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**THE NUCLEAR PROTEIN P30 SPECIFICALLY INTERACTS WITH A  
NUCLEAR MATRIX ATTACHMENT REGION FROM THE RAT  
GENOME**

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**Abstract:** In our previous study, a 454 bp DNA fragment was isolated from rat genomic DNA as an element which interacts with nuclear matrix proteins, i.e. a Matrix Associated Region (MAR). Computer analyses revealed that the right half of this fragment, named RME (Rat MAR Element), possesses a high matrix association potential and is likely to be responsible for the matrix association of the whole sequence. RME was used as a probe in an electrophoretic mobility shift assay (EMSA), and with the use of Southwestern blotting, a rat liver nuclear protein which binds specifically to it was identified. Its molecular mass was estimated by SDS-PAGE as 30 kDa (p30). Polyclonal antibodies raised against protein-RME complexes caused a super-shift of specific complexes in EMSA, and bound to p30 in nuclear extracts of rat liver in Western blotting. The immunofluorescence labelling of a rat embryonic fibroblast cell monolayer with anti-p30 antibody revealed a mainly intranuclear pattern of staining.

**Key Words:** MAR, Chromatin Organization, Protein-DNA Interactions, EMS

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Abbreviations used: MAR – Matrix Associated Region; bp – base pair; kDa – kilodalton; PMSF – phenylmethylsulfonyl fluoride; DTT – dithiothreitol; EMSA – electrophoretic mobility shift assay; poly(dI-dC) – poly deoxyinosinic-deoxycytidylic acid; TAE – Tris/acetate/EDTA buffer; DAPI – 4, 6 diamidinophenylindole; FITC – fluoresceine isothiocyanate; CUE – core unpairing element; SDS-PAGE – SDS-polyacrylamide gel electrophoresis.

## INTRODUCTION

Mammalian chromatin fibers are organized into loop domains with an average size of about 75 kb, and attached to a putative nuclear skeletal structure nuclear matrix [1-4]. Genomic DNA associates with the nuclear matrix via specific genomic elements called Matrix Associated Regions (MARs). MARs play a significant structural role, and are also important for the regulation of replication and transcription [2]. They frequently co-localize with transcription regulatory sequences such as enhancers [5, 6] and insulators [7]. MARs have been found within the introns of various genes [6, 8], as well as within the proximal and distal sites flanking the 5' and 3' ends of genes [2, 9]. Although there is no rigid MAR consensus sequence, some specific structural features are common to these elements. Various nuclear proteins can specifically interact with typical MAR structural elements, and define the diverse and still poorly understood biological functions of MARs [2]. MARs interact with structural nuclear proteins such as topoisomerase II [10], lamins [11] and SP120/SAF-A [12], as well as with regulatory proteins such as hnRNPA1 [13], MAR-BP1 [14], transcription factor Pit1 [15] and many others [2]. Several types of regulatory proteins are found both in a matrix-bound and in a soluble state [14, 16, 17]. The identification of MAR-binding proteins is helpful in understanding the complex structural and regulatory role of MARs in eukaryotic genomes.

In our previous study, we isolated a 454 bp DNA fragment from the rat genome (S77423 GenBank entry) as a DNA element interacting with rat nuclear matrix proteins, and showed that this sequence is a MAR *in vitro* [18]. In this study, using computer analyses, we found that the right half of this fragment, termed RME (Rat MAR Element) (220 bp, positions 235-454), showed a high matrix association potential. We used this RME fragment as a probe to search for soluble MAR-binding proteins, and found a 30 kDa protein (p30) that specifically interacts with this sequence.

## MATERIALS AND METHODS

### Preparation of nuclei and nuclear extract

Nuclei from liver cells of 2-month old rats were isolated as described previously [3]. Purified nuclei were extracted for 1 hr at 4°C in 10 mM Tris-HCl, pH 8.0, 0.1mM MgCl<sub>2</sub>, 0.2 mM PMSF, 0.5 mM dithiothreitol (DTT), 5% glycerol and 0.35 M NaCl, centrifuged for 10 min at 5000 g, and the supernatant nuclear extracts were collected.

