Biochimica et Biophysica Acta, 1063 (1991) 247–252

ADONIS 000527369100158O

BBAMEM 75189

Nucleotide and DNA uptake by *Neurospora crassa*: involvement of an uptake stimulating protein

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(Received 17 July 1990)
(Revised manuscript received 14 November 1990)

Key words: DNA uptake; DNA binding protein; Nucleotide uptake; (*N. crassa*)

The basal and DUSF (DNA-uptake-stimulating factor, described previously by Schablik and Szabó (1981) FEMS Microbiol. Lett. 10, 395–397) stimulated uptake of [^{3}H]DNA and radioactive nucleotides by *Neurospora crassa* (FGSC 1118, slime) cell-wall-less strain was studied. The uptake of [3H]DNA by the cells is a saturable and time-dependent process. The pH and temperature optimum for [^{3}H]DNA uptake are pH 7 and 27°C, respectively. Both basal and DUSF-stimulated uptake of [^{3}H]DNA are inhibited by 2,4-dinitrophenol and iodoacetic acid. The inhibition of the basal uptake of [^{3}H]DNA by cycloheximide is greater than the DUSF-stimulated uptake. The DUSF enhanced not only DNA-uptake but also that of the oligonucleotides and mononucleotides. DUSF binds both macromolecular [^{3}H]DNA and [^{14}C]AMP, and there might be competition between nucleotides and DNA for the binding to DUSF. Polyclonal antibodies prepared against DUSF inhibited both basal and DUSF-enhanced [^{3}H]DNA uptake. DUSF was detected by immunoblotting among the proteins isolated from purified *N. crassa* cell-membranes. DUSF might be a receptor protein for DNA and nucleotides in the cell-membrane of *N. crassa* and play a role in DNA and nucleotide uptake.

Introduction

The uptake of oligonucleotides and high molecular weight DNA into eukaryotic cells was established in several laboratories, as reviewed in [1–4], but the physiological uptake mechanism remained obscure. The understanding of the mechanism and the stimulation of the uptake may serve also practical purposes since antisense oligonucleotides specifically inhibit gene expression [5] or virus multiplication [6,7] and macromolecular DNA is used in genetic transformation [8]. A DNA-uptake-stimulating substance was discovered in the culture medium of *N. crassa* (FGSC 1118, slime) cell-wall-less mutant strain and was designated DNA-uptake-stimulating factor (DUSF) [9]. The DUSF was purified to homogeneity and proved to be a protein of 230000 Da and some of its properties were described [10]. DUSF seems to be the only known protein that facilitates DNA uptake. In this paper we report that DUSF also stimulates the uptake of oligonucleotides and mononucleotides into *N. crassa* and conditions which optimize the action of DUSF is described.

Materials and Methods

[^{3}H]DNA and labelled nucleotides

The production and extraction of [^{3}H]DNA from *Neurospora crassa* cell-wall-less mutant strain (FGSC 1118 (fz; sg; os-1, slime)) was carried out as described in Ref. 10. The relative molecular mass of isolated [^{3}H]DNA was $30 \cdot 10^6$. The absorption ratio at 260 nm and 280 nm was 2.0 with a specific radioactivity of 0.29 kBq/μg. The mixture of [^{3}H]oligonucleotides was produced by digestion of [^{3}H]DNA with bovine pancreas DNase I (Sigma) at 37°C for 2 h. [^{3}H]TMP and [^{14}C]AMP were purchased from Amersham.

Uptake of [^{3}H]DNA and labelled nucleotides

N. crassa (FGSC 1118, slime) cell-wall-less mutant cells were cultivated in Vogel's [11] liquid medium with the addition of 1.5% sucrose, 7.5% L-(−)-sorbose for 24 h at 27°C in a horizontal shaker incubator at 100 rpm.

Abbreviations: DUSF, DNA-uptake-stimulating factor; DNase I, deoxyribonuclease I; BSA, bovine serum albumin; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate. Enzyme: Deoxyribonuclease I (EC 3.1.21.1).

