Mutually Exclusive Interaction of a Novel Matrix Attachment Region Binding Protein and the NF-\(\mu\)NR Enhancer Repressor

IMPLICATIONS FOR REGULATION OF IMMUNOGLOBULIN HEAVY CHAIN EXPRESSION*

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The immunoglobulin heavy chain (IgH) intronic enhancer stimulates transcription from functional promoters in B lymphocytes but not other cell types. The observation that binding sites for the nuclear factor-\(\mu\) negative regulator (NF-\(\mu\)NR) enhancer repressor overlap nuclear matrix attachment regions (MARs) in this enhancer has lead to the hypothesis that the cell type specificity of the enhancer might be controlled by regulating nuclear matrix attachment (Scheuermann, R. H., and Chen, U. (1989) Genes & Dev. 3, 1255–1266). To understand the role of MARs in IgH enhancer regulation, we have identified a novel MAR-binding protein, MAR-BP1, from soluble nuclear matrix preparations based on its ability to bind to the MARs associated with the IgH enhancer. Purified MAR-BP1 migrates as a 33-kDa protein, and it can be found in nuclear matrix preparations from a number of different types of lymphoid cell lines. Although specific binding sites have been difficult to localize by chemical or enzymatic footprinting procedures, NF-\(\mu\)NR binding sites are critical for efficient MAR-BP1 binding. Indeed, binding of the IgH enhancer to either intact nuclear matrix preparations or to MAR-BP1 is mutually exclusive to NF-\(\mu\)NR binding. These results are consistent with a model for cell-type-specific regulation in which binding of the NF-\(\mu\)NR repressor to the IgH enhancer prevents nuclear matrix attachment in inappropriate cells by interfering with MAR-BP1/enhancer interaction.

Transcription of the immunoglobulin heavy chain (IgH) gene is influenced by at least two distinct classes of cis-acting elements, promoters and enhancers. The IgH intronic enhancer, located between the \(J_H\) elements and C\(\mu\) elements, stimulates transcription from promoters in a relatively distance- and orientation-independent manner, and acts as a cell type-specific regulator, being functional in B cells and nonfunctional in non-B cells (for review, see Staudt and Lenardo (1991)). This enhancer contains multiple DNA sequence motifs that function as interaction sites for sequence-specific DNA-binding proteins. Many of these sites have been demonstrated to be important for transcriptional regulation. The B cell specificity of the IgH enhancer is regulated by both positively and negatively acting B cell-specific enhancer elements as well as protein–protein interactions between B cell-specific and ubiquitously expressed nuclear factors.

We have identified negative elements in the IgH enhancer that are bound by a nuclear protein, nuclear factor-\(\mu\) negative regulator (NF-\(\mu\)NR). NF-\(\mu\)NR purified from a preB cell line binds to four sites flanking the enhancer core in a cooperative manner (Scheuermann, 1992). NF-\(\mu\)NR activity is expressed in non-B cells and appears to repress the enhancer in these inappropriate cells (Scheuermann and Chen, 1989). One intriguing aspect of NF-\(\mu\)NR binding is that the four binding sites in the IgH enhancer overlap two nuclear matrix attachment regions (MARs) (Cockerill et al., 1987).

MARs are AT-rich DNA sequences that remain attached to a salt-resistant nuclear structure referred to as the nuclear matrix or scaffold (Berezny and Coffey, 1974). MARs/scaffold attachment regions have been mapped to DNA segments that are several hundred base pairs in length (Gasser and Laemmli, 1986; Cockerill and Garrard, 1986), which anchor chromatin loops to the nuclear matrix (for review, see Laemmli et al., 1992). Juxtaposition or cohabitation of MARs with transcriptional regulatory elements suggests that MARs may influence transcription (Cockerill et al., 1987). Indeed, some MAR sequences have been shown to stimulate transcription in transfection studies (Stief et al., 1989; Xu et al., 1989; Phi-Val et al., 1990; Klehr et al., 1991; Bode et al., 1992). Furthermore, the importance of the IgH enhancer MARs to enhancer activity has recently been demonstrated in transgenic mouse experiments where constructs lacking these MARs were inactive (Forrester et al., 1994).

Several MAR-binding proteins have been identified and purified from nuclear extracts (von Kries et al., 1991, 1994; Tsutsui et al., 1993; von Kries et al., 1994). Some of these proteins require relatively long DNA fragments for efficient binding and may recognize certain structural features of DNA rather than a precise nucleotide sequence (Zhao et al., 1993). Others may have the properties similar to transcription factors (Dickinson et al., 1992; Bidwell et al., 1993), since they recognize specific sequences within DNA fragments. Proteins binding to MARs include lamin B1 and topoisomerase II, which are major components of the nuclear matrix (Luderus et al., 1992; Sperry et al., 1989); the yeast RAP-1 factor and HeLa SAF-A/nrNRP-U, which induce DNA loop formation (Hofmann et al., 1989; Rongig et al., 1992; Fackelmayer et al., 1994); and SATB1 and nucleolin, which seems to preferentially bind DNA with base-unpairing potential (Dickinson and Kohwi-Shigematsu, 1995).

