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Sequence-specific DNA-binding proteins are components of a nuclear matrix-attachment site

(histone gene promoter/transcription/transactivation factor/HeLa cells)

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ABSTRACT We have identified a nuclear matrix-attachment region within an upstream element of a human H4 histone gene promoter. Nuclear matrix proteins, isolated and solubilized from HeLa S_3 cells, were found to interact with sequence specificity at this matrix-attachment region. Several types of assays for protein–DNA interaction showed that the minimal sequence for the nuclear matrix protein–DNA interaction was 5′-TGACGTCCATG-3′; the underlined region corresponds to the core consensus sequence for ATF transcription factor binding. Two proteins with molecular masses of 43 and 54 kDa were identified by UV-crosslinking analysis as integral components of this protein–DNA complex. The molecular masses of these proteins and the ATF-binding site consensus sequence suggest that these proteins are members of the ATF family. Our results provide direct evidence for nuclear matrix localization of sequence-specific DNA-binding factors for an actively transcribed gene. The proximity of a strong positive transcriptional regulatory element to the matrix-attachment region of this gene suggests that the nuclear matrix may serve to localize and concentrate trans-acting factors that facilitate regulation of gene expression.

The functional diversity of the nuclear matrix is becoming increasingly apparent. The proteinaceous components are being better characterized, and evidence continues to accumulate for variations in nuclear matrix protein composition that reflect cell structure and function (1–3). The nuclear matrix, originally defined by Berezney and Coffey (4) as the insoluble skeletal framework within the nucleus, can maintain the structural integrity of the nucleus and provides anchor sites for DNA attachment (5, 6). These anchor sites have been reported to constrain DNA into loop structures of ≈60 kilobases (kb) (7, 8). It is generally acknowledged that the three-dimensional conformation of chromatin can affect transcriptional regulation. However, it remains to be established how the nuclear matrix supports this organization of chromatin, and more importantly, the biochemical composition and sequence specificity of these anchorage sites are only beginning to be defined (6). The nuclear matrix has also been reported to have a role in mRNA transcription and processing via its involvement in attachment and/or association with newly transcribed mRNA (9), ribonucleoprotein particles (10), pre-mRNA splicing machinery (11, 12), and steroid receptors (13, 14). To attribute structure–function relationships to these reported associations, it is essential to characterize the nuclear matrix proteins involved and the nature of their interactions.

The identification of regions of DNA attachment to the nuclear matrix [(matrix-attachment regions (MARs)] has been reported for several genes, including mouse immunoglobulin κ chain (15), rat α_2-macroglobulin (16), human interferon β (17), several developmentally regulated genes of *Drosophila melanogaster* (18), human β-globin (19), and the human apolipoprotein B gene (20). There are also data demonstrating that actively transcribed genes are preferentially associated with the nuclear matrix (21–25). However, information describing the sequence specificity and the protein components of these MARs is limited.

To understand the function of MARs, several laboratories have recently performed transcriptional studies with MAR elements. The importance of these elements in gene regulation is becoming increasingly apparent. For the chicken lysozyme gene Stief *et al.* (26) have described a MAR that can increase gene expression and is partially orientation dependent and positionally independent with respect to its effect on the lysozyme transcriptional enhancer. Phi-van *et al.* (27) demonstrated that a chicken lysozyme 5′ MAR can elevate expression of a heterologous promoter in different cell types. Schaack *et al.* (28) showed that adenovirus DNA is bound to the nuclear matrix throughout the infection and that this binding may play a role in viral transcription. Zenk *et al.* (29) have described the binding of a 480-base-pair (bp) cloned DNA fragment from the avian β-globin gene enhancer to the nuclear matrix; they also showed that topoisomerase II is not the protein tightly bound to the DNA. Finally, Blasquez *et al.* (30) found that deletion of the intronic immunoglobulin κ MAR led to a decrease in gene expression. Thus, a consensus appears to emerge that MARs can play an important role in regulating the level of gene expression.

