The EMBO Journal vol.11 no.9 pp.3431 - 3440, 1992

Characterization of SAF-A, a novel nuclear DNA binding protein from HeLa cells with high affinity for nuclear matrix/scaffold attachment DNA elements

Helmut Romig, Frank O.Fackelmayer, Andrea Renz, Uwe Ramsperger and Arndt Richter

Department of Biology, University of Konstanz, 7750 Konstanz, Germany

Communicated by R.Knippers

We identified four proteins in nuclear extracts from HeLa cells which specifically bind to a scaffold attachment region (SAR) element from the human genome. Of these four proteins, SAF-A (*s*caffold *a*ttachment *f*actor A), shows the highest affinity for several homologous and heterologous SAR elements from vertebrate cells. SAF-A is an abundant nuclear protein and a constituent of the nuclear matrix and scaffold. The homogeneously purified protein is a novel double stranded DNA binding protein with an apparent molecular weight of 120 kDa. SAF-A binds at multiple sites to the human SAR element; competition studies with synthetic polynucleotides indicate that these sites most probably reside in the multitude of A/T-stretches which are distributed throughout this element. In addition we show by electron microscopy that the protein forms large aggregates and mediates the formation of looped DNA structures.

Key words: DNA binding protein/loop formation/nuclear matrix/scaffold

Introduction

The nuclear matrix is an operationally defined insoluble structure which remains after treatment of isolated nuclei with DNase I and extraction of proteins with high salt solutions (Berezney and Coffey, 1974). This structure, which is the same size and shape as the original nucleus, contains the nuclear lamina, remnants of the nucleoli and an internal proteinaceous network. The scaffold is a similar structure, although it differs from the nuclear matrix with respect to the overall protein composition. Scaffolds remain when histones and many other nonhistone nuclear proteins are removed from isolated nuclei by the chaotropic agent lithium diiodosalicylate (Mirkovitch *et al.*, 1984). Both nuclear substructures contain, in addition to proteins, a low amount of nuclear DNA that is resistant to DNases or restiction enzyme digestion.

Several enzymes important in DNA replication or transcription have been identified as components of the nuclear matrix and of scaffolds. These enzymes include DNA polymerases (Smith and Berezney, 1980), RNA polymerases (Jackson and Cook, 1985; Roberge *et al.*, 1988; Dickinson *et al.*, 1990), topoisomerase II (Earnshaw *et al.*, 1985; Berrios *et al.*, 1985) and transcription factors (Getzenberg and Coffey, 1990). In addition, methods have been developed for the preparation of nuclear substructures under physiological salt conditions. These structures possess almost complete polymerizing activities when compared with the activities found in isolated nuclei and intact cells (Jackson and Cook, 1985). From these experiments it was concluded that the complex machinery necessary for replication of the genome and for precisely controlled transcription, including RNA processing and transport (for review see van Driel *et al.*, 1991), is bound to nuclear substructures. These studies also implied that nuclear substructures may play a central role in nuclear organization and may provide the structural basis for all processes of DNA metabolism.

Nuclear DNA has been found to be tightly associated with the proteinaceous nuclear substructures via specific, evolutionarily conserved DNA elements, which have been termed SARs (*s*caffold *a*ttachment *r*egions, Gasser and Laemmli, 1986) or MARs (*m*atrix *a*ssociated *r*egions, Cockerill and Garrard, 1986). We shall use the term SAR element in the following as the term 'matrix' is also used for other non-nuclear structures. SAR elements are AT-rich single copy fragments of 250 – 1500 bp. They often contain stretches of A and T residues and clusters of topoisomerase II consensus sequences (Gasser and Laemmli, 1986). Although SAR elements of one species exhibit strong affinity for the nuclear matrix prepared from other species, no conserved 'consensus sequence elements' have been found.

DNA in eukaryotic interphase nuclei is organized into loop domains with average lengths of 50 – 100 kb (for reviews see Gasser and Laemmli, 1987; Cook, 1990). The presence of loop domains and the existence of SAR elements has led to models which postulate that SAR elements are involved in the formation of these loop domains. It has been assumed that SAR elements are the specific sequences at which the genomic DNA is bound to the scaffold at the basis of these loops.

In many cases, SAR elements co-map with boundaries of actively transcribed chromatin domains (Mirkovitch *et al.*, 1984; Bode and Maass, 1988; Phi-Van and Strätling, 1988). Sippel and co-workers (Stief *et al.*, 1989) isolated cell lines which contained an artificial domain with a reporter gene. They were able to show that an SAR element from the chicken lysozyme gene, which they termed an A-element, mediated a significant stimulation of gene expression when cloned upstream and downstream of a reporter gene. In addition, they found that the expression of this reporter gene was independent of the site of integration into the genomic DNA when it was framed by A-elements. Similar results have been reported by Phi-Van *et al.* (1990) and Klehr *et al.* (1991). These studies implied that SAR elements are able to stimulate transcription of a gene and may protect a transcribed region from position effects from neighbouring sequences. However, SAR elements have also been identified within intron sequences (Cockerill and Garrard, 1986; Käs and Chasin, 1987; Brun *et al.*, 1990). The function of these SAR elements is unknown. As SAR elements have also been found close to origins of DNA replication (for reviews see

Cook, 1991; Hamlin *et al.*, 1991) and since it has been suggested that they are involved in illegitimate recombination (Sperry *et al.*, 1989), it may be that different types of SAR elements exist and that these elements have different functions in the organization of the genome.

Nuclear matrices and scaffolds contain many different nonhistone proteins. Only a few of these proteins have been purified and biochemically and functionally characterized. Among these proteins are the lamins A, B and C, topoisomerase II (sc1), the glycoprotein gp 188, which is a component of nuclear pore complexes, and sc2, a scaffold protein of unknown function. A new nuclear protein, ARBP, which specifically binds to matrix/scaffold attachment DNA regions, has recently been purified from chicken oviduct cells (van Kries *et al.*, 1991). In addition, Hakes and Berezney (1991) described seven abundant DNA binding proteins which are present in nuclear matrix prepared from rat cell nuclei. These proteins include lamins A and B and five novel proteins which have been named matrins D, E, F, G and matrin 4. The DNA binding proteins matrins F and G and matrin 3, a nuclear matrix protein with unknown function and properties, have been cloned recently (Belgrader *et al.*, 1991).

Since SAR elements may be involved in anchoring genomic DNA to nuclear substructures, it is necessary to identify the proteins mediating this anchorage and to purify and characterize them to understand better their biological functions. Here we report the identification of a novel DNA binding protein which is present in nuclei, in the nuclear matrix and in scaffolds derived from HeLa cells. This protein has an apparent molecular weight of 120 kDa and specifically binds to several SAR elements from different eukaryotic species. We have purified this protein, which we have named *s*caffold *a*ttachment *f*actor *A* (SAF-A) to homogeneity and we describe its biochemical properties.