Here we describe the identification and purification of a novel 33-kDa nuclear matrix protein, MAR-BP1, which binds to the IgH enhancer MARs. Binding of MAR-BP1 to the IgH enhancer MARs requires the four NF-\(\mu\)NR binding sites. Indeed, binding of MAR-BP1 and NF-\(\mu\)NR to IgH enhancer frag-
ments is mutually exclusive. These results are consistent with a model for cell type-specific regulation in which NF-κB binding to the IGH enhancer prevents nuclear matrix attachment by interfering with MAR-BP1/enhancer interaction.

MATERIALS AND METHODS

DNA Probes—The 1.0-kb Xbal restriction fragment containing the entire mouse IGH intronic enhancer and μκ2, a HindIII fragment from mouse Cμ, have been described previously (Scheuermann and Chen, 1989). The 3'-En fragment (309 bp) is derived from the enhancer by digestion with Xbal and EcoRI. The 5'-En fragment (381 bp) is derived by digestion with Xbal and PvuII. The MboI 5'-En fragment (301 bp) is prepared by digestion of 5'-En with MboII removing 80 bp from the 5'-end. The 0.92-kb κ MAR and 0.66-kb Drosophila histone spacer MAR were gifts from W. Garrard (U.T. Southwestern Medical Center). The mutant versions of the IGH enhancer containing NF-κB-binding site deletions have been described previously (Scheuermann, 1992).

Isolation of Nuclei and Nuclear Matrix—The WEHI-231 mature B cell line was grown as described previously (Scheuermann and Chen, 1989). Cells were lysed in a buffer containing Nonidet P-40, and nuclei were prepared by centrifugation through a sucrose cushion as described by Berezney and Coffey (1974). Nuclear matrix was prepared from the purified nuclei by a high salt extraction method (Cockerill and Garrard, 1986).

Preparation of Total Soluble Nuclear Matrix Proteins—Soluble nuclear matrix proteins were prepared by a denaturation-renaturation process. Generally, 50 mg of nuclear matrix was dissolved in 25 ml of Denaturation Buffer (100 mM NaCl, 20 mM Tris-HCl (pH 7.4), 8 mM urea). After a 10-min incubation at room temperature, insoluble material was removed by centrifugation at 10,000 × g for 15 min at 4°C. The supernatant was first dialyzed against Renaturation Buffer (100 mM NaCl, 20 mM Tris-HCl (pH 7.4), 1 mM phenylmethylsulfonlfuoride) containing 1 mM urea for 3 h and then against Renaturation Buffer without urea for an additional 3 h at 4°C. After 10 min of centrifugation at 4°C to clarify, the supernatant (soluble nuclear matrix) was frozen in liquid N2 and stored at −80°C.

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Matrix DNA Binding Assay—A standard in vitro MAR binding assay was performed as described previously (Cockerill and Garrard, 1986). Briefly, matrices were washed 3 times in Washing Buffer (50 mM NaCl, 1 mM MgCl2, 10 mM Tris-HCl (pH 7.4), 0.5 mM phenylmethylsulfonlfuoride, 0.25 mg/ml bovine serum albumin). Binding was performed by incubation of 10 μM of nuclear matrix (−5 μg of matrix protein) with 90 μl of Assay Solution (50 mM NaCl, 2 mM EDTA, 10 mM Tris-HCl (pH 7.4), 0.5 mM phenylmethylsulfonlfuoride, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 0.25 mg/ml bovine serum albumin, 1–2 ng of [32P]-end-labeled DNA fragments, and 20 μg of unlabeled, sonicated E. coli DNA) shaking for 1 h at 23°C. DNA fragments that interact with the matrix were separated from free DNA by 10 min of centrifugation at 10,000 × g at 4°C. After washing in 1 ml of Final Washing Buffer (50 mM NaCl, 2 mM EDTA, 10 mM Tris-HCl (pH 7.4), 0.25 mg/ml bovine serum albumin), the protein-DNA complexes were solubilized in 15 μl of Solubilizing Buffer (2 mM EDTA, 40 mM Tris acetate, 0.4 mg/ml proteinase K, 0.5% SDS, and 5 μg/ml sonicated salmon sperm DNA) and incubated overnight at 37°C. Resulting matrix-bound DNA fragments were resolved by electrophoresis on 15% agarose alongside a sample of probe DNA representing 25% of that used in the initial reaction.

