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Immunoglobulins anti-endonuclease 32 kDa from *Cucurbita pepo* var *patissonina* affect process of DNA synthesis

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Immunoglobulins anti-endonuclease 32 kDa inhibit DNA synthesis. We observed that low concentrations of IgGs (about 50 μg IgG per 1×10^6 cell nuclei) temporary inhibit DNA synthesis. This inhibition concerns only the synthesis of DNA bound to the nuclear matrix (associated with isolated nuclear matrix). Preincubation of cell nuclei of White bush with IgG generates longer DNA fragments than in controls. Involvement of the 32 kDa endonuclease or an endonuclease-65 kDa protein complex from the nuclear matrix in replication or structural organisation of replication is considered.

Nuclear matrix is known to play an important role not only in structural organisation of DNA [1, 2] but also in transcription, processing and transport of RNA [3, 4]. Evidence has been obtained that origins of replicons are sites of attachment of the DNA to the nuclear matrix and the chromosomal scaffold. The latter is another important nuclear structure, which is formed by a reorganisation of part of the nuclear matrix [5].

Our previous data indicate the presence of a 32 kDa endonuclease-18 kDa inhibitor complex in the nuclei of White bush seedlings [6]. This 18 kDa inhibitor was found later to be a cleavage product of actin (Szopa & Fahrni, unpublished). We have demonstrated that, in phytohormone-treated cells, the nuclear enzyme-inhibitor complex dissociates: inhibitor remains in the nucleoplasm, and endonuclease binds tightly to the nuclear matrix, what suggests a dynamic role of the endonuclease in cellular metabolism of intensively growing cells. We have also reported that, the nuclease-inhibitor complex in cell nuclei is accompanied by a 65 kDa protein [7, 8]. We have found that the nuclear matrix displays highly specific endonuclease activity. The endonuclease associated with the nuclear matrix is able to introduce only a single nick into the superhelical plasmid pBR 322 DNA form. The enzyme loses its high specificity when it is purified but reveals its activity towards DNA when is associated with the 65 kDa protein [9]. We have also demonstrated that IgG anti-endonuclease inhibits DNA synthesis *in vitro* [8]. Our latest results revealed that the plant nuclear matrix-associated endonuclease or the isolated endonuclease 32 kDa-protein 65 kDa complex showed a preference for recognising and digesting the plasmid containing the SAR DNA element [10].

[1]Abbreviations: SDS, sodium dodecyl sulphate; SAR, scaffold attached region; MAR, matrix attached region; ARS, autonomously replicating sequence; bp, base pair; PBS, phosphate buffered saline; TBE, Tris/borate/EDTA

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It seems that there are some functional similarities between our 32 kDa endonuclease (or the complex nuclease-protein 65 kDa) and the endonuclease purified from *Crithidia fasciculata* [11] which has been suggested to play a role in replication of kinetoplast DNA, and seems to be able to recognise specifically the ARS sequences from yeast.

In the following paper we tried to evaluate whether 32 kDa endonuclease is involved in DNA synthesis.

MATERIALS AND METHODS

Plant material. White bush seeds (*Cucurbita pepo* var. *patissonina*) were surface sterilised in 1% H_2O_2 solution, soaked in water for 2–3 h, sown in a moist germinating-bed and then allowed to germinate for 7 days in the dark at 20–24°C.

Isolation of cell nuclei and nuclear matrices. Preparation and purification of the cell nuclei was performed as described previously [6]. The nuclear matrices were isolated from purified White bush cell nuclei by the method of [12].

DNA electrophoresis. Electrophoresis of DNA in native conditions was carried according to [13] using a linear gradient of acrylamide from 3.5% to 8% in TBE buffer (Tris/borate/EDTA).

Preparations of IgG anti-endonuclease 32 kDa. IgGs anti-endonuclease were prepared by immunisation of a rabbit with four portions of 32 kDa endonuclease (150 μg of protein each) at two week intervals. The isolated IgG, recognised in Western blots only the 32 kDa endonuclease (1:3000 dilution) [9, 10]. Control IgGs, were prepared from the rabbit directly before immunisation.

DNA synthesis in vitro. For the assay of DNA synthesis *in vitro* the procedure of [14] was used. The DNA synthesis in the presence of IgG was preceded by preincubation of nuclear extract or cell nuclei with 10 ml of phosphate buffered saline (PBS) containing IgG for 5 min at 30°C.