### DNA

The 220 bp fragment (positions 235-454 of GenBank entry S77423) was amplified and labeled with [ $\alpha$ -<sup>32</sup>P]-dATP via the PCR method, with the specific primers 5'-GGATCACGGGTTTGTGGAG-3' and 5'-TTTCGGGTCCCTGTGCGCA-3' (BioTeZ), using the pMspI8 plasmid described in [18] as a template. The same method was used for amplifying and labeling the left half (positions 4-

230 of the GenBank entry S77423) with the specific primers 5'-AAGTCCCACACCTCGGATC-3' and 5'-TGGTGTCTCTGGCAGGGGT-3' (BioTeZ). PCR products were purified by electrophoresis in 0.5% agarose [19].

#### **Electrophoretic mobility shift assay (EMSA)**

Protein-DNA complex formation was detected using electrophoretic mobility-shift assays (EMSA) [20]. The reaction mixture contained a 3 ng labeled DNA probe, 0.2-5 µg of proteins (nuclear extract or a column fraction) and 0.5-3 µg of competitor DNA in buffer A (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 5% glycerol); the total volume was 20-70 µl and the final concentration of NaCl did not exceed 0.1 M. Poly(dI-dC) (Sigma, USA) was used as a non-specific competitor, and the non-labeled DNA probe as a specific competitor. The mixture was incubated for 30 min at room temperature, and protein-DNA complexes were resolved by electrophoresis (40 mA, 3 h) on 4% polyacrylamide gels in 0.5 x TAE buffer (1 x TAE buffer is 40 mM Tris-acetate pH 8.0, 2 mM EDTA) [19]. Finally, the gels were dried and exposed to XAR-5 film (Kodak, USA).

#### **Ion exchange chromatography on DEAE-Sepharose columns**

DEAE-Sepharose (Sigma, USA), packed in a 10 x 0.7 cm column, was equilibrated with buffer B (15 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF, 5% glycerol, 50 mM NaCl). The nuclear extract was diluted 7-fold with the same buffer to a NaCl concentration of 0.05 M and loaded onto the column. After washing with buffer B, proteins were eluted in the same buffer, with a gradient of NaCl concentration increasing by 0.1 M for each 1 ml step, 10 steps in all. All the procedures were carried out at room temperature. One ml fractions were collected and stored at -20°C. The proteins from the fractions were analyzed by EMSA and SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

#### **Southwestern blotting**

The proteins were separated on 12% polyacrylamide/SDS gels and electrophoretically transferred onto a Hybond-P (PVDF) membrane (Amersham, UK) [21, 22]. The membrane was incubated in binding buffer C (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 1mM DTT) with 2.5% BSA for 5 h at room temperature, and then in the same buffer with 0.25% BSA, 20 ng/ml <sup>32</sup>P-labeled DNA probe, and 5 µg/ml competitor DNA for an additional 5 hours. Sonicated *E. coli* DNA was used as a non-specific competitor. The membrane was finally washed three times with buffer C, dried, and exposed to XAR-5 film (Kodak, USA). Alternatively, gels were fixed in 50% ethanol and then stained with Coomassie Brilliant Blue (R-250) or silver [23].

#### **Polyclonal antibodies and Western blotting**

Several preparative EMSA gels were prepared in optimal reaction conditions to obtain RME-protein complexes (complex A, Fig. 2 C, lane 3), dried, and

autoradiographed. Slices of the gel containing RME-protein complexes were ground in liquid N<sub>2</sub> and suspended in a buffer (150 mM NaCl, 10 mM phosphate buffer (Na<sub>2</sub>HPO<sub>4</sub>)/(NaH<sub>2</sub>PO<sub>4</sub>), pH 7.5) with 0.1% Tween-20. A male guinea pig was injected intraperitoneally with such suspensions [24] and boosted after 14 days. Blood was taken on day 7 after the second boost, and serum was purified and stored at 4°C with sodium azide. Rat nuclear proteins were separated in 12% polyacrylamide/SDS gels [21], electrotransferred onto Hybond-P (PVDF) membrane (Amersham, UK), and immunoblotted using the standard methods [23]. Biotinylated goat anti-guinea pig antibodies (Sigma, USA) followed by streptavidin-conjugated alkaline phosphatase (Boehringer Mannheim, Germany) were used for the detection of primary antibodies.

