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Interaction of MAR-Sequences With Nuclear Matrix Proteins

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Abstract The recent discovery of DNA sequences responsible for the specific attachment of chromosomal DNA to the nuclear skeleton (MARs/SARs) was an important step towards our understanding of the functional and structural organization of eukaryotic chromatin [Mirkovitch et al.: Cell 44:273–282, 1984; Cockerill and Garrard: Cell 44:273–282, 1986]. A most important question, however, remains the nature of the matrix proteins involved in the specific binding of the MARs. It has been shown that topoisomerase II and histone H1 were capable of a specific interaction with SARs by the formation of precipitable complexes [Adachi et al.: EMBO J 8:3997–4006, 1989; Izaurralde et al.: J Mol Biol 210:573– 585, 1989]. Here, applying a different approach, we were able to "visualize" some of the skeletal proteins recognizing and specifically binding MAR-sequences. It is shown that the major matrix proteins are practically the same in both salt- and LIS-extracted matrices. However, the relative MAR-binding activity of the individual protein components may be different, depending on the method of matrix preparation.

The immunological approach applied here allowed us to identify some of the individual MAR-binding matrix proteins. Histone H1 and nuclear actin are shown to be not only important components of the matrix, but to be involved in a highly efficient interaction with MAR-sequences as well. Evidence is presented that proteins recognized by the anti-HMG antibodies also participate in MAR-interactions.

Key words: matrix proteins, MAR sequences, histone H1, nuclear actin, HMG proteins

Since the early 1950s it has been known that after removal of DNA and high salt extraction of nuclei "residual chromosomal proteins" remain as an insoluble residue [Mirsky and Ris, 1951]. They were assumed to constitute a "macromolecular skeleton" to which DNA was fixed [Mounty and Dounce, 1958]. The first electron microscopic observation of these nuclear skeletal structures was accomplished in 1960 [Georgiev and Chentsov, 1960] and later, the term "nuclear matrix" was introduced [Berezney and Coffey, 1974], referring to the highly structured residual framework consisting almost entirely of protein.

Since then, an overwhelming amount of results and studies has been published, describing the isolation and characterization of skeletal structures from both interphase nuclei and metaphase chromosomes. Despite the enormous experimental evidence, however, there are still ambiguities concerning the very existence of the nuclear matrix [reviewed by Cook, 1988] and the dependence of its appearance and composition upon the isolation procedure [Kaufman et al., 1986; Fey et al., 1986; Verheijem et al., 1988]. Currently, two terms are used referring to the nuclear skeleton: *matrix* or *scaffold,* depending solely on the method (high salt or LIS) employed for nuclear extraction [reviewed by Gasser, 1988]. Although both procedures have their advantages and disadvantages [Mirkovitch et al., 1984; Fey et al., 1986; Smith et al., 1987], what is important for us is that both types of residual structures recognize and specifically interact with the same class of DNA sequences. These sequences have been named "matrix-association regions" or "scaffold-association regions" (MARs/SARs, respectively) [Mirkovitch et al., 1984; Cockerill and Garrard, 1986; Gasser and Laemmli, 1986]. It is believed that MARs (SARs) are an evolutionarily conserved class of sequences that anchor the chromosomal loops (5–200 kbp) to the skeletal structures. According to a current hypothesis, these loops represent separately controlled domains having

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not only structural, but also functional significance [Gross and Garrard, 1987; Bodnar, 1988; Goldman, 1988].

A most important and difficult question remains the identification of the individual matrix proteins that interact specifically with the MARs (SARs). Many proteins have been implied to constitute the nuclear skeleton [reviewed by Verheijem et al., 1988; Nelson et al., 1986]. But it is only topoisomerase II and histone H1, for which besides their presence in the nuclear matrix [Berrios et al., 1985] or scaffold preparations [Earnshaw et al., 1985; Gasser et al., 1986] a specific interaction with MARs (SARs) has been demonstrated [Blasquez et al., 1989; Sperry et al., 1989; Adachi et al., 1989].

In the present study we have attempted to "visualize" the nuclear skeletal proteins that could be involved in a specific interaction with a MAR. While this paper was in preparation, results from two other laboratories appeared, exploiting similar approach and describing proteins interacting specifically with MAR-sequences [von Kries et al., 1991; Hakes and Berezney, 1991; Nakayasu and Berezney, 1991]. Their possible relation to the proteins observed here is discussed. The term *nuclear matrix* has been kept for both types of skeletal structures obtained after either LIS or high-salt extraction of interphase nuclei.

MATERIALS AND METHODS

Isolation of Nuclear Matrices

Nuclear matrices were prepared from freshly isolated nuclei of mouse liver or Ehrlich ascites tumor cells by two methods: by LIS extraction of DNase I digested nuclei [Mirkovitch et al., 1984], or by high-salt extraction of DNase I digested nuclei as described in Cockerill and Garrard [1986], with the only modification that Ca^{2+} in the digestion buffer was omitted in an attempt to avoid protease activation.

Nuclear proteins solubilized by the extraction treatments and the nuclear matrix proteins obtained as an insoluble residue were fractionated in SDS-polyacrylamide gels [Laemmli, 1972] without prior heating of the samples. Electrophoretically fractionated proteins were electroblotted onto nitrocellulose filters and processed further as described [Miskimins et al., 1985; Herlt et al., 1988]. The protein samples were loaded into wide gel wells, such that after blotting and cutting of the nitrocellulose sheets many strips containing identical protein profiles were obtained. The transfer of the proteins after electroblotting was checked by staining one nitrocellulose strip with amido black.

Detection of the DNA-Binding Proteins

Nitrocellulose strips containing the electroblotted proteins were soaked in matrix-binding buffer (MBB) [Cockerill and Garrard, 1986], containing 10 mM Tris (pH 7.4), 2 mM EDTA, 100 mM NaCl, and up to 2 mg/ml of bovine serum albumin (BSA). The high concentration of serum albumin prevented nonspecific "sticking" of the probe to the filters. After 30 min shaking in MBB, at room temperature, the preincubation continued for another 30 min in the presence of the desired amount of unlabeled competitor DNA. At the end of the preincubation, the labeled probe was introduced and the binding reaction was carried out for 60 min at room temperature with gentle shaking. Unbound radiolabeled probe was washed off the filters by three washings in MBB, containing 150–200 μg/ml *E. coli* DNA and an elevated (0.5 M) concentration of NaCl. After air-drying, the filters were exposed to X-ray film overnight.

