Acta Biochimica Polonica

Vol. 42 No. 2/1995

171–176

QUARTERLY

The paper was presented at the "First International Seminar on Nuclear Matrix"

Identification of the proteins responsible for SAR DNA binding in nuclear matrix of *Cucurbita pepo**

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Key words: *Cucurbita pepo* var. *patissonina*, endonuclease, gel shift assay, nuclear matrix, SAR DNA

The nuclear matrices from White bush (*Cucurbita pepo* var. *patisonina*) cell nuclei have been isolated using three methods: I, standard procedure involving extraction of cell nuclei with 2 M NaCl and 1% Triton X-100; II, the same with pre-treatment of cell nuclei with 0.5 mM $CuSO_4$ (stabilisation step); and III, method with extraction by lithium diiodosalicylate (LIS), and compared the polypeptide pattern. The isolated matrices specifically bind SAR DNA derived from human β-interferon gene in the exogenous SAR binding assay and in the gel mobility shift assay. Using IgG against the 32 kDa endonuclease we have found in the DNA-protein blot assay that this protein is one of the proteins binding SAR DNA. We have identified three proteins with molecular mass of 65 kDa, 60 kDa and 32 kDa which are responsible for SAR DNA binding in the gel mobility shift assay experiments.

The specific organization of chromatin in Eukaryotic cell nuclei ensures such fundamental processes as replication and transcription [1, 2]. The intranuclear network called nuclear matrix or nuclear scaffold has been isolated from a variety of non plant sources and characterised (for review see [3, 4]). Recent data concerning isolation of nuclear scaffolds or nuclear matrices from plants [5–8] point to morphological similarities between them and those of other Eukaryotes. Also DNA fragments which were found to play an important role in fastening of DNA loop domains to nuclear matrix and called SAR or MAR DNA (nuclear scaffold/matrix attachement region) [9–12] revealed the same structure and properties as these in plants [6]. Although SAR/MAR DNA have been well characterized, we know very little about proteins recognising and binding these sequences. Recently a few articles were published on isolation and characterisation of animal nuclear scaffold/matrix proteins binding SAR/MAR DNA [13–16] but studies on these proteins from plants are very seldom.

We have undertaken studies concerning this issue. Experiments performed in our laboratory demonstrated the presence of a 32 kDa endonuclease in nuclear matrix of White bush (*Cucurbita pepo* var. *patissonina*) cells and that the level of this protein is hormone dependent [5, 17]. We also have found that IgG anti-endonuclease inhibited the synthesis of the DNA associated with nuclear matrix ([5], Rzepecki & Szopa, unpublished). Recently, we demon-

*This work was supported by Grant No. 40088 9101 from the State Committee for Scientific Research.

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[1]Abbreviations: DTT, dithiothreitol; SAR, (nuclear) scaffold attached region; LIS, lithium diiodosalicylate; PMSF, phenylmethylsulphonylfluoride; SDS, sodium dodecyl sulphate.

strated that 32 kDa endonuclease binds to nuclear matrix *via* the 65 kDa protein [18, 19]. Our most recent experiments demnostarted that nuclear matrix proteins from *Pisum sativum* cells bound SAR DNA from the β-interferon gene in the exogenous SAR DNA binding assay and in the DNA-binding protein blot assay [20].

The aim of the present study was to investigate the specific binding of proteins from White bush (*Cucurbita pepo* var. *patissonina*) cell nuclei to SAR DNA. We also compare the polypeptide composition in preparations of nuclear matrices isolated by three different methods which were used in our laboratory for isolation of nuclear matrices from the other plant, *Pisum sativum* [20].

MATERIALS AND METHODS

Plant material. White bush (*Cucurbita pepo* var. *patissonina*) seeds were surface sterilised in 1% H_2O_2 solution, soaked in water for 3 h, sown in a moist germinating-bed and then allowed to germinate for 5 days in the dark at 22–24°C. Seedlings were harvested into liquid nitrogen and used immediately.

DNA constructs. Plasmid pCL was constructed by insertion of *Bam*HI-*Hin*dIII, 800 bp fragment of human-interferon SAR DNA into pTZ plasmid. The SAR character is due mainly to the AATATATTT-tract which is positioned in an appropriate environment for strand separation [21]. Plasmid pBLSIgH7 was constructed by insertion in *Bam*HI, *Hin*dIII site of the 150 bp fragment containing five repeats of 25 bp SAR motif from human β-interferon SAR DNA. Plasmids: pCL, pTZ and pBLSIgH7 were kindly provided by Prof. Jurgen Bode (GBF, Braunschweig, Germany).