In protein competition experiments, [32P]-labeled DNA fragments (∼2 ng) were preincubated with purified NF-κB, purified MAR-BP1, or soluble matrix proteins and 5 μg of sonicated E. coli DNA as a nonspecific competitor protein at room temperature. After the addition of 15 μg sonicated E. coli DNA and 5 μg nucleosomal matrix, the solution was incubated shaking for one hour at 23°C and processed as described above.

Mobility Shift Assays (MSAs)—DNA binding assays (Garner and Rezvini, 1981; Fried and Crothers, 1981) were performed as described previously (Scheuermann and Chen, 1989) with the following modifications. Binding reactions were done in 25 μl, total volume, containing −0.5 ng of [32P]-end-labeled DNA, 2 μg of poly(dI-dC), and protein fragments in Binding Buffer (10 μl HEPES (pH 7.9), 100 mM NaCl, 0.5 mM EDTA, 0.02% Tween, and 10% glycerol). After 25 min of incubation at room temperature, samples were electrophoresed through 4% native polyacrylamide gels in 0.5 × TGE (25 mM Tris base, 190 mM glycine, 1 mM EDTA) at 4°C. Free and complexed DNA was quantified following fixation with 10% methanol, 10% acetic acid and drying using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

UV Cross-linking—UV-cross-linking experiments were performed as described previously (Scheuermann, 1990). After UV irradiation, the samples were digested with 30 μg of DNase I at 23°C for 30 min and loaded on an 11% SDS-polyacrylamide gel.

Denaturing Gel Electrophoresis—Standard discontinuous SDS-polyacrylamide gel electrophoresis was used (Laemmlı, 1970).

RESULTS

Nuclear Matrix Binds Specifically to the IGH Enhancer—The IGH enhancer contains the DNA recognition sites for a variety of different nuclear proteins (Fig. 1A), including an octamer motif, several E boxes, and four binding sites for NF-κB (P1–P4). The 1.0-kb Xbal enhancer fragment can be subdivided into three pieces, the 5'-En, core, and 3'-En fragments by restriction enzyme digestion. The 5'-En and 3'-En fragments
have been found to specifically interact with the nuclear matrix (Cockerill et al., 1987) and thus contain MARs.

Nuclear matrix preparations from a B cell line were found to specifically bind IgH enhancer DNA in the presence of an excess of nonspecific E. coli competitor DNA (Fig. 1B; compare lane 2 with 3 and lane 8 with 9). In the same samples, the competitor DNA completely abrogated matrix binding to two control DNA fragments that lack MARs, the plasmid pUC19 (lane 3) and a fragment derived from the IgH Cμ region (lane 9). The κ MAR derived from the Ig light chain κ gene-C intron also binds to the nuclear matrix, apparently with a higher binding affinity (lane 6). MARs derived from a Drosophila histone gene (Dr MAR) and from an IgH chain variable region gene promoter (Webb et al., 1991) also bind to these nuclear matrix preparations with similar binding affinity as the enhancer MARs (data not shown).

Isolation of a Soluble Nuclear Matrix Protein That Binds to MAR Sequences—In order to further characterize this matrix/enhancer interaction, we have identified a MAR-binding protein isolated from nuclear matrix solubilized in 8 M urea. Total soluble nuclear matrix proteins were prepared by a denaturation-renaturation protocol as described under "Materials and Methods." Soluble matrix preparations were analyzed for the presence of specific MAR-binding proteins using MSAs. Using the 5'-En MAR-containing fragment, a single strong protein-DNA complex was observed (Fig. 2A, lane 2). The generation of this complex could be competed by a number of different DNA fragments containing MARs, the 5'-En fragment itself (lane 3), the entire 1.0-kb IgH enhancer (lane 4), 3'-En (lane 7), Dr MAR (lane 8), and κ MAR (lane 9); whereas the enhancer core fragment could not compete for this interaction, even at a 200-fold molar excess (lane 6). This interaction is not simply due to binding of DNA that has an A/T-rich character since poly(dA/dT) was not an effective competitor (lane 5).

Using the 3'-En MAR-containing fragment as a probe, a similar pattern was observed (Fig. 2B). Although several faint complexes were observed in addition to the major MAR-binding complex, they are nonspecific since they were competed by the enhancer core and poly(dA/dT). Our data indicate that the same MAR-binding protein is responsible for the complexes seen with 5'-En and 3'-En probes, and yet the complex formed with the 3'-En fragment migrates more slowly than the 5'-En complex. This may be due to the ability of this MAR-binding protein to bend the bound DNA fragments (data not shown). Bending tends to slow the mobility of protein-DNA complexes most dramatically when the binding site is situated in the center of the fragment (Kim et al., 1989). Perhaps the binding site for this MAR-binding protein is situated in the middle of the 5'-En fragment but close to one of the ends of the 5'-En fragment. Changes in mobility of bent DNA are also more dramatic with smaller DNA fragments.