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The cells were harvested by centrifugation (1500 rpm, 15 min, 27°C), washed three times in fresh medium, and then a cell suspension of 10^7 cells/ml was prepared. Incubation mixtures consisted of 1 ml cell suspension, 0.8 ml of effectors, 0.1 ml of DUSF (15 μg) or fresh medium and 0.1 ml of [^{3}H]DNA (5 μg) or labelled nucleotides. The incubation was carried out for 2 h with shaking in a water-bath at 27°C. During 2 h of incubation there was no significant degradation of macromulecular [^{3}H]DNA by DNases of the cells, as checked by agarose gel electrophoresis [13]. The uptake of polynucleotides was terminated by rapid cooling of the samples for 10 min at 0°C. Nonabsorbed [^{3}H]DNA was eliminated from the surface of the cells by addition of 0.25 mg DNase I (Sigma) for 10 min at 27°C. Thereafter the samples were cooled again to 0°C and the cells were washed four times in 0.5 M NaCl at 0°C, and dried in the centrifuge tubes. Then 1 ml 0.5 M $HClO_4$ was added to the cells and this mixture was placed in boiling water for 15 min. After centrifugation 0.2 ml samples of the supernatant were mixed with 1.5 ml Aquasol-2 scintillation liquid and the radioactivity was counted. Data shown in the figures and tables are the mean values ± S.D. of three separate determinations.

Preparation of DUSF

DNA-uptake-stimulating protein was isolated and purified from the culture medium of *N. crassa* cell-wall-less strain (FGSC 1118 (fz; sg; os-1, slime)) as described in Ref. 10. The purified DUSF was free from DNase contamination, as demonstrated by the lack of release of radioactive perchloric acid-soluble material from macromolecular [^{3}H]DNA when was incubated with DUSF [12].

Preparation of antibodies against DUSF

Rabbits were immunized by intramuscular injection with 85 μg of homogeneous DUSF six times at 1-week intervals. At the first immunization they were additionally injected with 0.5 ml of complete Freund adjuvant (Difco) and subsequently with 0.5 ml of incomplete adjuvant. Six days after the last injection the rabbits were bled and the serum was separated. The anti-DUSF immunoglobulins were purified from the serum by gel filtration on Sephacryl S300 (Pharmacia), followed by chromatography on DEAE-Sephadex A50 (Pharmacia). The specific immunreaction of anti-DUSF immunoglobulins with DUSF was controlled by Ouchterlony's immunodiffusion test [14]. The immunoprecipitates in the agarose gel were stained as in Ref. 15.

Isolation of cell-membrane, solubilization of membrane proteins and SDS-polyacrylamide gel electrophoresis

N. crassa (FGSC 1118, slime) 48-h-old cells were washed three-times with 10 mM $MgSO_4$, 380 mM sorbose containing 50 mM Tris-HCl buffer (pH 7.5). All subsequent steps were as described by Scarborough [16] with slight modification: (i) no concanavalin A was used for cell-aggregation and (ii) each solution contained 0.25 mM phenylmethylsulfonyl fluoride (PhMeSO_2F). The membrane ghosts were washed five times with 10 mM Tris-HCl (pH 7.5). The ghosts were solubilized by ultrasonication (3 × 20 s at 0°C, Braunsonic 300 S) in 5 mM EDTA, 0.25 mM PhMeSO_2F, 1% SDS containing 10 mM Tris-HCl (pH 7.5). The sonicated membrane was placed in a boiling water-bath for 5 min. Then it was centrifugated at 5000 rpm for 5 min at 0°C and the supernatant was dialyzed overnight against 5 mM EDTA, 0.25 mM PhMeSO_2F, 0.1% SDS containing