Isolation of cell nuclei. Preparation and purification of cell nuclei were performed as described previously [5].

Preparation of nuclear matrices. Nuclear matrices were isolated from purified White bush cell nuclei by three methods: the method involving treatment of nuclei with 2 M NaCl and 1% Triton X-100 (standard method); standard method with previous stabilisation with 0.5 mM $CuSO_4$ and an alternative method with the use of lithium diiodosalicylate (LIS) for extraction of histones as described in detail in the previous paper [20] except that 5 U of *Eco*RI or *Pst*I was used per 1 μg DNA in the *C. pepo* nuclear matrix preparation.

Electrophoresis and immunoblotings. Nuclear and nuclear matrix proteins were separated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis in 13% gels. Proteins were visualised by silver staining. The procedures for transfer of proteins onto nitrocellulose filters and formation of immune complexes were the same as described previously [5].

Endonuclease activity assay. Endonuclease activity was determined as described previously and plasmid pBR322 was used as substrate [5]. Cleavage products were subsequently resolved on 0.8% agarose gel in TPE buffer. After electrophoresis the gel was stained with ethidium bromide and photographed under UV light.

Exogenous SAR DNA binding assay. This assay was performed as described previously [20].

DNA-binding protein blot assay. Proteins transferred onto nitrocellulose filters were stained with Ponceau S (Sigma) and photographed. Nitrocellulose was washed with DNA binding buffer [20] and incubated for 1.5 h at 30°C with gentle agitation in DNA binding buffer containing 5% instant non-fat dry milk, followed by washing with binding buffer without non-fat milk. The binding buffer was replaced with the same buffer containing 20 ng/mL labelled probe DNA (3×10^8 c.p.m./μg DNA; *Hin*dIII-*Bam*HI, 800 bp fragment of pCL DNA containing β-interferon SAR DNA) and sonicated *E. coli* genomic DNA (2 μg/mL). Then the sample was treated as described previously [20].

Gel mobility shift assay. Nuclear matrices (60 μg protein) and nuclear matrices extracts (40 μg protein) were incubated with 50 ng pBLSIgH7 plasmid DNA digested with *Bam*HI, *Hin*dIII and labelled (random priming method, 3×10^8 c.p.m./μg DNA) for 30 min at 30°C in GMS buffer (20 mM Tris/Cl, pH 7.4, 2.5 mM $MgCl_2$; 0.1 mM EDTA; 50 mM NaCl; 0.1 mM DTT; 1 mM ATP; 0.1 mM PMSF, and 15% glycerol) containing dyes (0.15% bromophenol blue; 0.15% xylene cyanol) with 0.5 μg of sonicated *E. coli* genomic DNA as a competitor. After incubation samples were run on 6% polyacrylamide gel in 0.5 × TBE buffer at 4°C. Then gel

was dried and autoradiographed for 14 h at –70°C.

Preparation of nuclei and nuclear matrices extracts. Cell nuclei and nuclear matrices (5×10^7 cell nuclei or equivalent) were extracted with 3 volumes of buffer (50 mM Tris/Cl, pH 7.4; 2.5 mM $MgCl_2$; 0.1 mM EDTA; 0.1 mM DTT; 0.1 mM PMSF; 2 M urea and 15% glycerol) for 30 min at 4°C. Supernatant fractions were recovered by centrifugation at 15000 × *g* for 20 min and dialysed for 5 h against GMS buffer (20 mM Tris/Cl, pH 7.4; 2.5 mM $MgCl_2$; 0.1 mM EDTA; 50 mM NaCl; 0.1 mM DTT; 1 mM ATP; 0.1 mM PMSF and 15% glycerol). After checking conductivity, extracts were concentrated on Amicon filters and used immediately for further experiments.

Isolation of SAR DNA binding proteins in the gel mobility shift assay. Nuclear matrices and nuclear matrix extracts (60 μg protein) were incubated with labelled pBLSIgH7 plasmid DNA and resolved on polyacrylamide gel in the same way as in the gel mobility shift assay. After electrophoresis the gel was autoradiographed for 14 h at –70°C. Pieces of the gel, corresponding to the position of the shifted band of the SAR containing fragment of DNA and SAR containing DNA fragment from control lane, were cut with a razor blade, transferred to Eppendorf tube, homogenised and extracted with SDS gel loading buffer. Then the isolated proteins were resolved on 13% acrylamide-SDS gel and silver stained.

