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NUCLEAR MATRIX ATTACHMENT REGIONS AND TOPOISOMERASE II BINDING AND REACTION SITES IN THE VICINITY OF A CHICKEN DNA REPLICATION ORIGIN

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SUMMARY We have mapped nuclear matrix attachment regions (MARs), defined by their specific binding to nuclear matrices *in vitro*, and sites of topoisomerase II reaction, detected by DNA cleavage *in vitro* in the presence of the inhibitor VM-26, in the vicinity of the replication origin of the chicken α-globin gene domain. Two MARs are located close to the downstream end (in the direction of transcription) of a 3 kb fragment which includes the origin. These MARs contain sites for strong topoisomerase II binding and reaction. Our observations on this gene domain support two hypotheses concerning MARs in eukaryotic cells, namely that they are close to DNA replication origins and that they contain multiple topoisomerase II recognition sites.

A new class of sequence elements in the eukaryotic genome has been described recently, termed scaffold or matrix attachment regions (SARs or MARs; 1-9). These elements are characterised by their tenacious binding to nuclear scaffolds or matrices after digestion of the bulk of the DNA, which allows them to be isolated and cloned, and by their ability to bind to isolated nuclear scaffolds or matrices *in vitro* in a highly specific fashion. Their affinity for the nuclear matrix or scaffold has been proposed to play a role in the topological organisation of the genome. The SARs and MARs which have been studied contain multiple recognition sites for DNA topoisomerase II (EC 5.99.1.3), and are composed of relatively AT-rich sequences (4-9).

Recent observations suggest that MARs participate in the organisation of DNA replication origins in yeast cells, because autonomously replicating DNA sequence elements (ARSs), which contain putative replication origins, possess MAR activity, and deletion analysis shows that loss of MAR activity is correlated with decrease or loss of ARS function (9). In order to examine this question in higher eukaryotic cells, we have mapped the distribution of MARs in the upstream region of the chicken α-globin gene domain, where the replication origin is situated (10). We have also examined the relationship between the localisation of MARs and of sites of topoisomerase II binding and reaction *in vitro*.

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MATERIALS AND METHODS

Cell cultures and recombinant clones. AEV-transformed chicken erythroblasts (line LSCC clone A6: 11) were grown in suspension culture in DMEM with 10% fetal calf serum. The insertions of recombinant clones covering parts of the chicken α-globin gene domain, clone α5HR (12) and Hind3 clone 7 (13), were used to construct subclones for the present work, by standard protocols (14).

DNA binding to nuclear matrices. Nuclear matrices were prepared, and DNA binding *in vitro* was measured, as described in (6). Matrices from $2x10^7$ cells were used in each binding assay; sheared E.coli DNA (400 μg/ml) was used as a nonspecific competitor.

Topoisomerase II-mediated DNA cleavage. Topoisomerase II was purified to homogeneity from bovine thymocytes by a method derived from that described in (15) (Hancock, unpublished). ^{32}P-end-labeled DNA (0.05 μg) was mixed with 10-20 ng of enzyme in 150 μl of 20 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$, 50 or 150 mM NaCl, 1 mM dithiothreitol, 250 μg/ml ATP, and 5% glycerol. VM-26 was added to 10 μg/ml, and the reaction mixture was incubated for 15 min at 37°C. SDS was added to 0.5%, EDTA to 10 mM, and proteinase K to 500 μg/ml, and after incubation overnight at 37°C the DNA was purified by phenol and $CHCl_3$ extraction and analysed by electrophoresis in polyacrylamide or agarose gels.

Analytical procedures. Digestion of DNA with restriction enzymes, end-labeling, electrophoresis, autoradiography, and chemical DNA sequencing, were by standard methods (14).

RESULTS

MARs in the vicinity of the replication origin

To localise MARs near the replication origin of the α-globin gene domain, a 3 kb DNA fragment containing the origin (10) was divided into 10 subfragments, which were cloned (Fig. 1A). The insertions of these subclones were end-labeled, and a mixture of all the insertions was incubated with isolated nuclear matrices as described in (6). The DNA fragments which bound to the nuclear matrix, and the unbound fragments, were then isolated from the pelleted matrices