Our previous studies of the human FO108 histone H4 gene suggested that a nuclear matrix attachment site is located within the first 2000 nucleotides (nt) of the promoter. Pauli *et al.* (31) found, by "Southwestern" (Southern/immunoblot) analysis, that upstream fragments from this histone H4 promoter are preferentially associated with the residual nuclear structural proteins after histone removal. In addition, these upstream regions contain consensus sequences that have been associated with MARs—topoisomerase II cleavage sites as well as A-box- and T-box-rich sequences. In the present study a MAR within a strong positive transcriptional regulatory element in the upstream promoter of the human H4 histone gene (K.L.W., S.I.D., P. E. Kroeger, J.L.S., and G.S.S., unpublished data) was identified by the nuclear matrix gene reassociation technique of Cockerill and Garrard (15). Taking advantage of the ability to solubilize nuclear matrix proteins, we then showed that a subset of these proteins interacts specifically with sequences within the

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Abbreviations: MAR, matrix-attachment region; NMP-1, nuclear matrix protein 1; nt, nucleotide(s).

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MAR. In addition, the nuclear matrix proteins involved in the MAR interaction were partially characterized by UV-crosslinking experiments. Our studies provide direct evidence for localization of sequence-specific DNA-binding factors to the protein component of the nuclear matrix. We suggest that the nuclear matrix may serve to localize and concentrate trans-acting factors that participate in regulation of gene expression.

METHODS AND MATERIALS

Nuclear Matrix Isolation. The nuclear matrix was isolated according to the methods of Ornelles *et al.* (48) from HeLa S3 cells grown in suspension culture at 37°C. For protein–DNA interaction studies, the nuclear matrix preparation was further purified by removing the cytoskeletal intermediate-filament proteins (1). Identification of a MAR in the histone H4 gene promoter (32) was done as described by Cockerill and Garrard (15) for the immunoglobulin κ gene.

Analyses of Protein–DNA Interactions. Gel-mobility-shift assays were done according to Staudt *et al.* (33). Total nuclear extracts for transcription factor preparations were isolated according to the method of Dignam *et al.* (34). Site-directed base substitution was done according to the uracil-containing template procedure of Kunkel (35). Footprint analysis of the nuclear matrix protein 1 (NMP-1) protein–DNA complex was done by using the 1,10-phenanthroline copper(II) method (36, 37). The DNA was electrophoresed through a 6% polyacrylamide/7.5 M urea sequencing gel. Binding conditions for dimethyl sulfate protection experiments were essentially identical to those for the gel-mobility-shift assay, except that the normal 20-μl reaction was scaled up 5-fold. Maxam and Gilbert sequencing reactions (38) were electrophoresed in adjacent lanes of the gel as markers. For characterization of nuclear matrix proteins by DNA-affinity chromatography, the NMP-1 oligonucleotide was covalently coupled to Sepharose CL-2B beads as described by Kadonaga and Tjian (39). The sample fractions were desalted on PD-10 columns (Pharmacia), concentrated, and further analyzed by UV crosslinking according to Chodosh *et al.* (40).

RESULTS

Identification of a Nuclear MAR in the H4 Histone Gene Promoter. Previous studies have suggested the presence of a nuclear MAR in the distal promoter of the FO108 human H4 histone gene (31). Moreover, it is known that during exponential growth of HeLa S3 cells the histone H4 gene is actively transcribed (41). To determine more directly whether a specific region of the H4 histone gene is preferentially associated with the nuclear matrix when it is actively transcribed, we used an *in vitro* MAR assay (15). The H4 histone gene promoter along with pUC19 vector as an internal control was digested with restriction endonucleases, and after radiolabeling these fragments, ranging in size from 65 bp to 3900 bp, were added to nuclear matrices isolated from exponentially growing cultures of HeLa cells. The bound DNA fragments were reisolated and separated by PAGE. The results shown in Fig. 1 demonstrate that the 141-bp *Eco*RI–*Ban* II fragment from the promoter (−730 to −590 bp) is associated preferentially with the isolated nuclear matrix.