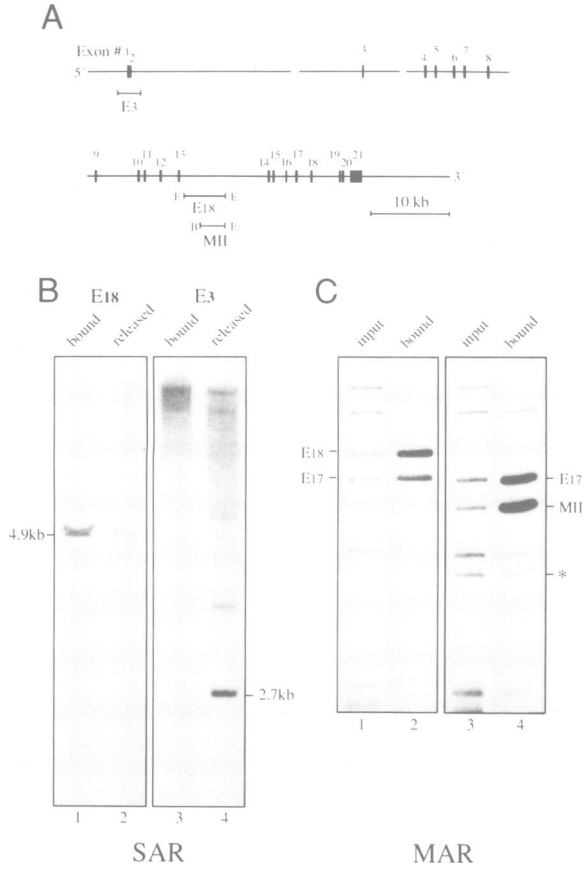

Fig. 1. Identification of SAR elements in the human topoisomerase I gene. (**A**) Structure of the human topoisomerase I gene (Kunze *et al.*, 1991). Vertical bars show the exon sequences and are numbered from 1 to 21. E_3 and E_{18} are two of the consecutively numbered *Eco*RI fragments which cover the gene locus. MII, *Bam*HI–*Eco*RI subfragment of E_{18}. E, *Eco*RI; B, *Bam*HI; kb, kilobase pairs. (**B**) Partitioning of E_3 and E_{18} with genomic DNA bound to (lanes 1 and 3) or released (lanes 2 and 4) from scaffolds. Equal amounts of DNA derived from the two fractions were separated on 0.7% agarose gels, the DNA was transferred to nitrocellulose membranes and the membranes were hybridized with radioactively labelled E_{18} or E_3 DNA. (**C**) Binding of the E_3 and E_{18} DNA fragments to the nuclear matrix. Purified phage λ DNA containing the *Eco*RI fragments E_{17} and E_{18} were digested with *Eco*RI (lanes 1 and 2) or with *Eco*RI and *Bam*HI (lanes 3 and 4) and end-labelled. 10 ng of labelled DNA fragments were incubated with nuclear matrix in the presence of a 10 000-fold excess of *E.coli* competitor DNA. Lane 1 (*Eco*RI digest) and lane 3 (*Eco*RI/*Bam*HI digest) show the labelled DNA fragments added to the nuclear matrix. The DNA was fractionated into matrix bound (lanes 2 and 4) and free DNA by low speed centrifugation. The asterisk indicates the 2 kb DNA fragment created when E_{18} is cut by *Bam*HI into MII (2.9 kb) and this non-SAR. E_{17}, *Eco*RI fragment adjacent to E_{18}.

Results

Identification of a nuclear matrix/scaffold attachment DNA element in the human topoisomerase I gene locus

We have determined the structure of the human type I DNA topoisomerase gene (Kunze *et al.*, 1991). To elucidate the

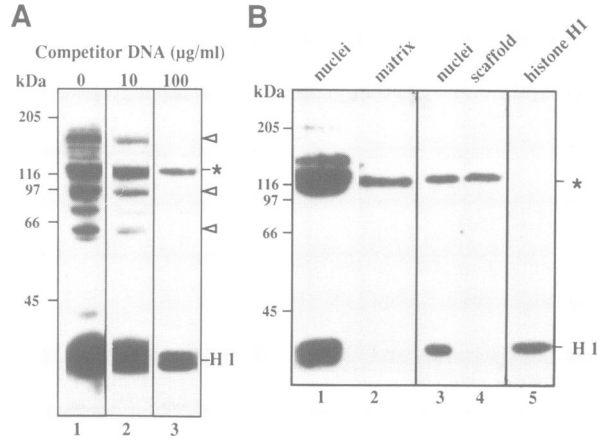

Fig. 2. Identification of nuclear proteins binding to MII. (**A**) Nuclear extracts (25 μg total protein) were separated on 8% SDS–polyacrylamide gels and the proteins were transferred to nitrocellulose membranes. The membranes were incubated with 10 ng of end-labelled MII DNA without (lane 1) or with 10 (lane 2) or 100 μg/ml (lane 3) of *E.coli* competitor DNA in a total volume of 3 ml. The asterisk indicates the 120 kDa protein with the highest affinity for MII DNA. The arrowheads indicate proteins with molecular weights of 165, 96 and 60 kDa. H1, histone H1. Molecular weight markers were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa) and ovalbumin (45 kDa). (**B**) 25 μg of total protein derived from nuclear extracts (lanes 1 and 3), nuclear matrix (lane 2) and scaffolds (lane 4) were separated on 8% SDS–polyacrylamide gels and the proteins were transferred to nitrocellulose membranes. The membranes were incubated with 10 ng of end-labelled MII DNA (lanes 1–6) with 100 μg/ml of *E.coli* competitor DNA in a total volume of 3 ml. Lane 5 shows 0.5 μg purified human histone H1 separated on the same gel and incubated with labelled MII DNA. The asterisk points to the 120 kDa protein. Markers are as in (A).

domain organization of the gene, we initiated experiments to localize nuclear matrix/scaffold attachment DNA elements. During this work we identified several DNA fragments which are able to bind to nuclear matrix preparations and which partition with scaffold bound DNA fragments (H.Romig and A.Richter, in preparation). Figure 1A shows the structure of the human topoisomerase I gene and the location of the DNA fragments that we used in the experiments presented below. The DNA fragment E_{18} is a 4.9 kb *Eco*RI subfragment of the gene located in intron 13. The E_{18} fragment bound efficiently to the nuclear matrix in a matrix DNA binding assay (Figure 1C, compare lanes 1 and 2); and in fact, E_{18} shows the highest affinity for the nuclear matrix of all topoisomerase I gene fragments tested.

Using the scaffold DNA binding assay we were able to show that this DNA fragment also partitions with scaffold bound DNA (Figure 1B). In this experiment we digested genomic DNA in histone depleted nuclei with *Eco*RI and separated the DNA into scaffold bound and scaffold released fractions. After agarose gel electrophoresis of identical amounts of DNA from both fractions, transfer of the DNA to nitrocellulose membranes and Southern hybridization with radioactively labelled E_{18} DNA as a probe, we found that there was ~9-fold more E_{18} fragment in the scaffold bound DNA fraction than in the scaffold released DNA fraction (Figure 1B, compare lanes 1 and 2). In contrast, the 2.7 kb *Eco*RI fragment E_3, which contains the promoter of the gene as well as exons 1 and 2, is a non-SAR DNA fragment. It does not bind to the nuclear matrix (data not shown) and partitions completely with DNA fragments released from scaffolds (Figure 1B, compare lanes 3 and 4).