Isolation of MAR-BP1—We further purified this MAR-binding protein from other soluble nuclear matrix proteins by a three-step chromatography procedure as described under "Materials and Methods." The protein composition of different purification fractions was assessed by SDS-polyacrylamide gel electrophoresis and silver staining (Fig. 3A). The polypeptide composition of the total nuclear matrix preparation (lane 2) is virtually identical to that of the soluble nuclear matrix preparation (lane 4), indicating that the renaturation process is effective at the solubilization of most matrix proteins; this soluble matrix preparation gives the protein-DNA complexes described in Fig. 2. The amount and composition of proteins in the denatured pellet is variable, indicating that the procedure may not be efficient for all matrix proteins. After DNA-cellulose affinity chromatography, a single major protein can be identified by silver staining (lane 6), with a molecular mass of ~33 kDa.

In order to determine if this protein is indeed responsible for MAR binding, UV-cross-linking experiments were performed to determine the molecular mass of the MAR-binding activity present in this purified fraction (Fig. 3B). Lanes 1 and 2 show that the same protein binds to both 5'-En and 3'-En fragments. The molecular mass of this labeled species is consistent with a 33-kDa protein bound to a small stretch of DNA predicted to remain following DNase digestion of the cross-linked complex. Cross-linking of the 5'-En fragment with purified NF-κB (see Materials and Methods) gives rise to a larger labeled species (lane 4), consistent with its 40-kDa molecular mass (Scheuermann, 1992). These results support the contention that although these binding activities interact with the same enhancer fragments, they are distinct. We have named this MAR-binding protein, MAR-BP1, for matrix attachment region binding protein 1.

The cell type specificity of MAR-BP1 expression was investigated by evaluating MAR binding activity in soluble nuclear matrix preparations isolated from preB, mature B, and mature T cell lines by MSA (Fig. 4). DNA-MAR-BP1 protein complexes were found using soluble matrix preparations from all six cell lines tested. At this point, the significance of lower MAR-BP1 expression in preB cell lines as compared with mature B cell lines is not clear, but it is a consistent finding with both preB cell lines examined.

Analysis of MAR-BP1 Binding Sites in the IgH Enhancer—Chemical and enzymatic footprinting approaches are fre-
MAR Binding and IgH Enhancer Regulation

Fig. 3. Purification of a soluble MAR-binding protein. A, purification fractions containing MAR-binding proteins were analyzed by SDS-polyacrylamide gel electrophoresis (10%) and silver staining. Lane 1 contains silver staining marker (Bio-Rad); lane 2 contains 1 μg of the nuclear matrix; lane 3 contains denatured nuclear matrix pellet; lane 4 contains 1 μg of soluble nuclear matrix protein; lane 5 contains 0.1 μg of the DEAE peak fraction; lane 6 contains 10 ng of the peak fraction from DNA-cellulose affinity chromatography. B, UV cross-linking of MAR-BP1. Internally labeled 5'-En (lanes 1, 3, and 4) or 3'-En (lane 2) fragments were used in MSAs, the wet gels were irradiated, specific complexes were isolated, and proteins were resolved by SDS-polyacrylamide gel electrophoresis. Samples for lanes 1 and 2 contained 0.1 μg of MAR-BP1 DEAE fraction; lane 3 contained no protein; lane 4 contained 10 ng of NF-κB/NR fraction F1. Molecular masses (kDa) of protein size markers are indicated. Similar results were obtained using total soluble nuclear extracts and DNA-cellulose column fractions.

Fig. 4. Cell type distribution of MAR-BP1. Soluble nuclear matrix proteins were prepared from the different cell lines as indicated. MAR-BP1 DNA binding activity was examined by MSA with 0.5 μg of protein and 0.5 ng of 5'-En probe. The arrow indicates the characteristic MAR-BP1 complex.

Although used to identify regions of DNA that are physically covered by the interacting protein. We used ortho-phenanthroline copper and DNase I footprinting and missing nucleoside approaches in attempts to define the binding sites for MAR-BP1 within the IgH enhancer without success (data not shown). These experiments were performed with soluble nuclear matrix preparations and with purified MAR-BP1 under conditions where >80% of the DNA was bound, as assessed by MSA. In the same experiments, purified NF-κB/NR gave strong protected regions corresponding to the binding sites described previously (Scheuermann and Chen, 1989), confirming that the techniques were working. The inability to identify precise binding sites for other MAR-binding proteins has been reported (e.g. Mielke et al. (1990) and Tsutsumi et al. (1993)). In the case of SATB1, the missing nucleoside approach identified binding sites, while DNase and chemical footprinting failed (Dickinson et al., 1992). It is thought that MAR binding may represent the combination of multiple low affinity interactions to achieve binding specificity, since the binding proteins do not footprint well and relatively long DNA fragments are required for high affinity binding. This may also be the case for MAR-BP1.