Identification of the 32 kDa protein binding to pCL DNA. Nuclear matrix and nuclear extracts (equivalent to 5×10^6 cell nuclei) were incubated with 0.8 μg of superhelical form of plasmid pCL DNA to generate OC DNA (relaxed, open circular form) for 30 min at 37°C (see endonuclease activity assay). Then the incubation mixture was resolved in 1% agarose gel in TPE buffer. As a control nuclear matrix, nuclear extracts and isolated OC form of pCL DNA (deproteinized after incubation with nuclear matrix) were also resolved in the same gel. Then the proteins and DNAs were transferred onto nitrocellulose filters by the method described previously [5]. Nitrocellulose filters were stained with anti-endonuclease 32 kDa IgG or probed for SAR binding activity with labelled *Hin*dIII, *Bam*HI fragment of pBLSgH7 DNA containing SAR DNA.

RESULTS

We have isolated nuclear matrices from purified White bush (*C. pepo* var. *patissonina*) cell nuclei using three different procedures: standard method with extraction by 2 M NaCl and 1% Triton X-100, standard method preceded by stabilisation with 0.5 mM $CuSO_4$, and an alternative method with the use of LIS for extraction of histones and nonhistone chromatin proteins and compared the polypeptide composition of nuclear matrices preparations isolated in different ways with that of purified cell nuclei (Fig. 1). The standard method (lane 1) and the standard method preceded by stabilisation step (lane 2) were equally efficient in removal of histones whereas the alternative method was less effective. The nuclear matrices isolated by LIS extraction (lane 3, 30 mM LIS; lane 4, 15 mM LIS) contained more histones, especially histone H1 (well visible, negatively stained double band at about 36 kDa).The amount of histones in the "alternative" nuclear matrix was

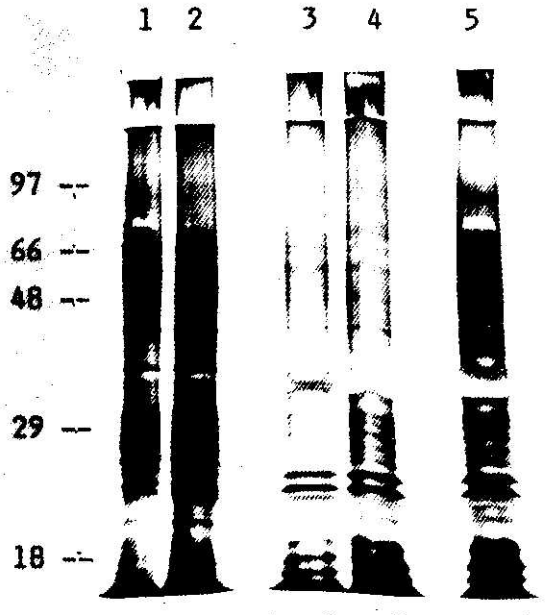

Fig. 1. Protein pattern of nuclear matrices and cell nuclei separated by SDS-polyacrylamide (13%) gel electrophoresis and stained with silver.
Lane 1, nuclear matrix isolated by high salt extraction. Lane 2, nuclear matrix isolated by high salt extraction preceded by stabilisation with 0.5 mM $CuSO_4$. Lanes 3 and 4, nuclear matrix isolated by LIS extraction (45 mM and 15 mM, respectively). Lane 5, isolated cell nuclei. The equivalent of 1×10^7 cell was loaded on each gel. The position of molecular mass markers (kDa × 10^{-3}) is indicated.

not visibly changed even on increasing LIS concentration up to 45 mM. Despite these differences, the polypeptide pattern of all three kinds of nuclear matrix preparations was similar and typical of the nuclear matrices isolated from plants [5, 6, 7, 20].