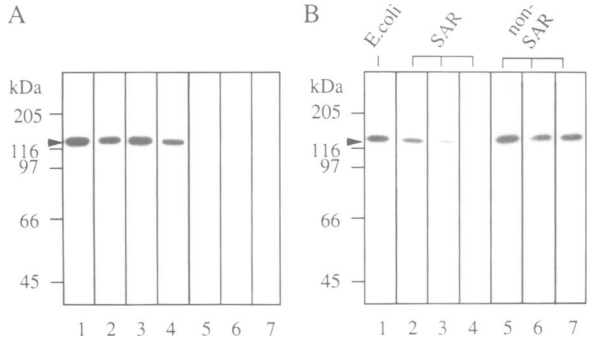

Fig. 3. Binding of homologous and heterologous DNA fragments to the 120 kDa protein. (**A**) Nuclear extracts (25 μg total protein) were separated on 8% SDS–polyacrylamide gels and the proteins were transferred to nitrocellulose membranes. The membranes were incubated in the presence of 20 μg/ml of *E.coli* competitor DNA with 10 ng of end-labelled DNA in a total volume of 3 ml. The labelled DNA fragments used were MII (lane 1), E4 (lane 2), B1X1 (lane 3), B4B5 (lane 4), E3 (lane 5), E7X4 (lane 6) and pUC18 (lane 7). The specific activities (c.p.m./μg) of the DNA fragments were 2.2×10^6 (lane 1), 4.3×10^6 (lane 2), 1.9×10^6 (lane 3), 3.4×10^6 (lane 4), 3.6×10^6 (lane 5), 2.9×10^6 (lane 6) and 2.2×10^6 (lane 7). (**B**) Competition of MII binding to the 120 kDa protein by SAR and non-SAR DNA. Nuclear extracts (25 μg total protein) were separated on 8% SDS–polyacrylamide gels and the proteins were transferred to nitrocellulose membranes. The membranes were incubated with 10 ng of end-labelled MII DNA fragment (total volume 3 ml) in the presence of 20 μg/ml of *E.coli* competitor DNA (lane 1), with 5 μg/ml (lane 2), 10 μg/ml (lane 3) and 20 μg/ml (lane 4) of SAR DNA or with 5 μg/ml (lane 5), 10 μg/ml (lane 6) and 20 μg/ml (lane 7) of non-SAR DNA. SAR and non-SAR DNA was prepared as described in Materials and methods. The arrowheads point to the 120 kDa protein; molecular weight markers were as in Figure 2.

Large DNA fragments may be nonspecifically enriched in the scaffold fraction and this could be the reason for the partitioning of the 4.9 kb E_{18} fragment with the nuclear scaffold. This is, however, unlikely since under the same experimental conditions, E_{12}, a DNA fragment from the gene which is three times as long as E_{18}, was not enriched in the nuclear scaffold bound fraction (data not shown).

We have shortened E_{18} and found that a 2.9 kb *Bam*HI–*Eco*RI fragment bound as well to the nuclear matrix as the entire E_{18} fragment (Figure 1C, compare lanes 3 and 4). However, further deletion of DNA sequences from the 5′ side and from the 3′ side of the 2.9 kb fragment gradually reduced its binding affinity for nuclear matrix preparations (data not shown). Obviously, the matrix binding DNA region is present on the 2.9 kb *Bam*HI–*Eco*RI subfragment, the MII human SAR element.

We have sequenced MII and found that the DNA fragment is AT-rich (69.7%) and contains several sequences which perfectly or imperfectly match with the topoisomerase II consensus sequence (Spitzner *et al.*, 1990), with A- and T-

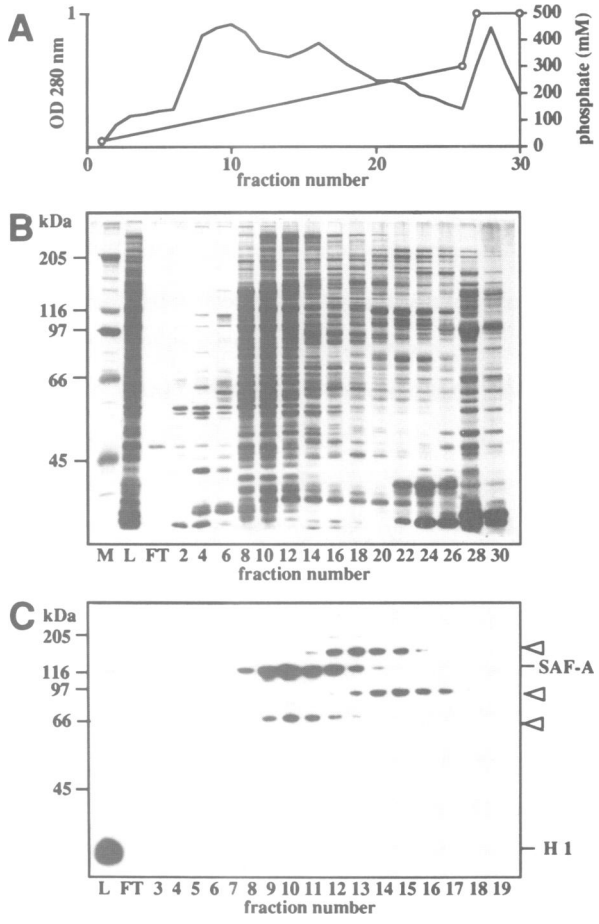

Fig. 4. Chromatographic separation of MII DNA binding proteins on hydroxylapatite. (**A**) Relative optical density measured at 280 nm. (**B**) Aliquots from the indicated fractions were separated on 10% SDS–polyacrylamide gels and the proteins were stained with silver. (**C**) Aliquots from the indicated fractions were separated on 8% SDS–polyacrylamide gels and the proteins were transferred to nitrocellulose membranes. The membranes were incubated with 50 ng of end-labelled MII DNA with 2.5 μg/ml of *E.coli* competitor DNA in a total volume of 10 ml. Molecular weight markers were as in Figure 2; L, protein loaded onto the column; FT, flow-through; SAF-A, scaffold attachment factor A (120 kDa); the arrowheads point to the three other proteins which bind to MII.

boxes (Gasser and Laemmli, 1986), with the sequence TATATTT (Bode *et al.*, 1991) and with the ARS consensus sequence (Amati and Gasser, 1988).

Identification of a 120 kDa protein that specifically binds to SAR elements

We have used MII as a DNA substrate to identify nuclear proteins binding to this DNA. We have purified from human cells a protein that specifically recognizes MII DNA as well as several other vertebrate SAR elements (see below). To identify proteins with affinity to MII we used a protein blot DNA binding assay which has been used for the purification of a SAR DNA binding protein from chicken oviduct cells by von Kries *et al.* (1991). Figure 2A shows those nuclear HeLa proteins which are active under the experimental conditions, and which bind MII DNA in the presence of high concentrations of competitor DNA. In the absence of competitor DNA, nuclear extracts contain several proteins binding the labelled MII DNA. Most prominent among these are proteins with apparent molecular weights of 165, 120, 96, 60 and 35 kDa (Figure 2A, lane 1). The binding of MII DNA to these proteins was sensitive to proteinase K but remained unaffected by DNase I or RNase A digestion. Addition of competitor DNA at concentrations of 10 or 100 μg/ml (Figure 2A, lanes 2 and 3) drastically reduced the binding activity of most of these DNA binding proteins. The two proteins which bind to MII DNA in the presence of a 30 000-fold excess of competitor DNA (Figure 2A, lane 3) have apparent molecular weights of 120 kDa and 35 kDa, respectively (Figure 2A, lane 3; Figure 2B, lanes 1 and 3). We have performed DNA binding experiments with purified human histone H1 and found that the protein which migrates corresponding to a protein with 35 kDa in the gel system used, showed specific MII binding activity (Figure 2B, lane 5). This strongly suggests that the MII binding protein observed in nuclear extracts may be histone H1. Similar results have been reported previously (Izaurralde *et al.*, 1989; von Kries *et al.*, 1991).

