REGULATION OF THE *AM-1* LOCUS IN NEUROSPORA: EVIDENCE OF INDEPENDENT CONTROL OF ALLELIC RECOMBINATION AND GENE EXPRESSION

D. E. A. CATCHESIDE

Research School of Biological Sciences, The Australian National University, Canberra, A. C. T., Australia

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GENES affecting the frequency of recombination between allelic differences have been found in *Neurospora crassa* (JESSOP and D. G. CATCHESIDE 1965; D. G. CATCHESIDE 1966; JHA 1967). In the two most extensively studied cases, crosses homozygous for *rec* (a general symbol for genes affecting recombination) exhibit allelic recombination frequencies an order of magnitude or more greater than those containing the rec^+ allele. The *rec* genes are specific, *rec-1* affecting recombination frequency between *his-1* (*histidine-1*) alleles and *rec-3* that between *am-1* (*amination-1*) alleles. There is no interaction between *rec-1* and *am-1* nor between *rec-3* and *his-1* (D. G. CATCHESIDE 1966). Neither *rec* gene is contiguous with the structural gene which it affects. Indeed, *rec-3* is located about 12 map units proximal to mating type in linkage group I whilst *am-1* is in linkage group V and *rec-1* is about 19 units distal to *his-1* also in linkage group V (D. G. CATCHESIDE 1966 and unpublished observations).

The dominant nature, specificity and remoteness of the rec^+ genes from their points of action has led to the proposition that they are regulatory genes (D. G. CATCHESIDE 1966; WHITEHOUSE 1966) analogous to those described in other organisms (MCCLINTOCK 1957; JACOB and MONOD 1961). Regulation by rec^+ is most simply envisaged as acting directly at the gene level by the agency of a gene product which prevents either the formation of hybrid DNA or the correction of any consequently mispaired bases. The polarized effect of *rec* on either or both of these processes (D. G. CATCHESIDE 1967) is most simply described in terms of a direct control at the gene level (D. E. A. CATCHESIDE 1967). This paper considers the possibility that the effects of *rec* genes on recombination frequency may reflect modifications of an integrated, dual purpose, control system acting at the gene level; D. G. CATCHESIDE (1966) and WHITEHOUSE (1966) have suggested that control of recombination and transcription may be integrated. The *rec-3*, *am-1* pair has been chosen for investigation as it has a number of convenient properties.

Mutants at the *am-1* locus are known to produce nicotine adenine dinucleotide phosphate-specific glutamate dehydrogenase (NADP-GDH; Enzyme Commission number 1.4.1.4) varieties which differ in properties from the wild-type enzyme (FINCHAM 1962; FINCHAM and STADLER 1965). Hence, the *am-1* locus

is concerned in the specification of NADP-GDH. The specific activity of the enzyme, in wild-type Neurospora, is known to be reduced by growth or incubation of the organism in the presence of exogenous urea, glutamate + ammonium nitrate or a number of other nitrogenous compounds (SANWAL and LATA 1962a; BARRATT 1963; TUVESON, WEST and BARRATT 1967). The effector is thought to be the ammonium ion (BARRATT 1963; STACHOW and SANWAL 1967). Some confidence that the decrease in specific activity reflects repression of enzyme production rather than enzyme inhibition has been provided by experiments with mixed extracts (SANWAL and LATA 1962c; D. E. A. CATCHESIDE unpublished) and by the presence of a low level of cross-reacting material (protein immunologically related to NADP-GDH) in wild type and in certain *am-1* mutants grown in urea supplemented medium (SANWAL and LATA 1962b).

A second glutamate dehydrogenase, nicotine adenine dinucleotide-specific (NAD-GDH; Enzyme Commission number 1.4.1.2), found in *Neurospora crassa* (SANWAL and LATA 1961) is induced under conditions which repress NADP-GDH. However, there is no evidence that regulation of the glutamate dehydrogenases is due to their interconversion (SANWAL and LATA 1962b) and recently a structural gene for NAD-GDH has been reported to be unlinked to *am-1* (AHMED and SANWAL 1967).