### **Immunofluorescence**

Rat embryonic fibroblast cells were grown on cover slips. The cells were fixed in 4% formaldehyde in KCM (135 mM KCl, 20 mM NaCl, 0.5 mM EDTA, 0.1% Triton X-100, 10 mM Tris-HCl, pH 8.0) and stored in this solution before use [25]. All the antibody reactions were carried out in KCM with 0.5-5% BSA as the blocking agent. The antibody reactions were performed for at least 1 h in a moist chamber at room temperature. The primary antibody was detected with fluoresceine isothiocyanate-conjugated (FITC) goat anti-guinea pig IgG (Sigma). The cells were counterstained with DAPI. A Zeiss Axioplan (Germany) fluorescence microscope was used for viewing and imaging.

### **Computer-based analyses**

To characterize transcription factors and MAR-binding proteins, we used the TRANSFAC 5.0 – PUBLIC [26] and S/MARt DB [27] databases, respectively. Prediction and analysis of the MARs were carried out using the BLAST (Basic Local Alignment Search Tool) [28] and MAR-Wiz 1.0 [29] programs.

## **RESULTS**

### **Characterization of the 454 bp DNA fragment (GenBank entry S77423)**

Computer analyses show that the RME sequence possesses a high matrix association potential (Fig. 1 A) and contains elements that are characteristic of MARs, i.e. topoisomerase II recognition sites (GTNWAYATTNATNNR) [30], origins of replication (ATTA, ATTTA) [31], AT-homooligonucleotide tracts [32, 33], inverted repeats [2], and fragments of kinked DNA that have generally been associated with the presence of copies of the TG, CA or TA dinucleotide separated by 2-4 or 9-12 nucleotides [34, 35]. RME possesses core unpairing elements (CUEs) (ATATWT) [36], and preferential topoisomerase II cleavage sites ((A/T)A(C/T)ATT) [37] (Fig. 1 B). Inverted repeats are able to form hairpin structures upon torsional strain on the DNA. Two alternative hypothetical hairpin structures which may form within the RME sequence (positions 345-409) are shown in Fig. 1 C. The left half of the 454 bp DNA fragment (positions 1-234) possesses a significantly lower matrix association

potential and contains few MAR structural features (Fig. 1 A, B). The presence of typical MAR structural elements within RME makes it a good probe to search for specific MAR-binding proteins.