Sequence-Specific Protein–DNA Interactions. Several approaches were pursued to address the possibility of sequence-specific interactions of the 141-bp H4 histone promoter fragment with nuclear matrix proteins. Initially, we used the gel-mobility-shift assay to determine whether any protein–DNA interactions existed within this MAR element. A radioactive DNA probe spanning the region of the promoter between the *Eco*RI and *Ban* II restriction sites was

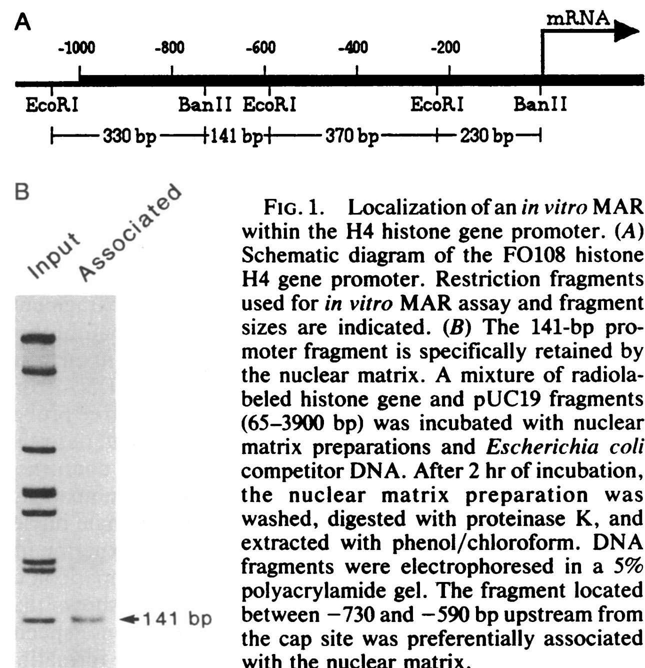

FIG. 1. Localization of an *in vitro* MAR within the H4 histone gene promoter. (*A*) Schematic diagram of the FO108 histone H4 gene promoter. Restriction fragments used for *in vitro* MAR assay and fragment sizes are indicated. (*B*) The 141-bp promoter fragment is specifically retained by the nuclear matrix. A mixture of radiolabeled histone gene and pUC19 fragments (65–3900 bp) was incubated with nuclear matrix preparations and *Escherichia coli* competitor DNA. After 2 hr of incubation, the nuclear matrix preparation was washed, digested with proteinase K, and extracted with phenol/chloroform. DNA fragments were electrophoresed in a 5% polyacrylamide gel. The fragment located between −730 and −590 bp upstream from the cap site was preferentially associated with the nuclear matrix.

incubated with isolated, solubilized nuclear matrix. The MAR probe formed one major complex, designated NMP-1, with the nuclear matrix proteins (Fig. 2).

To define more precisely the 5′ and 3′ boundaries of nuclear matrix protein interaction within the MAR, a bidirectional-deletion analysis was done by labeling either the *Eco*RI or the *Ban* II end of the 141-bp MAR fragment. The fragments were then digested to obtain progressively shorter probes (see Fig. 5), which were each used in gel-mobility-shift assays; the results are shown in Fig. 2. Full-length MAR probe labeled at either the *Eco*RI or *Ban* II site shows the NMP-1 protein–DNA complex (Fig. 2 *A* and *B*, respectively). Digestion of the *Eco*RI probe with either *Mnl* I (−708 nt) or *Hin*fI (−649 nt) did not disrupt