The 120 kDa protein (and the other DNA binding proteins described above) is a nuclear protein and is not present in cytosol or in low salt nuclear extracts (data not shown). The 120 kDa protein (Figure 2B, lanes 1 and 3) is also a protein component of the nuclear matrix (Figure 2B, lane 2) and of scaffolds (Figure 2B, lane 4). We therefore refer to the 120 kDa protein as SAF-A (*s*caffold *a*ttachment *f*actor A).

We have analysed whether the 120 kDa protein binds to other SAR elements. We found that the *Eco*RI restriction fragment E_4, a SAR element from the topoisomerase I gene present adjacent to the topoisomerase I gene promoter (Figure 3A, lane 2), the 2.9 kb B1X1 fragment (Figure 3A, lane 3) containing the SAR element from the 5′ side of the chicken lysozyme gene (Phi-Van and Strätling, 1988) and the 2.1 kb DNA fragment B4B5 (Figure 3A, lane 4) from the 3′ side of the gene were specifically bound by the 120 kDa protein. In contrast, linearized pUC18 or the DNA fragments E_3 and E7X1 (from chicken, see von Kries *et al.*, 1991) which are non-SAR DNA fragments, were not bound by the protein under the same conditions (Figure 3A, lanes 5, 6 and 7).

These experiments show that the 120 kDa protein has a high affinity for several vertebrate SAR elements and may be a general SAR DNA binding protein. This conclusion is supported by competition experiments shown in Figure 3B in which we compared the binding of labelled MII in the presence of increasing amounts of scaffold associated DNA (SAR DNA) or in the presence of DNA released from scaffolds by *Eco*RI digestion (non-SAR DNA). We found that the scaffold associated DNA fraction competed more efficiently (Figure 3B, lanes 2, 3 and 4) than released DNA (Figure 3B, lanes 5, 6 and 7).

Purification of SAF-A

Our purification procedure starts from an extract of HeLa cell nuclei and involves chromatography on hydroxylapatite, heparin–Sepharose and Mono-Q columns. The binding activity of SAF-A was measured throughout the purification by its ability to bind radioactively labelled MII DNA in the

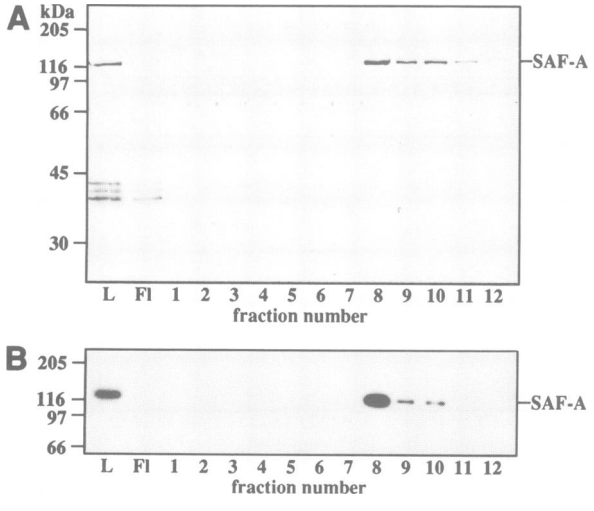

Fig. 5. Chromatographic separation of SAF-A on Mono-Q. (**A**) Aliquots from the indicated fractions were separated on 10% SDS–polyacrylamide gels and the proteins were stained with silver. (**B**) Aliquots from the indicated fractions were separated on 10% SDS–polyacrylamide gels and the proteins were transferred to nitrocellulose membranes. The membranes were incubated with 50 ng of end-labelled MII DNA with 25 μg/ml of *E.coli* competitor DNA in a total volume of 10 ml. Molecular weight markers were as in Figure 2; L, protein loaded onto the column; Fl, flow-through; SAF-A, scaffold attachment factor A.

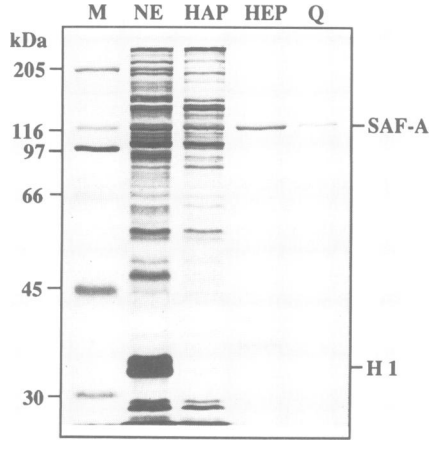

Fig. 6. Purification of SAF-A. Proteins present in nuclear extract (NE, 15 μg) and in active fractions after chromatography on hydroxylapatite (HAP, 10 μg), heparin–Sepharose (HEP, 1.5 μg) and Mono-Q (Q, 0.2 μg) chromatography were separated on a 7.5% polyacrylamide gel and the proteins were stained with silver. Molecular weight markers were as in Figure 2.

presence of competitor DNA. Initial experiments showed that 500 mM NaCl at pH 8.0 is necessary and sufficient to extract most of the protein from isolated nuclei.

Nuclear extracts (fraction NE) were prepared from $8-10 \times 10^9$ HeLa cells and loaded onto a hydroxylapatite column. Bound proteins were eluted with a salt gradient and aliquots of the fractions indicated in Figure 4 were analysed for protein composition (panels A and B) and DNA binding activity (panel C). The DNA binding reaction was performed in the presence of 2.5 μg/ml of competitor DNA. Under these low stringency conditions (500-fold excess of *Escherichia coli* DNA) the elution behaviour of SAF-A and the other MII DNA binding proteins can be followed simultaneously. SAF-A elutes between 100 and 130 mM potassium phosphate and is well separated from histone H1. Fractions containing >50% of the activity present in the peak fraction were combined (fraction HAP) and directly loaded onto a heparin–Sepharose column. Unbound proteins were washed off and the column was sequentially eluted in three steps. SAF-A eluted in the final gradient between 450 and 550 mM NaCl. Active fractions were combined (giving fraction HEP), diluted and applied to a Mono-Q column. The Mono-Q column was developed (see Materials and methods) and individual fractions were analysed for protein composition (Figure 5A) and DNA binding activity (Figure 5B). SAF-A eluted at 500 mM NaCl. Active fractions were combined (giving fraction Q) and stored at −70°C.

Figure 6 summarizes the purification of SAF-A. After chromatography on Mono-Q the protein was homogeneous and appeared as a single band which migrates corresponding to 120 kDa under denaturing conditions (Figure 6, lane Q). The purification described yielded 70–80 μg of homogeneous SAF-A protein from 8×10^9 HeLa cells with a recovery of 7–10%.

The purified SAF-A is free of topoisomerase I, topoisomerase II and single and double stranded nuclease activities (data not shown). We performed Western blotting experiments with antibodies against purified SAF-A, topoisomerases I and II and nucleolin (Figure 7). These studies clearly demonstrate that SAF-A is not a modified form of topoisomerase I or a degradation product of topoisomerase II. SAF-A is also not antigenically related to nucleolin (Figure 7). In addition, we show that neither topoisomerase I or II, nor their degradation products, bind MII DNA under the conditions of the protein blot DNA binding assay (Figure 7).

Preliminary experiments showed that a mouse antiserum, which recognized purified ARBP prepared from chicken oviduct cells, did not recognize purified SAF-A. At present, purified human ARBP is not available, but a protein with the same molecular weight as the chicken ARBP was recognized by the serum in nuclear extracts from HeLa cells (data not shown).