Labeling of the DNA Probes

The recombinant plasmid pBs MAR 3.1, containing a 345 bp Hind III–Dra I fragment of pG19/45 [Cockerill and Garrard, 1986] was used as a probe (a kind gift from Dr. W.T. Garrard, UT Dallas). The plasmid was labeled by nick-translation, or alternatively, the MAR-containing sequence was excised from the vector and 5′-end labeled by polynucleotide kinase. When total genomic DNA was used as a probe, it was sheared to 0.5–2 kbp and labeled by nick-translation. Equal amounts (1×10^7 cpm/ml) of radiolabeled probes were used in the binding reactions. Thus, the intensity of the bands reflected indirectly the strength and the specificity of the interaction.

Immunological Identification of Some MAR-Binding Proteins

Anti-nuclear actin antibodies were prepared and kindly provided by Dr. A. Uschewa, IMB Sofia [Valkov et al., 1989]. Antibodies against a mixed preparation of high mobility group proteins (HMGs) were kind gifts from Drs. S. Dimov and V. Roussanova, IMB Sofia; serum against topoisomerase II was a gift from Dr. Grosse, Heidelberg, Germany. Gel-fractionated

matrix proteins were probed with the antibodies by the Western blot technique [Towbin et al., 1979]. The nitrocellulose strips with the bound antibodies were subsequently interacted with the labeled MAR probes as described above. When necessary, immune complexes were visualized with a goat-antirabbit IgG horseradish peroxidase conjugate.

RESULTS

Interaction of the Proteins of LIS-Extracted Matrices With MARs

Preferential interaction of some matrix proteins with a MAR. The proteins solubilized by the LIS extraction and the insoluble matrix proteins were electroblotted onto nitrocellulose filters after fractionation in SDS-containing polyacrylamide gels (Fig. 1a,b). Interaction of preincubated filter strips containing the matrix proteins with a labeled (nick-translated) κ-immunoglobulin gene MAR-containing plasmid showed a specific binding of the plasmid to some of the proteins (Fig. 1c,d). From the matrix fraction, two proteins with apparent molecular masses of 45 Kd (p45) and 39 Kd (p39) are seen to bind the probe (lane d). Because a signal from the corresponding proteins of the soluble fraction was absent (lane c), these proteins could be considered true matrix proteins.

However, the strongest signal observed is with several lower molecular mass proteins present in the soluble fraction as well: one is with the mobility of histone H1 and two others with apparent mobilities corresponding to 28 Kd (p28) and 16 Kd (p16) proteins, respectively (Fig. 1c,d). It is important that these proteins, although present in only minor quantities in the matrix (as judged from the Coomassie-blue staining) bind MAR-DNA with a remarkable efficiency.

Since an apparent preference of the matrix proteins for binding single-stranded DNA has been reported [Comings and Wallack, 1978; Hakes and Berezney, 1991] and since nick-translated probes contain single-stranded regions, it was necessary to test the binding behavior of a double-stranded MAR. For this reason, the MAR-containing insert was cut from the plasmid, 5′-end-labeled by polynucleotide kinase and used as a binding probe. It may be seen that the same proteins bound the double-stranded MAR as efficiently as the nick-translated whole plasmid (Fig. 2A, lane a).

Exactly the same proteins bound the MAR when matrices isolated from actively dividing cells–Ehrlich ascites tumor cells were tested for their MAR-binding capacity (not shown). This result is in good agreement with the current understanding that some of the nuclear matrix proteins are conserved in different tissue types and in evolution [reviewed in Gasser, 1988; Blasquez et al., 1989].

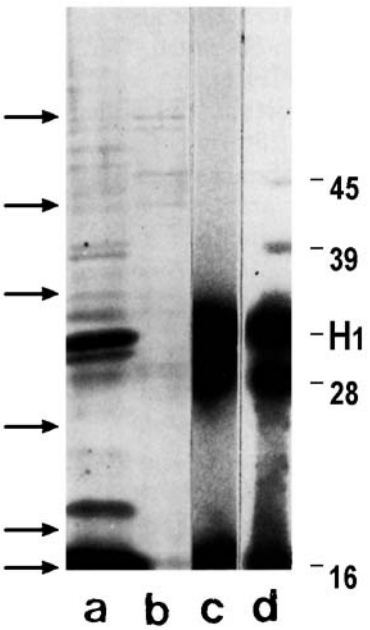

Fig. 1. LIS-extracted mouse liver nuclei. **Lane a:** Nuclear proteins solubilized by the extraction and recovered from the supernatant. **Lane b:** Nuclear matrix proteins recovered from the pellet. Fractionation in SDS-polyacrylamide gels and Coomassie blue staining. The arrows show the position of the protein molecular markers in the order 68 kD, 45 kD, 35 kD, 24 kD, 18 kD, and 14 kD. **Lane c:** The soluble nuclear proteins shown in lane a after electroblotting onto nitrocellulose filters and incubation with labeled MAR in the presence of 150 μg/ml of *E. coli* DNA. **Lane d:** The nuclear matrix proteins, corresponding to those shown in lane b, after interaction with a labeled MAR under the conditions described for lane c.

The specificity of the observed interaction was studied by testing the dependence of the protein binding activity on the sequence composition of the probe.

