GENETIC ANALYSIS OF THE GENE FOR N-ACETYLGLUCOSAMINIDASE IN *DICTYOSTELIUM DISCOIDEUM*

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ABSTRACT

Three independent mutations affecting N-acetylglucosaminidase in *Dictyostelium discoideum* were mapped by the parasexual system and found to lie on linkage group IV. These mutations as well as two others were found to be recessive and noncomplementing in heterozygous diploids. Thus they all appear to affect the *nagA* locus. Since two of the mutations give rise to thermolabile enzyme, this defines the structural gene for N-acetylglucosaminidase.

The enzyme is a homodimer of a 68,000 dalton subunit and thus would be expected to be determined by a single locus. The expression of this gene is regulated by the stages of development; however, it should be mentioned that none of the mutations fell in a separate locus that might determine a specific positive regulatory protein.

THE parasexual recombinational system of the cellular slime mold *Dictyostelium discoideum* has permitted over forty loci to be mapped onto seven linkage groups (NEWELL, RATNER and WRIGHT 1977; FREE, SCHIMKE and LOOMIS 1976). The wild-type strains of this organism are haploid and carry seven chromosomes, which can be distinguished by their size and banding pattern when stained with Giemsa (ZADA-HAMES 1977; ROBSON and WILLIAMS 1977). At a frequency of about 1 in 10^5, pairs of haploid cells fuse and form stable diploids; these, on rare occasions, will segregate a chromosome at random to generate aneuploid progeny. This process continues until the haploid complement is reached. Since the loss of chromosomes is random, linked markers will co-segregate, allowing them to be assigned to specific linkage groups. Mitotic crossing over has allowed several markers to be ordered on the chromosome (GINGOLD and ASHWORTH 1974; WILLIAMS, KESSIN and NEWELL 1974).

Some years ago, a series of mutant strains was isolated in which N-acetylglucosaminidase was either inactive or thermolabile (DIMOND, BRENNER and LOOMIS 1973). It was of interest to determine whether independently isolated mutations mapped in the same group and whether any cases of complementation could be found. If mutations inactivated a regulatory gene that was required for expression of the structural gene, then we would expect them to be complemented by structural gene mutations in heterozygous diploids.

MATERIALS AND METHODS

Strains: All strains were derived from the haploid NC-4. The isolation of the N-acetylglucosaminidase mutants (DBL series) from the axenic strain A3 has been previously reported (DIMOND, BRENNER and LOOMIS 1973). The genotypes of the other strains are given in Table 1. The cells were grown in association with *Klebsiella aerogenes* at 18° on SM medium (SUSSMAN 1966).

Isolation of growth temperature sensitive derivatives: Strains DBL 81, 211, 230, 239, and 251 were mutagenized, plated clonally and tested for ability to grow at 18° but not at 27° as previously described (LOOMIS 1969b). From several hundred clones replica plated, several isolates were recovered from each strain that were growth temperature-sensitive. One with a low ($< 10^{-6}$) reversion frequency was selected from each N-acetylglucosaminidase mutant for genetic analysis.

Diploid formation: Pairs of haploid strains each carrying a different recessive mutation leading to temperature-sensitive growth (tsg) were mixed in equal numbers and allowed to develop together (LOOMIS 1969b). Spores were spread on a lawn of *K. aerogenes* at 10^6 per plate, incubated 6 hr at 22° for phenotypic expression, and then shifted to 27°. After 5 to 7 days, plaques appeared at a frequency of about 10^{-5}. Several clones were picked and streaked on plates which were incubated at 27° for an additional 3 days. Each diploid strain studied was shown to be able to segregate white, methanol resistant progeny at a frequency of 10^{-3} to 10^{-4}.