If the *rec-3*$^+$ gene product is a repressor of transcription of the *am-1* gene, as well as of recombination between *am-1* alleles, it might be expected that NADP-GDH in *rec-3* strains would not be repressed under conditions which repress synthesis in *rec-3*$^+$ strains.

MATERIALS AND METHODS

Neurospora strains and growth conditions: Four strains of Neurospora were used (Table 1). Each was grown for 4 days on slopes of VOGEL's N minimal medium (VOGEL 1964) solidified with 1.2% agar and including 2% sucrose as a carbon source. Conidial suspensions were made in distilled water and approximately 5×10^5 conidia were inoculated into each culture flask. The 250 ml conical flasks, closed with Oxoid caps, each containing 50 ml of medium, were incubated for 48 hours on a reciprocating shaker at 25°C in the dark. Triplicates of each strain were grown on VOGEL's N minimal medium supplemented with 2% sucrose (VM medium), on VM supplemented with 0.25 M ammonium nitrate + 0.05 M monosodium glutamate (NG medium) and on VM (without ammonium nitrate) + 0.1 M urea (U medium). Mycelial pads were harvested by vacuum filtration, washed with distilled water and freeze dried.

Preparation of extracts: The dry pads were ground in a pestle and mortar with glass powder and extracted with 10 ml of 0.05 M, pH 6.8, sodium phosphate buffer containing 5 mM disodium EDTA and 5 mM 2-mercaptoethanol. Debris was removed by centrifugation and a sample of each supernatant was dialysed against three changes, each of 80 volumes, of extraction buffer. All extracts from a single experiment were dialysed simultaneously in the same vessel.

Enzyme assays: The NADP-specific GDH was assayed both by the oxidation of NADPH and by the reduction of NADP. The former is probably the biologically important reaction as those *am-1* mutants which produce an NADP-GDH able to reduce NADP but not oxidise NADPH nevertheless require a source of α-amino nitrogen for rapid growth (FINCHAM 1962). NAD-GDH was assayed by the reduction of NAD. The assays were linear over the activity range observed (Figure 1). Protein was determined by the method of LOWRY *et al.* (1951), the mean of three determinations being used.

The specific activity of GDH was determined 48 hours after inoculation, prior to the cessation of growth, as continuation of incubation beyond this period led to net loss of NADP-GDH activity.

TABLE 1

Known genetic constitution of Neurospora strains

Strain	Mating type	Known genetic constitution			
1534	A	*rec-3*+;	*cot*+;	*am-1*+,	*rec-1*
1535	a	*rec-3*;	*cot*+;	*am-1*+,	*rec-1*+
3819	a	*rec-3*;	*cot*;	*am-1*+,	*rec-1*
5911	A	*rec-3*+;	*cot*;	*am-1*+,	*rec-1*+

rec-3 is in linkage group I; *cot* C102t in IV; *am-1* and *rec-1* in V.
Strains 1534 and 1535 are the product of several generations intercrossing E5256A and E5297a.
Strains 3819 and 5911 are derived by complex pedigrees involving extensive back crossing to 1534 and 1535. Components of other genetic backgrounds have been introduced, necessarily with *cot* C102t, and incidentally by the use of an *inos* 37401 stock.

Mathematical treatment of results: Estimates of specific activity were converted into $\log_{10}$ form (Tables 2 and 4) to normalise the effect of errors which are likely to be proportional to the magnitude of specific activity. The effects attributable to known genetic differences (Table 3 and 5) were calculated by summing the entire data from the relevant column in Table 2 or 4. The significance of the calculated effect was estimated by comparing the variance $\frac{(\text{effect})^2}{\text{number of items}}$ with the error variance; this comparison is expressed in Tables 3 and 5 as the ratio F. In these analyses the seven effects account for seven of the 23 degrees of freedom. Thus F has 1 and 16 degrees of freedom and has the value 4.49 at the 0.05 level of significance and the value 8.53 at the 0.01 level.