Sequence specificity of the DNA bound by the nuclear matrix proteins. The fact that among the numerous proteins from both the supernatant and the matrix only a few bound the labeled MAR already suggested specificity of the interaction. The next question that was asked was whether this reaction is dependent upon the sequence composition of DNA. To test this, total, sheared, and labeled mouse genomic DNA was used as a substrate instead of the MAR-containing plasmid. The reaction was carried

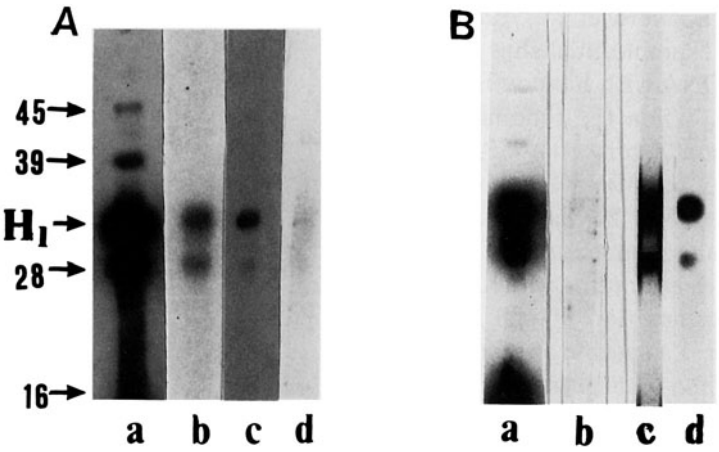

Fig. 2. **A:** Nuclear matrix proteins reacted with **(lane a)** isolated 5'-end labeled MAR-sequence; **(lane b)** nick-translated total mouse genomic DNA; **(lane c)** nick-translated empty vector. These reactions were performed in the presence of 150 μg/ml *E. coli* DNA. **Lane d:** Binding of a MAR in the presence of 10 μg/ml unlabeled pBs MAR 3.1 as a competitor (compare lanes a and d). **B: Lane a:** Binding of MAR to the matrix proteins in the presence of 35 μg/ml unlabeled total mouse DNA as a competitor. **Lane b:** Nick-translated mouse genomic DNA incubated with the matrix proteins in the presence of the same amount of unlabeled competitor as in lane a. **Lane c:** Binding of MAR to the matrix proteins in the presence of 0.5 M NaCl. **Lane d:** MAR-matrix binding in the presence of 100 mM phosphate buffer.

out under the same binding conditions, in the presence of 150 μg/ml of *E. coli* DNA. As shown in Fig. 2A, lane b, H1 and p28 do bind genomic DNA, but with a considerably lower affinity as compared to the MAR-containing probe (compare lanes a, b). An important observation is the complete failure of genomic DNA to bind to p39 and p45.

This result supports our supposition that the binding to p39 and p45, as well as the strong signal observed with H1 and p28 are a result of the specific interaction of the matrix proteins with the MAR-sequence. The poor binding of nick-translated "empty" vector to the matrix (lane c) is in agreement with this. The idea was supported further by a binding experiment in which a MAR-containing plasmid was used as a specific competitor: 10 μg/ml of unlabeled pBs MAR 3.1 abolished completely the binding of the MAR probe (Fig. 2A, lane d). These results allow us to conclude that the binding of the probe by the matrix proteins is due to the interaction with the MAR-sequence.

Since some nuclear DNA-binding proteins show a strong preference for mammalian DNA over prokaryotic DNA [Renz, 1975; Diez-Cabellero et al., 1989], it was necessary to confirm that the strong binding of labeled MAR to the matrix proteins was not simply due to the mouse origin of the sequence. For this reason the binding of the MAR was tested in the presence of an excess of total mouse DNA as a competitor. Before the introduction of the labeled MAR, the matrix proteins were allowed to interact with 35 μg/ml of total mouse genomic DNA as described in Methods. The subsequent incubation with the MAR in the presence of a vast excess of genomic DNA demonstrated that the MAR binding to matrix proteins p45 and p39 is highly specific; it showed also that MAR binds preferentially to H1 and p28 even in the presence of a 3,500-fold higher amount of mouse genomic DNA (Fig. 2B, lane a); by contrast, the binding of a control labeled probe of random mouse DNA was completely abolished in the presence of the same amount of unlabeled competitor (compare lanes a, b in Fig. 2B).

Stability of the MAR-interaction with the matrix proteins under different salt conditions. It is known that the DNA attachment to the matrix is resistant to high-ionic strength extraction conditions. To test the specificity of the MAR-matrix binding in terms of resistance to high-salt treatment, nitrocellulose strips with already bound MAR were subjected to subsequent washes with 2 M NaCl. No release of the matrix-bound MAR was observed, indicating high stability of the performed DNA-protein complexes (not shown).

The specificity of MAR interaction was tested further under binding conditions of elevated ionic strength. It is known that the specificity of the interaction of some proteins with DNA is strongly dependent upon the ion concentration

[Renz, 1975; Diez-Caballero et al., 1989] and that 0.5 M NaCl, for example, abolishes nonspecific interactions of DNA with histone H1 [Miskimins et al., 1985]. When the binding of the MAR to the blotted matrix proteins was carried out in the presence of 0.5 M NaCl, p28 and H1 still bound the DNA (Fig. 2B, lane c). Under these conditions total genomic mouse DNA did not bind to any of these proteins which further indicated that the interaction of p28 and H1 with the κ-gene MAR is a specific one, occurring under conditions when nonspecific binding is abolished [Wright et al., 1987].

The interaction of the matrix proteins with the MAR is weaker in the presence of 100 mM phosphate buffer probably as a result of the increased repulsion of the probe (Fig. 2B, lane d).

Interactions of the nuclear matrix protein with different MARs. To further characterize the specificity of the matrix-proteins interactions different MAR-sequences were tested as binding probes. Recently, we have identified matrix-association regions in the α-globin gene with a very high affinity for the matrix proteins [Avramova and Paneva, 1992]. Two adjacent attachment sites, differing in their binding affinity for the matrix (when tested by the in vitro assay) were localized: a strong MAR binding well to the matrix in the presence of even 300 μg/ml of *E. coli* DNA, and a weaker MAR, competed off completely by 200 μg/ml of bacterial DNA. These MAR-containing regions from the murine α-globin gene were tested for their capacity to be specifically recognized and bound by the matrix proteins. Lane a of Figure 3 illustrates that the α-globin gene MAR binds to exactly the same proteins as the κ-immunoglobulin gene MAR; in the presence of the same amount of competitor DNA the adjacent weak MAR is bound as well, although in lower amount (lane b), while the upstream region, located 1.3 kb from the strong MAR, binds in the same nonspecific fashion as total mouse DNA (compare lane c to Fig. 2A, lane b). These results provide good evidence that the proteins visualized by this approach seem to be involved indeed in an interaction with the MAR sequences. Therefore, p45, p39, H1, p28 and p16 are capable of binding MAR-containing DNAs of different origin with a notable preference over competitor DNA.