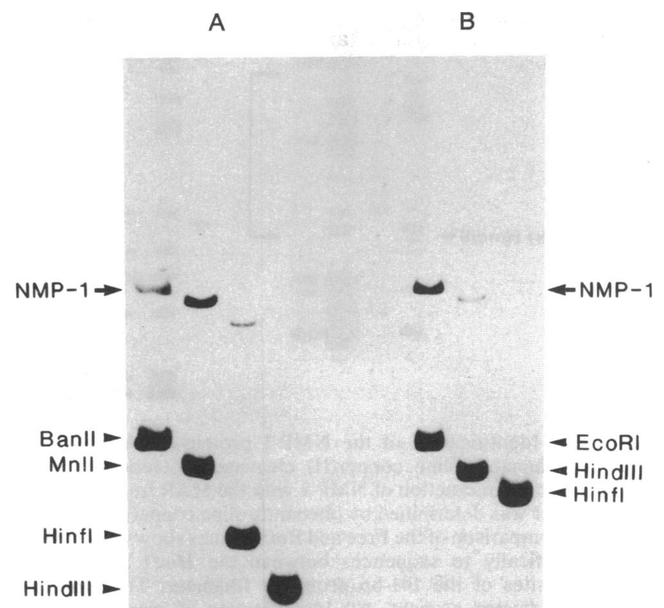

FIG. 2. Nuclear matrix protein–DNA interaction within the 141-bp distal promoter element. Gel-mobility-shift assays were used in conjunction with bidirectional-deletion analysis to determine the site of nuclear-matrix protein binding within the 141-bp MAR fragment. Probes were labeled at the *Eco*RI (*A*) or *Ban* II (*B*) sites. The NMP-1 interaction with the probe was localized to the region between the *Hin*fI and *Hin*dIII restriction sites (−649 to −628 bp).

the interaction, whereas digestion of the probe with *Hin*dIII (−628 nt) eliminated binding. As seen in Fig. 2*B*, the *Ban* II-labeled probe supported NMP-1 complex formation when digested with *Hin*dIII (−628 nt) but not when digested with *Hin*fI (−649 nt). These results clearly localize the protein–DNA interaction site between the *Hin*dIII and *Hin*fI restriction sites. Intensity of the NMP-1 complex is less when the probes are digested with either *Hin*dIII or *Hin*fI, suggesting that these sequences play a role in the stability of the complex.

To demonstrate sequence specificity of the protein–DNA interaction within the 141-bp H4 histone promoter fragment, the NMP-1 complex was subjected to 1,10-phenanthroline copper(II) cleavage analysis. Results of the footprint analysis are shown in Fig. 3*A*. The banding pattern of the DNA from the NMP-1 complex, when compared with that of free probe, revealed a protected region from −630 nt to −644 nt, between the *Hin*dIII and *Hin*fI restriction sites; this result confirmed the binding site identified by bidirectional-deletion analysis.

To define the NMP-1-binding sequence with single nucleotide resolution, dimethyl sulfate fingerprinting experiments were done; the results are shown in Fig. 3*B*. Comparison of the complexed (Bound) and free probe samples shows that three guanine residues (−636, −637, and −640) were specifically protected from methylation and cleavage by formation of the NMP-1–DNA complex. This nucleotide sequence falls between the *Hin*dIII and *Hin*fI restriction sites and coincides with the phenanthroline copper(II) footprint. These results show that NMP-1 specifically binds to the MAR within the minimal sequence motif 5′-TGACGTCCATG-3′. Analysis of the upper strand revealed guanine contacts at −633, −639, and −642 (data not shown). Interestingly, examination of the NMP-1-binding site revealed a core ATF transcription factor consensus sequence (42) within the interaction domain. Fig. 4 summarizes the guanine nucleotide contacts and the ATF-binding consensus sequence.

