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QUARTERLY

Interaction of the *Pisum sativum* nuclear matrix proteins with SAR DNA*

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We have isolated the nuclear matrices from *Pisum sativum* cell nuclei using three methods: i. standard procedure involving extraction of cell nuclei with 2 M NaCl and 1% Triton X-100; ii. the same with pretreatment of cell nuclei with 0.5 mM $CuSO_4$ (stabilisation step); and iii. method including lithium diiodosalicylate extraction. We compared the polypeptide pattern and residual DNA content of the nuclear matrices isolated. The nuclear matrices displayed a specific endonuclease activity which was due to the presence of a 32 kDa protein. The isolated nuclear matrices bound specifically the scaffold-attached (SAR) DNA derived from human β interferon gene, in the exogenous SAR binding assay. Using the DNA-protein binding blot assay we demonstrated the presence of two nuclear matrix proteins of 66 kDa and 62 kDa which bound specifically SAR DNA.

The chromatin of eukaryotic cell nuclei must be highly ordered and compact to make possible such fundamental processes as replication and transcription. Recent data [1] indicated that plant chromosome structure is consistent with the common organisation of DNA into 10 nm fibers which are then wound into 30 nm solenoids and then into chromatin loops. These loops are maintained by periodic attachment of solenoid structures, at their bases, to the intranuclear framework by nonhistone proteins [2]. This intranuclear network called nuclear matrix or nuclear scaffold has been isolated and characterised from a wide variety of sources (for review see: [3, 4]).

Recently isolation of nuclear scaffolds or nuclear matrices from plants was undertaken [5–8]. The accumulated data point to morphological similarities between plant nuclear matrices and those of other Eukaryotes. Also the DNA fragments which were found to play an important role in fastening of the DNA loop domains to nuclear matrix structure, and called SAR[1] or MAR DNA (nuclear scaffold/matrix attachment region) revealed the same structure and properties [6]. All the SAR/MAR DNA elements known have a high AT base pair content (>70%) and a topoisomerase II consensus sequence [9], and usually contain sequences related to the yeast autonomously replicating sequences (ARSs) [10, 11] as well as sequences related to T-box and A-box [6, 12]. Some SAR elements are able, when placed upstream and downstream of the gene in transgenic organ-

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[1]Abbreviations: SAR, scaffold attached region; LIS, lithium diiodosalicylate; PMSF, phenylmethyl sulfonylfluoride; DTT, dithiothreitol.

isms, to alleviate the commonly observed position effect on the expression of the gene [13].

Although SAR/MAR DNA are well characterised, we know very little about animal proteins and almost nothing about plant proteins recognising and binding these sequences. Recently von Kries *et al.* [14] have purified a 95 kDa protein from chicken which binds to MARs DNA in a cooperative fashion. Romig *et al.* [15] purified four proteins from HeLa cells, among which a 120 kDa protein called SAF-A showed the highest affinity for SAR DNA. Dickinson *et al.* [16] have isolated a human cDNA clone for the protein (called SATB1) expressed predominantly in thymus, and selectively bound to SAR sequences. It was also found [17] that lamin B1 (67 kDa protein) is responsible for binding of SAR DNA.

Experiments in our laboratory demonstrated the presence of a 32 kDa endonuclease in nuclear matrix of White bush cells (*Cucurbita pepo* var. *patissonina*). The nuclear matrix associated endonuclease showed high specific activity towards plasmid pBR322 DNA as a substrate [5], while the isolated 32 kDa endonuclease showed a DNase I-like activity. Recently, we have demonstrated that this 32 kDa endonuclease binds to nuclear matrix *via* a 65 kDa protein. The isolated: 32 kDa endonuclease-protein 65 kDa complex mimics specific activity of the nuclear matrix associated endonuclease [18, 19]. We also found that the anti-endonuclease IgG inhibits synthesis of DNA associated with nuclear matrix [5].

In the present study we compare the polypeptide composition and DNA content of nuclear matrices from *Pisum sativum* cells isolated by three different methods. We demonstrate the presence of endonucleolytic activity in nuclear matrix of pea cells which shows similar properties to that observed in *C. pepo* nuclear matrix. We also demonstrate that the pea nuclear matrix preparation binds specifically SAR DNA from the human β interferon gene. Moreover, the particular proteins which bind specifically SAR DNA have been identified.