TABLE 1

Genotype of strains

Strain	Linkage group I	II	IV	VI
XM101	*cycA*	*whi acrA tsgD*	*bwn*	$manA_2$
HL500	*cycA*	*whi acrA tsgD*	$nagA_{81}$	$manA_2$
HL501	*cycA*	*whi acrA tsgD*	$nagA_{211}$	$manA_2$
DL500	$\frac{+}{cycA}$	$\frac{+\ +\ +}{whi\ acrA\ tsgD}$	$\frac{+\ nagA_{81}}{bwn\ +}$	$\frac{+}{manA_2}$
DL501	$\frac{+}{cycA}$	$\frac{+\ +\ +}{whi\ acrA\ tsgD}$	$\frac{+\ nagA_{211}}{bwn\ +}$	$\frac{+}{manA_2}$
DL502	$\frac{+}{cycA}$	$\frac{+\ +\ +}{whi\ acrA\ tsgD}$	$\frac{+\ nagA_{239}}{bwn\ +}$	$\frac{+}{manA_2}$
DL503	$\frac{+}{cycA}$	$\frac{+\ +\ +}{whi\ acrA\ tsgD}$	$\frac{+\ nagA_{251}}{bwn\ +}$	$\frac{+}{manA_2}$
DL504	$\frac{+}{cycA}$	$\frac{+\ +\ +}{whi\ acrA\ tsgD}$	$\frac{nagA_{211}}{nagA_{81}}$	$\frac{+}{manA_2}$
DL506	$\frac{+}{cycA}$	$\frac{+\ +\ +}{whi\ acrA\ tsgD}$	$\frac{nagA_{230}}{nagA_{211}}$	$\frac{+}{manA_2}$
DL507	$\frac{+}{cycA}$	$\frac{+\ +\ +}{whi\ acrA\ tsgD}$	$\frac{nagA_{239}}{nagA_{211}}$	$\frac{+}{manA_2}$
DL508	$\frac{+}{cycA}$	$\frac{+\ +\ +}{whi\ acrA\ tsgD}$	$\frac{nagA_{251}}{nagA_{211}}$	$\frac{+}{manA_2}$

Selection of methanol resistant segregants: Clones of diploid cells were grown at 22° and allowed to form fruiting bodies so as to allow segregation of temperature-sensitive haploids. About 10^5 spores were then plated on SM agar plates, which were made 2% in methanol by spreading 0.8 ml of methanol on each plate that contained 40 ml SM agar. After incubation at 22° for 5 days, methanol resistant (*acrA*) plaques appeared that were picked to straight SM agar. About 50–80% of these clones were white and temperature-sensitive for growth, indicating that they were haploid for the linkage group II markers *whi*, *acrA* and *tsgD*.

Scoring for markers: The spore-color markers *whi* (white) and *bwn* (brown) were visually scored a week after each clone had formed fruits on SM agar at 22°. Temperature sensitivity for growth (tsg) was tested by ability to form plaques on bacterial lawns following 4 days incubation at 27°. Cycloheximide resistance (*cycA*) was scored on SM plates containing 450 μg/ml cycloheximide following inoculation with a toothpick previously inserted into a plaque of a haploid strain.

Scoring enzyme markers: Haploid clones were grown on SM agar and allowed to develop to the slug stage. About 5×10^6 cells were collected on a sterile loop and suspended in 1 ml of 0.1 M acetate buffer pH 5 containing 0.1% Nonidet P-40 detergent to lyse the cells.

α-Mannosidase-1 was scored by adding 0.25 ml extract to 0.25 ml of p-nitrophenyl-α-mannoside (10^{-2} M) containing 5mM cysteine to inhibit α-mannosidase-2. Following incubation at 35° for 60 min, 0.5 ml of 1 M Na_2CO_3 was added. Extracts from strains carrying the *manA*+ allele gave rise to yellow product, while those from *manA* strains remained colorless.

N-acetylglucosaminidase was scored by adding 0.25 ml of extract to 0.25 ml of p-nitrophenyl-β-D-N-acetylglucosaminide (2×10^{-2} M). After incubation at 22° for 60 min, 0.5 ml of 1 M Na_2CO_3 was added. Extracts of *nagA*+ strains gave rise to yellow product, while those from *nagA* strains were colorless. Segregants of DL 500 made either wild-type N-acetylglucosaminidase or temperature-sensitive enzyme. The latter was scored before and after heating the extract to 50° for 3 hr. This treatment had little effect on wild-type N-acetylglucosaminidase, but inactivated more than 95% of the activity in strains carrying $nagA_{81}$.