RESULTS

Although there is less growth after 48 hours in NG (ammonium nitrate- glutamate) medium than in VM (minimal), there is no significant effect of genetic differences on the growth achieved (Table 3). Estimations of the specific activity of NADP-GDH by oxidative and reductive assays agree quantitatively (Table 2). The degree of repression achieved with U (urea) medium (Tables 4 and 5) was not as great as that with NG medium (Tables 2 and 3).

If repression of NADP-GDH were prevented in *rec-3* strains it would be expressed in these analyses as a positive interaction between *rec-3* and treatment similar, in magnitude, to the effect of treatment alone. The relative magnitude of these effects (Table 6) is such that in *rec-3* strains repression by treatment is of the order of 93–97% of that in *rec-3*+ strains. The effect of *rec-3* on repression of NADP-GDH production is, therefore, at most small; the observed effect may be due to unknown genetic differences between the strains examined. Such differences are likely in heterothallic organisms even amongst inbred lines such as those used in these experiments. The effect of the *rec-3*, treatment interaction on NAD-GDH specific activity is insignificant.

The experiments are confounded by known genetic differences. Effects of *rec-3*, *rec-1* and *cot* are individually confounded by the first order interaction between the remaining pairs and *rec-3* is confounded by mating type. However, there is no biologically sound reason to attribute to *rec-1* or mating type a role which could interfere with GDH specific activity. However, the primary bio-