MATERIALS AND METHODS

Plant material. *P. sativum* 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 grow 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 β-interferon SAR DNA into pTZ plasmid. The SAR character is due mainly to the AATATATTT-tract which is positioned in surroundings appropriate for strand separation [20]. Plasmids: pCL and pTZ were kindly provided by Prof. Jurgen Bode (GBF, Braunschweig, Germany).

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

Preparation of nuclear matrices. Nuclear matrices were isolated from purified pea cell nuclei by three methods: i. the standard method involving nuclei treatment with 2 M NaCl and 1% Triton X-100; ii. the same with additional pretreatment with 0.5 mM $CuSO_4$ (stabilisation step); and iii. method with the use of lithium diiodosalicylate (LIS) for extraction of histones. Briefly: the isolated cell nuclei were washed twice with buffer A (20 mM Tris/Cl, pH 7.4; 80 mM NaCl; 1 mM EDTA; 0.5 mM spermidine; 0.1 mM PMSF) and stabilised (in first procedure this step was omitted) by incubation at 30°C for 30 min in buffer B (20 mM Tris/Cl, pH 7.4; 80 mM NaCl; 0.5 mM $CuSO_4$; 0.5 mM spermidine; 0.1 mM PMSF). The nuclei were then incubated for 20 min at 30°C in buffer C (20 mM Tris/Cl, pH 7.4; 2.0 M NaCl; 0.2 mM $MgCl_2$; 0.5 mM spermidine; 0.1 mM PMSF) and D (20 mM Tris/Cl, pH 7.4; 1% Triton X-100; 5.0 mM $MgCl_2$; 0.5 mM spermidine; 0.1 mM PMSF) followed by washing (3×) with buffer E (20 mM Tris/Cl, pH 7.4; 80 mM NaCl; 5 mM $MgCl_2$; 0.5 mM spermidine; 0.1 mM PMSF), and finally incubated with *Eco*RI or *Pst*I (3 U per 1 μg DNA in nuclear matrix preparation) for 2 h at 37°C. After digestion with the restriction enzyme nuclear matrix was washed twice in buffer E and used immediately for further experiments.

Alternatively, nuclear matrix was prepared by incubation for 20 min at 30°C in CALT buffer (20 mM Tris/Cl, pH 7.4; 80 mM NaCl; 1 mM EDTA; 80 mM LiCl; 0.5 mM spermidine; 0.1 mM PMSF) containing 30 mM LIS, followed by washing three times with buffer E and digestion with *Eco*RI or *Pst*I and washing, as mentioned above. The isolated nuclear matrix was used immediately for further experiments.

Isolation of DNA. Nuclear matrices (equivalent to 8×10^7 cell nuclei) were incubated in 50 mM Tris/Cl buffer, pH 7.4, containing 5 mM EDTA, 0.5% SDS, 1 mg/mL proteinase K, and 0.5 mM spermidine at 56°C for 2 h. After extraction with phenol-chloroform (twice) and chloroform (once), DNA was precipitated with ethanol and dissolved in TER buffer (10 mM Tris/Cl, pH 7.0; 2 mM EDTA; 50 μg/mL RNase). The DNA preparations were analysed spectrophotometrically and on 1% agarose gel in TPE buffer (0.09 M Tris/phosphate; 0.002 M EDTA). After electrophoresis the gel was stained with ethidium bromide and photographed under UV light.

Electrophoresis and immunoblotting. Nuclear and nuclear matrix proteins were separated by sodium dodecyl sulphate (SDS) polyacrylamide gel (13%) electrophoresis. Proteins were visualised by silver staining. Procedures for electrophoretic transfer of proteins onto nitrocellulose filters and formation of immune complexes were the same as described previously [5]. Histone proteins were identified by immunoblots using rabbit anti-calf thymus histones IgG.