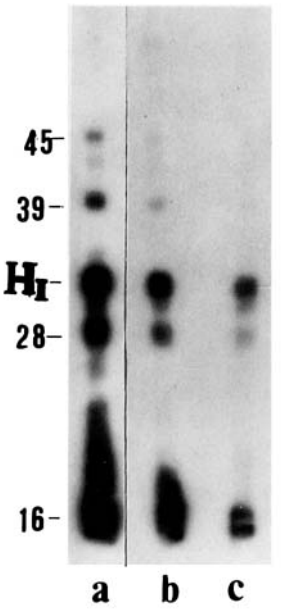

Fig. 3. Lane a: Nuclear matrix proteins interacted with a MAR-containing fragment from the α-globin gene locus (fragment E) from Avramova and Paneva [1992]. **Lane b:** Binding of the weaker MAR (fragment D). **Lane c:** Binding of a fragment located 1.3 kbp from the attachment site (fragment A). Binding reactions were carried out in the presence of 120 μg/ml *E. coli* DNA.

Interaction of Salt-Extracted Matrices With the MAR

The earlier and most widely used method for nuclear matrix preparation involved extraction of digested nuclei with 2 M NaCl. Since it has been demonstrated that independently of the extraction procedure, both LIS- and salt-prepared skeletal structures recognize and precipitate the same DNA sequences [Cockerill and Garrard, 1986; Gasser and Laemmli, 1986; Smith et al., 1987], it was of interest to see which salt-matrix proteins would be involved in the specific binding of the MAR.

Although no great difference between the corresponding precipitated or solubilized proteins obtained by the two techniques (as judged by the Coomassie-blue staining) was observed, there were some differences in the profile of the matrix proteins interacting with the MAR (Fig. 4A, lane a). A notable difference was the lower binding of the probe to H1, p28, and especially to p39 relative to the other proteins in the salt-matrix. In addition, a higher molecular mass protein (p55) and a protein with a mobility slightly less than H1 (not observed in the LIS-matrix) also showed a capacity to bind the MAR.

Identification of Some of the MAR-Binding Proteins

The two-dimensional high-resolution fractionation of nuclear matrix proteins reported recently [Stuurman et al., 1990; Dworetzky et al., 1990; Berezney, 1991] provided significant information about the complexity of their protein composition. By the method described here and in Stuurman et al. [1990], Dworetzky et al., [1990], and Berezney, [1991], the capacity of individual protein bands, or spots, to specifically interact with the MARs was demonstrated. Although very useful, these approaches cannot provide information as to what the nature of the individual MAR-interacting proteins could be. In the experiments described below, an effort to identify some of these proteins was made.

A protein with a molecular mass of about 45–48 kDa has been observed as a matrix component of different nuclear types studied [Stuurmant et al., 1990; Dworetzky et al., 1990; Berezney, 1991] and here we showed that such a protein is involved in the specific interaction with the MARs. Earlier, it had been shown that nuclear actin (a protein with an electrophoretic mobility in this region) was present in the nuclear matrix [Clark and Rozenbaum, 1979; Nakayasu and Ueda, 1986; Valkov et al., 1989]. What has not been shown, however, is whether nuclear actin was capable to specifically bind a MAR, especially after having undergone the fractionation and blotting procedures described above. To test this, purified nuclear actin was gel-fractionated, electroblotted, and reacted with the labeled probe. As shown in Figure 4B, lane b, it has the capacity to bind a MAR with a preference over the unlabeled competitor DNA.

Recently, the ability of histone H1 to form highly specific precipitable complexes with SARs has been observed [Adachi et al., 1989; Izaurralde et al., 1989]. Since the mobility of one of the matrix proteins coincided with that of histone H1, total purified mouse liver histones were tested for their capacity to bind a MAR when blotted onto nitrocellulose after gel fractionation. In Figure 4C, it may be seen that from all five histones, H1 efficiently bound the MAR in the presence of a large excess of competitor. One of H1 variants, as well as one of the core histones, also bound the MAR although with lower affinity.

It is necessary to specifically point out that the fact that the purified proteins interact with MARs does not imply that these are the same proteins which we observe as the MAR-binding proteins in the matrix. In an attempt to somehow identify these particular proteins we tested the capacity of specific antibodies to prevent MAR interactions.

Binding of the MAR to the nuclear matrix proteins in the presence of specific antibodies. Electroblotted, fractionated matrix proteins were reacted with highly specific anti-nuclear actin antibodies [Valkov et al., 1989]. The subsequent MAR binding of such pretreated matrix proteins is shown in Figure 4A, lane d: there is an almost complete disappearance of the signal from p45 in the antibody-treated sample as compared to the untreated sample (Fig. 4A, lane a), or to the blot treated with preimmune sera (lane c). It is interesting to note also the effect of anti-actin antibody on the MAR binding of p28. This result could indicate either the existence of common immunological determinants between p45 and p28, or that a degradation product of actin coincided with another MAR-binding protein with the same electrophoretic mobility as p28. The experiment described below supported the second possibility.

Earlier it had been suggested that the HMGs (especially HMG-17) could be found in the nuclear matrix [Reeves and Chang, 1983; Shahyona et al., 1984]. However, there has been no evidence as to whether any of the HMG proteins has the capacity to interact with a MAR. We, therefore, tested the effect of antibodies against all four HMGs upon the binding of the MAR. Figure 4A, lane e shows that the signal from p28 is strongly reduced but not completely abolished by preincubation of the proteins with anti-HMG antibodies. In fact, the effect of the anti-HMG antibodies upon p28 is comparable to that of the anti-actin antibodies, suggesting that p28 represents two different proteins. This was further confirmed by the complete abolishment of p28 binding activity after treatment of the matrix proteins with a mixture of the two antibody samples (Fig. 4A, lane f).