Since the precise mapping of MAR sites was problematic, we decided to concentrate on approaches to investigate the relationships between NF-κB/NR binding sites and MAR-BP1 binding using MSAs. The 381-bp 5'-En, containing the NF-κB/NR binding sites P1 and P2, can be subdivided into smaller fragments by restriction enzyme digestion (Fig. 5A). No binding could be demonstrated with purified MAR-BP1 using fragments derived from either end of the 5'-En fragment (Fig. 5B, 5-P and X-R). Specific binding could be demonstrated to a subfragment containing the middle region (R-P). This result suggests that the putative MAR-BP1 binding site lies within the 185-bp region between the RsaI and the Spel sites; this region also contains the NF-κB/NR binding sites P1 and P2. The fact that the binding affinity for this subfragment was only ~20% of that of the whole fragment suggests that either the binding site had been partially disrupted by restriction enzyme cleavage or that the length requirement for high affinity binding discussed above is coming into play. Similar results were obtained in experiments using 3'-En fragments as probes, i.e. only the whole 309-bp fragment had MAR-BP1 binding activity; any further digestion of this fragment lead to a dramatic loss of binding activity (data not shown).

Since MAR-BP1 binds to both the 3'-En fragment and to subfragments of 5'-En containing NF-κB/NR binding sites, the importance of the four NF-κB/NR sites P1-P4 was investigated. In MSA experiments using the 5'-En fragment as a probe, IgH enhancer fragments in which all four NF-κB/NR binding sites had been deleted were considerably less effective at competition than the wild-type enhancer (Fig. 5C). In addition, deletion of either P1 or P2 from the 5'-En fragment, or deletion of either P3 or P4 from the 3'-En fragment significantly reduced their ability to compete with the respective wild-type fragments for MAR-BP1 binding (Fig. 5D). These results indicate that NF-κB/NR binding sites are essential for MAR-BP1 binding, due to either their identity or extensive overlap.

To further analyze the influence of NF-κB/NR sites on MAR-BP1 binding, we measured the DNA binding affinity using 5'-En fragments with or without either P1 or P2 site deletion (Fig. 5E). The calculated dissociation (Kd) constant for binding to the intact 5'-En fragment is 1.5 × 10⁻⁸ M. However deletion of either P1 or P2 site dramatically decreases the MAR-binding affinity by a factor of 5 or more. This reduction in affinity is not simply due to a shortening of the DNA probe size, since removal of 80 bp from the 5' end by MboI digestion had no effect on binding affinity. Taken together these results indicate that NF-κB/NR binding sites play a critical role for MAR-BP1 binding.
A comparison of the binding affinities for MAR-BP1 and other DNA-binding proteins (Table I) indicates that although the affinity of MAR-BP1 for MAR fragments is lower than that measured for several transcriptional regulatory proteins like NF-κB, LEF-HMG, SP1, and MLTF, it is similar to the affinity measured for another MAR-binding protein, nucleolin.