Enzyme assay: N-acetylglucosaminidase was assayed as previously described (Loomis 1969a). One unit is defined as that amount which produces 1 nmole of p-nitrophenol per minute. Protein was assayed by the method of Lowry *et al.* (1951).

RESULTS

Mapping nagA: To assign the structural gene coding for N-acetylglucosaminidase to a linkage group, the segregation pattern was followed for a diploid formed between a strain wild-type for N-acetylglucosaminidase (XM101) and strain DBL81. This latter strain forms thermolabile N-acetylglucosaminidase (Dimond, Brenner and Loomis 1973). Strain XM101 is wild-type for N-acetylglucosaminidase and carries a marker for cycloheximide resistance on chromosome *I*; a growth temperature-sensitive mutation (*tsgD*), methanol resistance (*acrA*) and white (*whi*) on chromosome *II*; a marker that gives brown pigment (*bwn*) on chromosome *IV*; and $manA_2$ allele on chromosome *VI* (Free, Schimke and Loomis 1976). Since diploids arise only infrequently, it was first necessary to isolate a growth temperature-sensitive derivative of strain DBL81. By replica-plating a mutagenized culture of this strain to plates incubated either at 18° or 27°, growth temperature-sensitive strains were recovered at a frequency of 1%. One of these, strain DBL81–7, was crossed with strain XM101. Diploids were recovered as plaques growing at the nonpermissive temperature (27°) at the frequency of 10^{-5}. One of these diploids, strain DL500, was chosen for further study.

TABLE 2

Cycloheximide resistant segregants of strain DL500

Phenotype	*nag*+ *bwn*	nag_{s1} *bwn*+	Total
cyc A+	7	7	14
cyc A	12	13	25
Total	19	20	39

Cells of strain DL500 were grown for 20 generations at the permissive temperature of 22° to allow segregation of haploids. Plates containing 2% methanol were then spread with 10^5 spores of this population. Methanol-resistant segregants carrying the second chromosome of strain XM101 were recovered at a frequency of 5×10^{-4}. Thirty-nine white, methanol-resistant, temperature-sensitive segregants were analyzed for the other markers.

There were 19 segregants which formed wild-type N-acetylglucosaminidase and 20 which formed thermolabile enzyme (Table 2). Half of the cycloheximide sensitive segregants were wild-type for N-acetylglucosaminidase and half had thermolabile enzyme (Table 2). Likewise, half of the cycloheximide resistant segregants had thermolabile N-acetylglucosaminidase and half did not. Clearly, the gene affecting N-acetylglucosaminidase (*nagA*) segregated independently of linkage groups I and II.

Of the 18 segregants showing wild-type α-mannosidase, 11 were also wild-type for N-acetylglucosaminidase, and of 21 that were α-mannosidase negative, 8 were wild-type for N-acetylglucosaminidase (Table 3). Thus, the *manA* gene on chromosome *VI* segregated independently of *nagA*. However, all 19 of the segregants showing wild-type N-acetylglucosaminidase were brown and all 20 showing thermolabile enzyme were nonbrown (Tables 2 and 3). On this basis, the $nagA_{s1}$ gene is on linkage group IV along with *bwn*.