In the same time the antibodies against the HMGs specifically abolished also the MAR-binding activities of p55 and p16 (Fig. 4A, lane e,f). This might indicate some relatedness among the proteins but this fact needs further studies.

Topoisomerase II is considered the major protein component of the nuclear skeleton [Berrios et al., 1985; Earnshaw et al., 1985; Gasser et al.,

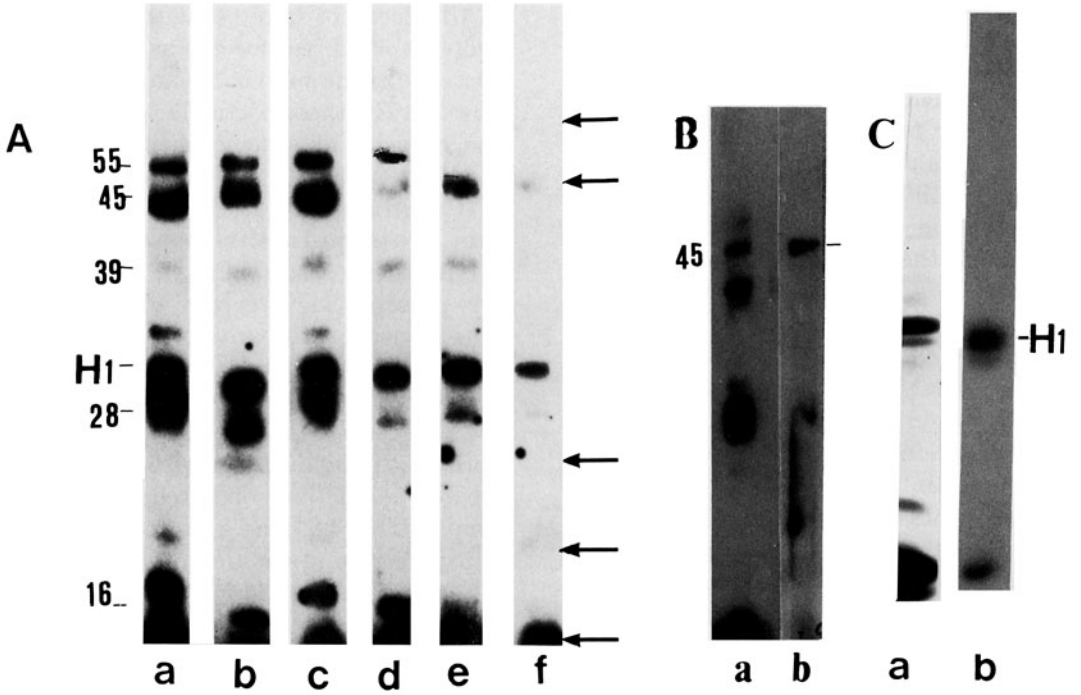

Fig. 4. **A:** Nuclear matrix proteins obtained by 2 M NaCl extraction of mouse liver nuclei and reacted with MAR in the presence of 150 μg/ml *E. coli* DNA **(lane a)**; the same matrix proteins incubated with anti-topo II antibodies prior to reaction with labeled MAR **(lane b)**; the same proteins pretreated with preimmune sera before MAR binding **(lane c)**; MAR binding of the matrix proteins pretreated with anti-actin antibodies **(lane d)** ; matrix proteins pretreated with anti-HMG antibodies **(lane e)** and with a mixture of anti-actin and anti-HMG antibodies **(lane f)** prior to incubation with the MAR. **B:** Nuclear matrix proteins incubated with a MAR shown as a control **(lane a)** and purified nuclear actin **(lane b)**, electroblotted and reacted with the MAR under the same conditions as in lane a. **C:** Purified histones fractionated in SDS gels stained with Coomassie blue **(lane a)**. **Lane b:** The same proteins after blotting and interaction with the MAR.

1986] capable of a specific interaction with MARs/SARs [Sperry et al., 1989; Adachi et al., 1989]. However, no high molecular mass protein that could correspond to topo II has been seen to bind specifically the MAR under the conditions described above. This result is in full agreement with the results from two other laboratories [von Kries et al., 1991; Hakes and Berezney, 1991; Nakayasu and Berezney, 1991]: applying the same approach, in neither of the matrix preparations were topo I or topo II seen to interact with a MAR probe. However, it is known that topo II may display several lower molecular weight proteolytic fragments recognized by anti-topo II antibodies that are similar in electrophoretic behavior to the MAR-binding matrix proteins visualized here [Earnshaw et al., 1985; Mirkovitch et al., 1988]. The possibility that some of these MAR-binding proteins could in fact represent degradation forms of topoisomerase II was checked by testing their binding activity in the presence of anti-topoisomerase II antibodies. The MAR-binding capacity of the matrix proteins pretreated with anti-topo II antibodies (Fig. 4A, lane b) shows that there is practically no change in the pattern of MAR binding after the antibody treatment is compared to the untreated sample (lane a), or to a control sample pretreated with preimmune sera (Fig. 4Ac). This result suggests that none of the observed proteins was derived from topo II.

DISCUSSION

The specific binding of DNA sequences to proteins fractionated and electroblotted onto nitrocellulose filters has been reported on several occasions [Miskimins et al., 1985; Hertl et al., 1988; Wright et al., 1987]. Of particular importance for us are the results obtained with a DNA probe from the promotor region of the transferrin receptor gene, shown to bind specifically to nuclear proteins with molecular masses between 85 and 105 Kd [Miskimins et al., 1985]. The different proteins recognized by the κ-gene MAR reported in this study point to the reliability of the method and support the conclusion for a specificity of the observed interactions.

An important modification of the experimental approach described here was the employment of BSA and competitor DNA in both the preincubation and the binding mixtures. Thus, coating with an inert protein and saturation with nonspecific DNA were allowed to take place before the introduction of the specific probe. The observed binding of the labeled sequences, therefore, reflected the displacement of unspecifically bound DNA by the more preferred fragments. This modification was necessitated by previous findings that only an excess of unlabeled competitor DNA would allow for high-affinity interactions to be resolved [Hertl et al., 1988; Diez-Caballero et al., 1989; Izaurralde et al., 1989].