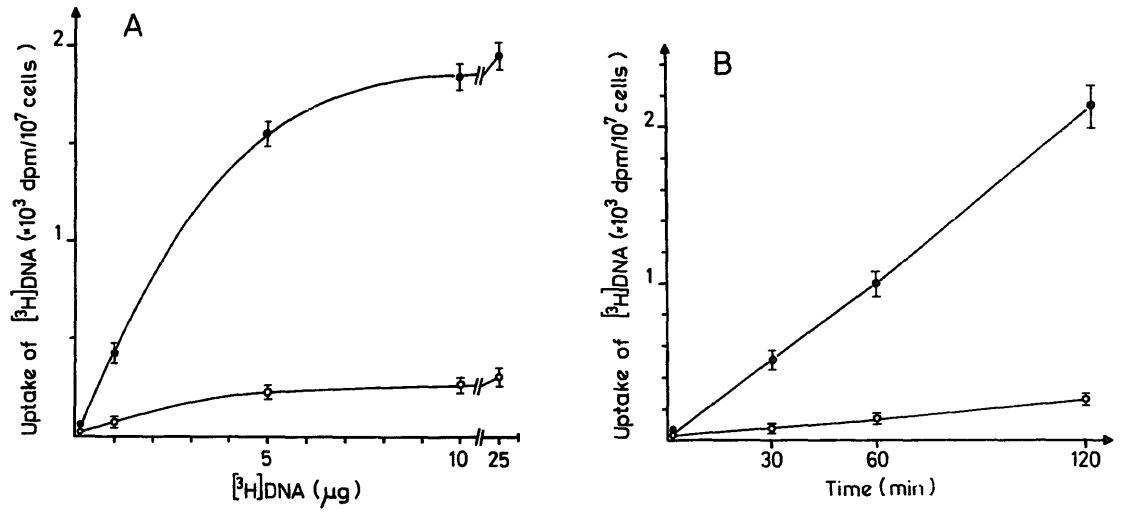

Fig. 1. The saturation (A) and time (B) curves of [^{3}H]DNA uptake by *N. crassa* slime cells. The [^{3}H]DNA uptake by the cells was measured at different concentrations of [^{3}H]DNA after two h of incubation (A) and at 5 μg [^{3}H]DNA after 30, 60 and 120 min of incubation (B), in the absence (○——○) and in the presence (●——●) of DUSF (15 μg) as described in Materials and Methods.

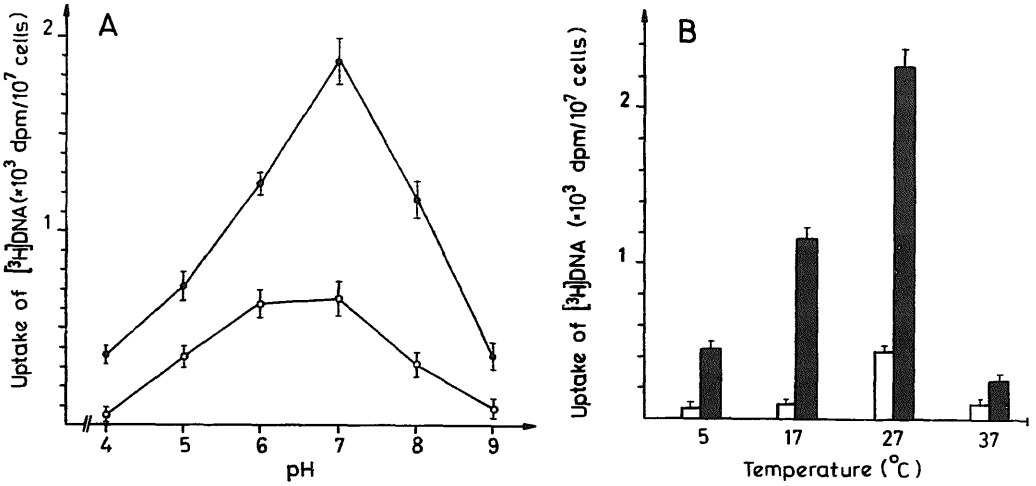

Fig. 2. The effect of pH (A) and temperature (B) upon [^{3}H]DNA uptake by *N. crassa* slime cells. The measurement of [^{3}H]DNA uptake (5 μg) by cells was carried out at various pH values (A) and temperatures (B) in the absence (○——○; white bars) and in the presence (●——●; black bars) of DUSF (15 μg) as described in Materials and Methods.

Tris-HCl (pH 7.5) buffer at 4°C, followed by lyophylization. SDS-polyacrylamide gel electrophoresis of the solubilized membrane proteins was carried out in 10% slab gel according to Laemmli [17].