Preparation of samples of DNA to loading on polyacrylamide gels. When DNA synthesis was completed the samples were chilled in an ice bath, then SDS, EDTA and proteinase K were added to the final concentration of 0.5%, 10 mM, 50 μg/ml, respectively, and the samples were incubated for 2 h at 50°C. After extraction twice with phenol, twice with phenol/chloroform mixture and once with chloroform, DNA was precipitated with ethanol and dissolved in the gel loading buffer (20 mM Tris/HCl, pH 8, 0.5 mM EDTA, 30%, v/v, glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol).

Synthesis of DNA on endogenous nuclear DNA and separation of the products by gradient polyacrylamide gel electrophoresis. Sonicated cell nuclei (8×10^6) were preincubated for 5 min with IgG anti-endonuclease (400 μg) or with control IgG (400 μg). Then cofactors for DNA synthesis were added and the incubation was continued for 20 min at 30°C. Next, the samples were incubated for 2 h at 50°C with proteinase K, extracted twice with phenol, twice with phenol-chloroform mixture and once with chloroform. After precipitation with ethanol, DNA was dissolved in the gel-loading buffer and resolved by electrophoresis (3.5%–8% acrylamide gradient). After electrophoresis the gel was cut into pieces (0.5 cm) and radioactivity was counted in BETAmatic. The radioactivity of each piece was expressed as a percentage of the total radioactivity of the lane.

RESULTS

We have reported previously that IgG anti-endonuclease inhibits DNA synthesis *in vitro* and nicking activity of the nuclear matrix [7]. Since for the latter activity our 32 kDa endonuclease is responsible [8] it was interesting to examine the effect of IgG anti-endonuclease on synthesis of "total" DNA and DNA bound to the nuclear matrix.

Generally, literature data are in agreement that replication origins are located in the sequence of bases occupying 30 nm of chromatin fibres bound to nuclear matrix [1]. In consequence replication should begin at DNA sequences bound to the nuclear matrix and proceed along chromatin fibers extended from the nuclear matrix. To check this possibility we led the reaction of DNA synthesis in isolated nuclei of White bush using [^{3}H]TTP. After different periods of incubation the amount of [^{3}H]TMP incorporated into DNA was measured directly in the nuclei or after isolation of nuclear matrix. The direct measurements — so called "total" DNA synthesis — represents replication of the bulk nuclear DNA,

while measurements of the incorporation into DNA bound to the nuclear matrix represents replication of the DNA which remained associated with the nuclear matrix after its isolation [15]. As shown in Fig. 1 in the first four minutes of incubation, incorporation of [^{3}H]TMP into "total" DNA and DNA-bound to the nuclear matrix was the same. After this period, incorporation of [^{3}H]TMP into "total" DNA increased with the time of incubation, whereas incorporation into matrix DNA remained almost the same. These results strongly support the hypothesis that the first regions of replicated DNA are the DNAs bound to nuclear matrix.

To investigate the effect of IgG on synthesis of the "total" DNA and nuclear matrix-bound DNA we have chosen a suboptimal amount of IgG anti-endonuclease (50 μg IgG per 1×10^6 cell nuclei) which does not inhibit significantly DNA synthesis [8].

As shown in Fig. 2, incorporation of [^{3}H]TMP into "total" DNA and matrix-bound DNA in the presence of IgG anti-endonuclease is the same in the first four minutes of the reaction but by about 50% lower than incorporation into the

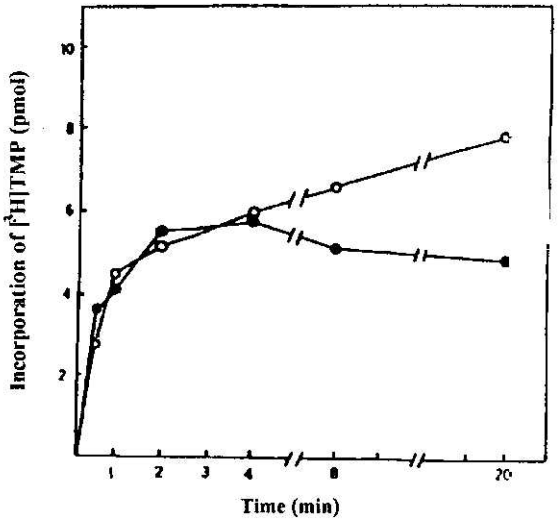

Fig. 1. Time dependence of a synthesis of a "total" DNA and DNA bound to the nuclear matrix.
DNA synthesis was assayed as incorporation of [^{3}H]TMP into acid insoluble (5% trichloroacetic acid) product according [14]. Cell nuclei (3×10^6) were incubated at 30°C. Empty circles present the incorporation of the [^{3}H]TMP into the "total" DNA (whole nuclear DNA). Black circles present the incorporation of the [^{3}H]TMP into nuclear matrix-bound DNA (DNA associated with nuclear matrix after its isolation). Each data point represents the average of a minimum of two determinations, each done in triplicate. The standard error of the mean for each data point was less than 3%.