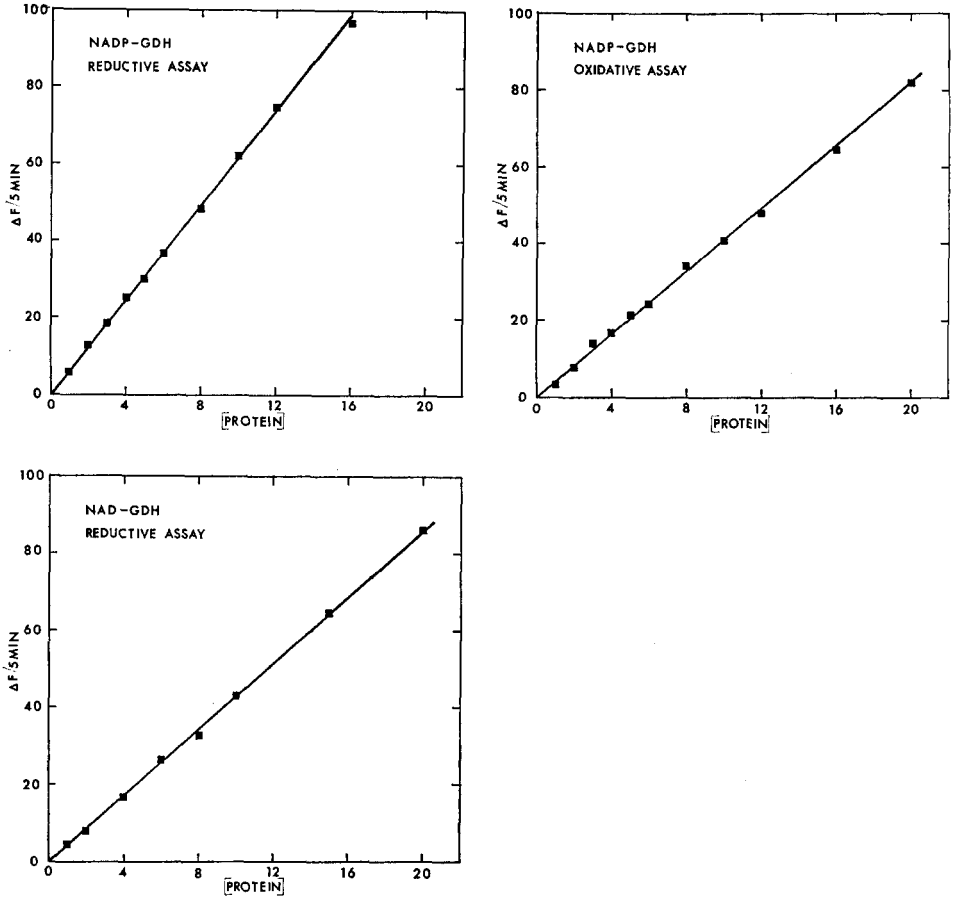

FIGURE 1.—Collinearity of protein concentration and enzyme activity.

The reaction mixtures contained, in a total volume of 1.5 ml, 100 μmoles Tris-HCl buffer, 10 μmoles disodium EDTA and 10 μmoles 2-mercaptoethanol; the pH was adjusted to 8.4 with HCl. Reductive assays contained 0.24 μmoles NADP or 0.27 μmoles NDA. Following 3 mins preincubation at 37°C, 50 μmoles monosodium glutamate were added to initiate the reaction. The increase in fluorescence (excitation 352 mμ, fluorescence 458 mμ) was recorded for 1½–2 mins. The oxidative assay contained 0.10 μmoles NADPH and 20 μmoles α-keto (glutarate (pH adjusted to 8.4 with NaOH). Following 3 mins preincubation at 37°C, 30 μmoles ammonium chloride were added and the decrease in fluorescence was recorded for 1½–2 mins. Initial velocities were calculated as Δf/5 mins; in the reductive assays one unit is equivalent to the reduction of 12 mμmoles/ml of coenzyme and in the oxidative assay to the oxidation of 63 mμmoles/ml. (The absolute specific activities of GDH in strain 5911 grown on minimal medium for 48 hours are, for the reduction of NADP: 0.34 IU/mg protein, for the oxidation of NADPH: 0.77 IU/mg, and for the reduction of NAD: 2.2 mIU/mg.)

chemical lesion due to *cot* C102t, a temperature sensitive colonial mutant expressed at 34°C, could have a residual effect at 25° altering effector metabolism or transport, and hence GDH specific activity. The most serious problem is the effect of unknown genetic heterogeneity, within the four strains used, capable of affecting GDH specific activity. The most obvious case of this is the generally higher level of both NADP-GDH and NAD-GDH activities in strain 1534. The

TABLE 2

Dry weight and glutamate dehydrogenase specific activities of mycelia grown in VM and NG medium

Medium	Strain	Dry weight of mycelium (mg)‡	$\log_{10}$ specific activity (arbitrary units)		
			NADP-GDH		NAD-GDH
			Reductive assay	Oxidative assay	Reductive assay
VM*		258.0	4.1110	4.0235	2.2252
	1534	289.6	4.0254	3.9442	2.2544
		265.2	4.0693	4.0206	2.2207
		273.7	3.9179	3.8198	2.0634
	1535	265.8	3.9296	3.8402	1.9591
		285.9	3.9388	3.8733	1.8845
		273.5	3.9179	3.8693	2.0742
	3819	274.6	3.9628	3.8990	2.0784
		272.2	3.9058	3.8494	2.0291
		275.9	3.9185	3.8816	2.0651
	5911	261.3	3.9231	3.8986	2.0302
		295.7	3.9274	3.8775	2.0829
NG†		217.0	2.7653	2.7087	3.2355
	1534	226.0	2.7275	2.6305	3.1847
		189.4	2.8623	2.7916	3.1776
		212.3	2.7205	2.7404	2.9608
	1535	213.7	2.7060	2.6944	3.0301
		205.3	2.7358	2.7151	2.9492
		187.1	2.8347	2.7849	2.9904
	3819	206.6	2.9217	2.8655	3.0932
		186.7	2.9167	2.8907	2.9797
		186.5	2.8042	2.7456	3.1223
	5911	205.8	2.8366	2.7472	3.0590
		189.6	2.8366	2.8158	3.1921

* VOGEL's minimal medium supplemented with 2% sucrose.
† VM supplemented with 0.25 M ammonium nitrate + 0.05 M monosodium glutamate.
‡ Mycelium from 50 ml shaken cultures grown at 25°C for 48 hours.