Protein blotting, immunostaining and gold staining of protein blot

The separated membrane proteins were transferred onto nitrocellulose (Hybond-C, Amersham) sheets by bidirectional capillary diffusion as described by Reinhart [18]. Then the nitrocellulose sheets were blocked with 2% bovine serum albumin for 1 h at 4°C. The blots were developed with anti-DUSF as primary antibodies (rabbit), then with peroxidase-antiperoxidase complex conjugated anti-rabbit immunoglobulins (goat) as secondary antibody (Amersham), for 1 h at 37°C. The peroxidase reaction was carried out by incubating for several minutes in 0.1 M Tris-HCl, pH 7.5 buffer containing 0.5 mg/ml *p*-phenylenediamine dihydrochloride, 1 mg/ml pyrocatechol and 0.01% H_2O_2 [19]. Between and after using of the immunoglobulins the blots were washed with 10 mM Tris-HCl (pH 7.5), 150 mM

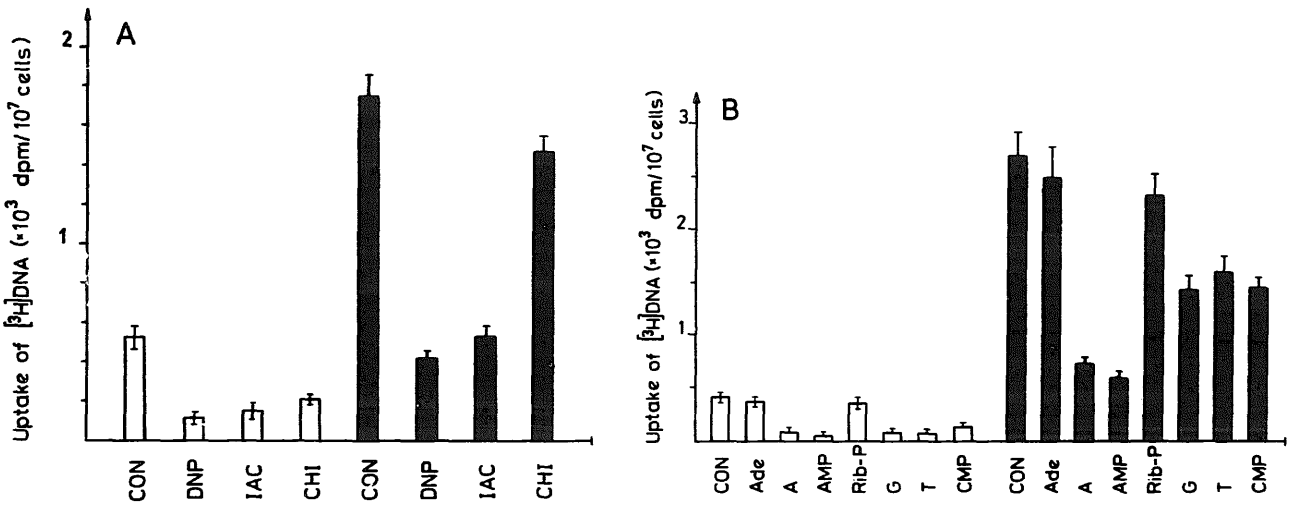

Fig. 3. The effect of 2,4-dinitrophenol, iodoacetic acid, cycloheximide (A), some nucleotides and their constituents (B) upon [^{3}H]DNA uptake by *N. crassa* slime cells. The measurement of [^{3}H]DNA uptake (5 μg) by *N. crassa* cells was carried out in the absence (white bars) and in the presence (black bars) of DUSF (15 μg) as described in Materials and Methods. Symbols of figure A: DNP (2,4-dinitrophenol) 10^{-3} M; IAC (iodoacetic acid) 10^{-3} M; CHI (cycloheximide) 10^{-4} M; CON (control). Symbols of figure B: Ade (adenine) 10^{-4} M; A (adenosine) 10^{-4} M; AMP (adenosine 5′-phosphate) 10^{-4} M; G (guanosine) 10^{-4} M; T (thymidine) 10^{-4} M; CMP (cytidine 5′-phosphate) 10^{-4} M; Rib-*P* (ribose 5′-phosphate) 10^{-3} M; CON (control).

TABLE I

The effect of DUSF and unlabelled DNA in excess upon the uptake of digested [^{3}H]DNA and radioactive nucleotides by N. crassa slime cells

The uptake of radioactive nucleotides by the cells was measured as described in Materials and Methods. The applied quantities of radioactive materials in the incubation mixtures were as follows: Digested [^{3}H]DNA, $8.3 \cdot 10^{-11}$ M; [^{3}H]TMP, $1.3 \cdot 10^{-11}$ M; [^{14}C]AMP, 10^{-11} M. The effect of unlabelled DNA, $1.6 \cdot 10^{-9}$ M isolated. from *N. crassa* alone or with 15 μg DUSF upon the uptake of nucleotides was measured.