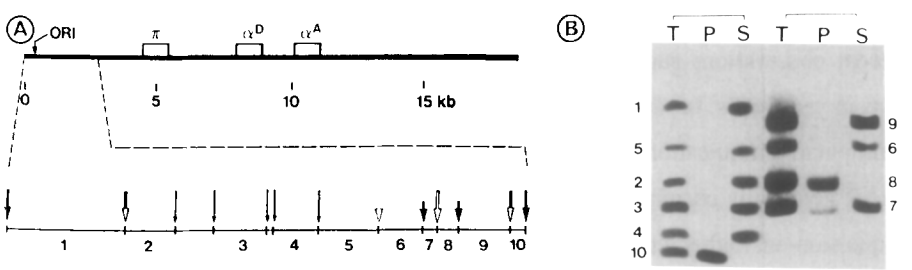


FIGURE 1 (A), a map of the chicken α-globin gene domain, containing the embryonic π and the adult α^D and α^A genes and the replication origin (ORI). Below, the ten fragments which were subcloned and employed to detect MARs; restriction sites are described in (10, 12, 13). (B), binding of end-labeled fragments to nuclear matrices *in vitro* detected by polyacrylamide gel electrophoresis. T, initial mixture of fragments; P, fragments bound to and recovered from pelleted nuclear matrices; S, fragments not bound and recovered from the supernatant.

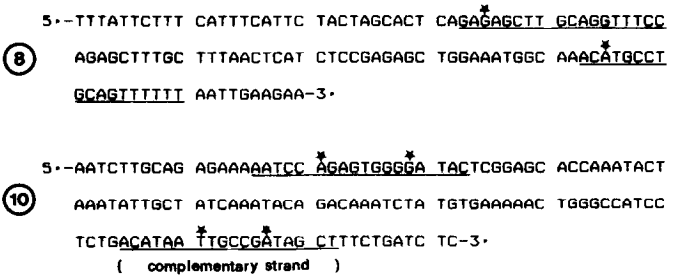


FIGURE 2 The DNA sequences of fragments 8 and 10 (Fig. 1B) which contain MARs. Underlined, avian topoisomerase II consensus sequences (16); (*), mismatches.

or from the supernatant, respectively, and separated by electrophoresis in polyacrylamide or agarose gels. The initial mixture of end-labeled fragments served as a control.

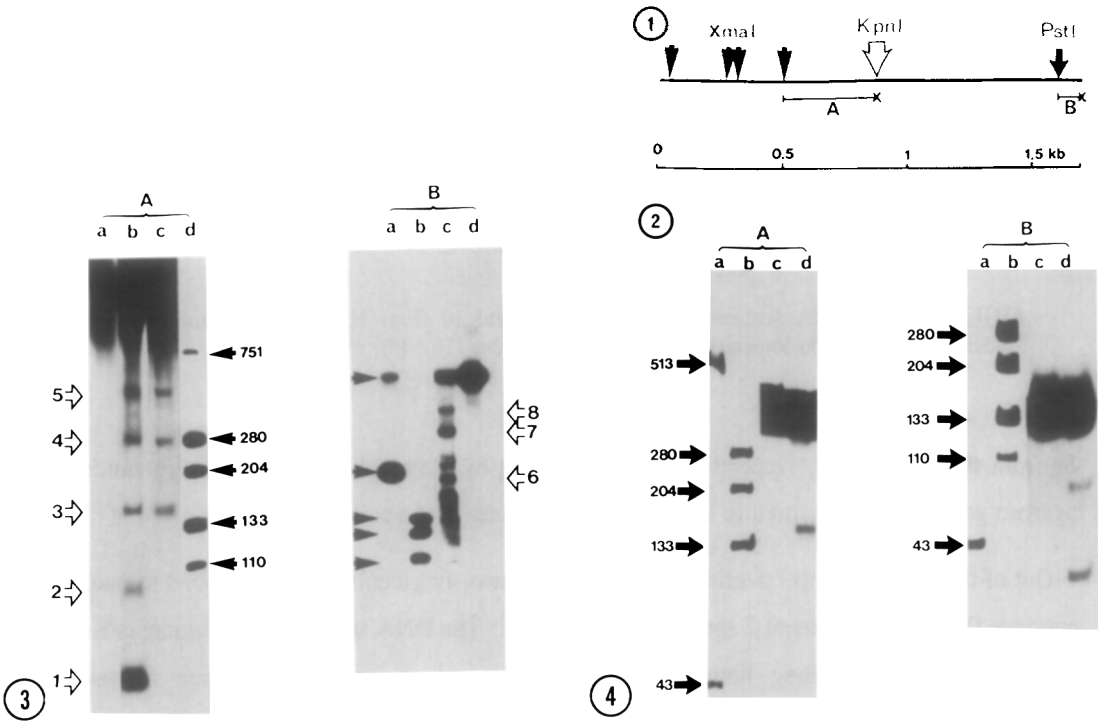
Out of the ten fragments covering this region only two, fragments 8 and 10, bound to nuclear matrices (Fig. 1B). Fragment 7 showed weak binding. The DNA sequences of fragments 8 and 10 were determined: they both contain the consensus recognition sequence for avian topoisomerase II (16) and are rich in AT (Fig. 2).

