The EMBO Journal vol.4 no.10 pp.2525-2532, 1985

# Mutants of *Polysphondylium pallidum* altered in cell aggregation and in the expression of a carbohydrate epitope on cell surface glycoproteins

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Communicated by G.Gerisch

**Mutants of the cellular slime mold *Polysphondylium pallidum* have been selected using a cell sorter and a fluorescent-labeled monoclonal antibody, mAb 293. This antibody blocks cell adhesion when applied as Fab, and recognizes a carbohydrate epitope containing L-fucose. This epitope is expressed on the cell surface and is present on >10 membrane glycoproteins of different apparent mol. wts. Twenty mutants were obtained which did not bind mAb 293 when tested at 2 h of starvation. After longer periods of starvation the epitope became detectable in the mutants. In all these mutants aggregation patterns were atypical. Generally streams of cells that were radially orientated around aggregation centers were missing or were much shorter than in wild-type. Genetic analysis demonstrated that aberrant aggregation was linked to the alteration in carbohydrate epitope expression. One mutant was unstable and gave rise to subclones in which almost no antibody binding was observed, even after 24 h of starvation, and only few aggregation centers with no streams or very short ones were formed. These results indicate that the capability of the cells to aggregate is correlated with the exposure on their surfaces of the carbohydrate epitope recognized by mAb 293, whose function in development remains to be established.**

*Key words:* fucosylation/contact sites/cell sorting/lectins/cellular slime molds

## Introduction

*Polysphondylium pallidum* is a cellular slime mold, like *Dictyostelium discoideum,* whose cells aggregate after ~7 h of starvation, forming large streams radially orientated around aggregation centers. The system responsible for aggregation of *P. pallidum* cells differs from that of *D. discoideum* such that cells of the two species sort out into separate aggregates not only because they respond to different chemoattractants (Shimomura *et al.*, 1982), but also because of species-specific membrane-membrane recognition (Gerisch *et al.*, 1980).

Cell surface glycoproteins and their participation in cell adhesion have been studied in *P. pallidum* (Bozzaro and Gerisch, 1978; Steinemann *et al.*, 1979; Bozzaro *et al.*, 1981) in parallel to similar work on *D. discoideum* (Müller and Gerisch, 1978; Murray *et al.*, 1981, 1983; Ochiai *et al.*, 1982; Yoshida *et al.*, 1984; Bertholdt *et al.*, 1985). The adhesion blocking activity of polyclonal Fab from antisera raised against whole membrane fractions of aggregation-competent *P. pallidum* cells was neutralized to 80% by a purified carbohydrate fraction (Toda *et al.*, 1984a). The carbohydrate was obtained by hydrazinolysis of two glycoproteins that form a double band in the 64-kd region after SDS-polyacrylamide gel electrophoresis. Fab of a monoclonal antibody, mAb 293, raised against this pair of glycoproteins completely blocked adhesion of suspended *P. pallidum* cells and was neutralized by the purified carbohydrates (Toda *et al.*, 1984a). These observations prompted us to select mutants that are defective in the carbohydrate epitope recognized by mAb 293.

In immunoblots mAb 293 recognized not only the 64-kd pair but also between 10 and 20 other glycoproteins of lower and higher mol. wts. Binding of the antibody to all these glycoproteins was completely blocked by free L-fucose (Toda *et al.*, 1984b). This result does not mean that the antibody recognized only L-fucose residues, but indicates that the affinity of the anti-