Effectors added	The uptake of radioactive materials (dpm/10^7 cells)		
	digested [^{3}H]DNA	[^{3}H]TMP	[^{14}C]AMP
None	114± 8	404± 81	1648± 15
Unlabelled DNA in excess	31± 10	289± 23	1158± 35
DUSF	2278±106	1049±108	6389±274
DUSF+ unlabelled DNA in excess	1253± 83	635± 90	4472±185

NaCl, 0.05% Tween-20 buffer. The gold staining of the protein-blot was done with the method of Yamaguchi [20].

Results and Discussion

The effects of DNA concentration, pH and temperature upon [^{3}H]DNA uptake

The basal and the stimulated uptake of [^{3}H]DNA by DUSF into *N. crassa* cells proved to be a saturable (Fig. 1A) and time-dependent (Fig. 1B) process. The influence of pH upon [^{3}H]DNA uptake was studied (Fig. 2A). The optimal basal DNA uptake occurred between pH 6 and 7, while the stimulatory effect of DUSF has a sharp peak at pH 7. At lower values (pH < 7) the decreased uptake cannot be considered as a consequence of cell destruction or denaturation of DUSF because the cultivation of *N. crassa* and the production of DUSF take place at pH 5. Fig. 2B shows that the uptake of [^{3}H]DNA is maximal at 27°C and it is drastically decreased at higher or lower temperatures. The DUSF stimulated uptake of [^{3}H]DNA is not influenced by lowering the temperature to 17°C or 5°C as in the case with basal uptake. A considerable amount of [^{3}H]DNA can be taken up by the stimulated cells even at that low temperature.

The influence of 2,4-dinitrophenol, iodoacetic acid and cycloheximide upon [^{3}H]DNA uptake

Fig. 3A shows that both the uncoupler 2,4-dinitrophenol that inhibits ATP synthesis by terminal oxydation and iodoacetic acid, an inhibitor of glycolysis, diminished [^{3}H]DNA uptake. It seems that DNA uptake or a part of the process is energy-requiring. Cycloheximide strongly inhibited the uptake of [^{3}H] DNA by cells in the absence of DUSF, but had much less effect upon the DUSF-stimulated uptake. This may indicate the involvement of de novo synthesized proteins in the DNA uptake process, one of which might be DUSF itself.

The effect of nucleotides and nucleosides upon [^{3}H]DNA uptake

Fig. 3B shows that AMP, CMP, guanosine and thymidine inhibited [^{3}H]DNA uptake both by untreated and by DUSF treated cells. The components of AMP, adenine and ribose 5′-phosphate were not inhibitors while adenosine strongly decreased [^{3}H]DNA uptake. These results indicate that it is not the purine base (adenine) or ribose 5′-phosphate moiety separately, but it is a composite molecule, the nucleoside (adenosine) or nucleotide (AMP) that inhibited [^{3}H]DNA uptake. The uptake mechanism of nucleotides and of DNA may have common components and that possibility prompted us to determine the uptake of [^{14}C]AMP, [^{3}H]TMP and a mixture of ^{3}H-labelled oligonucleotides by *N. crassa*. The ^{3}H-labelled oligonucleotides were prepared by DNase I digestion of [^{3}H]DNA as described in Materi-

TABLE II

The binding of [^{3}H]DNA and [^{14}C]AMP by DUSF

Nitrocellulose (Hybond-C, Amersham) discs of the same diameter (12 nm) were soaked in 2% bovine serum albumine (BSA) or in 0.7 mg/ml DUSF containing 10 mM sodium phosphate buffer (pH 7.0) for 60 min. The DUSF loaded discs were placed into 2% BSA containing solution for 30 min. One group of these discs were soaked in unlabelled AMP, ($5 \cdot 10^{-4}$ M) the other in unlabelled DNA, ($1.7 \cdot 10^{-8}$ M) solution for 30 min. The discs were immersed into [^{3}H]DNA, ($3.3 \cdot 10^{-10}$ M) or [^{14}C]AMP, ($2 \cdot 10^{-11}$ M) containing buffer solution for 30 min. The unbound radioactivity was thoroughly washed out. The washed discs were solved in 0.5 ml ethyl acetate, then 3 ml of Aquasol-2 scintillation liquid was added and the radioactivity was counted. Every step was carried out at 4°C and the values are the average of three parallels.