Physical properties of SAF-A

We have determined the sedimentation coefficient of the purified SAF-A protein by centrifugation in linear glycerol gradients with aldolase, haemoglobin and cytochrome *c* as sedimentation standards. In solutions containing 500 mM NaCl, SAF-A sedimented as a monomeric protein with an apparent sedimentation coefficient of $4.8S_{20,w}$. Lowering the salt concentrations resulted in the formation of large aggregates which pelleted to the bottom of the gradients. The isoelectric point of SAF-A as determined by two-dimensional gel electrophoresis was 5.0 ± 0.2.

DNA binding properties of purified SAF-A

When we determined the DNA binding activity of purified SAF-A by a filter retention assay in the absence of competitor DNA, 1 ng of MII DNA was bound by 10 ng of purified SAF-A (Figure 8A). The binding of SAF-A to MII DNA is apparently noncooperative, with a Hill coefficient of 0.97. Filter retention experiments performed in the presence of increasing amounts of unlabelled MII DNA or *E.coli* DNA as competitor showed that purified SAF-A specifically binds to MII. At a 10-fold excess of *E.coli* competitor DNA the amount of MII DNA bound was reduced to 70% (Figure 8B) and even with a 100-fold excess of competitor 30% of input MII DNA was bound by the protein. At these concentrations

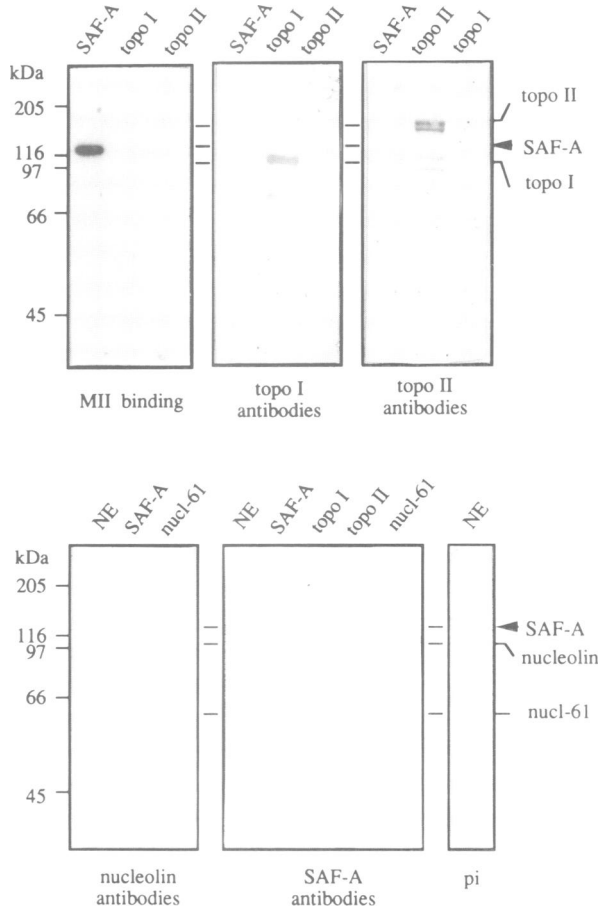

Fig. 7. SAF-A is not related to topoisomerases I and II or nucleolin. 25 μg of HeLa cell nuclear extract (NE) or 500 ng each of purified SAF-A (SAF-A), topoisomerase I (topo I), topoisomerase II (topo II) or the 61 kDa proteolytic fragment of nucleolin (nucl-61) were separated on 10% SDS–polyacrylamide gels. The proteins were transferred to nitrocellulose membranes and the membranes were analysed in the protein blot DNA binding assay as described (MII binding) or with antibodies against topoisomerase I (topo I antibodies), topoisomerase II (topo II antibodies), nucleolin (nucleolin antibodies) and SAF-A (SAF-A antibodies). topo II: 180 kDa and 170 kDa forms of topoisomerase II. nucl-61: 61 kDa fragment of nucleolin. The horizontal bars between the panels indicate the positions of topoisomerase II, SAF-A and topoisomerase I (upper panels) or SAF-A, nucleolin or the 61 kDa fragment of nucleolin (lower panels).

of competitor DNA 10% and 2% of residual binding was observed with labelled non-SAR pUC18 DNA as substrate. Thus, the filter retention experiments performed with the purified protein confirm the results obtained in the protein blot DNA binding experiments.

We have also used in competition studies a series of synthetic polynucleotides including poly(dA−dT), poly (dG−dC), poly(dA)·poly(dT) and poly(dA−dC)·poly (dT−dG). We found that poly(dA−dT) and poly(dA)·poly (dT) competed for binding of SAF-A to the human SAR element, whereas poly(dG−dC) and poly(dA−dC)·poly (dT−dG) did not compete. The double stranded alternating poly(dA−dT) was as efficient a competitor as unlabelled MII DNA (Figure 8C) and, at an input ratio of 0.6 (MII DNA to competitor DNA; w/w), <25% of labelled MII was bound by SAF-A. In contrast, poly(dA)·poly(dT) competed less well and at input ratios of 5 and 10, 60% and 35% of the labelled MII DNA was bound by the protein respectively (Figure 8C). Most of the experiments shown in Figure 8C were performed with commercially available polynucleotides which we sheared to an average length of 500−3000 nucleotides. Under the conditions of the filter retention experiments, with unsheared polynucleotides we observed the formation of high molecular weight structures which did not enter agarose gels, presumably due to the base pairing of single stranded regions of individual molecules. When we used these high molecular weight polynucleotide chains, the extent of competition was largely unaffected in the cases of poly(dA−dT), poly(dG−dC) and poly(dA−dC)·poly (dT−dG). In contrast, the unsheared poly(dA)·poly(dT) competed very efficiently for binding of SAF-A to MII DNA. In fact in this case poly(dA)·poly(dT) was the most efficient competitor tested.

Our attempts to identify by gel retardation experiments individual binding sites for SAF-A which might be present on MII failed as SAF-A forms large DNA−protein complexes which do not enter the gel. We also performed deletion experiments to localize binding sites of SAF-A using the protein blot DNA binding assay. For this purpose, we used a series of labelled DNA subfragments of MII DNA. We found that any subfragment tested showed a significant but reduced binding affinity when compared with the affinity of the complete MII DNA (data not shown). This is in agreement with the results obtained in matrix DNA binding experiments which also showed that any subfragment had a reduced affinity for nuclear matrix preparations.