To answer the question whether nuclear matrices isolated from White bush cell nuclei are able to bind specifically SAR DNA we have used plasmid pCL containing 800 bp SAR DNA flanking human β-interferon gene in view of its well defined properties and known nucleotide sequence [21]. The assay for specific interaction of DNA with nuclear matrix proteins called: the exogenous SAR DNA binding assay involves incubation of nuclear matrices with two labelled *Bam*HI-*Hin*dIII restriction fragments: of 800 bp and the remaining 2871 bp vector fragment of the pCL plasmid DNA, in the presence of an excess of nonspecific competitor DNA (sonicated *E. coli* DNA). In such tests an exogenously added SAR DNA sequence competes with an endogenous genomic SAR sequence for binding to the nuclear matrix proteins. After incubation, the reaction mixture is centrifuged and both the pellet and the supernatant are analysed for the presence of an endogenously added labelled SAR DNA. Only pelleted DNA (associated with the nuclear matrix pellet) is supposed to react with the nuclear matrix proteins. Figure 2 demonstrates specific interaction of the nuclear matrix proteins with SAR DNA fragment. In the absence of *E. coli* genomic DNA as nonspecific competitor (Fig. 2, lanes 1 and 2), the nuclear matrix pellet showed the presence of both DNA fragments, 800 bp SAR DNA and the large vector fragment (lane 1). In the presence of *E. coli* DNA (lanes 3 and 4) only the 800 bp SAR DNA fragment was found in the pelleted nuclear matrix (lane 3). Increasing amounts of unlabelled SAR DNA fragment added to the incubation mixture (lanes 5 and 6, 50 ng; lanes 7 and 8, 100 ng) competed with the labelled SAR DNA fragment for the nuclear matrix proteins in the pellet fraction (lanes, 5 and 7, respectively). In the absence of nonspecific competitor 150 ng of unlabelled SAR DNA fragment added competed almost completely with the labelled SAR fragment (lane 9) but had no effect on nonspecific binding of the vector DNA.

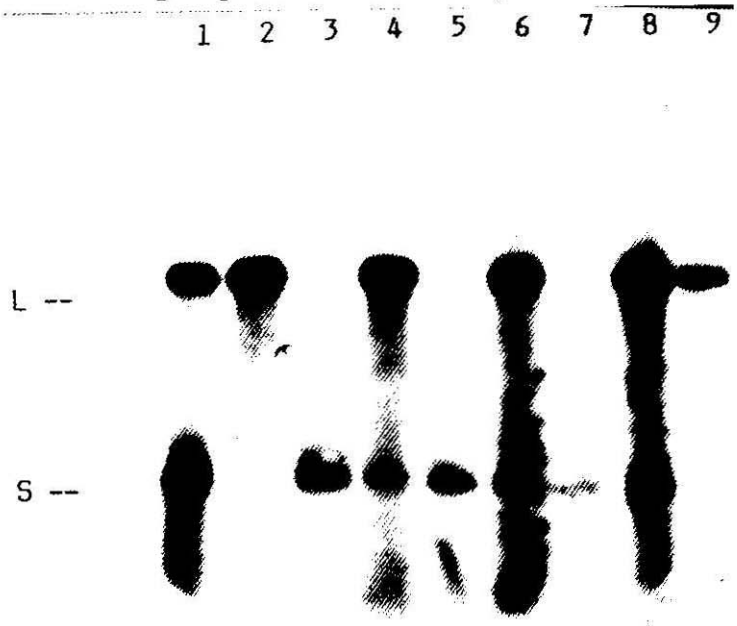

Fig. 2. Specific SAR DNA binding to nuclear matrix in the exogenous SAR binding assay.
Nuclear matrix was isolated from cell nuclei (3×10^8) by the high salt method. The plasmid pCL DNA containing 800 bp human β-interferon SAR was digested with *Bam*HI and *Hin*dIII, labelled (multipriming method) and incubated with nuclear matrix in the presence or absence of sonicated *E. coli* genomic DNA as a nonspecific competitor, and in the presence or absence of unlabelled 800 bp SAR DNA as specific competitor. After centrifugation the pellets (lanes: 1, 3, 5, 7, 9) and supernatants (lanes: 2, 4, 6, 8) were investigated for the presence of the labelled DNA fragments by agarose gel electrophoresis followed by autoradiography. Lanes 1 and 2, without nonspecific DNA competitor. Lanes 3 and 4, with 0.5 μg of the *E. coli* DNA as a nonspecific competitor. Lanes 5, 6 and 7, 8, with 0.5 μg of *E. coli* genomic DNA in the presence of 50 ng and 100 ng, respectively, of the unlabelled SAR DNA fragment. Lane 9 is the pellet fraction with 150 ng of the unlabelled SAR DNA fragment in the absence of *E. coli* DNA. L, position of the vector (large) fragment of the plasmid DNA. S, position of the 800 bp SAR DNA fragment.