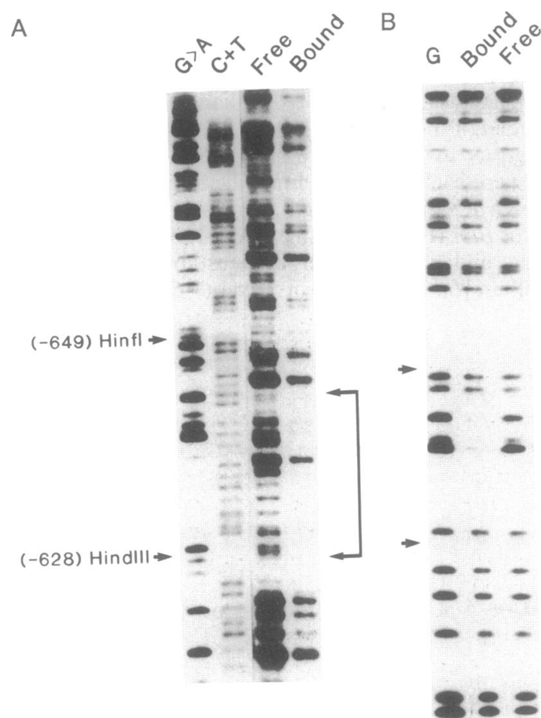

FIG. 3. Identification of the NMP-1 protein–DNA interaction site. (*A*) Phenanthroline copper(II) cleavage protection. The sequence-specific interaction of NMP-1 with the MAR fragment of the H4 promoter was determined by phenanthroline copper(II) footprint analysis. Comparison of the Free and Bound lanes shows that NMP-1 binds specifically to sequences between the *Hin*fI and *Hin*dIII restriction sites of the 141-bp promoter fragment. The protected region is indicated at right. (*B*) Identification of specific guanine residue contacts. Single-nucleotide interactions of NMP-1 on the lower strand were established at guanine residues −636, −637, and −640 by dimethyl sulfate protection analysis of the gel-shifted protein–DNA complex. The recognition-binding sequence is GGACGTCA (lower strand), which contains the core consensus sequence for the ATF family of transcription factors (underlined). Lanes: G, guanine sequence ladder; Bound, probe complexed with protein; Free, free probe.

NMP-1 Binds to an ATF Consensus Sequence. Site-directed base substitution and competition assays were done to define further the binding-site requirement for the NMP-1 complex. Formation of the NMP-1 complex was specifically reduced by an oligonucleotide containing the NMP-1-binding site sequence (−653 nt to −627 nt), but was not changed by an oligonucleotide containing the Sp1 consensus binding site (data not shown). To verify that the ATF-related sequence identified by dimethyl sulfate protection and footprint analysis (see above) was required for NMP-1 binding, oligonucleotide site-directed mutagenesis was used to introduce a mutation in which 5′-TGA*CG*-3′ was altered to 5′-TGA*TC*-3′. When this mutated DNA probe was used with nuclear matrix extracts in the gel-mobility-shift assay (Fig. 5*A*), the NMP-1 complex was formed with the wild-type probe but was not found with the mutated probe. These results indicate that the ATF consensus sequence is necessary for formation of the NMP-1 complex with the MAR DNA fragment. The lower bands seen in this and other NMP-1 gel-shift assays appear to be breakdown products of the NMP-1 complex, as they are specifically reduced by an NMP-1 oligonucleotide and are not formed when the NMP-1 mutated probe is used.

When transcription factor extracts prepared by the method of Dignam *et al.* (34) were used with the MAR probe, a more complex pattern of protein–DNA interactions was seen (Fig. 5*B*). To determine whether these complexes were related, we used the mutated MAR probe. Fig. 5*B* shows that the mutated probe did not support formation of the NMP-1 like complex from the soluble extract. Further studies have shown that the NMP-1-like complex from the soluble extract has the same pattern of guanine residue contacts with the MAR probe as does the NMP-1 complex (K.L.W., S.I.D., J.L.S., G.S.S., unpublished data). These results indicate that the NMP-1 protein(s) can be isolated by high salt extraction of nuclei, perhaps by releasing these proteins from the nuclear matrix.

Proteins of 43 kDa and 54 kDa Are Involved in MAR Binding. To characterize the protein composition of the NMP-1 complex, we carried out UV-crosslinking experiments to label NMP-1 proteins selectively based on their specific binding to the MAR in the H4 histone gene promoter. Thus it was possible to determine the molecular masses of the nuclear matrix proteins involved in binding. The probe was prepared by synthesizing the complementary strand of the 141-bp MAR fragment in the presence of bromodeoxyuridine triphosphate and [α-^{32}P]dCTP. The labeled probe was incubated with nuclear matrix proteins and then UV-irradiated for increased amounts of time. The complex pattern of

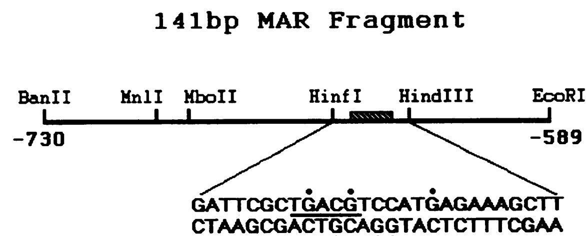

FIG. 4. Schematic representation of the NMP-1-binding element. The guanine nucleotide contacts are shown for both strands (•), and the ATF consensus sequence is underlined.