NADP-GDH in this strain is repressed to a level similar to that in other strains, resulting in an increased treatment effect. Significant variance, except that due to treatment, is largely attributable to these properties of strain 1534.

DISCUSSION

The selective advantage of specific systems concerned solely with the regulation of recombination, within single genes or perhaps groups of genes, might appear to be biologically unimportant, and would seem difficult to reconcile with the consequent demand for extra genetic material. An attractive possibility is that such systems are pleiotropic and are responsible for some other, more essential, regulatory function which also acts at the gene level, perhaps regulation

TABLE 3

Effects upon yield and GDH specific activities attributable to known genetic differences between strains and estimates of significance

Source of variation	Dry weight of mycelium		NADP-GDH Reductive assay		NADP-GDH Oxidative assay		NAD-GDH reductive assay	
	Calculated effect*	F	Calculated effect*	F	Calculated effect*	F	Calculated effect*	F
(*rec-3*) or (*rec-1*, *cot*)	—2.6	0.002	—0.3990	5.0	—0.2434	1.3	—1.7576	49.
(*cot*) or (*rec-3*, *rec-1*)	—86.4	1.9	+0.1966	1.2	+0.3228	2.3	—0.3486	1.9
T (treatment)†	—865.4	190.	—13.8796	6000.	—13.6666	4100.	+12.0074	2300.
(*rec-3*, T) or (*rec-1*, *cot*, T)	—2.6	0.002	+0.4048	5.1	+0.7466	12.	—0.1780	0.5
(*cot*, T) or (*rec-3*, *rec-1*, T)	—116.4	3.5	+1.0696	36.	+0.8152	15.	+0.1462	0.3
(*rec-3*, *cot*) or (*rec-1*)	—25.6	0.17	+0.8254	21.	+0.6284	8.7	+1.1444	21.
(*rec-3*, *cot*, T) or (*rec-1*, T)	+24.8	0.16	—0.0484	0.07	—0.2016	0.9	—0.4492	3.2

* The effects, in arbitrary units, were calculated from the results presented in Table 2.
† The treatment effect is that attributable to growth in NG medium in comparison with growth in VM medium.

TABLE 4

NADP-GDH specific activity of mycelia grown in VM and U medium

Strain	$\log_{10}$ NADP-GDH specific activity (arbitrary units) reductive assay	
	VM medium*	U medium†
	4.1044	3.1744
1534	4.0844	3.1981
	4.0892	3.1661
	3.9191	3.2686
1535	3.9271	3.2577
	3.9782	3.2405
	3.8343	3.1065
3819	3.8814	3.0997
	3.9501	3.1836
	3.9130	3.2232
5911	3.9430	3.2117
	3.9134	3.0770

The mycelium was grown in 50 ml shaken cultures for 48 hours at 25°C.
* VOGEL's minimal medium supplemented with 2% sucrose.
† VM, less ammonium nitrate, supplemented with 0.1 M urea.

TABLE 5

Effects on NADP-GDH specific activity attributable to known genetic differences between strains and estimates of significance

Source of variation	Calculated effect*	F
(*rec-3*) or (*rec-1*, *cot*)	—0.4511	4.8
(*rec-1*) or (*rec-3*, *cot*)	—0.0003	0.0002
T (treatment)†	—9.3305	2100.
(*rec-3*, T) or (*rec-1*, *cot*, T)	+0.6633	10.
(*rec-1*, T) or (*rec-3*, *cot*, T)	—0.7003	12.
(*rec-3*, *rec-1*) or (*cot*)	—1.0709	27.
(*rec-3*, *rec-1*, T) or (*cot*, T)	+0.2635	1.6

* The effects, in arbitrary units, were calculated from the results presented in Table 4.
† The treatment effect is that attributable to growth in U medium in comparison with growth in VM medium.