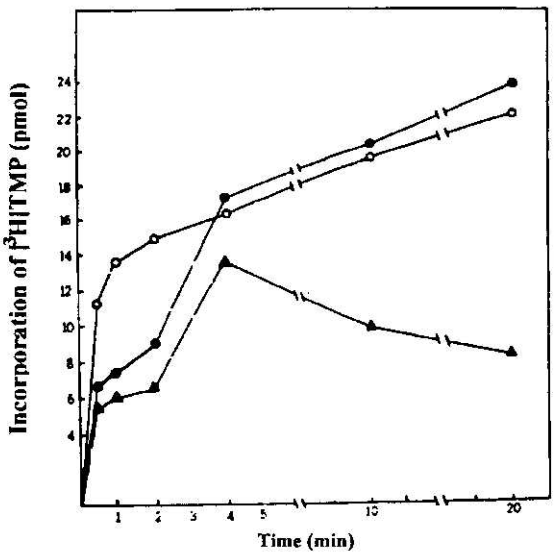

Fig. 2. Effect of IgG anti-endonuclease on synthesis of the "total" DNA and the DNA bound to the nuclear matrix.
DNA synthesis was assayed as incorporation of [^{3}H]TMP into acid insoluble (5% trichloroacetic acid) product. Sonicated cell nuclei (8×10^6) were preincubated for 5 min at 30°C with IgG (400 μg) anti-endonuclease (black circles and triangles) or with control IgG (400 μg) (empty circles) and then cofactors for DNA synthesis were added followed by incubation at 30°C for indicated time. Empty and black circles present the incorporation of the [^{3}H]TMP into the "total" DNA (whole nuclear DNA). Black triangles present the incorporation of the [^{3}H]TMP into nuclear matrix bound DNA (DNA associated with nuclear matrix after its isolation). Each data point represents the average of a minimum of three determinations, each done in triplicate. The standard error of the mean for each data point was less than 5%.

"total" DNA in the presence of control IgG. At longer periods of incubation, incorporation of [^{3}H]TMP into the "total" DNA in the presence of IgG anti-endonuclease as well as control IgG is the same and increases with the incubation time, whereas incorporation of the [^{3}H]TMP into nuclear matrix DNA in the presence of IgG anti-endonuclease remains stable or even slightly diminished. This suggests, that IgG anti-endonuclease, in such an amount (50 μg IgG per 1×10^6 cell nuclei) is able to inhibit temporarily DNA synthesis, and that the process of this inhibition is localised in nuclear matrix or nuclear matrix-bound DNA. It should be pointed out that the higher amounts of IgG (> 150 μg IgG per 1×10^6 cell nuclei) irreversibly diminished DNA synthesis both in the "total" DNA and the DNA bound to nuclear matrix [8].

IgG anti-endonuclease influence the size of newly synthesised DNA fragments

To evaluate whether IgG anti-endonuclease has any effect on the size of newly synthesised DNA fragments we carried out the following experiments. Cell nuclei were preincubated with IgG anti-endonuclease (50 μg per 1×10^6 cell nuclei) or with control IgG and then all cofactors for DNA synthesis were added and the reaction was carried out for 20 min at 30°C. Then we analysed the products of the DNA synthesis by polyacrylamide gradient gel electrophoresis under native conditions.

As shown in Fig. 3, in the control experiments the highest radioactivity was found in the piece of the gel laying at 4.5 cm from the start. This distance represents the migration of the DNA of about 200 bp what would suggest that the radioactivity come from the Okazaki fragments. Treatment of the cell nuclei with IgG anti-endonuclease resulted in generation of DNA fragments with higher molecular weight than that from the control nuclei and the highest radioactivity corresponds to the size of about 450 bp.