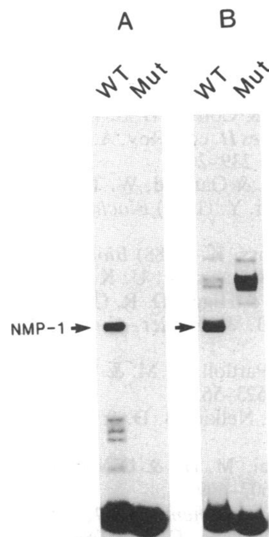

FIG. 5. Protein–DNA interactions of the MAR fragment with nuclear matrix proteins and high salt nuclear extracts. Gel-mobility-shift analyses show that the NMP-1 interaction is found in high salt extracts of isolated nuclei (*B*) as well as in the nuclear matrix protein fraction (*A*). Site-directed mutation of the NMP-1-binding site (TGA*CG*TCC to TGA*TC*TCC) abolished the NMP-1 interaction in both the nuclear matrix (*A*) and high salt nuclear extracts (*B*). Lanes: WT, wild-type MAR fragment used as probe; Mut, probe with mutation of the ATF consensus sequence.

crosslinked protein bands seen on the gel (Fig. 6*A*) was both UV and protein dependent because no bands were formed in control reactions in which protein or UV irradiation was omitted. To determine which of the multiple bands comprised the NMP-1 complex, NMP-1 and nonspecific oligonucleotides were used as competitors in the binding reaction. Proteins corresponding to 43 kDa and 54 kDa were specifically eliminated by excess unlabeled NMP-1 oligonucleotide, indicating that they are part of the NMP-1 complex.

The NMP-1 complex was partially purified from the nuclear matrix protein fraction by affinity chromatography with

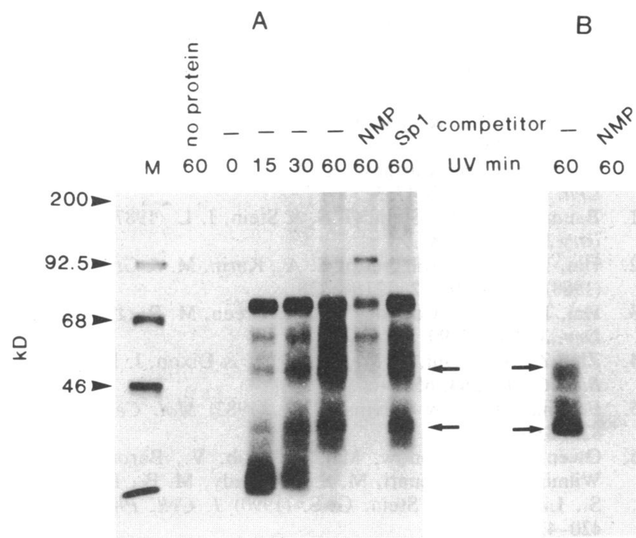

FIG. 6. Characterization of NMP-1 by UV crosslinking to the MAR fragment. Nuclear matrix extracts (*A*) or partially purified NMP-1 fraction from an oligonucleotide-affinity column (*B*) were UV-crosslinked with the distal MAR element. The period of UV illumination is indicated in minutes. The lower two bands in *A* (arrows) are the only ones specifically eliminated by the NMP-1 oligonucleotide. These two bands are formed with the partially purified fraction (*B*) and represent proteins of 43 and 54 kDa.

the NMP-1 oligonucleotide covalently coupled to Sepharose beads. Fractions containing NMP-1-binding activity were pooled. When this partially purified NMP-1 fraction was used for UV-crosslinking analysis (Fig. 6*B*), the same protein bands were identified, and both were specifically eliminated by the NMP-1 competitor oligonucleotide. These results clearly demonstrate that two nuclear matrix proteins of 43 and 54 kDa are involved in formation of the NMP-1 complex with an ATF-related sequence in the MAR fragment of the H4 histone gene promoter.

DISCUSSION

The nuclear matrix has been postulated to play a fundamental role in the structural organization of DNA within the nucleus. Furthermore, two lines of evidence indicate that the nuclear matrix may be involved in transcriptional regulation of gene expression: (*i*) Composition of a subset of the nuclear matrix proteins has been shown to be cell-type specific (1, 3). (*ii*) A functional relationship between nuclear matrix proteins and transcriptional control is further suggested by progressive modifications in the complement of these proteins in parallel with changes in gene expression during cell differentiation (2).