SAF-A aggregates and forms specific loops with MII DNA

We analysed protein−DNA complexes formed between purified SAF-A and MII DNA by electron microscopy. Typical images of these complexes are shown in Figure 9. We observed two types of protein complexes bound to DNA: small non-filamentous aggregates of SAF-A with an average diameter of 35 nm attached to 1−3 MII DNA molecules (Figure 9A) and filamentous aggregates. The filamentous aggregates formed by SAF-A had an apparent width of 35 ± 4 nm and were present either as small circles with a diameter of ~140 nm or as filaments (Figure 9B) up to 1 μm in length (not shown). In both types of filamentous SAF-A complexes we observed many DNA molecules bound to the protein. An interesting feature of these protein−DNA complexes was the ability of SAF-A to induce the formation of DNA loops. No such looped structures were observed

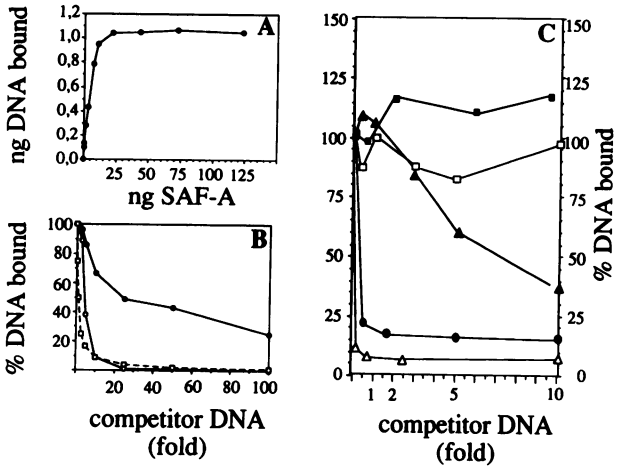

Fig. 8. DNA binding properties of purified SAF-A. (**A**) DNA filter retention assay performed with increasing amounts of SAF-A and 1.7 ng labelled MII DNA. (**B**) DNA filter retention experiments were performed with 3.5 ng SAF-A and 1.7 ng of labelled MII DNA (squares and closed circles) or the same amount of labelled pUC18 DNA (open circles) in the presence of increasing amounts of unlabelled MII DNA (squares) or sheared *E.coli* DNA (circles). The amount of DNA bound in the absence of competitor is set as 100%. (**C**) DNA filter retention experiments with 3.5 ng SAF-A and 1.7 ng of labelled MII DNA in the presence of increasing amounts of poly(dA−dT) (filled circles), sheared poly(dA)·poly(dT) (filled triangles), high molecular weight poly(dA)·poly(dT) (open triangles) poly(dG−dC) (filled squares) and poly(dA−dG)·poly(dT−dC) (open squares). The amount of DNA bound in the absence of competitor is set as 100%. All data points represent the mean of six individual experiments performed with two preparations of SAF-A.

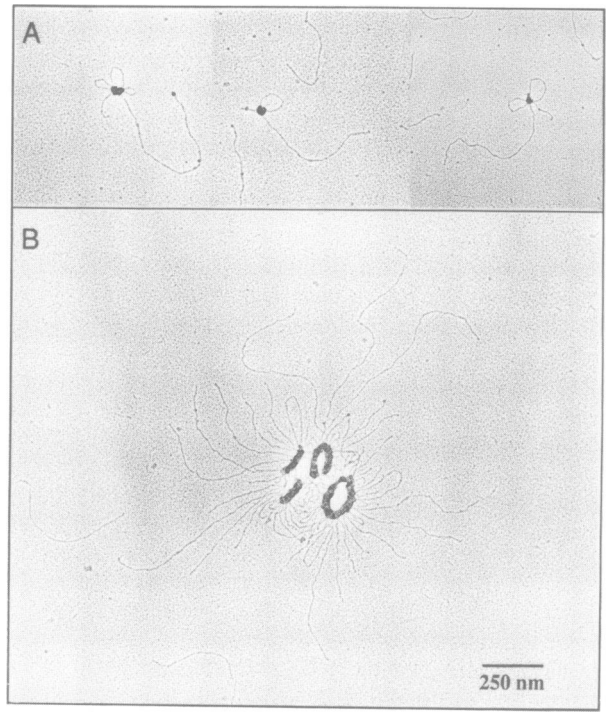

Fig. 9. Purified SAF-A reconstitutes loops with MII DNA. Electron micrographs of typical SAF-A/MII complexes. (**A**) MII DNA molecules bound to nonfilamentous SAF-A multimers. (**B**) MII DNA molecules bound to filamentous multimers of SAF-A.

with MII DNA alone or when serum albumin, topoisomerases I, II or SV40 large T-antigen were used instead of SAF-A (not shown).

A 120 kDa SAR DNA binding protein is present in different species and tissues

The SAR elements used in our experiments were identified by their ability to bind to nuclear matrix *in vitro*. It has been shown by others that nuclear matrices prepared from cells of different species are interchangeable with respect to their ability to bind SAR DNA from heterologous sources (Cockerill and Garrard, 1986; Phi-Van *et al*, 1988). In fact, a protein with the molecular weight and the binding properties of SAF-A was present in extracts prepared from human, mouse and bovine cells and was even detected in an extract from *Xenopus laevis* oocytes (Figure 10). An SAF-A related protein was found in roughly comparable amounts in the nuclei of all mouse tissues analysed including lung, liver, heart and kidney (Figure 10).

Discussion

We have identified in intron 13 of the human DNA topoisomerase I gene a DNA element, the MII fragment, which possesses high affinity for nuclear matrix preparations and for scaffolds. We identified four nuclear proteins which bind to this MII fragment. Of these, SAF-A showed the highest binding affinity for MII DNA and is a component of the nuclear matrix and of scaffolds. This protein shows strong and selective binding of MII DNA and binds to several other SAR elements from vertebrate cells including homologous SAR elements from the topoisomerase I gene and two SAR elements from the upstream and downstream regions of the chicken lysozyme gene domain. In contrast, several non-SAR DNA fragments including DNAs which are as AT-rich as SAR elements were not bound by SAF-A.

We have purified SAF-A to homogeneity. SAF-A is a double stranded DNA binding protein which selectively binds SAR DNA from vertebrate cells. The protein has an apparent molecular weight of 120 kDa and sedimented in sucrose gradients containing high salt concentrations as a monomeric protein with $4.8S_{20,w}$. Lowering the salt concentrations resulted in the formation of large aggregates, a property which reflects the self-polymerizing ability of the protein and which may be related to its structural functions. SAF-A is an abundant protein: we calculated that human HeLa cells contain $\sim 1 \times 10^6$ copies of this protein per nucleus. A protein with the same apparent molecular weight and the MII DNA binding properties of SAF-A was also present in nuclei prepared from mouse and bovine cells and in extracts prepared from *X.laevis* oocytes.

The protein was identified and purified using a protein blot DNA binding assay. In this assay SAF-A shows specific binding to SAR elements from vertebrate cells. Filter retention experiments confirmed these results and showed that the purified protein specifically interacts with MII DNA in presence of high amounts of competitor DNA.

What determines the binding specifity of SAF-A? MII DNA shares several structural features with other known SAR elements: the fragment is AT-rich and contains a number of perfect and imperfect matches with repeats of the topoisomerase II consensus sequence (Spitzner *et al.*, 1990), with A- and T-boxes (Gasser and Laemmli, 1986), with the sequence TATATTT which may be part of an unwinding element (Bode *et al.*, 1991) and with the ARS consensus sequence (Amati and Gasser, 1988). Deletion experiments showed that shortening of MII resulted in a gradual loss of the affinity of MII for SAF-A and indicated that neither of these motifs alone can be responsible for the binding of MII. AT-richness on the other hand is also not sufficient for the binding of a particular DNA to SAF-A as an AT-rich non-SAR fragment and fragments from *Saccharomyces cerevisiae* containing the AT-rich ARS1 or CEN3 sequences (Amati and Gasser, 1988) were not bound or were only poorly bound by the protein. This may indicate that SAF-A recognizes structural features of AT-rich DNA elements rather than unique sequence elements. This notion is supported by the competition studies with synthetic polynucleotides. In these experiments we found that poly(dA−dT) competed most efficiently for binding of SAF-A to MII DNA. poly(dA)·poly(dT) was less efficient and poly(dG−dC) or poly(dA−dC)·poly(dT−dG) did not compete for binding of SAF-A to MII DNA. Interestingly, the same polynucleotides which competed for binding of SAF-A to the human SAR element in our experiments are very efficient in the nucleation of histone H1 and topoisomerase II assembly on these artificial SAR-like elements (Izaurralde *et al.*, 1989).