Binding of the Nuclear Matrix or MAR-BP1 and NF-κB to the IgH Enhancer Is Mutually Exclusive—We have proposed a model for NF-κB function in which binding of NF-κB sites for MAR-BP1 binding. To further address this hypothesis, we examined the effect of NF-κB binding on the association of the MAR-BP1 binding requires NF-κB sites. A. 381-bp 5'-En fragment (X-P) was digested with restriction enzymes and subfragments isolated as indicated. B. MAR-BP1 binding to the probes described in A was evaluated by MSA, quantified by PhosphorImaging, and plotted as a percentage of total probe in the reaction mix. C. Different NF-κB sites deletion mutants were used in MSA competition experiments to test the effect of NF-κB binding sites on MAR-BP1 binding. 32P-labeled 5'-En fragment was incubated with 5 μl of MAR-BP1 DEAE fraction in the presence of a 10−100 fold molar excess of competitor DNA from wild-type or mutant IgH enhancer containing deletions of NF-κB binding sites P1, P2, P3, and P4 (mut). D. Wild-type, P1, or P2 deletion mutant (d1-5'En and d2-5'En) 5'-En DNAs were used as competitors at a 50-fold molar excess in binding reactions containing labeled 5'-En probe (lanes 1–6) as described above. Wild-type, P3, or P4 deletion mutants were used in competition experiment containing labeled 5'-En probe (lanes 7–11). E. Binding affinities of MAR-BP1 to wild-type or mutant 5'-En fragments. Binding of varying concentrations of MAR-BP1 to DNA probes (25 pm) was measured by MSA. The DNA probes included the 381-bp 5'-En fragment (open squares), the 301-bp MboI 5'-En fragment (open squares), the 350-bp d1-5'En fragment (closed circles), and the 330-bp d2-5'En fragment (open circles). The dissociation constant (Kd) was calculated from the protein concentration required for a 50% shift of the probe (Koudelka, et al., 1987; Dickinson and Kohwi-Shigematsu, 1995) to be $1.3 \times 10^{-8}$ M for both the 5'-En and the MboI 5'-En fragments. The affinities for the d1-5'En and d2-5'En fragments were estimated to be at least 5-fold lower than that of the wild-type 5'En fragment based on the relative amounts of protein needed to bind 10% of the respective probes.
IgH enhancer with the nuclear matrix in vitro. Purified NF-\(\mu\)NR was preincubated with the IgH enhancer followed by binding analysis to insoluble nuclear matrix preparations as described in Fig. 1B. Binding of NF-\(\mu\)NR to the IgH enhancer prevents its interaction with the nuclear matrix (Fig. 6A), in support of this model. In contrast, neither total soluble nuclear matrix protein (Fig. 6B, lane 3) nor purified MAR-BP1 (lane 4) was found to inhibit binding of the enhancer to insoluble nuclear matrix; rather, stimulation of binding was observed. This may be due to the ability of soluble matrix proteins like MAR-BP1 to bind insoluble matrix through protein-protein interactions, bringing down additional enhancer DNA.

The requirement of NF-\(\mu\)NR sites for MAR-BP1 binding suggests that the interaction of these two proteins might be mutually exclusive. To address this possibility, a kinetic experiment was performed to examine NF-\(\mu\)NR binding to a mixture of free DNA and DNA-MAR-BP1 complexes (Fig. 7). With time, the amount of NF-\(\mu\)NR-DNA complex increases until equilibrium is reached (by -15 min). The increase in NF-\(\mu\)NR-DNA complex is balanced by a decrease in the amount of free probe remaining (lanes 5 and 6). In contrast, the amount of MAR-BP1-DNA complex remains unchanged. This result indicates that MAR-BP1 binding prevents NF-\(\mu\)NR interaction. However, if excess NF-\(\mu\)NR is used and the reaction is allowed to proceed for 60 min, well beyond the half-life of the MAR-BP1-DNA complex (25 min), NF-\(\mu\)NR will replace MAR-BP1 (Fig. 8). This result is consistent with the higher affinity of NF-\(\mu\)NR for this fragment. Thus, in cells where both NF-\(\mu\)NR and MAR-BP1 are present (e.g. non-B cells) the enhancer would be occupied by NF-\(\mu\)NR.

**DISCUSSION**

MAR-BP1 is a Novel Nuclear Matrix-derived MAR Binding Protein—MAR-BP1 was isolated from nuclear matrix samples prepared by the high salt method from a mature B cell line, and migrates as a 33-kDa protein on SDS-polyacrylamide gels. The question arises as to the relationship between MAR-BP1 and other identified nuclear matrix proteins. In one study, the protein composition of a highly purified nuclear matrix preparation from rat liver contained 12 major proteins including lamins A, B, and C, the B-23 nucleolar protein, an hnRNP protein, and eight other unidentified proteins termed nuclear matrixins (Hakes and Berezney, 1991; Berezney, 1991). In addition to these major proteins, a number of minor proteins have also been purified from the nuclear matrix. Many of these proteins appear to be cell-type specific (Fey and Penman, 1988).

A group of proteins have been identified that bind to MARs in vitro. The 67-kDa lamin B1 has been found to bind MAR sites (Luderus et al., 1992). The 116-kDa yeast protein RAP-1 binds to the silent mating-type locus in the region of the transcriptional silencer (Hofmann et al., 1989). The 95-kDa protein ARBP was isolated based on its ability to bind to MARs from the chicken lysozyme gene and was found to bind the IgH enhancer MAR as well (von Kries et al., 1991). SAF-A/hnRNP-U, a 120-kDa protein isolated from HeLa cells, binds to a scaffold attachment region element from the topoisomerase I gene (Ronom et al., 1992; Fackelmayer et al., 1994). SATB1 was cloned as a gene encoding a thymus-specific protein that binds to an A/T-rich motif found in many MARs, including those flanking the IgH enhancer (Dickinson et al., 1992). While the sequence of the cloned SATB1 gene predicts a molecular mass
MAR Binding and IgH Enhancer Regulation

The interaction of many MAR-binding proteins differ from the recognition sites of nuclear transcription factors in that a relative large fragment (~200 bp) is required for high affinity binding (von Kries et al., 1991, 1994; Tsutsui et al., 1993; Luderus et al., 1994). In the case of MAR-BP1, this would explain why removal of the Xbal-Rsal region from the 5’-end of the 5’-En fragment reduces binding affinity by a factor of 4 and yet has no intrinsic affinity for MAR-BP1 itself (Fig. 5).