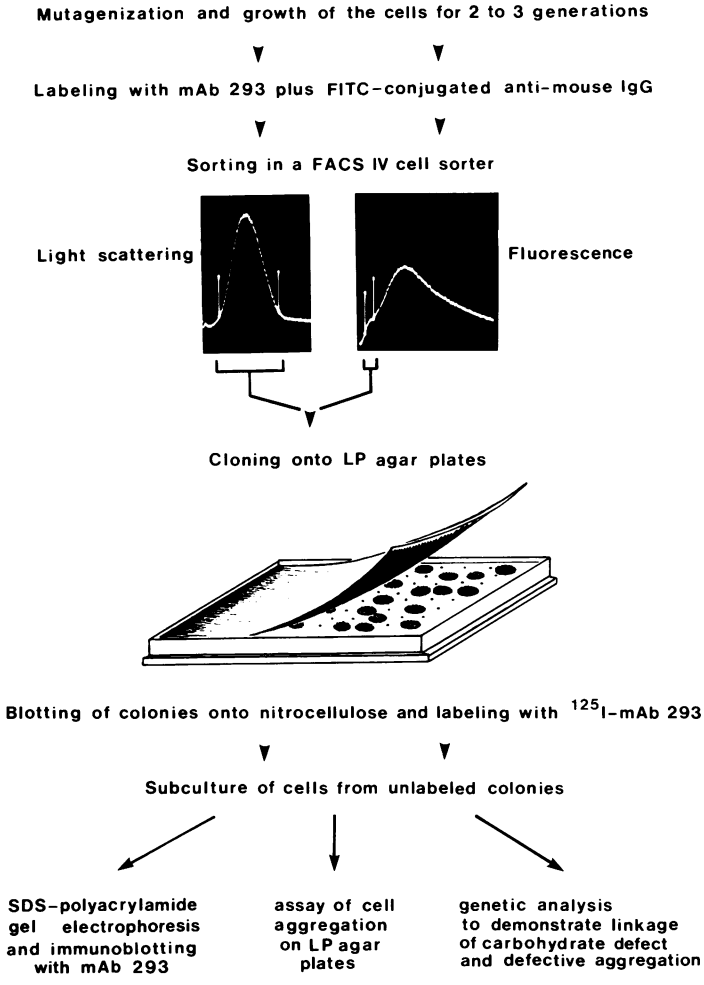


**Fig. 1.** Experimental protocol for the selection and analysis of mutants defective in ep 293 expression. In most of the experiments, cell-sorter selection of the cells was repeated after several generations of growth before the cells were cloned onto lactose-peptone (LP) agar. The vertical white lines in the photograph show the boundaries of the windows from which cells were selected. Intensity of light scattering as a measure of cell size, and of fluorescence are plotted on the abscissa, relative numbers of cells on the ordinate. The window of fluorescence corresponds to the autofluorescence intensity of the cells. The photograph was taken during a second run of selection where unlabeled cells were already enriched.

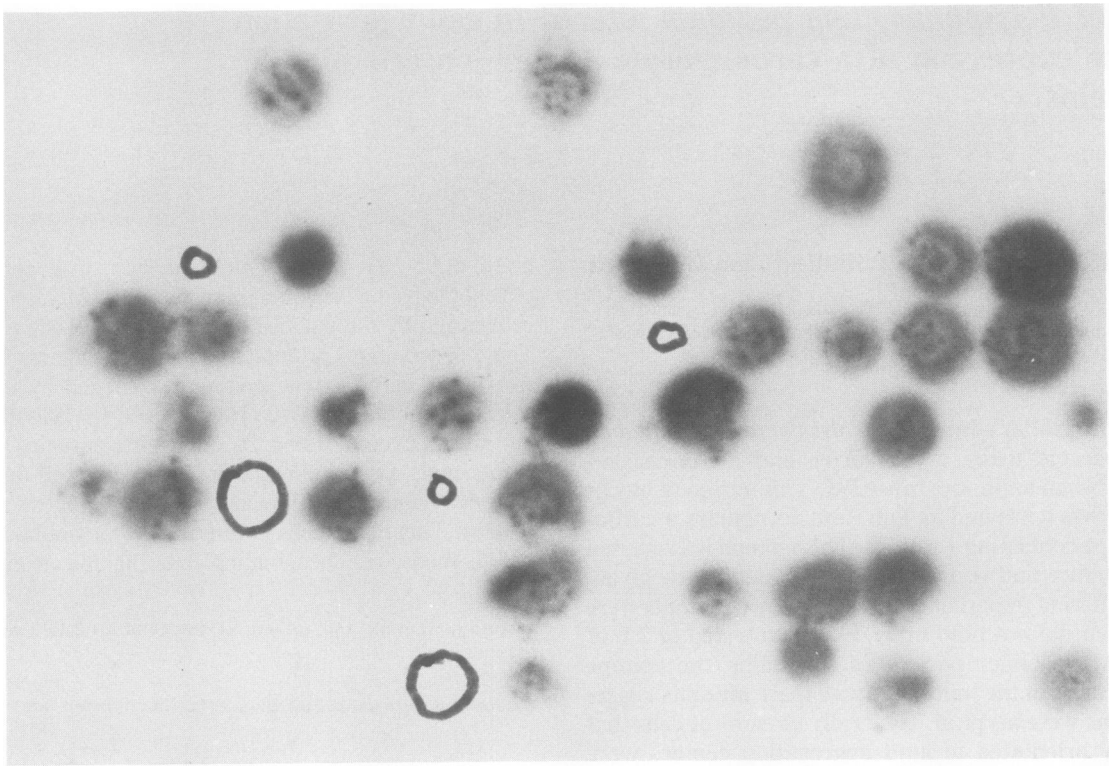

**Fig. 2.** Colony blot labeled with [$^{125}$I]mAb 293. Approximate sizes of colonies at unlabeled positions are indicated. The two larger unlabeled colonies were the origins of two mutants; the three smaller colonies proved to be wild-type; they were too young to be detectably labeled.

**Table I.** Yield of mutants after one or two runs of sorting in a FACS IV cell sorter

| Experiment no. | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Mutagen | U.V. | MNNG | U.V. | MNNG |
| Presorted | Yes | No | Yes | Yes |
| Ratio of sorted to total cells | $3.4 \times 10^{-3}$ | $2.4 \times 10^{-3}$ | $5.7 \times 10^{-3}$ | $1.1 \times 10^{-2}$ |
| Ratio of growing to sorted cells | 0.47 | 0.30 | 0.19 | 0.65 |
| No. of clones examined | 89 | 85 | 108 | 124 |
| No. of stable mutants | 5 | 2 | 6 | 7 |
| Designation of mutants | PN6002; 6003; 6004; 6005; 6006 | PN6007; 6008 | PN6009; 6010; 6011; 6012; 6013; 6014 | PN6015; 6016; 6017; 6018; 6019; 6020; 6021 |

body to L-fucose was high enough to prevent its binding to the complete carbohydrate chains, where it recognizes an epitope that includes not only L-fucose but also other sugars. Methylation analysis revealed that L-fucose was always in a terminal position at the non-reducing ends of the protein-linked oligosaccharide chains (Toda *et al.*, 1984b). Therefore, mAb 293 could be used to select, in a cell sorter, mutants in which either the expression of the whole set of glycoproteins recognized by this antibody is defective, or in which synthesis, transfer to proteins, or processing of the carbohydrate residues is blocked.