One key issue in defining the structural and functional properties of the nuclear matrix relative to transcriptional regulation is to provide a link between the MARs and the transcriptional activity of a gene. In other words, can the MAR serve as a positive regulator of transcription when the gene is associated with the nuclear matrix? We have approached the problem by first localizing a MAR within the first 1 kb of the FO108 human H4 histone gene promoter. The ability to solubilize the protein components of the nuclear matrix provided the basis for directly studying protein–DNA interactions by using the defined MAR. The physical association with protein components of the nuclear matrix was characterized by using several criteria, including gel-mobility-shift assay, phenanthroline copper(II) and dimethyl sulfate protection experiments, and site-directed mutagenesis. These studies clearly demonstrate that nuclear matrix proteins bind with sequence specificity to the human histone H4 gene promoter.

The minimal sequence motif for NMP-1 interaction within the MAR was determined to be 5′-<u>TGACG</u>TCCATG-3′; the TGACG element represents the core consensus sequence for the ATF family of transcription factors (42). This fact raises several interesting possibilities. (*i*) The MAR lies within a strong activator region of the H4 histone promoter identified by both *in vivo* and *in vitro* transcriptional analysis of deletion mutants (ref. 32; K.L.W., P. E. Kroeger, J.L.S., G.S.S., unpublished data). (*ii*) The molecular masses of the proteins involved in the NMP-1 complex, 43 kDa and 54 kDa, are similar to those of two previously characterized ATF transcription factors (43), in addition to binding to the ATF core consensus sequence. Thus, our results indicate that some members of the ATF transcription factor family are nuclear matrix proteins. When nuclear transcription factor extracts were prepared by high salt extraction, similar NMP-1 binding was observed with the MAR fragment. This result suggests that the nuclear matrix could, in fact, be localizing and/or concentrating transcription factors that are solubilized from the nuclear matrix during high salt extraction. Interestingly, Zhu *et al.* (44) purified from a high salt extract a 43-kDa ATF-related transcription factor required for rat somatostatin gene expression that recognizes the sequence 5′-<u>TGACGTC</u>A-3′, a seven of eight match with our NMP-1 element.

At least two classes of DNA-binding proteins appear to be associated with the nuclear matrix, both contributing to the transcriptional properties of active genes. The attachment

region-binding protein recently described by von Kries *et al.* (6) displays characteristics consistent with a role for this protein in the generation of functional chromatin loop domains. However, the attachment region-binding protein does not recognize a defined sequence; rather, it is an abundant nuclear protein that binds 200- to 350-bp MARs containing multiple T-rich sequence motifs. Thus the attachment region-binding protein appears to serve primarily a structural role in matrix–chromatin interactions.

Another protein that may serve primarily a structural role in matrix association is the adenovirus terminal protein. Schaack *et al.* (28) have shown by mutational analysis that the terminal protein, which is covalently attached to the 5′ ends of adenovirus DNA, mediates the tight binding of the DNA to the nuclear matrix. Terminal protein mutants also display reduced transcriptional efficiency for the adenovirus early genes, which cannot be complemented by coinfection with wild-type virus. These results suggest that association of DNA to the nuclear matrix may have a critical role in adenovirus transcription. Interestingly, Feldman and Nevins (45) have shown that the adenovirus $E1A_a$ transcription regulatory protein also is present in the nuclear-matrix fraction in infected cells. However, it is unclear whether $E1A_a$ has a direct structural role in attachment of adenovirus DNA to the matrix.

NMP-1 appears to represent a second type of DNA-binding protein associated with the nuclear matrix. NMP-1 exhibits sequence-specific interactions with an H4 histone promoter regulatory element contained within a MAR. Thus, NMP-1 may be representative of a class of nuclear matrix-associated factors directly involved in transcriptional control. The NMP-1 complex was also found in nuclear matrix preparations from proliferating osteoblasts in which the H4 histone gene is actively transcribed (46, 47), but this complex was no longer seen in differentiated cells after down-regulation of histone gene transcription (unpublished observations). Taken together, these findings suggest that the nuclear matrix could serve as a site for assembly of factors that render the promoter competent to support the initiation of transcription.

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