In any case, it is clear that deletion of either P1 or P2 on the 5’-En fragment, or P3 or P4 on the 3’-En fragment had profound effects on MAR-BP1 binding suggesting a requirement for cooperative interactions. It is possible that these sequences serve as nucleation sites for a more extensive covering of the region by multiple MAR-BP1 proteins. This could explain why it has proven difficult to footprint these, or any, DNA regions in MAR-BP1 complexes.

MARs and Transcription Regulation—The major findings from this work can be summarized as follows. 1) The IgH enhancer repressor protein, NF-µ NR, is able to inhibit attachment of the IgH enhancer to the nuclear matrix. This is true of matrices isolated by the high salt method (Fig. 6A) or the L1S method (data not shown). 2) A 33-kDa DNA-binding protein, MAR-BP1, has been isolated from nuclear matrix preparations of different lymphoid cell types, which bind specifically to MAR segments derived from a number of different DNA loci. 3) Although MAR-BP1 binding sites overlap NF-µ NR binding sites in the IgH enhancer, the proteins differ in molecular weight, DNA binding characteristics and cell-type-specific expression profiles. 4) NF-µ NR and MAR-BP1 binding are mutually exclusive.

Taken together, these results provide support for a model of NF-µ NR-mediated enhancer regulation involving nuclear matrix attachment previously proposed (Schuermann and Chen, 1989). In this model (see Fig. 9) an important aspect of IgH enhancer function would be to attach the heavy chain locus to the nuclear matrix in appropriate cells (B lymphocytes). This would bring the gene into regions of the nucleus that contain high concentrations of transcription factors, RNA polymerase, and topoisomerases. However, in inappropriate cells (other than B lymphocytes) NF-µ NR would be expressed and would bind to its recognition sites flanking the enhancer, thereby preventing nuclear matrix attachment.

R.-T. Zong and R. H. Scheuermann, unpublished results.

Fig. 8. NF-µ NR displaces DNA-bound MAR-BP1. MAR-BP1 DEAE fraction (20 ng, lane 2; 25 ng, lane 3; 30 ng, lane 4; 40 ng, lanes 5–9) was incubated with the labeled 5’-En fragment for 30 min at room temperature to reach equilibrium, at which time purified NF-µ NR was added at 2 ng (lane 6), 3 ng (lane 7), 3.5 ng (lane 8), and 4 ng (lane 9). Incubation was continued for an additional 60 min on ice before MSA analysis.

If of 86,000, the protein migrates to a position of ~103,000 in SDS/polyacrylamide gels even when translated in vitro (Nakagomi et al., 1994).

Based on its size, MAR-BP1 is distinct from these members of the MAR-binding family of nuclear proteins. However, two proteins that have been found to bind MARs have similar sizes, NF-µ NR and histone H1. A variety of observations presented here indicate that MAR-BP1 and NF-µ NR are distinct. Complexes formed by NF-µ NR and DNA containing its recognition sites form characteristic complexes that migrate very slowly in MSA gels. Complexes with MAR-BP1 migrate more rapidly. NF-µ NR binding generates complexes that are easily analyzed by DNase I footprinting, whereas MAR-BP1 complexes are not. The most convincing argument that they are distinct is that UV-cross-linking reveals proteins of different sizes.

MAR-BP1 also appears to be distinct from histone H1. Purified histone H1 from the same B cell line has different DNA-binding/competition characteristics and antigenicity as compared with MAR-BP1.

Our results describe the identification of a novel MAR-binding protein that specifically binds to MAR sites associated with the IgH intronic enhancer. Although we cannot rule out the possibility that other matrix proteins are involved in the association between the nuclear matrix and the IgH enhancer in B cells, MAR-BP1 shows the strongest MAR-binding activity in cells, MAR-BP1 binding can be competed by double-stranded P4 DNA, it was unaffected by either single-stranded P4 oligonucleotide.

It now appears that MAR-binding proteins can be divided into two groups. One group included proteins like nucleolin (Dickinson and Kowhi-Shigematsu, 1995) and Lamin B (Hakes and Berezney, 1991), which have a propensity to bind single-stranded DNA. The other group includes SATB1 and MAR-BP1, which bind double-stranded DNA, possibly through the recognition of a particular tertiary structure such as a narrow minor groove.