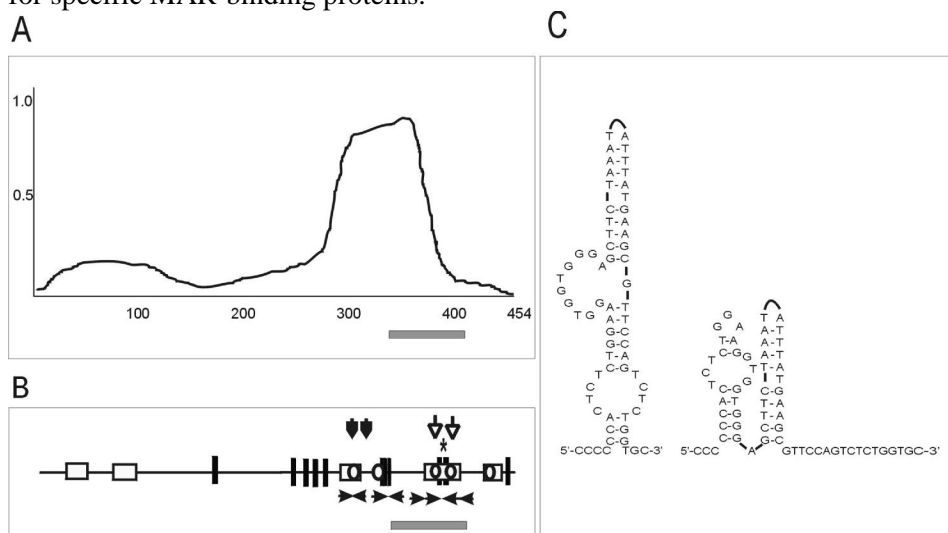


Fig. 1. Characterization of the 454 bp DNA fragment. A. A diagram of the matrix association potential of the 454 bp DNA fragment, drawn using the MAR-Wiz 1.0 program. The figures on the X-axis denote positions on the DNA sequence in bp. The calculated matrix association potential of the DNA sequence is shown on the Y-axis. B. Patterns characteristic of MARs:  $\square$ , topoisomerase II recognition sites;  $\circ$ , origins of replication;  $\bar{\text{I}}$ , AT-homooligonucleotide tracts,  $\blacktriangleright$ , inverted repeats;  $\blacktriangledown$ , kinked fragments  $\downarrow$ , core unpairing elements (CUEs); \*, preferential topoisomerase II cleavage site. C. Inverted repeats in the RME sequence (positions 345-409, denoted with a gray box in Fig.1 A, B) which could form hairpin structures; two alternative hairpin structures are shown.

### Detection of RME-binding activity in nuclear extracts from rat liver

We used the RME fragment as a probe to search for soluble nuclear proteins that interact specifically with MARs. EMSAs were used to detect complex formation between labeled RME DNA and proteins from liver nuclear extracts. One major complex (A, arrowhead on Fig. 2 A, lanes 1-3) and other complexes of lower mobility were formed in the presence of poly(dI-dC) as a non-specific competitor. An excess of non-specific or specific competitor DNA was added to the shift assay in order to examine the specificity of these complexes. All the complexes were clearly seen when a 1000-fold molar excess (3000 ng) of poly(dI-dC) was used as a non-specific competitor (Fig. 2 A, lane 3). By contrast, a 20-fold molar excess of a specific competitor (the RME fragment itself) caused the complete disappearance of complex A, but did not influence the stability of lower mobility complexes (Fig. 2 A, lane 4), confirming the high

specificity of complex A formation. Therefore, only the protein involved in complex A formation seems to be responsible for specific interactions with the RME sequence. When the left half of the 454 bp DNA fragment was used as a DNA probe, no specific complexes with proteins from liver nuclear extract were observed (Fig. 2 B).