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

SDS polyacrylamide gel electrophoresis with immobilised DNA. Preparation of the gel was the same as in the case of SDS-PAGE with the exception that, before polymerisation, heat denatured calf thymus DNA was added (to the final concentration of 20 μg/mL of the gel) to the resolving gel. After electrophoresis, gels were washed 4 times for 20 min with 50 mM Tris/Cl, pH 7.0 buffer, then twice with buffer containing 2.5 mM $MgCl_2$ and 2.5 mM $CaCl_2$ followed by incubation for 20 h at 24°C with a fresh portion of the buffer. Then the gel was stained with ethidium bromide and photographed under UV light. Polypeptides showing nuclease activity are revealed by this procedure as dark bands due to digestion and washing off of DNA in this place.

Exogenous SAR DNA binding assay. Nuclear matrices (100 μg protein) were incubated for 30 min at 30°C with 50 ng of labelled (random priming method) DNA fragment and different amounts (up to 5 μg) of sonicated *E. coli* genomic DNA in a total volume of 150 μL in binding buffer (20 mM Tris/Cl, pH 7.4; 5 mM $MgCl_2$; 80 mM NaCl; 0.1 mM DTT; 0.5 mM spermidine; 0.1 mM PMSF). The nuclear matrices were pelleted at $3000 \times g$ for 5 min and washed with binding buffer. Supernatant and pellet fractions were incubated in 200 μL of the binding buffer enriched in EDTA, SDS and proteinase K (10 mM, 1% and 1.0 mg/mL, respectively) for 2 h at 56°C. Samples were extracted twice with a phenol-chloroform mixture and once with chloroform, followed by precipitation of DNA with ethanol. Whole amounts of the pellet and supernatant DNA fractions were run on 1% agarose gel in TPE buffer. After electrophoresis the gels were dried and autoradiographed for 10–12 h at –70°C.

DNA-binding protein blot assay. Cell nuclei, nuclear matrices and nuclear extracts (40, 30 and 20 μg of protein, respectively) were resolved upon SDS-PAGE (13% gel) followed by transfer of proteins onto nitrocellulose filters. The proteins were then stained with Ponceau S and photographed. Nitrocellulose was washed with DNA binding buffer (20 mM Tris/Cl, pH 7.4; 1 mM EDTA; 80 mM NaCl) and incubated for 1.5 h at 30°C with gentle agitation in this buffer containing 5% instant nonfat dry milk. The latter buffer was replaced with the binding buffer containing 20 ng/mL labelled DNA probe (3×10^7 c.p.m./mg DNA; *Hin*dIII-*Bam*HI, 800 bp fragment of pCL DNA containing β-interferon SAR DNA) and sonicated *E. coli* genomic DNA (0.3 μg/mL). Then the filter was incubated for 1 h at 30°C followed by five washings for 5 min each with 0.5% instant nonfat dry milk. The DNA-protein complexes were visualised by autoradiography for 10–12 h at –70°C. In the control experiments, a large fragment of *Hin*dIII–*Bam*HI pCL DNA (vector fragment) was labelled and used in the same way for detection of unspecific interaction of proteins with DNA.

RESULTS

Nuclear matrices were isolated from purified *P. sativum* cell nuclei by three procedures. Figure 1 illustrates the polypeptide pattern of three

types of nuclear matrix preparations isolated under various conditions, and polypeptide composition of the isolated cell nuclei. Subsequently we determined histone bands by Western blots using anti-calf thymus histones IgG (not shown). The most important difference we have observed is the low level of histone H1 which is present only in trace amounts in the nuclear matrix isolated by the high salt extraction procedure (Fig. 1, lane 3) and is present in higher amounts when either of the two other isolation methods is applied (Fig. 1, lanes: 4 and 5). Thus, the high salt extraction method seems to be the most suitable for extraction of histones. There is also another difference, the presence of a group of proteins (24–27 kDa) in nuclear matrices isolated by LIS extraction and by the high salt method with the stabilisation step. With the exception of the proteins indicated the polypeptide pattern of all three of nuclear matrix preparations is similar and typical of nuclear matrices (scaffolds) isolated from other plant cells [5–7].