The binding sites for several MAR-binding proteins differ from the recognition sites of nuclear transcription factors in that a relative large fragment (~200 bp) is required for high affinity binding (von Kries et al., 1991, 1994; Tsutsui et al., 1993; Luderus et al., 1994). In the case of MAR-BP1, this would explain why removal of the Xbal-Rsal region from the 5’-end of the 5’-En fragment reduces binding affinity by a factor of 4 and yet has no intrinsic affinity for MAR-BP1 itself (Fig. 5).

In any case, it is clear that deletion of either P1 or P2 on the 5’-En fragment, or P3 or P4 on the 3’-En fragment had profound effects on MAR-BP1 binding suggesting a requirement for cooperative interactions. It is possible that these sequences serve as nucleation sites for a more extensive covering of the region by multiple MAR-BP1 proteins. This could explain why it has proven difficult to footprint these, or any, DNA regions in MAR-BP1 complexes.

MARs and Transcription Regulation—The major findings from this work can be summarized as follows. 1) The IgH enhancer repressor protein, NF-µ NR, is able to inhibit attachment of the IgH enhancer to the nuclear matrix. This is true of matrices isolated by the high salt method (Fig. 6A) or the L1S method (data not shown). 2) A 33-kDa DNA-binding protein, MAR-BP1, has been isolated from nuclear matrix preparations of different lymphoid cell types, which bind specifically to MAR segments derived from a number of different DNA loci. 3) Although MAR-BP1 binding sites overlap NF-µ NR binding sites in the IgH enhancer, the proteins differ in molecular weight, DNA binding characteristics and cell-type-specific expression profiles. 4) NF-µ NR and MAR-BP1 binding are mutually exclusive.

Taken together, these results provide support for a model of NF-µ NR-mediated enhancer regulation involving nuclear matrix attachment previously proposed (Schuermann and Chen, 1989). In this model (see Fig. 9) an important aspect of IgH enhancer function would be to attach the heavy chain locus to the nuclear matrix in appropriate cells (B lymphocytes). This would bring the gene into regions of the nucleus that contain high concentrations of transcription factors, RNA polymerase, and topoisomerases. However, in inappropriate cells (other than B lymphocytes) NF-µ NR would be expressed and would bind to its recognition sites flanking the enhancer, thereby preventing nuclear matrix attachment.
This model implies that MARs are transcriptional regulatory elements. The first indications that MARs might be involved in transcription came from the observation that MARs are frequently found near cis-acting transcriptional regulatory elements (e.g., Gasser and Laemmli, 1986; Cockerill and Garrard, 1986; Cockerill et al., 1987). Analysis of the effects of MARs on transcriptional activity has revealed an interesting characteristic of MARs that distinguish them from promoter and enhancer elements. MARs have been found to stimulate transcription from defined promoters in a number of systems, but only when the test construct is stably integrated into a chromosomal context (e.g., Blasquez et al., 1989; Xu et al., 1989; Klehr et al., 1990). In addition to increasing the level of transcription, MARs seem to provide for copy number-dependent and position independent transcription (Phi-Van et al., 1990; McKnight et al., 1992). Although locus control regions can have similar effects, it is not clear whether they are the same as MARs or similar (Dillon and Grosveld, 1993).

Some evidence suggests that MARs might play a role in tissue-specific expression regulation. Genes that are actively transcribed are preferentially associated with the nuclear matrix, while genes that are not expressed are not bound (Gerdes et al., 1994). In addition, it has been found that the β-globin gene is associated with the nuclear matrix in reticulocytes but not in thymocytes, whereas the malic enzyme gene is attached in thymocytes but not reticulocytes (Brotherton et al., 1991). This observation demonstrates that tissue-specific nuclear matrix attachment correlates with tissue-specific transcriptional expression.

In the case of the IgH intronic enhancer, a recent publication provides strong support for the importance of these MAR regions in transcriptional enhancement (Forrester et al., 1994). The ability of the IgH enhancer to stimulate expression of a rearranged Ig heavy chain transgene was examined in preB cell lines isolated from transgenic mice in which the transgene contained a wild-type enhancer or an enhancer with MAR site deletions. While all preB cell lines from mice containing the wild-type enhancer exhibited high level transgene expression, transgene expression was absent in lines from MAR deletion mutants. These results indicate that, in mice, IgH enhancer activity requires intact MARs. As NF-κB is only expressed in non-B cells and can prevent nuclear matrix attachment in vitro, the cell-type-specificity of the IgH enhancer may in part be controlled by the negative regulation of nuclear matrix attachment.

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