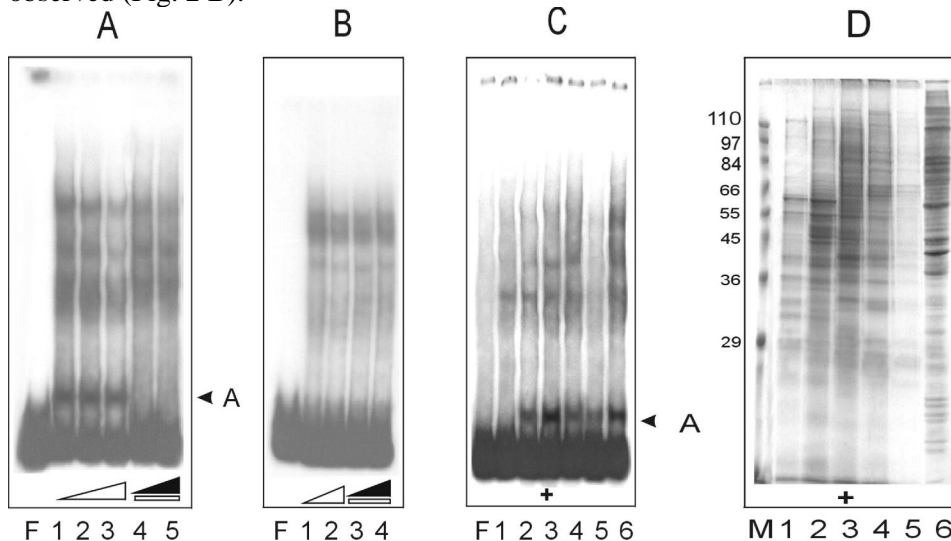


Fig. 2. Detection and partial purification of the liver nuclear protein that specifically interacts with the RME sequence. A. Detection of complexes between RME DNA and liver nuclear proteins using EMSA. One  $\mu\text{g}$  of proteins from liver nuclear extract was incubated with 3 ng of labeled RME in the presence of 1000, 2000, or 3000 ng of poly(dI-dC) (lanes 1-3, open triangle) or 50, 100 ng of non-radioactive RME DNA as a specific competitor together with 500 ng of poly(dI-dC) (lanes 4-5, filled triangle). The arrowhead denotes the position of the specific complex A. Lane F, probe DNA alone. B. Detection of complexes between the left half of the 454 bp DNA fragment (positions 4-230) and liver nuclear proteins using EMSA. One  $\mu\text{g}$  of proteins from liver nuclear extract was incubated with 3 ng of labeled DNA probe in the presence of 1000 and 2000 ng of poly(dI-dC) (lanes 1-2, open triangle) or 50, 100 ng of non-radioactive DNA probe as a specific competitor together with 500 ng of poly(dI-dC) (lanes 3-4, filled triangle). Lane F probe DNA alone. C. Detection of complexes between RME DNA and liver nuclear proteins fractionated by DEAE-Sepharose column chromatography. Each lane contained 3 ng labelled RME and 2000 ng of poly(dI-dC). Lanes 1-5, 5  $\mu\text{l}$  aliquots of the fractions eluted with 0.2, 0.3, 0.4, 0.5 and 0.6 M NaCl, respectively. Lane 6, 1  $\mu\text{g}$  of protein from non-fractionated extract. Lane F, probe DNA alone. The arrowhead denotes complex A. D. SDS-PAGE analysis of proteins in DEAE-Sepharose chromatography fractions (lanes 1-5) and total nuclear extract (lane 6). Lane M, molecular mass markers (kDa). The fraction enriched in specific RME binding activity is indicated by "+".

#### Molecular mass of the RME-binding protein

RME-binding proteins were partially purified by step-wise DEAE-Sepharose column chromatography. The fraction eluted with approximately 0.4 M NaCl

(0.4DE) contained maximal RME-binding activity (arrowhead on Fig. 2 C, lane 3) and was used in further experiments. The polypeptide profile of the corresponding chromatography fractions is shown in Fig. 2 D. In order to reveal proteins that specifically interact with the RME sequence, we used Southwestern blotting. Fig. 3 A shows that when labeled RME is incubated with electrophoretically separated proteins from the 0.4DE fraction, the major detected protein has a molecular mass of about 30 kDa (p30) (arrowhead on Fig. 3 A, lane 1). We supposed that p30 participates in the formation of the specific complex A. In order to confirm this hypothesis, we determined the molecular mass of the protein involved in the formation of complex A. A preparative EMSA gel was made under optimal reaction conditions. Excised pieces of gel containing RME-protein complex A were incubated in SDS-PAGE buffer at room temperature for 30 min, then incubated at 95°C for 5 min and loaded on 12% SDS-PAGE gel. A protein with a molecular mass of about 30 kDa (p30) was the only one found in such experiments (arrowhead on Fig. 3 B, lane 1), indicating that this protein is responsible for the specific interaction with RME and the formation of the high mobility complex A in our EMSA experiments.

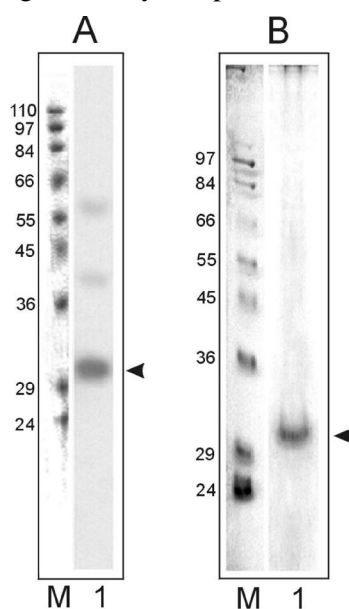


Fig. 3. Determination of the molecular mass of the RME-binding protein. A. Detection of a putative RME-binding protein by Southwestern blotting. Fifty  $\mu$ g of proteins from the DEAE-Sepharose eluted with 0.4 M NaCl were separated on SDS-PAGE, electrotransferred onto a PVDF membrane and probed with labeled RME DNA in the presence of a non-specific competitor (lane 1). Lane M, molecular mass markers (kDa). The arrowhead denotes the major RME-binding protein. B. SDS-PAGE analysis of proteins present in complexes with RME. The material from a fragment of a native gel containing the major protein-RME complex (complex A) was subjected to SDS-PAGE and stained with silver. Lane M, molecular mass marker (kDa). The arrowhead denotes the protein of 30 kDa which is responsible for specific complex formation.