The content of DNA was found to be the highest in the nuclear matrix prepared by the standard procedure with the stabilisation step

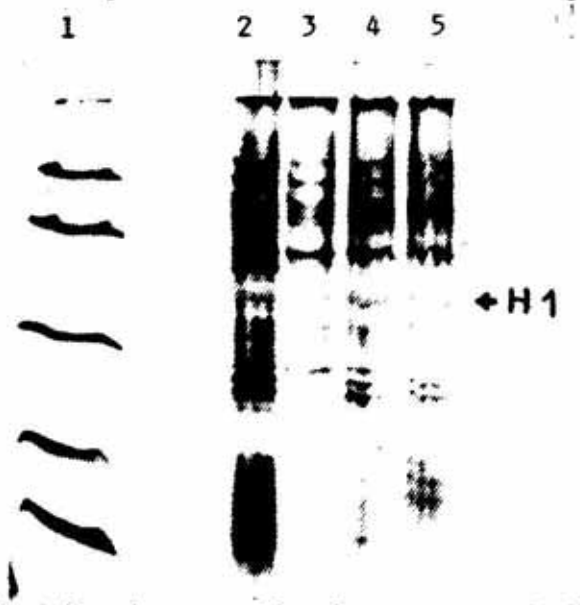

Fig. 1. Protein pattern of nuclear matrices and cell nuclei separated on 13% SDS-polyacrylamide gel and stained with silver.
Lane 1, Molecular mass markers (in kDa): 66, 48.5, 29, 18.4 and 14..2; lane 2, isolated cell nuclei (1×10^7 cell nuclei); lane 3, nuclear matrix isolated by high salt extraction (equivalent to 5×10^6 cell nuclei); lane 4, nuclear matrix isolated by high salt extraction preceded by stabilisation with 0.5 mM $CuSO_4$ (equivalent to 5×10^6 cell nuclei); lane 5, nuclear matrix isolated by LIS extraction (equivalent to 5×10^6 cell nuclei).

(22.75 ± 1.3 μg per nuclear matrix equivalent of 10^9 cell nuclei) while the lowest amount (6.5 ± 0.6 μg per nuclear matrix equivalent of 10^9 cell nuclei) in the nuclear matrix isolated by the standard procedure. The nuclear matrix isolated by LIS extraction shows an intermediate DNA quantity (11.7 ± 0.6 μg per nuclear matrix equivalent of 10^9 cell nuclei). To determine the quality of the DNAs associated with nuclear matrix preparations we resolved DNAs isolated from these preparations on 1% agarose gel (Fig. 2).

As nuclear matrices isolated from plant cells show endonuclease activity [5, 6, 19] we analysed nucleolytic activity of the nuclear matrices isolated from *P. sativum* cell nuclei in the test with pBR322 as a substrate and by SDS-polyacrylamide gel electrophoresis through gel with immobilised DNA. Incubation of the superhelical form of pBR322 DNA with nuclear matrices led to generation only of the open circular form (not shown). This result is similar to those obtained with *C. pepo* nuclear matrix [5, 19]. To ascertain which of the matrix proteins is responsible for this specific endonucleolytic activity, nuclear matrix proteins were tested on SDS-polyacrylamide gel electrophoresis with

Fig. 2. Agarose gel electrophoresis of DNA isolated from different nuclear matrix preparations.
Nuclear matrices were isolated from cell nuclei by three methods and served as the source of DNA isolated and resolved on 1% agarose gel in TPE buffer. Lane 1, Molecular mass DNA markers: λ DNA digested with *Eco*RI and *Eco*RV; lane 2, DNA associated with nuclear matrix isolated by high salt procedure (equivalent of 1.2×10^8 cell nuclei was applied); lane 3, DNA isolated from nuclear matrix purified by standard procedure with a previous stabilisation step (equivalent of 9.4×10^8 cell nuclei was applied); lane 4, DNA associated with nuclear matrix isolated by extraction with LIS (equivalent of 4.7×10^8 cell nuclei was applied).

immobilised DNA. Figure 3 shows nuclease activity of the nuclear matrices isolated by standard procedure and with LIS extraction compared to deoxyribonuclease I activity. In both nuclear matrix preparations there is a well visible nuclease band of 30 kDa. This result is similar to those obtained for *C. pepo* nuclear matrix where a 32 kDa endonuclease was found [5]. Preliminary data based on Western blots analysis of pea nuclear matrix proteins probed with IgG against endonuclease from *C. pepo* demonstrate that these proteins are not immunologically related.