The specificity of the MAR interaction with the proteins of the nuclear matrix, therefore, is based on the following observations: 1) only a few of the numerous protein bands obtained after blotting of fractionated proteins in the presence of an excess of BSA bound the MAR probe; 2) the MAR bound mainly to proteins present in very low quantities in the matrix preparation; 3) the binding to the matrix proteins occurred in the presence of an excess of both nonspecific (*E. coli*) and of the more specific (total mouse) competitor DNAs (under the same conditions a nonspecific probe displayed a significantly lower binding); 4) unlabeled MAR-containing plasmid abolished the binding and, therefore, it acted as a specific competitor; 5) only the MAR-sequence was involved in the binding to the matrix proteins, since the contribution of the empty vector was insignificant and an isolated end-labeled insert alone recognized the same proteins as did the whole plasmid; 6) DNA fragments from a different genomic location containing matrix-attachment sequences bound to the same matrix proteins, while an upstream flanking region (shown not to contain attachment sites) bound less efficiently and in a nonspecific way (Fig. 3).

Matrix proteins p39, p45, and p55 are true skeletal proteins, because they were not found in the soluble fraction. It should be noted, however, that the relative importance of these proteins as representatives of the nuclear matrix is different in the salt as compared to the LIS preparations. Thus, protein p55 which binds preferentially the MAR in salt matrices is reproducibly missing from LIS matrices, while p39 is much better represented in the latter case.

The matrix proteins interacting specifically with MAR are similar in nuclei of different origins, suggesting that these particular proteins probably belong to the common set of nuclear matrix proteins defined as the "minimal matrix" [Stuurman et al., 1990].

From the results presented it is clear that one MAR recognizes and is capable of binding to several proteins of the matrix. This is in agreement with an earlier conclusion that the "κ-gene MAR contains multiple and possibly overlapping binding sites for nuclear matrix proteins" [Blasquez et al., 1989]. However, we do not know whether all of these proteins do come in contact with this region around the κ-immunoglobulin gene in vivo.

Although by high-resolution two-dimensional gel electrophoresis the nuclear matrix protein components have been fractionated into individual spots and although some of these proteins were visualized as MAR binding [Hakes and Berezney, 1991; Nakayasu and Berezney, 1991; this study], a most important remaining question is their further identification.

Of the matrix proteins obtained by either extraction method, the protein with the highest capacity for MAR binding seems to be histone H1. It is extracted from chromatin by both high salt above 0.3 M KCl [Long et al., 1979] and LIS treatments [Mirkovitch et al., 1984]. In agreement with these findings only traces of H1 could be detected in both types of our matrix preparations. Nevertheless, the very small amount of H1 remaining with the matrix interacts with the MAR with such a preference that it cannot be neglected when evaluating the proteins constituting the nuclear matrix. It is important to note also that when the binding to H1 is less specific, it is completely abolished at salt concentrations above 0.4 M NaCl [Miskimins et al., 1985; Wright et al., 1987; our observations]. On the other hand, the binding of MAR to H1 in the presence of 0.5 M NaCl (Fig. 2B, lane c) and the resistance of the complex to multiple washings in the presence of up to 2 M NaCl support the conclusion that histone H1 is capable of interaction with MAR-containing DNAs.

The preference of histone H1 for a subfraction of the mammalian genomic DNA [Renz, 1975; Diez-Caballero et al., 1989] and even to a discrete region flanking the rat albumin gene has been reported earlier [Sevall, 1988]. It has been even suggested that dispersed preferential binding sites for H1 exist in the mammalian genome

[Renz, 1975; Diez-Caballero et al., 1989]. This speculation is particularly interesting in view of the suggestion that SARs may control the conformation of chromatin domains through their highly specific association with H1 [Izaurralde et al., 1989]. In the latter case the specificity of the interaction was demonstrated by the formation of specific precipitable complexes between isolated histone H1 and several SARs. Here, by another approach we demonstrate the capacity of histone H1 to specifically interact with the fragments anchoring the chromosomal DNA to the skeletal structures (Fig. 4C, lanes a,b).

Although earlier studies have suggested that nuclear actin is a component of the nuclear matrix [Clark and Rozenbaum, 1979; Nakayasu and Ueda, 1986; Valkov et al., 1989], its ability to preferentially bind MAR-sequences has not been known. The specific interaction between different MARs and a matrix protein with the mobility of actin (Fig. 1d; Fig. 2, lane a; Fig. 3a), the ability of purified nuclear actin to bind a MAR (Fig. 4C, lane b) and the specific abolishment of the p45 MAR binding by anti-actin antibodies (Fig. 4A, lane d) provided strong evidence for the following conclusions: i: nuclear actin is one of the proteins constituting the nuclear matrix independently of the procedure employed for matrix isolation, and ii: nuclear actin is a matrix protein capable of a specific recognition and binding of MARs, which implies its possible involvement in the attachment of genomic DNA to the nuclear matrix.

Some of the visualized MAR-binding proteins displayed mobilities coinciding with those of HMG 1 and 2 (p28) and of HMG 14 and 17 (p16). Antibodies against the four HMGs completely inhibited the binding capacity of p16 and p55, while that of p28 was reduced (Fig. 4A, lanes e,f). The fact that pre-treatment with anti-actin antibodies brought about the same effect on p28 binding as did the anti-HMG antibodies could be explained if one assumes that p28 is composed of two different proteins with similar electrophoretic mobilities. One of these proteins is recognized by the anti-actin antibodies (possibly representing a degradation product of actin), while the other is recognized and inactivated by the anti-HMG antibodies. This is supported further by the result shown in Figure 4A, lane f, obtained after a treatment with a mixture of both anti-HMG and anti-actin antibodies.