### Cellular distribution of the RME-binding protein

Polyclonal antibodies were raised against the RME-protein complex A cut from preparative EMSA gels. Immune serum tested using supershift assays produced a significant supershift of the DNA-protein complexes, while pre-immune serum altered the binding only slightly (Fig. 4 A). Immune serum added to the DNA fragment in the absence of protein extract did not affect its mobility (data not shown). Thus, the antibodies reacted with RME-binding protein but not with DNA. Nuclear extract from liver cells (Fig. 2 D, lane 6) was tested via immunoblotting. The immune serum at a dilution of 1:2000 recognized a protein with an apparent molecular mass of about 30 kDa (arrowhead on Fig. 4 B, lanes 4-6). Neither pre-immune serum nor secondary antibody alone produced a signal.

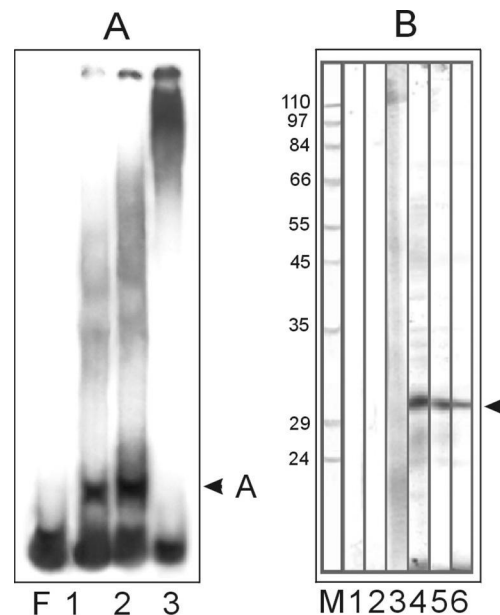


Fig. 4. Antibodies raised against the major RME-protein complex recognize protein p30. A. Analysis of RME DNA-protein complexes, using EMSA in the presence of anti-p30 antibodies. Lanes 1-3 contain 3 ng of labeled RME, 2000 ng of poly(dI-dC) and 5  $\mu$ l of proteins from the DEAE-Sepharose fraction eluted with 0.4 M NaCl. Pre-immune serum or immune serum (400-fold dilution) was added to the reaction mixture in lanes 2 and 3, respectively. B. Immunodetection of a RME-binding protein. Total liver nuclear proteins were separated electrophoretically, electrotransferred onto PVDF membranes and then probed with different immunoreagents. Lane/: lane 1, streptavidin-alkaline phosphatase alone; lane 2, biotinylated goat anti-guinea pig antibodies; lane 3, preimmune serum diluted 1000-fold; lanes 4-6, guinea pig serum against RME-binding protein from rat liver diluted 1000-, 1500-, or 2000-fold, respectively. Lane M, molecular mass markers (kDa). The arrowhead denotes the major protein of 30 kDa (p30).

An immunofluorescence assay with these antibodies was performed on a rat embryonic fibroblast cell monolayer. Cells were stained with DAPI to differentiate heterochromatin and euchromatin (Fig. 5 A). Staining with antibodies revealed a mainly intranuclear location of p30. Together with diffused nuclear staining, a number of bright dots were observed, mostly in euchromatin regions. Although the nuclei were heavily stained with antibody, some dots were also observed in the cytoplasm (Fig. 5 B).