Radioactive materials bound	Radioactivity bound (dpm/nitrocellulose disc; treatment of the nitrocellulose discs			
	BSA control	DUSF/BSA	DUSF/BSA/unlabelled AMP in excess	DUSF/BSA/unlabelled DNA in excess
[^{3}H]DNA	263± 5	1125±120	377±27	not done
[^{14}C]AMP	510±10	1086± 53	not done	519±15

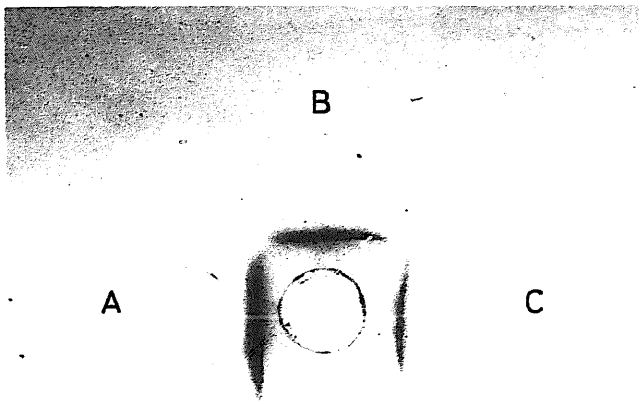

Fig. 4. The immundiffusion test of anti-DUSF antibodies in agarose gel. The middle well contained 50 μg of purified anti-DUSF immunoglobulins and wells A, B, C contained 1.4 μg, 0.7 μg and 0.35 μg of homogeneous DUSF protein, respectively. The immunoprecipitates were visualized by staining as described in Ref. 15.

als and Methods. Table I shows: (i) that DUSF increases the uptake of nucleotides: (ii) "Cold" DNA (in excess, 100 μg) inhibited both the basal and the DUSF-stimulated uptake of nucleotides. Fig. 3B and Table I indicate that there might be competition for uptake between DNA and nucleotides.

Purified DUSF binds macromolecular [^{3}H]DNA and [^{14}C]AMP

The capacity of purified DUSF to bind macromolecular [^{3}H]DNA and [^{14}C]AMP was determined with DUSF immobilized to nitrocellulose membrane (as described in legend of Table II). As it is shown in Table II DUSF binds both [^{3}H]DNA as well as [^{14}C]AMP. It can be seen that DNA competes effectively with AMP binding though further experiments are needed for quantitative analyses.

Experiments with polyclonal anti-DUSF immunoglobulin

Polyclonal antibodies were prepared against DUSF as described in Materials and Methods. Fig. 4 shows the specificity of the precipitation between purified (homogeneous) DUSF and partially purified antibodies. The effect of anti-DUSF immunoglobulins upon [^{3}H]DNA uptake was determined. Table III shows the inhibitory effect of the antibodies upon both basal and DUSF-stimulated [^{3}H]DNA uptake. The inhibition of the basal [^{3}H]DNA uptake is more pronounced than the DUSF-stimulated one. We assume that the cell-membrane of *N. crassa* containing DUSF by reacting with the anti-DUSF immunoglobulins became inaccessible to DNA and that caused the cessation of the basal uptake. The presence of DUSF among the proteins of the isolated cell-membrane of *N. crassa* was demonstrated by im-