In *P. pallidum* sexual development can be induced by co-culture of two haploid strains with opposite mating types (Francis, 1980). Under these conditions macrocysts are formed. These are thick-walled aggregates of cells in which zygote formation and meiosis is carried out. Thus sexual recombination can be used in *P. pallidum* for linkage analysis of mutants (Francis and Rupar, 1983). In the present paper we report on the selection and genetic analysis of 20 mutant strains in which delayed expression of the carbohydrate epitope recognized by mAb 293 was associated with aberrations in the pattern of cell aggregation. This epitope will be designated ep 293.

## Results

### *Selection of mutants*

After mutagenesis with nitrosoguanidine (MNNG) or ultraviolet irradiation (u.v.), cells of strain PN600 were grown on bacteria for 2–4 generations, then washed and shaken in non-nutrient buffer, and labeled after 2 h of starvation with mAb 293 and FITC-conjugated sheep anti-mouse IgG. Unlabeled cells were selected by use of a FACS IV cell sorter. They were either directly cloned on agar plates using a single-cell deposition system in combination with the cell sorter, or were regrown on bacteria, starved, labeled with antibodies and sorted for a second time. The cloned cells were allowed to grow into colonies of 0.5–1 cm diameter which contained $1-2 \times 10^5$ cells (Figure 1). Position and size of the colonies formed were marked on the bottom of the plates before the colonies were blotted onto nitrocellulose

**Table II.** Phenotypic characteristics of wild-type PN600 and mutant strains with defective ep 293 expression

| Strain | Generation time | Aggregation | Other characteristics |
|---|---|---|---|
| PN600 | 3.3 | Normal | – |
| PN6002 | 4.0 | Atypical | – |
| PN6003 | n.d. | Atypical | Macrocysts not germinating |
| PN6004 | n.d. | Atypical | – |
| PN6005 | n.d. | Atypical | – |
| PN6006 | n.d. | Atypical | – |
| PN6007 | 3.8 | Atypical | – |
| PN6008 | n.d. | Atypical | – |
| PN6009 | 3.5 | Atypical | – |
| PN6010 | n.d. | Atypical | – |
| PN6011 | 3.7 | Atypical | – |
| PN6012 | n.d. | Atypical | – |
| PN6013 | n.d. | Atypical | – |
| PN6014 | 4.2 | Atypical | – |
| PN6015 | n.d. | Atypical | – |
| PN6016 | n.d. | Atypical | No macrocysts |
| PN6017 | 3.3 | Strongly reduced | Numerous microcysts; no macrocysts |
| PN60181 | 3.2 | Strongly suppressed | Derived from unstable mutant PN6018 |
| PN60183 | 3.1 | Strongly suppressed | Derived from unstable mutant PN6018 |
| PN6019 | 3.8 | Atypical | – |
| PN6020 | 4.0 | Atypical | No macrocysts |
| PN6021 | 3.2 | Atypical | No macrocysts |

filters, labeled with [$^{125}$I]mAb 293 and autoradiographed (Figure 2). Serial dilution of wild-type cells showed that $10^4$ cells gave a noticeably weaker signal than $10^5$ cells, and $10^3$ cells could barely be detected. This indicates that clones of mutant cells which produce one tenth or less of the normal amount of antibody-binding sites could be easily distinguished from wild-type.

After blotting, few cells remained on the original plates, but these were enough for starting new cultures from clones that showed weak or no expression of ep 293 on the blots. Cells from each clone of interest were recloned, one subclone grown up in suspension culture, and its cells harvested after 2 h of starvation. Glycoproteins were labeled with mAb 293 after SDS-polyacrylamide gel electrophoresis of total cellular proteins and blotting onto nitrocellulose filters. In four independent mutagenesis experiments a total of 406 clones were screened, from which 20 proved to be mutant strains that did not significantly express ep 293 after 2 h of starvation (Table I). At least six mutants were the outcome of independent mutagenic events, since we obtained mutants in each of the four experiments, and in the fourth experiment two mutants, PN6017 and PN6018, were distinguishable from the others as reported in the next section.

*Phenotypic characteristics of the mutants*

With the exception of PN6018 all mutants grew well in suspension cultures with *Escherichia coli* B/r as a food bacterium. Generation times were determined for nine of the mutants and found to be close to 3.3 h, the generation time of wild-type PN600 (Table II). This result indicates that ep 293, which was not expressed during the growth phase in the mutants, was not important for the uptake of bacteria or for cell division. PN6018 grew very slowly in suspension. Because this mutant was unstable and gave rise to faster growing subclones, its generation time could not exactly be determined. Two of its subclones, PN60181 and PN60183, were selected and included in Table II.

Shape of both aggregates and fruiting bodies was estimated on lactose-peptone agar with *E. coli* B/r as food bacterium. Aggregation in all mutants differed from that in wild-type. Aggregation designated 'atypical' in Table II was characterized as follows. The aggregates often had no streams, or had a wheel-like appearance due to the formation of stubby streams of uniform length instead of the long branching streams seen during aggregation of the wild-type (Figure 3). Even though a single aggregate of a mutant may be like wild-type in appearance, the collection of aggregates on a plate allowed unequivocal recognition of the mutants. Many of the mutant amoebae remained as a smooth lawn between the aggregates, and the fruiting bodies formed were often smaller and had fewer branches than those of the wild-type.