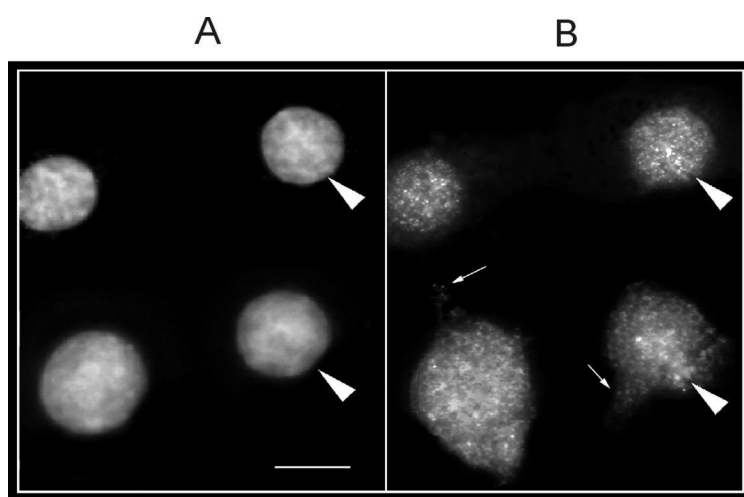


Fig. 5. Immunolocalization of the p30 in rat embryonic fibroblasts. A. Staining of DNA. B. Immunofluorescence localization of p30. The main p30 location in the euchromatin domains is marked with arrowheads; arrows denote a signal in the cytoplasm. Scale bar = 10  $\mu$ m.

## DISCUSSION

Computer analyses carried out with the help of the MAR-Wiz 1.0 and BLAST programs revealed that RME contains a number of structural elements which were typical of MARs, among them 4 potential binding sites for topoisomerase II. RME contains a preferential topoisomerase II cleavage site which, together with numerous AT-rich tracts, could be a target for cleavage by topoisomerase II, making it very likely that RME and topoisomerase II could interact *in vivo*. We found that the region of inverted repeats within the RME sequence included CUEs capable of initiating local unwinding of DNA, which could play a structural or a regulatory role. The possible formation of hairpins in the region of CUEs within a MAR may facilitate the appearance of single stranded DNA structures.

We find that the RME sequence possesses some structural features typical of MARs. However, it is clear that MARs can exhibit their structural or regulatory

functions only in complexes with specific MAR-binding proteins. In this study, we aimed to find out if the RME sequence could specifically interact with nuclear proteins. Using an EMSA strategy, we separated a DNA-protein complex A (Fig. 2 C, lane 6), which formed as a result of specific interactions between the RME sequence and a rat liver nuclear protein. The molecular mass of the protein involved was estimated by SDS-PAGE as 30 kDa, and Southwestern blotting revealed a protein with the same molecular mass. A polyclonal serum against p30 was raised and used for the investigation of the intracellular localization of p30 in rat embryonic fibroblasts, revealing that protein p30 is mainly located in the nucleus. A small amount of p30 was found in the cytoplasm, thus raising the possibility that p30 could shuttle between the nucleus and the cytoplasm.

Protein p30 could be one of the MAR-binding proteins already known. A component of hnRNP, hnRNPA1, has been shown to interact with MARs within the major histocompatibility complex class II genes, and to activate their transcription [13]. HnRNPA1 is found in the matrix-bound state as well as in the soluble state [16], and its molecular mass is about 34 kDa [38]. RME contains several ATTT motifs that could interact with hnRNPA1 [39]. The intracellular localization of p30 is similar to that of hnRNPA1, which has been shown to constantly shuttle between nucleus and cytoplasm [17]. The protein p30 detected in this study could therefore be hnRNPA1. Another protein, MAR-BP1, with a molecular mass of about 33 kDa, binds the MAR within the intronic enhancer of the IgH gene, and is found in the nuclear matrix as well as in the soluble nuclear fraction, and probably has a function in the tissue-specific transcription regulation of the IgH gene [14]. Luniak and Timchenko detected the nuclear matrix protein p28 in the soluble nuclear fraction which interacts with the DNA fragment DARC146, which supplies autonomous replication in mammalian cells [40]. Taking into account the similarity of the molecular masses, we cannot rule out the possibility that protein p30 may be identical to protein p28 or protein MAR-BP1.

Our working hypothesis is that protein p30 binds to the RME sequence *in vivo*, and may be involved in MAR functions. Using antibodies against p30, we are going to examine if p30 may be identical with one of the proteins described above, and obtain p30 in amounts sufficient for microsequencing.

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