In Fig. 4 we show the results of a similar experiment. The only difference was that plasmid pBR 322 DNA was added to the preincubation mixture. Also in this case we observed

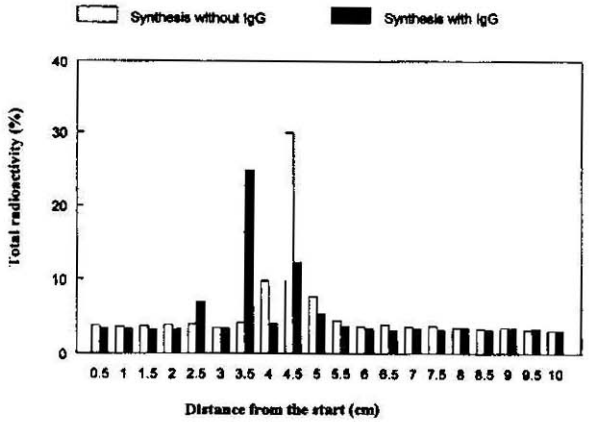

Fig. 3. Polyacrylamide gel electrophoresis (3.5%–8% acrylamide gradient) of products of DNA synthesis on endogenous nuclear DNA.
Sonicated cell nuclei (8×10^6) were preincubated for 5 min with IgG anti-endonuclease (400 μg) (black bars) or with control IgG (400 μg) (white bars). Then cofactors for DNA synthesis were added followed by incubation for 20 min at 30°C. Further procedure as described in Materials and Methods. Each data point represents two determinations, each done in triplicate. The standard error of each data point is less than 5%.

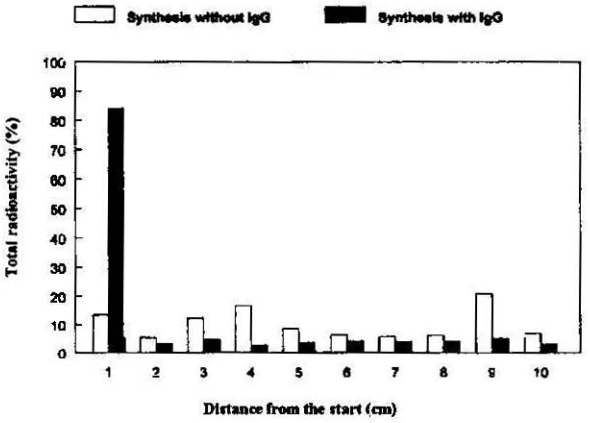

Fig. 4. Polyacrylamide gel electrophoresis (3.5%–8% acrylamide gradient) of products of DNA synthesis in sonicated cell nuclei with plasmid pBR 322 DNA.
Sonicated cell nuclei (8×10^6) were preincubated for 5 min with IgG anti-nuclease (400 μg) (black bars) or with control IgG (400 μg) (white bars) in the presence of 5 μg DNA pBR 322. Further procedure as described in Materials and Methods. Each data point represents two determinations each done in triplicate. The standard error of each data point is less than 5%.

generation of longer DNA fragments when cell nuclei were incubated with IgG anti-endonuclease. The highest peak of radioactivity in Fig. 4 corresponds to the length of pBR 322 DNA in the gel.

DISCUSSION

We reported previously that, in phytohormone-treated cells, nuclear matrices were enriched in 32 kDa endonuclease [6, 7] and that this enzyme in complex with 65 kDa protein was responsible for specific nicking activity of the nuclear matrix [8, 9]. We also demonstrated that IgG anti-endonuclease inhibited DNA synthesis *in vitro* [8].

Recent literature data suggest, that DNA fragments bound to nuclear matrix (especially SAR or MAR DNA) have at least a structural function in organisation of DNA; some authors also suggest a very important role of DNA fragments bound to nuclear matrix in the replication origin and regulation of DNA expression [1, 2]. In the present study we evaluated whether endonuclease itself or complexed (*via* 65 kDa protein) with nuclear matrix takes part in the process of DNA synthesis. The effect of temporary inhibition of DNA synthesis by the

low amount of IgG anti-endonuclease (50 μg IgG per 1×10^6 cell nuclei) and the fact that this inhibitory effect concerns only synthesis of the matrix DNA (DNA bound to the nuclear matrix after its isolation) strongly support our hypothesis. The latter seems to be supported also by generation of longer DNA fragments by IgG anti-nuclease in comparison to control IgG. It should be pointed out that at higher amounts of IgG anti-32 kDa endonuclease (150 μg per 1×10^6 cell nuclei) we observed that the DNA synthesis was irreversibly diminished.