Two mutant strains were clearly distinguished from the majority of mutants (Table II). One of them, PN6017, extensively produced microcysts after starvation; i.e., most of the amoeboid cells of the mutant were converted into rounded cells that were covered by a wall. Only the few remaining cells aggregated and formed fruiting bodies on agar plates. The early conversion of cells into microcysts appears to be the reason why PN6017 showed strongly reduced aggregation. The second mutant was the slowly growing PN6018 which showed atypical aggregates similar to the majority of mutants. However, two stable fast growing subclones of that mutant, PN60181 and PN60183, only sporadically formed aggregates on agar plates; most of the cells remained unaggregated. Stream formation around the centers was strongly suppressed in these subclones.

*Linkage of atypical aggregation to the ep 293 defect as revealed by meiotic recombination*

For genetic recombination, all mutants were co-cultured with PN100, a wild-type strain of opposite mating type. Macrocysts, the structures in which zygotes are formed and meiosis proceeds, were obtained from 16 crosses (Table II). Macrocysts from 15 of these crosses germinated, and the morphology of the progeny was determined on lactose-peptone agar with *E. coli* B/r (Table III). The unstable mutant PN6018 gave rise to three classes of progeny: those with normal, atypical and no aggregation; therefore it has not been included in Table III. In the remaining 14 crosses which were successful, progeny with normal and atypical aggregation were obtained. A large amount of progeny, 910 out of 1018, were wild-type in their aggregation characteristics and, with the exception of one progeny strain from PN6002, also in the shape of their fruiting bodies. This result suggests that the mutations determining the aggregation behavior were of negative selective value with respect to survival or hatching of the haploid cells that had been formed by meiosis in the macrocysts.

That the mutant progeny were genuinely haploid products of a zygote rather than leftover parental cells was determined by testing a number of them for mating type. Approximately half were of mating type opposite to the mutant parent. This result is expected since mating type acts as if controlled by two alleles of one gene (Francis, 1980).

Progeny of four crosses were probed in immunoblots to see whether ep 293 expression followed morphological appearance (Table III). All of the 21 progeny from these crosses which were classified as mutant on the basis of morphology failed to react with mAb 293 at 2 h of development, whereas 14 progeny of wild-type morphology did react. Figure 4 shows these results with progeny of mutant PN6002. Progeny no. 5, which was not included in Figure 4, showed normal aggregation and ep 293 expression but had fruiting bodies with curled stalks. Such fruiting bodies were not observed in any of the other strains and were