We conclude from our results that no single unique binding site for SAF-A is present on MII. Either multiple motifs are distributed over the whole fragment or structural properties rather than sequence motifs are responsible for binding of DNA to SAF-A. The competition studies with synthetic polynucleotides indicate that these motifs or structures most probably reside in the multiple arrays of A/T nucleotides which are distributed throughout the MII fragment.

One intrinsic property of SAF-A molecules is that they interact with each other, forming large aggregates. This property is observed in glycerol gradients performed at low salt concentrations and could be the reason for the drastic losses of the protein during our initial attempts to purify SAF-

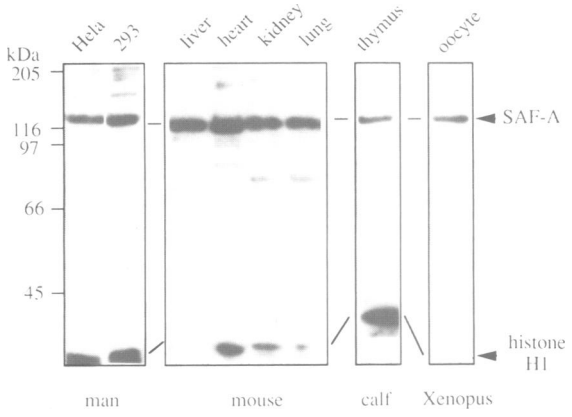

Fig. 10. A 120 kDa protein which binds MII DNA is present in several vertebrate species and in different tissues. Extracts were prepared from human (HeLa and 293), mouse (liver, heart, kidney and lung tissue) and calf tissue cells (thymus) and from *Xenopus laevis* oocytes (Richter *et al.*, 1980). 25−100 μg of total protein were separated on 10% SDS−polyacrylamide gels and the proteins were transferred to nitrocellulose membranes. The membranes were incubated with 10 ng of end-labelled MII DNA with 20 μg/ml of *E.coli* competitor DNA in a total volume of 3 ml. Molecular weight markers were as in Figure 2. SAF-A, 120 kDa protein related to SAF-A; H1, histone H1.

A. In addition, by electron microscopy, we observed globular or filamentous structures formed by SAF-A. These structures are multimeric forms of the protein and could contain 100 or more SAF-A molecules.

As SAF-A binds to all subfragments of MII analysed, it is quite likely that SAF-A mediates loop formation with MII DNA via protein−protein interactions between SAF-A molecules bound to different sites of the DNA molecule. The ability of SAF-A to interact specifically with SAR elements and to form complexes by protein−protein interactions is an essential requirement for a protein which may be involved in the formation of functional chromatin loops.

Two lines of evidence support our conclusion that SAF-A is in fact one of the proteins which are involved in the binding of specific DNA fragments to the nuclear matrix and to scaffolds. First, SAF-A was detected as a protein component of the nuclear matrix and of scaffolds. Secondly, we observed during our experiments that there is a direct correlation between the affinity of a given DNA for the nuclear matrix and the affinity of this fragment for SAF-A. The high affinity MII SAR of the topoisomerase I gene partitions almost exclusively with scaffolds and nuclear matrix preparations, and showed the strongest binding affinity for SAF-A. Other DNA fragments with significant but lower affinities for nuclear matrix and scaffold also had lower affinities for the isolated SAF-A protein. On the other hand, none of the non-SAR fragments tested bound to SAF-A. Thus, the affinity with which an individual DNA fragment binds to the nuclear matrix *in vitro* is proportional to the binding affinity of the same DNA fragment to purified SAF-A, strongly suggesting that SAF-A is a matrix/scaffold protein which mediates the binding of SAR DNA.

Numerous different nonhistone proteins are present in the nuclear matrix and in scaffolds and only a few of these proteins have been identified and functionally characterised. SAF-A is an abundant nuclear protein as are topoisomerases I and II. Our experiments, however, clearly showed that SAF-A is not related immunologically to these proteins and that it is functionally different, as these proteins do not bind MII DNA under conditions where SAF-A specifically binds to MII.

Recently, a new nuclear protein that binds cooperatively and specifically to matrix/scaffold attachment DNA regions has been purified from chicken oviduct cells (van Kries *et al.*, 1991). This protein (ARBP) has an apparent molecular weight of 95 kDa and is a constituent of the internal nuclear network. As in the case of SAF-A, ARBP binds to a variety of homologous and heterologous SAR elements but no unique sequence element responsible for binding could be identified. SAF-A is not related to ARBP as the purified protein differs in molecular weight and was not recognized by antisera raised against ARBP. Hakes and Berezney (1991) described several new proteins of unknown function which they identified in the nuclear matrix prepared from rat cell nuclei. Three of these proteins have been cloned recently (Belgrader *et al.*, 1991). SAF-A is apparently not related to these proteins as it is different in molecular weight and in its biochemical properties. Hofmann *et al.* (1989) described the purification of RAP-1, a DNA binding protein from yeast cells, which has an apparent molecular weight of 116 kDa under denaturing conditions and which promoted the formation of looped structures with DNA fragments containing the silencers that flank the mating type gene locus HML. RAP-1 (repressor−activator binding protein; Shore and Nasmyth, 1987) binds to the upstream region of many yeast genes and acts either as an activator or as a repressor of transcription (Devlin *et al.*, 1991). In addition it has been shown recently that RAP-1 may also be involved in meiotic but not mitotic recombination at some gene loci in yeast (White *et al.*, 1991). More generally, RAP-1 has been shown to affect DNA bending (Vignais *et al.*, 1989), association of DNA with scaffolds (Hofmann *et al.*, 1989) and nucleosome positioning (Devlin *et al.*, 1991). Though its molecular weight appears to be slightly higher, several biochemical properties of SAF-A are reminiscent of RAP-1. SAF-A is present in the nuclear matrix and in the scaffolds, and the purified protein reconstitutes looped structures with a human SAR element. However, further studies are necessary to determine whether SAF-A may be structurally or functionally related to RAP-1.

It is assumed that SAR elements are involved in a variety of DNA metabolism processes (Gasser and Laemmli, 1987; Bodnar, 1988; Cook, 1991; Hamlin *et al.*, 1991). The large number of SAF-A molecules present per nucleus points to a structural function of this protein. As nuclear substructures may play a central role in all processes discussed above it is at present premature to speculate whether SAF-A is involved in one or the other of these processes.

Materials and methods

Material

E_3, E_4, E_{17} and E_{18} are *Eco*RI fragments from the human DNA topoisomerase I gene locus; they have been described in Kunze *et al.* (1991). MII is a 2.9 kb *Eco*RI−*Bam*HI subfragment of E_{18}. B1X1, B4B5 and E7X4 are fragments from the chicken lysozyme gene locus (Phi-Van and Strätling, 1988). The ARS1- and the CEN3-containing DNA fragments from yeast were derived from pYe(CEN3)30 by *Eco*RI or *Eco*RI/*Bam*HI digestion, respectively (Amati and Gasser, 1988). The 61 kDa proteolytic fragment of nucleolin, topoisomerase I and topoisomerase II (Sapp *et al.*, 1985; Strausfeld and Richter, 1989) were purified as described. Antibodies against homogeneously purified topoisomerases I and II and nucleolin (Sapp *et al.*, 1986) were raised in rabbits and purified by protein A−Sepharose chromatography. Heparin−Sepharose and FPLC Mono-Q columns were from Pharmacia (Freiburg, Germany), hydroxylapatite (Bio-Gel HTP) and protein assay reagents from Bio-Rad. Molecular weight markers for SDS−PAGE, sedimentation standards, lithium diiodosalicylate and sheared *E.coli* DNA were from Sigma (München). Polynucleotides were obtained from Sigma (München) and were sheared to a length of 500−2500 nucleotides by sonication. Radioactive nucleotides and restriction nucleases were from Amersham (Braunschweig) and enzymes from Boehringer (Mannheim). Membranes and filters were obtained from Schleicher & Schuell. All other reagents were purchased from Merck (Darmstadt) and were of analytical grade.