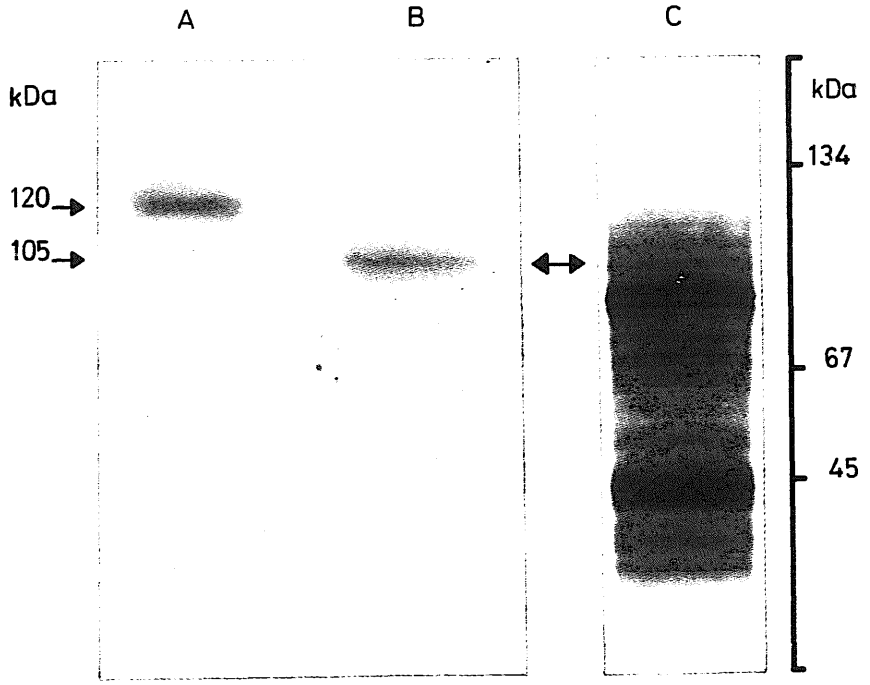

Fig. 5. Detection of DUSF by immunoblotting among the cell-membrane proteins of *N. crassa* (slime) cells. Cell-membranes were isolated and solubilized as described in Materials and Methods. The SDS/polyacrylamide gel electrophoresis of DUSF (2.5 μg, lane A) and the solubilized membrane proteins (85 μg, lane B) took place in 10% slab gel (2 mm). The separated proteins were transferred onto nitrocellulose sheets and DUSF was indicated by immunostaining (see in Materials and Methods). Membrane proteins blotted were visualized by gold staining on the nitrocellulose sheet (lane C). As molecular weight standards: ovalbumin (egg, 45000), serum albumin (bovine, 67000) and crosslinked serum albumin (bovine, dimer, 134000) were applied.

TABLE III

The effect of anti-DUSF antibodies upon [^{3}H]DNA uptake by N. crassa slime cells

The uptake of [^{3}H]DNA (5 μg) by cells was measured in the absence (−) and in the presence (+) of DUSF (20 μg) as described in Materials and Methods. The quantity of immunoglobulins in the incubation mixtures were the same (0.45 mg).

	[Uptake of [^{3}H]DNA (dpm/10^7 cells)]	
	control antibody	anti-DUSF antibody
−DUSF	680± 2	19± 4
+DUSF	4180±114	2378±112

munoblotting (Fig. 5). One of the minor bands of the blotted membrane proteins stained with gold (lane C) coincides with the single band (lane B) detected with immunostaining. The fact that the membrane protein reacting with anti-DUSF antibody is about 15 kDa less than the standard DUSF preparation (lane A) might be the result of proteolysis during the solubilization of the membrane proteins. It seems that DUSF is an integral part of the cell-membrane. The above experiments show that DUSF binds DNA and nucleotides and stimulated the uptake of DNA and nucleotides into the *N. crassa* slime cells, but further experiments are needed to determine how DNA and nucleotides penetrated the cell. Although the extracellular macromolecular DNA was not degraded (not shown) even after 2 h of incubation with the *N. crassa* cells, it can't be excluded that during penetration through the cell-membrane DNA (and nucleotides) were degraded.

DUSF is not unique as DNA-binding protein in eukaryotes [21]. There are recent publications about the uptake of the undegraded oligonucleotides by mammalian cells [22,23], and an oligonucleotide binding protein was isolated [22] and its presence in the cell membrane was also shown [23], but we are not aware of data about its stimulatory role in oligonucleotide or DNA uptake.