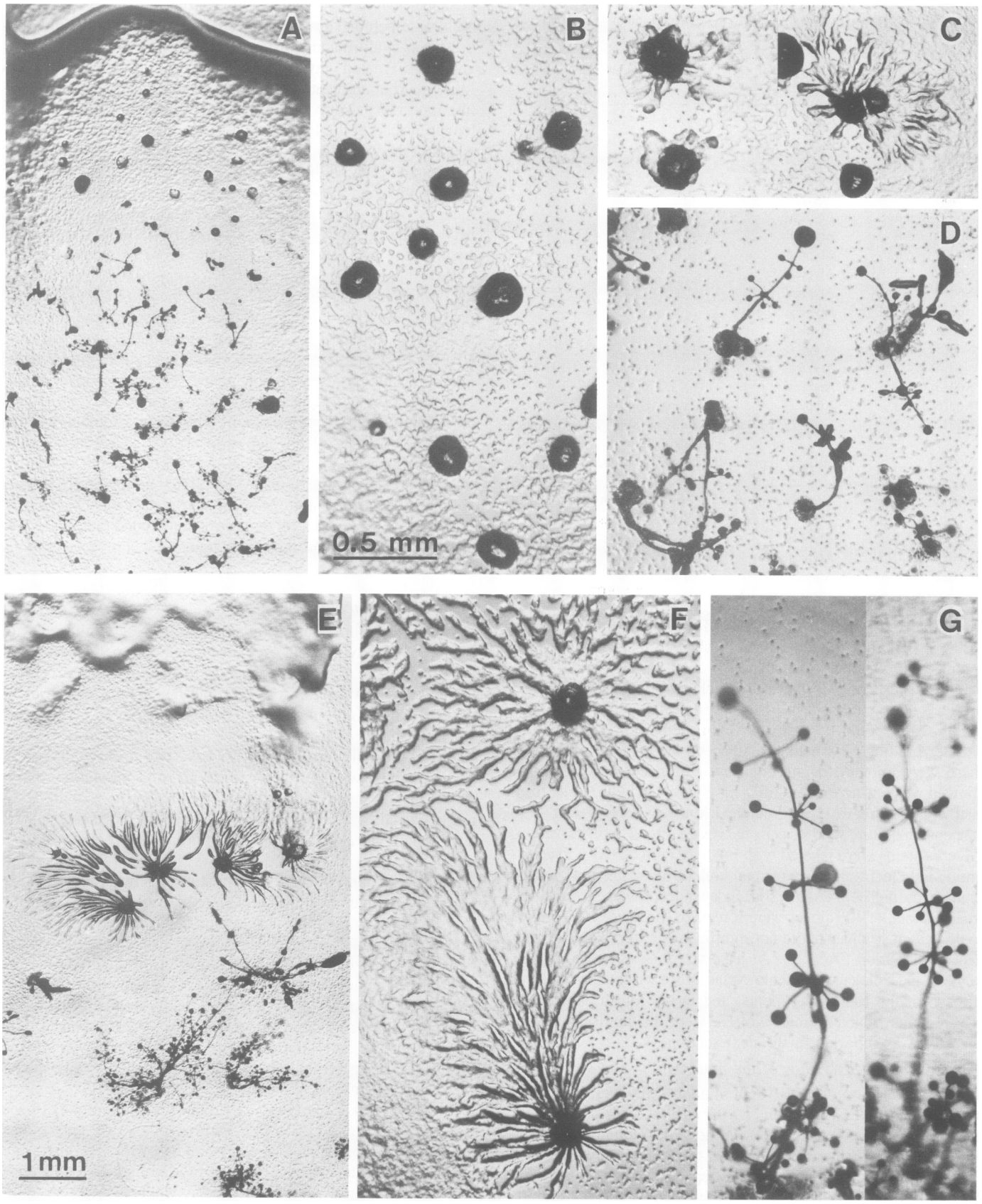


**Fig. 3.** Aggregation and fruiting body formation of mutant PN6002 (**A**–**D**) and wild-type PN600 (**E**–**G**). PN6002 exemplifies the developmental defects seen on LP agar in mutants whose aggregation was designated as atypical in Table II. **A,E,** sectors from colonies with the growth zone on top, aggregates below this zone, and fruiting bodies on the bottom. **B,C,F,** aggregates. Those of the mutant usually lacked streams, as shown in **B**. Exceptionally streams were formed, as they are in the aggregates in **C**. But these streams remained much smaller than in typical wild-type aggregates, as they are shown in **F**. **D,G,** fruiting bodies. The scale in **E** belongs to **A** and **E**; that in **B** to the other parts of the figure.

probably the outcome of a spontaneous mutation. We conclude that in each of the four mutants the atypical aggregation is due to mutation of the same gene that causes the defect in ep 293 expression. We have not yet performed crosses to determine whether the same gene is mutated in all four strains, at least three of which were derived from independent mutagenic events as shown in Table I.

**Table III.** Aggregation and ep 293 expression in meiotic recombinants

| Mutant number | Aggregation of progeny | | ep 293 expression in progeny | |
|---|---|---|---|---|
| | Normal | Atypical | With normal aggr. | With atypical aggr. |
| PN6002 | 143 | 6 | 7/7 | 0/5 |
| PN6004 | 26 | 0 | n.d. | – |
| PN6005 | 63 | 16 | n.d. | n.d. |
| PN6006 | 58 | 19 | n.d. | n.d. |
| PN6007 | 55 | 13 | 3/3 | 0/5 |
| PN6008 | 52 | 13 | n.d. | n.d. |
| PN6009 | 121 | 4 | n.d. | 0/4 |
| PN6010 | 65 | 5 | n.d. | n.d. |
| PN6011 | 66 | 25 | 4/4 | 0/7 |
| PN6012 | 49 | 1 | n.d. | n.d. |
| PN6013 | 77 | 2 | n.d. | n.d. |
| PN6014 | 65 | 3 | n.d. | n.d. |
| PN6015 | 47 | 0 | n.d. | – |
| PN6019 | 23 | 1 | n.d. | n.d. |

*Delayed expression of ep 293 in the majority of mutants*

Since our mutants were selected at 2 h of starvation, it remained open whether they were absolutely devoid of ep 293, or whether expression of this epitope was only delayed to occur at later stages of development. To clarify this point, all mutants were cultivated in suspension in order to assay them by immunoblotting for expression of ep 293 after 22 – 24 h of starvation. All mutants with atypical aggregation, as defined above, expressed the epitope after that period.

In order to test whether the mutant antigens recognized in the immunoblots were exposed on the cell surface, cells of mutant PN6002 were labeled at 0, 6 and 24 h of starvation with mAb 293 and FITC-conjugated sheep anti-mouse IgG. Fluorescence intensity was compared with that of labeled wild-type PN600 cells in a FACS IV cell sorter (Figure 5). While wild-type cells were already strongly labeled at 0 h, labeling of the mutant cells was negligible at this stage. After 6 h of starvation a fraction of the mutant cells was labeled, and after 24 h substantial labeling of all cells was obtained.

*Continually suppressed ep 293 in strongly aggregation-deficient strains*

As reported earlier in this paper, aggregation was strongly suppressed in two subclones, PN60181 and PN60183, of the unstable mutant PN6018. To test whether this aggregation deficiency is paralleled by suppression of ep 293, both subclones were cultivated in suspension and samples of starved cells were collected during a period of 24 h. As shown for PN60183 in Figure 6,