TABLE 6

Effect of rec-3 *on the repression of NADP-GDH*

Effect	NG medium		U medium
	Reductive assay	Oxidative assay	Reductive assay
Treatment*	—13.8796 ± 0.876	—13.6666 ± 1.0455	—9.3305 ± 1.007
rec-3, treatment	+0.4048 ± 0.876	+0.7466 ± 1.0455	—0.1780 ± 1.007

The data are abstracted from Tables 3 and 5.
* The treatment effect is that attributable to growth in NG or U medium in comparison with growth in VM medium.

of gene activity by control of the access of DNA-dependent RNA polymerase to the gene. A feature which is difficult to reconcile with this latter view is the apparent frequency of occurrence of genetic variants (*rec*) of the systems which control recombination frequency, amongst inbred laboratory stocks, which presumably reflects considerable heterogeneity in the wild population. Such variation would be expected to have effects upon the regulation of messenger production which might seriously disturb the regulation of enzyme level within the cell. In such a pleiotropic system the lack of an active regulatory element, due to mutation in a *rec* gene, would be expected to lead to constitutivity and lack of repressibility of the enzyme coded by the gene affected by *rec*. The observed effect of *rec-3* on recombination frequency between *am-1* alleles and its lack of effect upon the repressibility of NADP-GDH suggests that *rec-3*$^+$ does not produce a repressor of transcriptive activity of the *am-1* gene.

The site of repression control of NADP-GDH in Neurospora is at present under investigation. A mutation within the *am-1* gene, *am-1* 47305 (also known as *am*^{2}), affects the intracellular level of NADP-GDH protein under repressive conditions; this mutant appears less repressible than wild type, however, NAD-GDH induction is unaltered (D. E. A. CATCHESIDE, unpublished work). This can be considered as an indication of translation level control or specific destruction of preformed enzyme. Alteration of a control mechanism concerned with the destruction of a specific messenger (BRENNER 1965) in this strain is unlikely, as the mutant site of *am-1* 47305 is approximately centrally placed in the *am-1* locus. It must, therefore, be supposed not to be in an operator segment which, for control of messenger destruction, would be expected to be peripheral to the portion of the messenger carrying the structural information for the enzyme.

Regulation of translation or specific enzyme destruction does not preclude regulation of transcription of the *am-1* gene. Indeed, the *am-1* 47305 mutation does not prevent repression but reduces it an order of magnitude under conditions which repress wild-type NADP-GDH two orders of magnitude. The remaining repression may reflect impairment of a single regulatory circuit or a dual-level control of NADP-GDH: control both of transcription and of translation or destruction.

Control of recombination and transcription can be described in terms of theories involving different combinations of common regulatory elements (D. E. A. CATCHESIDE 1967). That *rec-3* does not affect repression of NADP-GDH indicates that it does not specify an element common to regulatory circuits controlling recombination and transcription.

It should be pointed out that these investigations were carried out in vegetative, non-meiotic, cultures. The effect of transcription rate upon the frequency of recombination, which has been examined in *Escherichia coli* (SHESTAKOV and BARBOUR 1967; HELLING 1967; and HERMAN 1968), has not been tested here. Instead, the effect of a gene known to derepress meiotic recombination, specifically at the *am-1* locus, has been tested for its effect upon the known repressibility of the *am-1* gene product in non-meiotic cultures.

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SUMMARY

A mutant (*rec-3*) which alters the frequency of recombination between alleles at the *amination-1* locus (*am-1*) in *Neurospora crassa* has no effect upon the repressibility of the NADP-specific glutamate dehydrogenase which is specified by the *am-1* gene.

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