No conclusion regarding the nature of p55 can be made as yet. Proteins of the nuclear matrix with molecular masses in this range have been reported [Long et al., 1979; Shahyona et al., 1984; Kaufman et al., 1986; Hakes and Berezney, 1991], but none has been identified yet. The recognition of this protein by the anti-HMG antibodies might indicate its relatedness to the HMGs and this fact deserves further attention. It is interesting to mention in this regard an earlier observation that two matrix proteins from neuronal nuclei—an HMG and a 55 kD protein—were found to be the substrates for the same protein modifying activity [Shahyona et al., 1984]. This fact might also point to a relatedness between these matrix proteins. Earlier, by comparing the two-dimensional peptide patterns of sperm nuclear matrix proteins, a strong internal homology among the proteins involved in the sperm skeleton was found, irrespective of substantial differences in their molecular masses [Avramova and Tasheva, 1987a,b]. These facts together with the recent suggestion that the nuclear matrix proteins (matrins) might "compose a broad family of structural proteins in the nucleus with potential subfamilies" [Nakayasu and Berezney, 1991] are of a particular interest, since they tentatively suggest that common principles might be responsible for the origin and diversity of nuclear skeletal proteins.

By two-dimensional gel electrophoresis, approximately 12 MAR-binding proteins were resolved [Nakayasu and Berezney, 1991]. Five of them belong to already identified nuclear proteins (see below); the other eight—termed *matrins*—consist of proteins with molecular masses of 125, 105, 76–60, 42–48, and 15 kDa, respectively. It may be tentatively suggested that MAR-binding proteins p55, p45, p39, and p16 correspond to some of the matrins described by Hakes and Berezney [1991] and Nakayasu and Berezney [1991], but such a possibility has still to be verified.

The low-molecular mass DNA-binding protein (ca 15 kDa) was identified as a residual component of the hnRNP core proteins [Hakes and Berezney, 1991]. However, the abolishment of MAR binding after pretreatment of the matrix proteins with HMG antibodies argues in favour of an idea that this band is composed of more than one DNA-binding protein. This protein is immunologically cross-related to two other higher molecular mass MAR-binding proteins.

Three of the MAR-binding proteins described by Nakayasu and Berezney [1991] were identified as the nuclear lamins. We, however, did not

observe any MAR binding to the lamins; moreover, isolated nuclear lamina were shown not to be able to bind and precipitate neither the κ-immunoglobulin [Sperry et al., 1989] nor the α-globin gene MARs [Avramova and Paneva, 1992] in an in vitro binding assay. The reason for this discrepancy is not clear at present, but it should be pointed out that our results are in agreement with previous reports demonstrating that the lamins are not involved in the attachment and specific interaction with SARs [Izaurralde et al., 1988]. They are in accordance also with recent observations that chromosomal DNA does not seem to be anchored to the nuclear periphery as previously considered [Paddy et al., 1990].

Summarizing, we could say that the specific interaction of some nuclear matrix proteins with MARs has been demonstrated. The major matrix proteins are principally the same in both salt and LIS matrices, although differences in the relative MAR-binding activity of the individual protein components was observed. What we consider a most important result, however, is the identification of some of the MAR-binding proteins. By testing the MAR binding in the presence of specific antibodies, the involvement of nuclear actin, histone H1, HMGs, and a protein (p55) recognized by the HMG antibodies in MAR recognition was demonstrated. Finally, it should be pointed out that some MAR-binding proteins may be underrepresented in the pictures obtained by this approach due to the irreversible denaturation of some proteins in SDS-containing medium. This in particular could explain the lack of a signal from topo II.

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REFERENCES

Adachi Y, Käs E, Laemmli UK (1989): Preferential cooperative binding of DNA topoisomerase II to scaffold associated regions. EMBO J 8:3997–4006.

Avramova Z, Paneva E (1992): Matrix attachment sites in the murine α-globin gene. Biochem Biophys Res Commun 182:78–85.

Avramova Z, Tasheva B (1987a): Proteins of the sperm nuclear matrix of a bivalvia mollusc; analysis by peptide mapping. Compt Rend Acad Sci Bulg 40:101–104.

Avramova Z, Tasheva B (1987b): Tightly bound nonprotamine proteins from ram sperm nuclei studied by one- and two-dimensional peptide mapping. Mol Cell Biochem 74: 67–75.

Berezney R (1991): A heuristic model for investigating genomic organization and function in the cell nucleus. J Cell Biochem 47:109–123.

Berezney R, Coffey D (1974): Identification of a nuclear protein matrix. Biochem Biophys Res Commun 60:1410–1417.

Berrios M, Oscheroff N, Fischer PA (1985): In situ localization of DNA topoisomerase II, a major polypeptide component of the Drosophila nuclear matrix fraction. Proc Natl Acad Sci USA 82:4142–4146.

Blasquez VC, Sperry AO, Cockerill PN, Garrard WT (1989): Elements that organize chromosomal loops in metaphase chromosomes. In "DNA-Protein Interactions in Transcription." New York: Alan R. Liss, pp 273–286.

Bodnar JW (1988): A domain model for eukaryotic DNA organization: Molecular basis for cell differentiation and chromosome evolution. J Theor Biol 132:479–507.

Clark TG, Rozenbaum JL (1979): An actin filament matrix in hand-isolated nuclei of X. laevis oocytes. Cell 18:1101–1108.

Cockerill PN, Garrard WT (1986): Chromosomal loop anchorage of the kappa immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites. Cell 44:273–282.

Comings DE, Wallack AS (1978): DNA-binding properties of nuclear matrix proteins. J Cell Sci 34:233–246.

Cook PR (1988): The nucleoskeleton: Artefact, passive framework or active site? J Cell Sci 90:1–6.

Diez-Caballero T, Aviles FX, Albert A (1989): Specific interaction of histone H1 with eukaryotic DNA. Nucleic Acids Res 9:1383–1393.

Dworetzky SI, Fey EG, Penman S, Liau JB, Stein JL, Stein GS (1990): Progressive changes in the protein composition of the nuclear matrix during rat osteoblast differentiation. Proc Natl Acad Sci USA 87:4605–4609.

Earnshaw WC, Halligan N, Cooke C, Heck MMS, Liu LF (1985): Topoisomerase II is a structural component of mitotic chromosome scaffolds. J Cell Biol 100:1706–1715.

Fey EG, Krochmalnic G, Penman S (1986): The nonchromatin substructures of the nucleus: The ribonucleiprotein (RNP)-containing and RNP-depleted matrices analysed by sequential fractionation and resinless section electron microscopy. J Cell Biol 102:1654–1665.

