1 cleaved with BamHI and HindIII; lane 3, pUC-H-1-H1 cleaved with HindIII; lane 4, pUC-H-1-Xho2 cleaved with HindIII and EcoRI; lane 5, pUC-Xho4-H1 cleaved with EcoRI and HindIII; lane 6, pUC-HaeIII-SacI cleaved with BamHI and SacI; lane 7, pUC-HaeII-SacI cleaved with BamHI and SacI; lane 8, pJAT71-E1-E2 cleaved with EcoRI and XbaI; lanes M, 123 bp ladder.

A DNA fragment 3' adjacent to the matrix-binding fragment shows strong DNA curvature
The subfragment HaeII-SacI (and the two larger fragments P1-P2 and E1-X1 that contain this subfragment) showed a large mobility reduction in 6% polyacrylamide gels (k-factors of 1.47, 1.41, and 1.53, respectively; Table I). This subfragment bound only weakly to nuclear matrix (see Fig. 2).
A mobility reduction in polyacrylamide gels is also observed
SacI is in good agreement with the experimentally found one. by the wedge model for the downstream half of fragment Haell- the end (bp 660, see Fig. 4). Thus the strong curvature calculated DNA is plotted for the sequence starting with position 301 to the downstream half of fragment Haell-SacI, while the upstream of Ulanovsky and Trifonov (28) calculates a strong curvature in polyacrylamide gel of fragment Haell-SacI (and the two larger cruciform formation is even enlarged (31). In the presence of due to curvature or cruciform formation can be easily separated: portion (base pairs 1 to about 300 in Fig. 4) is straight. In Fig. 5: Two-dimensional projection of the (non-planar) path of the DNA in the second half of subfragment Haell-Sacl, base pairs 301 to 660 (end), as calculated by an algorithm due to the model of Ulanovsky and Trifonov (28). This model assumes that the dinucleotide daA forms a particular wedge angle, while all other dinucleotides are flat (a wedge angle of zero).

Strongly curved DNA fragments of synthetic origin hardly bind to nuclear matrix

The strongly curved subfragment Haell-Sacl hardly binds to the nuclear matrix (see Fig. 2), while the 5’ abutting strongly binding subfragment is non-curved. Obviously, curvature is not a prerequisite for binding to the nuclear matrix of the chicken lysozyme 5’ MAR.

We further tested this finding using synthetic DNA fragments which are strongly curved. We chose the sequence d(GACAAATCTC)8 cloned into pK4A108 and its straight control sequence d(GACAGGACTC)8 (pK1A108) (22) as well as the sequence d(GCAAAAATAGGCAAAAAT(A/G)G)2 cloned in APO (a kind gift of Dr. M. Gartenberg).

The fragments of pK4A108 and APO containing phased longer runs of daA exhibit significant retarded mobilities in polyacrylamide gels (results not shown; 22). The sequence d(GACAAAAACTC)8 of plasmid pK4A108 bound very weakly to matrices, and the sequences from APO and pK1A108 showed not binding affinity (Fig. 2C). The slightly higher binding affinity of the sequence from pK4A108 relative to that from pK1A108 might well be attributed to the higher AT-content of the former sequence. Yet both sequences have the same purine-pyrimidine sequence and are flanked by identical vector sequences. In the matrix-DNA fragment binding experiment shown in Fig. 2, the fragment from pK1A108 contains additional 172 bp vector DNA.

These results confirm that sequence-directed DNA curvature is not a physical characteristic determining strong binding to nuclear matrix. From a variety of Drosophila DNA fragments that contain scaffold attachment regions, Homberger (16) found all of them curved. From this experimental finding it cannot directly be concluded that matrix (=scaffold) attachment sites themselves are curved. In these cases it might well be, as it is in the chicken lysozyme case analyzed here, that the curved DNA sequence is adjacent to the binding site. A DNA fragment containing a matrix binding site together with a curved region would show a retardation in polyacrylamide gels. With high probability, however, the curved region would not be placed in the center of the fragment. Thus, in most cases the mobility reduction would be rather small (25) as observed by Homberger (16).

GENERAL REMARKS

We have shown that the chicken lysozyme 5’ matrix attachment region contains a non-curved high-affinity matrix binding site for DNA structures other than curved sequences, for example for DNA fragments containing a cruciform structure (30). The different origins of anomalous mobility in polyacrylamide gels due to curvature or cruciform formation can be easily separated: the addition of 1 μg/ml ethidium bromide to the samples as well as the gel running buffer reduces the migration anomaly due to DNA curvature nearly completely (26), while that due to cruciform formation is even enlarged (31). In the presence of 1 μg/ml ethidium bromide the anomalous mobility in a 6%

### Table I

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Size from DNA sequencinga</th>
<th>Apparent size from polyacrylamide gel</th>
<th>k-factorb</th>
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<tr>
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<td>1324</td>
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<td>P1-P2</td>
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<tr>
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<td>490</td>
<td>0.94</td>
</tr>
<tr>
<td>H1-HaeII</td>
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<td>460</td>
<td>0.99</td>
</tr>
<tr>
<td>Haell-Sacl</td>
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</tr>
<tr>
<td>El-X1</td>
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<td>1.53</td>
</tr>
<tr>
<td>X1-E2</td>
<td>570</td>
<td>540</td>
<td>0.96</td>
</tr>
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</table>

* a Values given accommodate, that some restriction enzyme cleavages are not directly at the fragment ends, and thus include a few base pairs of the polylinker.

b The general error of the k-factor is about ± 0.02.

c Determined from the agarose gel in Fig. 3.
3’ followed by a strongly curved DNA sequence. DNA curvature is not a property determining strong matrix binding. From two strongly curved DNA fragments of synthetic origin, one does not bind to matrix while the other binds only weakly. The strongly curved subfragment of the chicken lysozyme MAR adjacent to the high-affinity binding site also hardly binds to matrix. The strong DNA curvature 3’ adjacent to the matrix binding site might have interesting functions. First, it might help to guide the DNA away from the nuclear matrix or, second, serve as a preferred binding site for nucleosomes (33, 34). Previous studies on the terminus of SV40 DNA replication have shown that this region contains a sharp sequence-directed curvature and forms unusually stable nucleosomes in vitro (35, 36). Third, the curved DNA sequence might be a significant element playing an integral role in the positive effects MARs exert on gene expression. Recent experiments have shown that the chicken lysozyme MAR (i.e. the B-1-XI fragment) can confer elevated, position-less dependent expression to stably transfected genes (14, 15). Fourth, the DNA curvature 3’ adjacent to the matrix binding site also hardly binds to matrix. From two MARs have to be studied to learn more about the general nature of the sequence arrangement placing a strongly curved DNA sequence downstream a MAR.

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