The linkage assignment was also determined for two other independent mutations inactivating N-acetylglucosaminidase. Growth temperature-sensitive mutants of strains DBL211 and DBL239 were mated with strain XM101, and diploids DL501 and DL502 were recovered from the respective matings. Methanol-resistant (*acrA*) segregants were selected on plates containing 2% methanol. From 94 such segregants of strain DL501, 63 were negative for N-acetylglusoaminidase and none of these were brown, while the others were

TABLE 3

α-Mannosidase segregants of strain DL500

Phenotype	*nag*+ *bwn*	nag_{s1} *bwn*+	Total
man A+	11	7	18
man A	8	13	21
Total	19	20	39

TABLE 4

Methanol resistant segregants of strains DL501 and DL502

Phenotype	*nag*+ *bwn*	nag_{81} *bwn*+
Segregants of strain DL501	31	63
Segregants of strain DL502	8	8

wild-type for N-acetylglucosaminidase and were brown (Table 4). Forty methanol-resistant segregants of strain DL501 were recovered, of which 11 were brown and the rest nonbrown. Eight of each class were tested for N-acetylglucosaminidase. All of the brown segregants were found to be wild-type for the enzyme, and all the nonbrown segregants were found to be lacking the enzyme. It appears that both of these mutations, $nagA_{211}$ and $nagA_{239}$, map to chromosome *IV*.

Dominance of nagA+: The accumulation of N-acetylglucosaminidase was tested in the diploids heterozygous for *nagA* to determine whether any of the mutations were dominant. To study this in strain DBL251, which forms thermolabile enzyme (DIMOND, BRENNER and LOOMIS 1973), a growth temperature-sensitive derivative was isolated, crossed with strain XM101, and a diploid of these strains, DL502, was isolated. In this strain as well as in diploids heterozygous for $nagA_{81}$, $nagA_{211}$, and $nagA_{239}$, the wild-type allele was expressed (Table 5).

TABLE 5

Haploid strains		NAG A locus	N-acetylglucosaminidase (units/mg protein)
XM101		*nagA*+	193
DBL81–7		$nagA_{81}$	12.5
DLB211–1		$nagA_{211}$	2.5
DBL230–5		$nagA_{230}$	3.6
DBL239–1		$nagA_{239}$	2.5
DBL251–1		$nagA_{251}$	122
HL500		nag_{81}	11.7
HL501		nag_{211}	4.0
Diploid strains	**Parental strains**	**NAG A locus**	**N-acetylglucosaminidase (units/mg protein)**
DL500	DBL81–7;XM101	$nagA_{81}$ /*nagA*+	173
DL501	DBL211–1;XM101	$nagA_{211}$/*nagA*+	96.2
DL502	DBL239–1;XM101	$nagA_{239}$/*nagA*+	153
DL503	DBL251–1;XM101	$nagA_{251}$/*nagA*+	220
DL504	DBL211–1;HL500	$nagA_{211}$/$nagA_{81}$	7.8
DL505	DBL230–5;HL500	$nagA_{230}$/$nagA_{81}$	8.8
DL506	DBL230–5;HL501	$nagA_{230}$/$nagA_{211}$	2.1
DL507	DBL239–1;HL501	$nagA_{239}$/$nagA_{211}$	1.58
DL508	DBL251–1;HL501	$nagA_{251}$/$nagA_{211}$	68