We think that DUSF might be a cell-membrane receptor of DNA and nucleotides in *N. crassa* and the mechanism of uptake is probably receptor mediated endocytosis. Our above results (the temperature dependence of the rate of uptake, the inhibition by energy depletion, the receptor specificity) are concordant with the conclusion of Loke [22] and Yakubov [23], who also suggested that the uptake of oligonucleotides took place by receptor mediated endocytosis. Although DNA is taken up by $CaCl_2$ and heat-shock [24] or poly(ethylene glycol) [25] treated spheroplasts of *N. crassa*, we assume that there exists also a physiological process which catalyzes the uptake of polynucleotides into the cell.

Acknowledgements

The authors are indebted to Miss Cecilia Nagy for her excellent technical help. This work was supported by a grant from the Hungarian Academy of Sciences.

References

1 Stebbing, N. (1979) Cell Biol. Int. Rep. 6, 485–502.
2 Mishra, N.C. and Tatum, E.L. (1973) Proc. Natl. Acad. Sci. USA 70, 3875–3879.
3 Mishra, N.C., Szabó, G. and Tatum, E.L. (1973) in The role of RNA in reproduction and development (Niu, M.C. and Segal, S.J., eds.), pp. 259–268.
4 Schablik, M., Szabolcs, M., Kiss, Á., Aradi, J., Zsindely, A. and Szabó, G. (1977) Acta Biol. Acad. Sci. Hung. 28, 73–279.
5 Heikkila, R., Schwab, G., Wickstrom, E., Loke, S.L., Pluznik, D.H., Watt, R. and Neckers, L.M. (1987) Nature (London) 328, 445–449.
6 Zamecnik, P.C. and Stephenson, M.L. (1978) Proc. Natl. Acad. Sci. USA 75, 280–284.
7 Zamecnik, P.C., Goodchild, J., Taguchi, Y. and Sarin, P.S. (1986) Proc. Natl. Acad. Sci. USA 83, 4143–4146.
8 Mishra, N.C. (1985) Adv. Genet. 23, 73–178.
9 Schablik, M. and Szabó, G. (1981) FEMS Microbiol. Lett. 10, 395–397.
10 Tóth, Gy., Békési, I., Schablik, M. and Szabó, G. (1987) Eur. J. Biochem. 162, 199–202.
11 Vogel, H.J. (1956) Microbiol. Genet. Bull. 13, 42–43.
12 Chow, T.Y.-K. and Fraser, M.J. (1983) J. Biol. Chem. 258, 12010–12018.
13 Maniatis, T., Fritsch, E.F. and Sambrook, H. (1982) Molecular Cloning. A laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
14 Ouchterlony, Ö. (1967) in Handbook of Experimental Immunology (Weir, D. M., ed.), pp. 655–706, Blackwell Science. Publishers, Oxford and Edinburgh.
15 Crowle, A.J. and Cline, L.J. (1977) J. Immunol. Methods 17, 379–381.
16 Scarborough, G.A. (1975) J. Biol. Chem. 250, 1106–1111.
17 Laemmli, U.K. (1970) Nature (Lond.) 227, 680–685.
18 Reinhart, M.P. and Malamud, D. (1982) Anal. Biochem. 123, 229–235.
19 Hanker, J.S., Yates, P.E., Metz, C.B. and Rustioni, A. (1977) Histochem. J. 9, 789–792.
20 Yamaguchi, K. and Asakawa, H. (1988) Anal. Biochem. 172, 104–108.
21 Bennett, R.M., Gabor, G.T. and Merritt, M.M. (1985) J. Clin. Invest. 76, 2182–2190.
22 Loke, S.L., Stein, C.A., Zhang, X.H., Mori, K., Nakanishi, M., Subasinghe, C., Cohen, J.S. and Neckers, L.M. (1989) Proc. Natl. Acad. Sci. USA 86, 3474–3478.
23 Yakubov, L.A., Deeva, E.A., Zarytova, V.F., Ivanova, E.M., Ryte, A.S., Yurchenko, L.V. and Vlassov, V.V. (1989) Proc. Natl. Acad. Sci. USA 86, 6454–6458.
24 Case, M.E. Schweizer, M., Kushner, S.R. and Giles, N.H. (1979) Proc. Natl. Acad. Sci. USA 76, 5259–5263.
25 Schweizer, M., Case, M.E., Dykstra, C.C., Giles, N.H. and Kushner, S.R. (1981) Proc. Natl. Acad. Sci. USA 78, 5086–5090.