Sites of topoisomerase II binding and reaction near the replication origin

The sites of action of topoisomerase II *in vitro* in this region were mapped by employing the agent VM-26 to trap functioning enzyme molecules, whose denaturation during DNA purification results in DNA cleavage (17). The 1.7 kb insertion from the α5HR clone was labeled at the downstream end, and incubated with purified topoisomerase II in the presence or absence of VM-26. In order to distinguish sites of weak and strong binding of the enzyme, two different concentrations of NaCl (50 and 150 mM) were used. The cleavage products were separated on polyacrylamide or agarose gels, and the positions of the cleavage sites were calculated.

Specific cleavage products were observed in the presence of VM-26 (Fig. 3, open arrows), but none were seen in its absence (not shown). The shortest cleavage products (1 and 2) were seen after incubation with 50mM NaCl, but not with 150 mM NaCl. We surmised that this phenomenon arose because a minimum length of DNA is neccessary for stable binding of topoisomerase II to its recognition sequence, and this interpretation was supported by the observation that some sites which were cleaved at both 50 and 150 mM NaCl in the entire 1.7 kb fragment were cleaved only at 50 mM NaCl in shorter subfragments (not shown).

The positions of the major sites of topoisomerase II-mediated cleavage were determined more precisely by using subfragments of the α5HR insertion and analysing the cleavage products in



FIGURES 3 (left) and 4 (right) The products of *in vitro* topoisomerase II-mediated cleavage of fragment α5HR and of two subfragments.

FIGURE 3 (A), analysis in a 6% polyacrylamide gel after incubation with: a, no enzyme; b, topoisomerase II in 50 mM NaCl; c, topoisomerase II in 150 mM NaCl. d, markers (length in bp). (B), analysis in a 1.8% agarose gel; a, b, markers (length in bp); c, incubated with topoisomerase II in 50 mM NaCl; d, no enzyme. The cleavage products are labeled 1-8 (open arrows) in order of increasing length.

FIGURE 4 (1), the positions of subfragments A and B. (2), cleavage products; a, b, markers (length in bp); c, incubation with no enzyme; d, with topoisomerase II in 50 mM NaCl.

polyacrylamide gels (Fig. 4). The exact position of some of these sites was determined on DNA sequencing gels. The resulting map of topoisomerase II sites in this region, and the DNA sequence of some of them, are shown in Fig. 5.

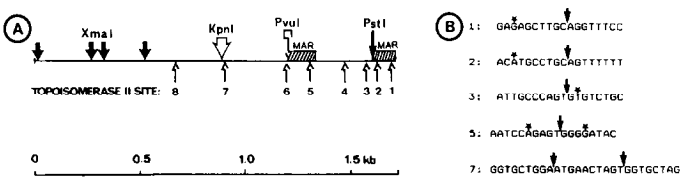


FIGURE 5 (A), a map of the two MARs and sites of topoisomerase II binding and reaction in DNA fragment α5HR. Sites 1-8 correspond to fragments 1-8 in Fig. 3. (B), the DNA sequences of sites 1, 2, 3, 5, and 7; arrows, cleavage sites; (*), mismatches with the consensus sequence for avian topoisomerase II (16).

DISCUSSION

We conclude from these observations that two MARs are situated in the vicinity of the origin of replication of the chicken α-globin gene domain. We suggest that this may be a general phenomenon in higher eukaryotic cells, because MARs are also located near to replication origins in the DHFR domain in chinese hamster cells (18). Several sites for topoisomerase II binding and reaction *in vitro* also occur near to the replication origin of the α-globin gene domain. Although it is not certain that these sites are accessible in chromatin *in vivo*, we have observed and mapped topoisomerase II sites in the same region by exposing growing cells to VM-26 (Razin and Hancock, in press). The topoisomerase II sites in the vicinity of this replication origin may be of functional importance for DNA replication itself or for its initiation, or may simply reflect the fact that MARs are in close proximity to the origin.

Our data add to those currently available concerning the consensus sequence for topoisomerase II binding and reaction. Three of the five cleavage sites which were analysed at the sequence level correspond to the consensus sequence in (16) with a single mismatch. One further sites fits the consensus sequence with two mismatches, while the sequence which contains two very close sites (sequence 7, Fig. 5B) does not show homology.

We further conclude from these experiments that both MARs in the upstream area of the chicken α-globin gene domain contain strong sites for topoisomerase II binding and reaction, extending and agreeing with studies on MARs in other cells (4-8). Further work will be directed to understanding the functional significance of the presence, adjacent to the replication origin of the α-globin gene domain, of MARs and topoisomerase II sites.

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