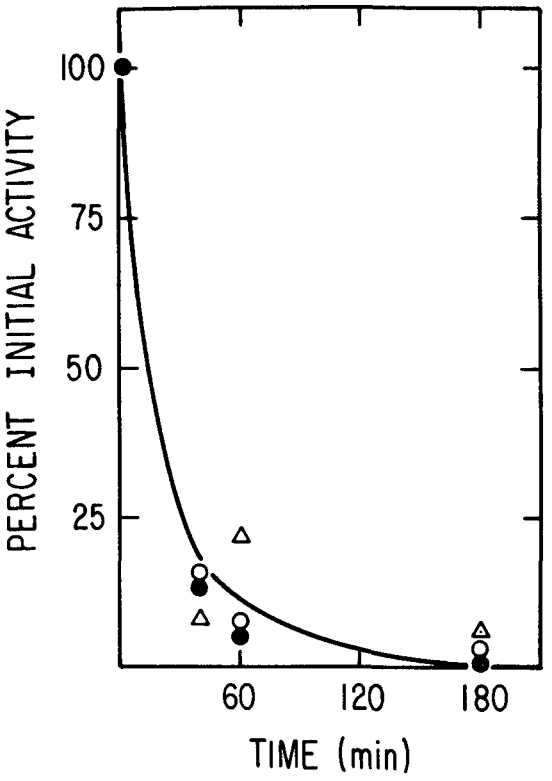

FIGURE 1.—Thermolability of N-acetylglucosaminidase. Cells of strains HL500 (●); DL504 (○); and DL505 (△) were lysed by sonication in 0.2 M acetate buffer pH5. Enzyme activity was assayed after 0, 40, 60 and 180 min incubation at 55°. Wild-type enzyme is stable under these conditions.

The recessive character of the mutation in strain DBL211–1 was further determined in a diploid strain isolated from a cross of this strain and a growth temperature-sensitive segregant of strain DL500. This segregant (HL500) carries the *cycA*, *whi*, *acrA*, *tsgD* and *manA* markers of parental strain XM101 and the $nagA_{81}$ marker of strain DBL81, which renders N-acetylglucosaminidase thermolabile. The diploid (DL504) isolated from a cross of strains HL500 and DBL211–1 expressed N-acetylglucosaminidase at the level of the $nagA_{81}$ parent strain, and this activity was thermolabile (Figure 1). Likewise a diploid formed between a growth temperature-sensitive derivative of strain DBL230 and strain HL500 was isolated to determine whether the mutation in strain DBL230, which results in lack of accumulation of N- acetylglucosaminidase, is recessive. The diploid, DL505, accumulated thermolabile N-acetylglucosaminidase, indicating that the mutation in strain DBL230 is recessive (Figure 1).

Alleles of nagA: Complementation among the N-acetylglucosaminidase mutations was tested in diploids formed from pairs of mutant strains. A growth temperature-sensitive segregant (HL501) of strain DL501, which carried the

cycA, *whi*, *acrA*, *tsgD*, and *manA* markers of parental strain XM101 and the $nagA_{211}$ marker of parental strain DBL211–1, was crossed with the temperature-sensitive derivatives of strains DBL81, DBL230, DBL239 and DBL251. The accumulation of N-acetylglucosaminidase was determined in diploids recovered from these crosses (Table 5). It is apparent that all of the mutations are non-complementing alleles in the *nagA* locus. Diploid strain DL508 carries the $nagA_{251}$ mutation, which does not inactivate N-acetylglucosaminidase but renders it thermolabile (DIMOND, BRENNER and LOOMIS 1973). The enzyme present in strain DL508 is as thermolabile as that in parental strain DBL251 ($t_{1/2}$ at 55° of 30 min).

DISCUSSION

The structural gene coding for N-acetylglucosaminidase maps to chromosome *IV* since a mutation, $nagA_{81}$, giving rise to thermolabile enzyme co-segregates with the wild-type allele of brown on this linkage group. Moreover, another mutation, $nagA_{251}$, which confers partial thermolability and reduces the substrate affinity of the enzyme, fails to complement $nagA_{211}$, which was shown to map to linkage group IV as well. It is therefore highly unlikely that these alterations in enzyme properties result from posttranslational modifications.

Two other mutations, $nagA_{230}$ and $nagA_{239}$, also fail to complement $nagA_{211}$ and show that three independent isolates lacking all measureable activity, as well as two temperature-sensitive isolates, each carry mutations in a single locus, *nagA*. Since the enzyme is a homodimer consisting of a pair of identical subunits of 68,000 daltons (EVERY and ASHWORTH 1973; DIMOND, BRENNER and LOOMIS 1973), only a single structural locus is expected. The results give no evidence for a positive regulatory protein that might be required for transcription or translation of the *nag* gene. If such a protein were about the same size as the enzyme and not required for expression of vital genes, it would be very unlikely for it not to have sustained a mutation in our collection of N-acetylglucosaminidase mutants. Since both the accumulation of N-acetylglucosaminidase during aggregation and the cessation of synthesis during the slug stage are controlled, these processes appear to be regulated, but the mechanisms are presently unknown.

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