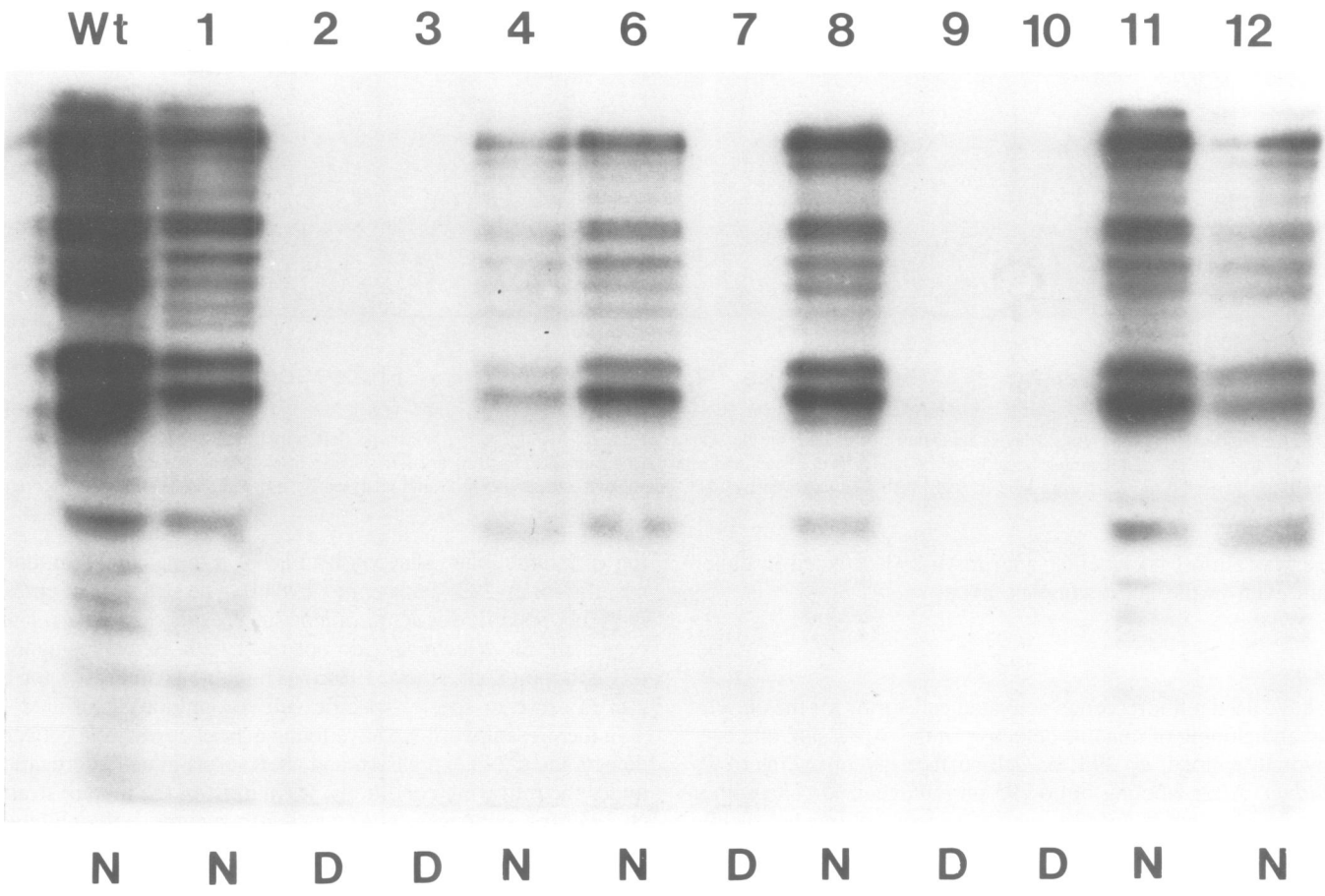


**Fig. 4.** Progeny with normal and defective ep 293 expression from crosses of mutant PN6002 with PN100, a wild-type strain of opposite mating type. Cells were harvested at 2 h of starvation, and total proteins from $1 \times 10^6$ cells of each strain were subjected to SDS-polyacrylamide gel electrophoresis, blotting and labeling with [$^{125}$I]mAb 293 followed by autoradiography. Wt, wild-type PN600. 1 – 12, numbers of progeny strains (no. 5 has not been included for reasons given in the text). N, strains developing normally on LP agar plates. D, strains with atypical aggregation and defective fruiting body formation.