To study whether nuclear matrices isolated from *P. sativum* cell nuclei are able to bind specifically SAR DNA, we used the "exogenous SAR binding assay" and the pCL plasmid DNA containing SAR DNA flanking human β-interferon gene, due to its well defined properties and known nucleotide sequence [20]. After incubation the reaction mixture was centrifuged and both pellet and supernatant were analysed for the presence of exogenously added SAR DNA. Only pelleted DNA is supposed to have reacted with nuclear matrix proteins. Figure 4 demonstrates the interaction of nuclear matrix proteins with SAR DNA fragment. In the absence of *E. coli* genomic DNA as a nonspecific competitor, the nuclear matrix pellet showed the presence both of DNA fragments, 800 bp SAR DNA and the large vector fragment (lane 1), whereas only vector DNA was found in supernatant (lane 2). In the presence of *E. coli* competitor DNA (lanes 3–8) only the SAR DNA fragment was found in the

Fig. 3. SDS-polyacrylamide gel electrophoresis of isolated nuclear matrix proteins with immobilised DNA.
Lane 1, deoxyribonuclease I (3 μg) as molecular mass standard. Lane 2, nuclear matrix (equivalent to 8×10^6 cell nuclei) isolated by standard procedure, and, lane 3, nuclear matrix (equivalent to 8×10^6 cell nuclei) isolated by LIS (30 mM) extraction.

nuclear matrix pellet (lanes: 3, 5 and 7). Competition by 300 ng of unlabelled SAR DNA fragment added to the labelled SAR fragment from nuclear matrix pellet fraction exceeded 90% (not shown). In the exogenous SAR binding assays the nuclear matrix isolated by LIS extraction showed the highest specific binding activity and the matrix isolated by high salt extraction with stabilisation with 0.5 mM $CuSO_4$, the lowest activity (not shown).

To answer the question which proteins are involved in specific SAR DNA binding, the DNA-binding protein blot assay was used (Fig. 3). Labelled SAR DNA was bound to cell nuclei and nuclear matrix proteins of 66 kDa and 62 kDa whereas labelled vector DNA was bound to proteins of about 18 kDa from *P. sativum*. Strong bands of

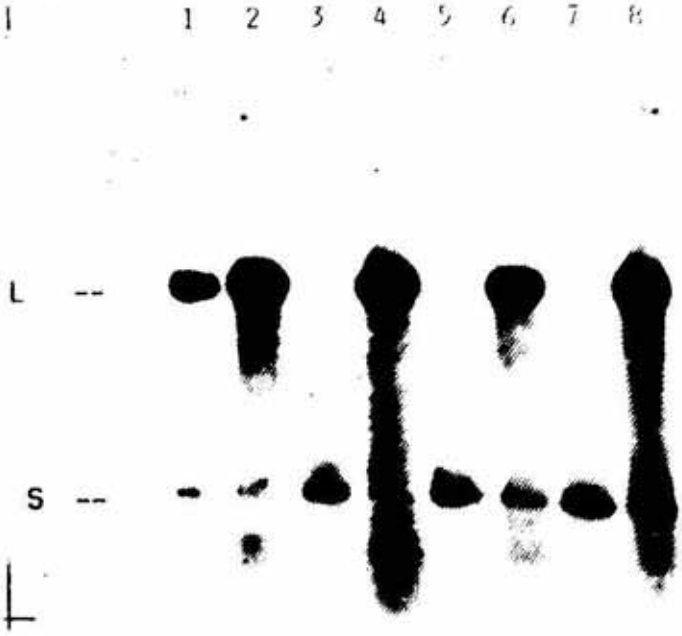

Fig. 4. Specific SAR DNA binding to nuclear matrix in the exogenous SAR binding assay.
Nuclear matrix was isolated from cell nuclei (2×10^7) by the high salt method. The plasmid pCL DNA containing 800 bp human β-interferon SAR was digested with *Bam*HI, *Hin*dIII, labelled (multi-priming method) and incubated with nuclear matrix in the presence or absence of sonicated *E. coli* genomic DNA as competitor. After centrifugation the pellets (lanes 1, 3, 5, 7) and supernatants (2, 4, 6, 8) were investigated. Lanes 1 and 2, without competitor. Lanes 3 and 4, with 1 μg *E. coli* DNA; lanes: 5, 6 and 7, 8 with 2 μg and 4 μg *E. coli* DNA, respectively. L, position of large vector DNA fragment. S, position of 800 bp SAR DNA.

about 18 kDa are due to nonspecific binding of vector DNA to histones. Only two proteins of 66 kDa and 62 kDa were found to bind SAR DNA both in nuclear matrix and cell nuclei.