Protein blot DNA binding assay

The protein blot DNA binding assay was performed essentially as described by von Kries *et al.* (1991) with *E.coli* DNA as competitor. End-labelled MII DNA was used as substrate at specific activities of $3-6 \times 10^6$ c.p.m./μg of DNA. The competitor concentrations used are indicated in the figure legends.

Matrix DNA binding assay

Matrix DNA binding assays were performed as described by Cockerill and Garrard (1986) with *E.coli* DNA as competitor. The restriction enzyme fragments used were end-labelled with Klenow polymerase and had specific activities of $3-6 \times 10^6$ c.p.m./μg of DNA. The competitor concentrations used are indicated in the figure legends.

Scaffold DNA binding assay

The preparation of scaffold and scaffold associated DNA was performed as described for *Drosophila* cell scaffolds with a heating step (20 min, 42°C) prior to the extraction with lithium diiodosalicylate (Mirkovitch *et al.*, 1984).

DNA filter retention assay

1.7 ng of radioactively labelled MII DNA was incubated with purified SAF-A as indicated in the figure legends in a buffer containing 10 mM Tris−HCl pH 8.0, 80 mM NaCl, 2 mM $MgCl_2$ and 0.05 mg/ml bovine serum albumin for 30 min at room temperature. Samples were filtered through prewetted GF51 glass fibre filters (Schleicher & Schuell) and washed with a total of 3 ml of incubation buffer without DNA. The amount of filter bound DNA was determined by liquid scintillation counting.

Purification of SAF-A

All purification steps were carried out at 4°C. All buffers up to fraction HAP contained 10 mM β-mercaptoethanol and a mixture of protease inhibitors, including 10 mM $Na_2S_2O_5$, 1 μM pepstatin, 1 μM aprotinin and 1 μM leupeptin. All buffers from fraction HAP contained 10 mM β-mercaptoethanol and 10 mM $Na_2S_2O_5$. A typical preparation starts with a total of $8-10 \times 10^9$ HeLa cells. Cells were collected by centrifugation (500 *g*, 5 min, 0°C) and washed three times in PBS buffer. The final cell pellet was resuspended in 80 ml of 10 mM Tris−HCl pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl and, after swelling for 10 min on ice, the cells were broken by 20 strokes in a loose fitting Dounce homogenizer. Nuclei were collected by centrifugation (750 *g*, 10 min, 0°C) and the nuclear pellet was washed three times with a total of 240 ml of the same buffer. The washed nuclei were resuspended and extracted into 80 ml of 10 mM Tris−HCl pH 8.0, 500 mM NaCl. Nuclear debris was pelleted by high speed centrifugation (150 000 *g*, 30 min, 0°C), and the supernatants were combined yielding 75 ml of nuclear extract (fraction NE). The nuclear extract was directly loaded at a flow rate of 0.5 ml/min onto a hydroxylapatite column (50 ml volume) equilibrated in 10 mM Tris−HCl pH 8.0, 500 mM NaCl. The column was washed with 150 ml of equilibration buffer and bound proteins were eluted with a gradient (170 ml) from 10 mM Tris−HCl pH 8.0, 500 mM NaCl to 10 mM Tris−HCl pH 8.0, 500 mM potassium phosphate. Fractions (4 ml) containing the specific DNA binding activity were combined (fraction HAP) and loaded at a flow rate of 0.25 ml/min onto a heparin−Sepharose column (10 ml volume) equilibrated with 10 mM Tris−HCl pH 8.0, 400 mM NaCl, 100 mM potassium phosphate. After washing the resin with equilibration buffer, bound proteins were sequentially eluted in three steps at a constant flow rate of 0.25 ml/min. First, with a nonlinear gradient (40 ml) from 500 mM NaCl to 2500 mM NaCl in 100 mM potassium phosphate, 10 mM Tris−HCl pH 8.0; secondly, with 40 ml of 10 mM Tris−HCl pH 8.0, 250 mM NaCl and thirdly, with 40 ml of 50 mM CAPS pH 9.8, 250 mM NaCl. Finally the column was eluted with a linear gradient from 250 to 1250 mM NaCl in 50 mM CAPS pH 9.8. Active fractions were combined (fraction HEP) and diluted 5-fold with ice-cold 10 mM β-mercaptoethanol, 10 mM $Na_2S_2O_5$. After centrifugation (20 000 *g*, 30 min, 0°C) the supernatant was immediately applied to a FPLC Mono-Q column (1 ml volume) equilibrated with 10 mM CAPS pH 9.8, 100 mM NaCl at a flow rate of 0.5 ml/min. The column was washed with 10 ml of 20 mM Tris−HCl pH 7.5, 100 mM NaCl and eluted with a linear gradient from 100 to 1000 mM NaCl in the same buffer. Active fractions were combined (fraction Q).

Electron microscopy of DNA protein complexes

150 ng of MII DNA were incubated with 30 ng of purified SAF-A in 30 μl of 10 mM triethanolamine−HCl pH 7.5, 80 mM NaCl, 1 mM $MgCl_2$ for 30 min at room temperature. DNA−protein complexes were fixed by adding 3 μl 1% glutardialdehyde for 15 min at 37°C. After chilling on ice, 5 μl aliquots were spread with benzalkonium chloride and visualized by rotary shadowing with tungsten as described by Schiedner *et al.* (1990).

Other methods

SDS−PAGE of proteins was performed according to Laemmli (1970). Protein gels were stained as described by Wray *et al.* (1980). Western transfer was performed according to Towbin *et al.* (1979) using alkaline phosphatase coupled secondary antibodies and two-dimensional polyacrylamide gel electrophoresis was as described by O'Farrell (1975). Protein concentrations were determined using the Bio-Rad protein assay kit with bovine serum albumin as a standard. Glycerol gradients (5−35% glycerol in 10 mM Tris−HCl pH 8.0, 500 mM NaCl) were centrifuged at 49 000 r.p.m., 0°C for 20 h in a Beckman SW55.1 rotor. DNA was labelled and separated by agarose and non-denaturing polyacrylamide gel electrophoresis according to standard protocols (Sambrook *et al.*, 1989). Autoradiography was performed using Kodak X-ray films.

Acknowledgements

We thank Dr W.H.Strätling for generously giving plasmid DNAs containing various fragments from the chicken lysozyme gene locus and antisera against ARBP, Dr M.Sapp for antibodies against nucleolin and for the purified 61 kDa protein, Dr S.Gasser for plasmid Ye(CEN3)30, Dr R.Knippers for encouragement and critical reading of the manuscript and B.Baumgärtner for providing mouse cell extracts. This work was supported by the Deutsche Forschungsgemeinschaft through SFB156 and a scholarship of the Boehringer Ingelheim Fonds to H.R.

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Received on March 23, 1992; revised on May 18, 1992