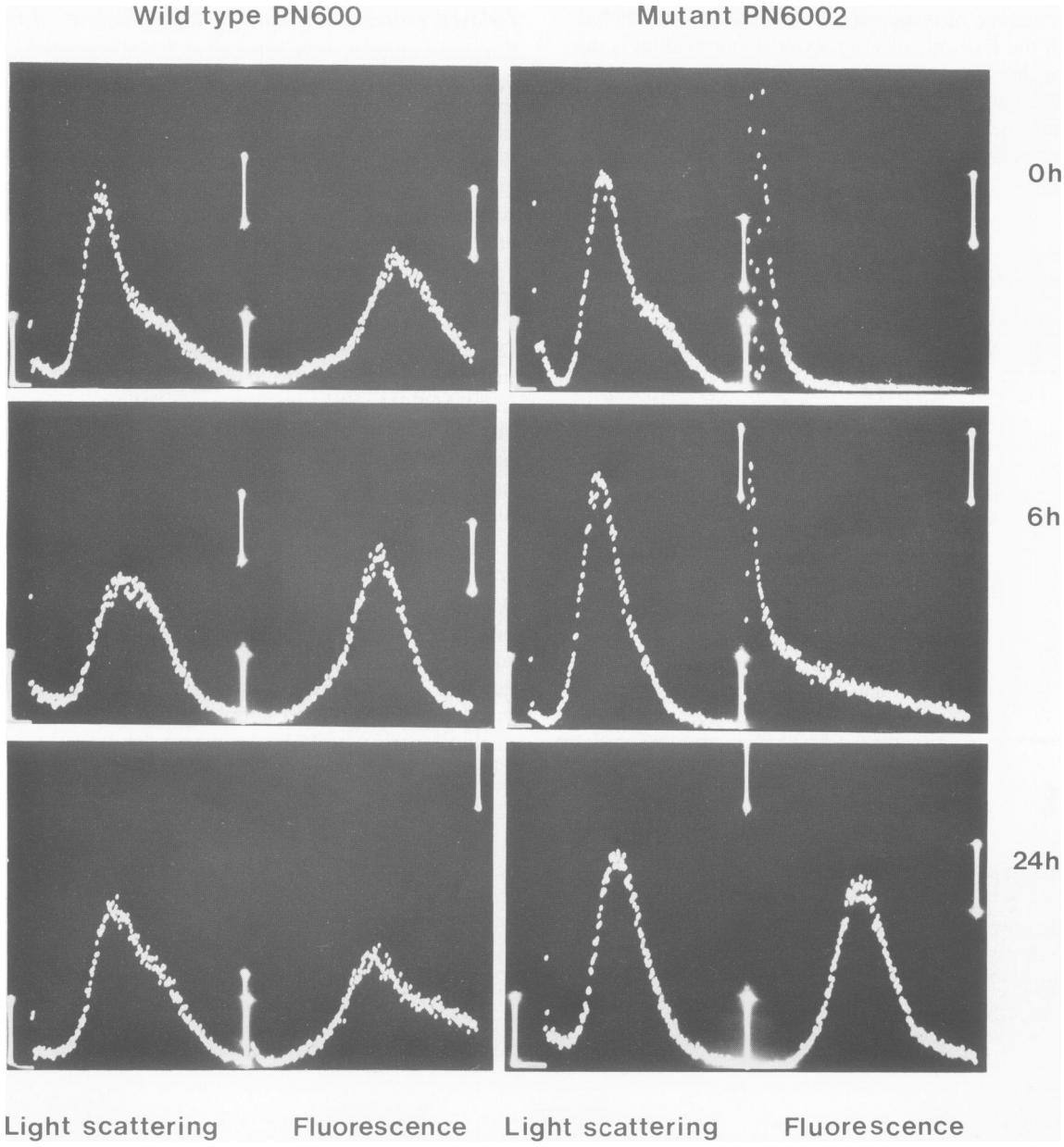


**Fig. 5.** Cell sorter scans indicating delayed appearance of ep 293 on the surface of mutant PN6002 cells. Wild-type **(left panel)** and mutant **(right panel)** cells were washed free of remaining bacteria at the late growth phase, and either used immediately (0 h) or after shaking in phosphate buffer for 6 or 24 h. At the times indicated the cells were labeled with mAb 293 followed by FITC-conjugated anti-mouse IgG, and analysed by use of a FACS IV cell sorter as indicated in Figure 1.

there was almost no labeling with mAb 293 detected in either strain even in the latest samples taken.

## Discussion

We have used a fluorescence-activated cell sorter for the enrichment and cloning of mutants defective in the expression of a carbohydrate epitope, ep 293, on cell surface glycoproteins of *P. pallidum*. After labeling of ep 293 on mutagenized cells with a monoclonal antibody and two runs of selection of unlabeled cells in a FACS IV cell sorter, ~5% of the clones obtained were defective in the expression of the epitope (Table I). Mutant clones were easily identified by blotting of the colonies onto nitrocellulose filters and labeling with iodinated antibody (Figure 2). The interest in these mutants resides in a potential function of ep 293 in cell aggregation, as has been suggested by the blocking of intercellular adhesion by Fab of a monoclonal antibody specific for ep 293 (Toda *et al.*, 1984a). The structure of ep 293 is highly specific for *P. pallidum* since even cells of a related organism, *D. discoideum*, do not react with antibodies against this epitope (Toda *et al.*, 1984a). Thus one prerequisite for ep 293 to serve in species-specific cell recognition is fulfilled.

In the present work we have found a strict correlation between defects in ep 293 expression and aberrations in cell aggregation under the following conditions. (i) In most of the mutant strains ep 293 expression was delayed rather than completely inhibited; aggregation of the mutants was atypical in that no or very short steams of cohering cells were formed around the aggregation centers. (ii) In some subclones derived from an unstable mutant, ep 293 was almost completely suppressed and, accordingly, only very few rudimentary aggregates were formed. (iii) By sexual reproduction recombinant progeny strains were obtained which

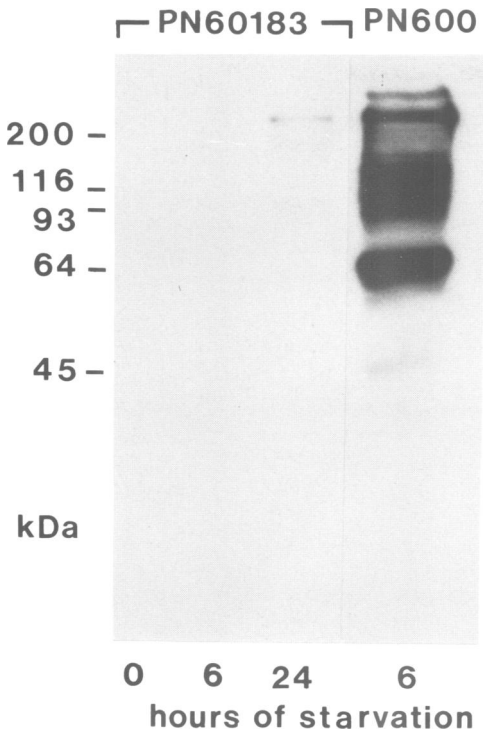


**Fig. 6.** Autoradiograph showing suppression of ep 293 in the strongly aggregation-deficient mutant PN60183. Mutant cells were washed at the late growth phase, and either used immediately (0 h) or after 6 or 24 h of shaking in non-nutrient buffer. For comparison, wild-type PN600 cells were harvested after 6 h of starvation. Total cell proteins were labeled with [$^{125}$I]mAb 293 after SDS-polyacrylamide gel electrophoresis and blotting onto nitrocellulose. 30 μg of protein were applied to each lane.

exhibited either wild-type or mutant aggregation behavior and showed, respectively, normal or defective ep 293 expression.