Our hypothesis is based on the fact that 32 kDa endonuclease is tightly associated with nuclear matrix *via* 65 kDa protein [8, 9] and is able to recognise and bind to the SAR DNA [10, 16]. In this model 32 kDa endonuclease together with 65 kDa protein may be a component of a larger protein complex bound to the DNA and fixing DNA (chromatin) loops in nuclear matrix skeleton structure by interactions with SAR or MAR sequences of the DNA. The sequences of the replication origins are located in nuclear matrix-associated DNA (which remained associated with nuclear matrix after its isolation) and replication complexes are also associated with nuclear matrix just behind the bases of the DNA loops fixed on the nuclear matrix. Therefore, we suggest that IgG anti-32 kDa endonuclease covering protein complex fastening of the DNA loops in the nuclear matrix disturb a movement of the replication fork or a movement of the DNA through the replication complex. This disturbance disappears when the DNA localised in the DNA (chromatin) loops undergoes the replication process. This hypothesis is under further investigation in our laboratory.

REFERENCES

1. Van der Velden, H.M.W. & Wanka, F. (1987) The nuclear matrix — Its role in the spatial organization and replication of eukaryotic DNA. *Mol. Biol. Reports* **12**, 69–77.
2. Verheijen, R., van Venroy, W. & Ramaekers, F. (1988) The nuclear matrix: structure and composition. *J. Cell Sci.* **90**, 11–36.
3. Zeitlin, S., Parent, A., Silverstein, S. & Efstratiadis, A. (1987) Pre-mRNA splicing and the nuclear matrix. *Mol. Cell. Biol.* **7**, 111–120.
4. Smith, H.C., Hams, S.G., Zillmann, M. & Berget, S.M. (1989) Evidence that a nuclear matrix protein participates in premessenger RNA splicing. *Exp. Cell Res.* **182**, 52I–533.
5. Klehr, D., Maass, K. & Bode, J. (1991) Scaffold-attached regions from the human interferon β domain can be used to enhance the stable expression of genes under the control of various promoters. *Biochemistry* **30**, 1264–1270.
6. Wisniowska, J., Rudnicki, K. & Szopa, J. (1987) The presence of nuclease-inhibitor complex in White bush cell nuclei. Effect of phytohormones. *J. Plant Physiol.* **129**, 407–413.
7. Rudnicki, K., Rzepecki, R. & Szopa, J. (1988) Rearrangement of the nuclease-inhibitor complex components within cells of White bush treated with phytohormone. *J. Plant Physiol.* **132**, 658–663.
8. Rzepecki, R., Bulaj, G. & Szopa, J. (1989) Endonuclease tightly associated with plant nuclear matrix affected DNA synthesis *in vitro*. *J. Plant Physiol.* **134**, 364–369.
9. Rzepecki, R., Szmidzinski, R., Bode, J. & Szopa, J. (1992) The 65 kDa protein affected endonuclease tightly associated with plant nuclear matrix. *J. Plant Physiol.* **139**, 284–288.
10. Rzepecki, R. (1994) The complex of the endonuclease 32 kDa and 65 kDa protein fiom nuclear matrix preferentially recognize plasmid with SAR DNA element. *J. Plant Physiol.* **144**, 479–484.
11. Linial, M. & Shlomai, J. (1988) A unique endonuclease from *Crithidia fasciculata* which recognizes a bend in the DNA helix. *J. Biol. Chem.* **263**, 290–297.
12. Pieck, A.C.M., Van der Welden, H.M.W., Rijken, A.A.M., Neis, J.M. & Wanka, F. (1985) Protein composition of the chromosomal scaffold and interphase nuclear matrix. *Chromosoma* **91**, 137–144.
13. Maniatis, T., Fritsch, E.F. & Sambrook, J. (1989) *Molecular cloning. Laboratory manual.* Cold Spring Harbor Laboratory Press, New York.
14. Tsutsui, K., Tsutsui, K., Sakurai, H., Shohmori, T. & Oda, T. (1986) Levels of topoisomerase II and DNA polymerase are regulated independently in developing neuronal nuclei. *Biochem. Biophys. Res. Commun.* **138**, 1116–1122.
15. Smith, H.C., Puvion, E., Buchholtz, L.A. & Berezney, R. (1984) Spatial distribution of DNA loop attachment and replicational sites in the nuclear matrix. *J. Cell Biol.* **99**, 1794–1802.
16. Rzepecki, R., Markiewicz, E. & Szopa, J. (1995) Identification of *Cucurbita pepo* nuclear matrix

proteins binding to SAR DNA. *Acta Biochim. Polon.* **42**, 171–176.