Gasser SM (1988): Nuclear scaffold and the higher order folding of eukaryotic DNA. In Kahl G (ed): "Architecture of Eucariotic Genes." Wienheim: VCH Verlagsgeselschaft, pp. 461–471.

Gasser SM, Laemmli UK (1986): The organization of chromatin loops; characterization of a scaffold attachment site. EMBO J 5:511–517.

Gasser SM, Laroch T, Falques J, Boy de la Tour E, Laemmli UK (1986): Metaphase chromosome structure: Involvement of topoisomerase II. J Mol Biol 188:613–629.

Georgiev GP, Chentsov YS (1960): The structure of the nucleus. An electron-microscopic investigation of isolated nuclei. Proc Acad Sci USSR 132:199–201.

Goldman MA (1988): The chromatin domain as a unit of gene regulation. BioEssays 9:50–55.

Gross DS, Garrard WT (1987): Poising chromatin for transcription. Trends Biochem Sci 12:293–297.

Hakes DL, Berezney R (1991): DNA binding properties of the nuclear matrix and individual matrix proteins. Evidence for salt-resistant DNA binding sites. J Biol Chem 266:11131–11140.

Herlt M, Schwarz HP, Neumann R, Eggers HJ (1988): Detection of DNA-protein binding in Western blots by phosphorus-labeled and biotinylated DNA-probes. BioTechniques 6:324–331.

Izaurralde E, Käs E, Laemmli UK (1989): Highly preferential nucleation of histone H1 assembly on scaffold-associated regions. J Mol Biol 210:573–585.

Izaurralde E, Mirkovitch J, Laemmli UK (1988): Interaction of DNA with nuclear scaffolds in vitro. J Mol Biol 200:111–125.

Kaufmann SH, Fields AP, Shaper JH (1986): The nuclear matrix: Current concepts and unanswered questions. In Jasmin G, Simard R (eds): "Methods and Achievements in Experimental Pathology; Nuclear Electron Microscopy," Vol. 12. Basel: Karger, pp. 141–171.

Laemmli UK (1972): Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Long BH, Huang CY, Pogo AO (1979): Isolation and characterization of the nuclear matrix in Friend erythroleukemia cells: Chromatin and hnRNA interactions with the nuclear matrix. Cell 18:1079–1090.

Mirkovitch J, Gasser SM, Laemmli UK (1988): Scaffold attachment of DNA loops in metaphase chromosomes. J Mol Biol 200:101–109.

Mirkovitch J, Mirault M-E, Laemmli UK (1984): Organization of the higher order chromatin loop: Specific DNA attachment sites on nuclear scaffold. Cell 44:273–282.

Mirsky AE, Ris H (1951): The composition and structure of isolated chromosomes. J Gen Physiol 34:475–492.

Miskimins WK, Roberts MP, McClelland A, Ruddle F (1985): Use of a protein blotting procedure and a specific DNA-probe to identify nuclear proteins that recognize the promoter region of the transferrin receptor gene. Proc Natl Acad Sci USA 82:6741–6744.

Mounty KL, Dounce AL (1958): The property and the enzymatic degradation of deoxyribonucleoprotein from liver cell nuclei. J Gen Physiol 41:595–608.

Nakayasu H, Berezney R (1991): Nuclear matrins: Identification of the major nuclear matrix proteins. Proc Natl Acad Sci USA 88:10312–10316.

Nakayasu H, Ueda K (1986): Preferential association of acidic actin with nuclei and nuclear matrix from mouse leukemia L5178Y cells. Exp Cell Res 163:327–336.

Nelson WG, Pienta KJ, Barrack ER, Coffey DS (1986): The role of the nuclear matrix in the organization and function of DNA. A Rev Biophys Chem 15:457–475.

Paddy MR, Belmont AS, Saumwebel H, Hamid D, Sedat J (1990): Interphase nuclear envelope lamins form a discontinuous network that interacts with only a fraction of DNA. Cell 62:89–106.

Reeves R, Chang D (1983): Investigations of the possible functions for glyco-sylation in the high-mobility group proteins; evidence for a role in nuclear matrix association. J Biol Chem 258:679–687.

Renz MC (1975): Preferential and cooperative binding of histone H1 to chromosomal mammalian DNA. Proc Natl Acad Sci USA 72:733–736.

Sevall JS (1988): High-resolution analysis of histone H1 binding site in a rat albumin gene. Biochemistry 27:5038–5044.

Shahyona N, LeVine H III, Bronson D, Cuatrecassas P (1984): Ca^{2+} Calmodulin-dependent protein kinase in neuronal nuclei. J Biol Chem 259:9341–9344.

Smith HC, Ochs RL, Lin D, Chinault AC (1987): Ultrastructural and biochemical compositions of nuclear matrices prepared by high salt or LIS-extractions. Mol Cell Biochem 77:49–61.

Sperry AO, Blasquez VC, Garrard WT (1989): Dysfunction of chromosomal loop attachment sites; illegitimate recombination linked to matrix-association regions and topoisomerase II. Proc Natl Acad Sci USA 86:5497–5501.

Stuurman N, Maijne AML, van der Pol A, de Long L, van Driel R, van Renswoude J (1990): The nuclear matrix from cells of different origin. Evidence for a common set of matrix proteins. J Biol Chem 265:5460–5465.

Towbin H, Staehelin T, Gordon J (1979): Electrophoretic transfer of proteins from gels to nitrocellulose sheets; procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.

Valkov NI, Ivanova MJ, Uschewa AA, Krachmarov CP (1989): Association of actin with DNA and the nuclear matrix of Guerin ascites tumor cells. Mol Cell Biochem 87:47–56.

Verheijem R, Venrooij W, Ramaekers F (1988): The nuclear matrix: Structure and composition. J Cell Sci 90:11–36.

von Kries JP, Buhrmester H, Strätling WH (1991): A matrix scaffold attachment region binding protein: Identification, purification and mode of binding. Cell 64:123–135.

Wright JM, Wierma PA, Dixon GH (1987): Use of protein blotting to study the DNA-binding properties of histone H1 and H1-variants. Eur J Biochem 168:281–285.