DISCUSSION

We have obtained by three different methods three kinds of nuclear matrix preparations from *P. sativum* cell nuclei, and compared their protein composition. Purified nuclear matrices consisted of a variety of nonhistone proteins and only trace amounts of histones were similar to those obtained from other plant systems [5–7]. The most important exception was the very high content of histone H1 in the nuclear matrices isolated by extraction with NaCl and Triton X-100 preceded by stabilisation, and in those isolated by LIS extraction. It is interesting that the amount of histone H1 retained in nuclear matrix preparations did not correlate with the amount of residual DNA found in nuclear matrix. An increase in LIS concentration in extraction buffer (up to 50 mM) did not change visibly the amount of histone H1 retained.

We demonstrated that all three preparations of nuclear matrices showed endonuclease activity highly specific towards plasmid DNA. This result is in agreement with the results obtained with other plant nuclear matrices [5, 7, 19, 21]. Also the polypeptides responsible for this endonuclease activity in *P. sativum* (30 kDa) and *C. pepo* (32 kDa) are similar in size but are not immunologically related.

Moreover, we have found that the nuclear matrix isolated by the standard method binds specifically SAR DNA from human β-interferon gene (Fig. 4). The lower amount of 800 bp SAR DNA fragment (Fig. 4: lanes 1 and 2) was probably due to endonucleolytic cleavage by a nuclear matrix associated endonuclease (these samples were incubated without competitor DNA) which, as we suggest, revealed a preference in recognition and digestion of SAR DNA [21].

In the DNA-binding protein blot assay (Fig. 5) we identified two nuclear matrix proteins of 66 kDa and 62 kDa which are responsible for binding of SAR DNA to nuclear matrix. The molecular mass of the larger protein (66 kDa) is similar to that of *C. pepo* nuclear matrix protein (65 kDa) showing SAR DNA binding properties (Rzepecki *et al.*, unpublished) and is also similar to that of lamin B1 protein from rat (67 kDa) which has been found to bind specifically SAR DNA sequences. This possibility is currently investigated in our laboratory.

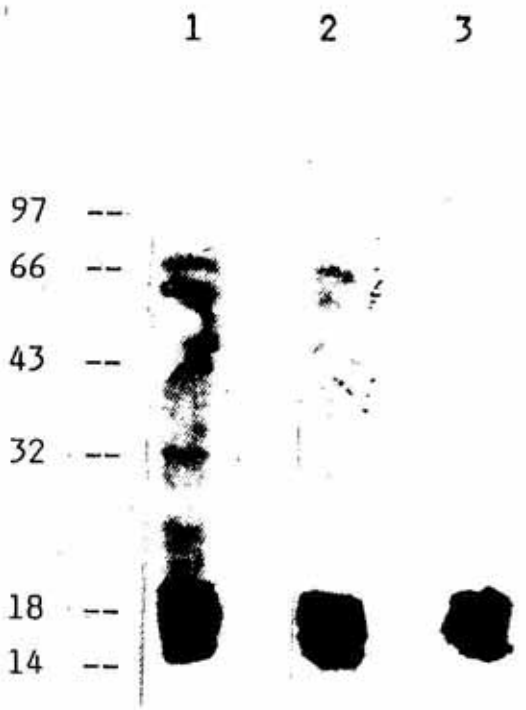

Fig. 5. Identification of SAR DNA binding proteins in the DNA-binding protein blot assay.
Proteins from cell nuclei (lane 1)(5×10^7 cell nuclei) and nuclear matrix prepared by the high salt procedure (lanes 2 and 3) (equivalent of 2×10^7 cell nuclei) were separated on 13% SDS-polyacrylamide gel. Proteins were transferred onto nitrocellulose filter and probed with labelled SAR sequence (lanes 1 and 2), or vector DNA (lane 3) in the presence of *E. coli* genomic DNA (300 ng/mL) as competitor. The position of molecular mass markers (kDa) is indicated.

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