To determine which of the nuclear matrix proteins are involved in the specific SAR DNA binding we have used the gel mobility shift assay procedure. Figure 3 shows the polypeptide composition of the nuclear matrix extract used in this experiment (lane 1) and that of polypeptides associated with the shifted band of the SAR DNA fragment (lane 2). Lane 3 demonstrates the result of a control experiment in which a piece of the gel containing the unshifted SAR DNA band (from the control lane — not incubated with nuclear matrix extract) was extracted. Two strong protein bands corresponding to

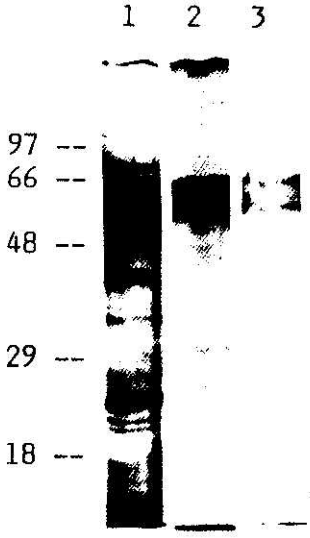

Fig. 3. Identification of proteins forming a stable complex with SAR DNA fragment in the gel mobility shift assay.
Nuclear matrix extract was incubated with *Bam*HI-*Hin*dIII digested and labelled pBLSIgH7 plasmid DNA and resolved on polyacrylamide gel electrophoresis in the same conditions as for the gel mobility shift assay procedure. After electrophoresis the gel was autoradiographed for 14 h at 70°C. Pieces of the gel, corresponding to the position of the shifted band of the SAR DNA, and the SAR DNA fragment from the control lane (without incubation with nuclear matrix extract) were cut out, homogenised and extracted with SDS gel loading buffer. Then the isolated proteins were resolved on 13% polyacrylamide-SDS gel followed by silver staining. Lane 1, protein pattern of the nuclear matrix extract used to form stable complex with SAR DNA. Lane 2, proteins extracted from the gel with the shifted band of SAR fragment. Lane 3, control experiment in which a piece of the gel containing the unshifted band of SAR fragment (not incubated with nuclear matrix proteins) was extracted. The position of molecular mass markers (kDa $\times 10^{-3}$) is indicated.

molecular mass of 65 kDa and 60 kDa are visible and together with a less distinct band of 32 kDa. This last band is identical with that of the 32 kDa endonuclease found to interact with SAR DNA as revealed by the Western blotting experiments with anti-endonuclease 32 kDa IgG (not shown).

DISCUSSION

We have isolated the nuclear matrices prepared by three different procedures and compared their polypeptide composition. The polypeptide patterns of the resulting preparations were generally similar to each other and typical of nuclear matrices isolated from plant cells [5, 6, 7, 20]. The only difference between the preparations analysed was the relatively high amount of histone H1 in the nuclear matrix isolated by LIS extraction, and the presence of a group of proteins of 21–26 kDa in this preparation. This result confirmed those recently obtained for pea nuclear matrices [20], in which higher amounts of histone H1 were found in the nuclear matrix preparation isolated by LIS extraction and by the high salt method with a stabilisation step. The nuclear matrices from pea prepared by those two methods also showed the presence of a group of 24–27 kDa proteins.

We have demonstrated that White bush nuclear matrix is able to bind specifically SAR DNA from human β-interferon gene and this binding competes in over 90% by addition of 100 ng of unlabelled SAR DNA fragment.

The analysis of proteins complexed with the shifted band of the SAR DNA fragment in the gel mobility shift assay revealed the presence of three proteins with molecular mass of 65 kDa, 60 kDa, and 32 kDa. Since the 32 kDa protein was found to be immunologically related to the 32 kDa endonuclease (as we demonstrated by Western blot analysis, data not shown) isolated from the same source [18], we suggest that it is the same protein. The finding that 32 kDa endonuclease is one of the SAR DNA binding proteins seems very interesting and confirms to some extent our earlier results that anti-endonuclease IgG inhibits incorporation of labelled TMP into DNA in isolated cell nuclei [5] and that this process is observed only in the case of the DNA associated with nuclear matrix (Rzepecki & Szopa, unpublished). The two others proteins: of 65 kDa and 60 kDa have been recognised on Western blots by IgGs raised against nuclear matrix 6 M urea extracts (not shown) and are similar in size to pea proteins (of 66 kDa and 62 kDa) found to bind SAR DNA [20]. Both *C. pepo* 65 kDa and *Pisum sativum* 66 kDa proteins are similar in size to lamin B1 (67 kDa), the protein from rat liver cells which was found to bind SAR DNA [16]. The possibility that these plant proteins are related to the lamin polypeptide is currently under investigation in our laboratory.

Identification of the 65 kDa protein in *C. pepo* cells as responsible for SAR DNA binding is also interesting in relation to our recent finding [19] that the endonuclease 32 kDa–protein 65 kDa complex preferentially recognises the plasmids containing SAR DNA element. The possi-

bility that these 65 kDa proteins are identical is also investigated in this laboratory.

We thank Prof. J. Bode (GBF, Braunschweig, Germany) for the kind gift of pCL and pBLSIgH7 plasmids.

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