The correlation between defective expression of ep 293 and atypical cell aggregation might be due to a requirement for this carbohydrate structure in normal aggregation. However, it is possible that the relationship is indirect, i.e., aggregation behavior and ep 293 expression might be controlled by common regulator genes that are involved in the overall programming of development. In a previous study we have reported that wild-type cells contain ep 293 already during the growth phase, which suggested that it was constitutive (Toda *et al.*, 1984a). If this were the case, regulatory genes would probably not be implicated in ep 293 expression. The mutations would more likely affect an enzyme directly involved in the synthesis or processing of the carbohydrates carrying ep 293. However, the finding that ep 293 is expressed in the mutants only after many hours of starvation suggests that a regulatory mechanism exists, and that the delay of expression in the mutants is caused by inappropriate timing of the regulation.

Another point that remains to be clarified is whether expression of the whole set of glycoproteins that carry ep 293 is delayed in the mutants, or whether these proteins pre-exist in a form that is devoid of ep 293. Since L-fucose, a sugar which is typical for complex-type carbohydrate chains, occupies a terminal position of ep 293 (Todal *et al.*, 1984b), processing similar to conversion of carbohydrates from the high-mannose to the complex type might produce the structure recognized by mAb 293.

## Materials and methods

*Preparation of the monoclonal antibody and labeling of glycoproteins*

Monoclonal antibody 25-293-2, abbreviated mAb 293, was prepared as described previously (Toda *et al.*, 1984a, 1984b), and iodinated using the chloramine-T method. SDS-polyacrylamide electrophoresis in 10% gels was carried out according to Laemmli (1970), and transfer to BA 85 nitrocellulose filters (Schleicher and Schuell, Dassel, FRG) and immunolabeling according to Towbin *et al.* (1979) as specified by Stadler *et al.* (1984). For cell-surface labeling, $1 \times 10^7$ washed cells were suspended in 100 μl solution containing 100 μg mAb 293 in phosphate-buffered saline (PBS) pH 7.0, and incubated for 15 min on ice. The cells were washed three times with PBS at 4°C, incubated for 15 min on ice with a 1:50 dilution of FITC-conjugated sheep anti-mouse IgG (Institute Pasteur Production), washed again and analysed using a FACS IV Cell Sorter (Becton-Dickinson).

*Suspension cultures*

Wild-type strain PN600 of *P. pallidum* and mutants derived from it were grown under continuous illumination at 23 ± 1°C in suspensions of $1 \times 10^{10}$ *E. coli* B/r cells per ml in 17 mM Soerensen phosphate buffer pH 6.0 in Erlenmeyer flasks on a rotary shaker at 150 r.p.m. (Bozzaro *et al.*, 1978). For starvation at the late growth phase, cells were grown up to densities of $7-8 \times 10^6$/ml, at which densities $1-2 \times 10^9$ bacteria/ml were left, washed and resuspended in the phosphate buffer at $1 \times 10^7$ cells/ml, and shaken as described. The suspension cultures were used for mutagenesis, for the determination of generation times by counting the cells in a hemocytometer, for immunofluorescence labeling and for examining ep 293 expression by SDS-polyacrylamide gel electrophoresis and immunoblotting.

*Agar plate cultures*

For evaluating the morphology of aggregates and fruiting bodies, wild-type and mutants were cultivated under continuous illumination at 23 ± 1°C on streaks of *E. coli* B/r on LP agar containing 0.1% lactose, 0.1% peptone, 2% agar and in some experiments, as that shown in Figure 3, 17 mM Soerensen phosphate buffer pH 6.0.

*Selection of mutants*

For mutagenesis by u.v. irradiation or treatment with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), PN600 cells were grown in suspension cultures and washed. Cells were treated with 3 mg/ml MNNG in 17 mM phosphate buffer pH 7.0 at 23°C for 15–20 min in darkness. This resulted in a relatively high survival of ~20%, at which double mutations are expected to be infrequent. After mutagenesis the cells were washed twice in phosphate buffer containing 0.1 M KCl. This relatively high molarity shrank the cells which appeared enlarged and rounded after MNNG treatment, and greatly increased viability. The washed cells were grown in suspension cultures for 2 days before selection was started by which time they had passed through 2–4 divisions.

For selection of mutants with defective ep 293 expression, mutagenized cells were labeled with mAb 293 and FITC-conjugated sheep anti-mouse IgG antibodies as described above. Unlabeled cells were sorted out and cloned using a FACS-IV cell sorter equipped with a single-cell deposition system. 12 × 8 cm microtiter plates without wells were filled with LP agar, the agar was covered by a lawn of *E. coli* B/r, and 96 droplets containing sorted cells were deposited per plate. After growth of the cloned cells the colonies formed were blotted onto a nitrocellulose filter, labeled with [$^{125}$I]mAb 293 and autoradiographed. Sufficient cells remained on the agar surface after blotting to start new cultures from clones which proved to be defective in antibody binding.

*Genetic recombination*

Crosses were performed using the macrocyst sexual cycle as described by Francis (1980). The mutants made in strain PN600 were crossed with wild-type strain PN100 (Francis and Rupar, 1983), and haploid progeny were obtained by picking sori from individual germinating macrocysts.

## Acknowledgements

D.Francis received a grant as a guest professor of the Max-Planck-Gesellschaft during the first part of this work. K.Toda was a post-doctoral fellow of the Max-Planck-Gesellschaft.

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*Received on 1 July 1985*