Chromosome instability related to gene suppression in *Aspergillus nidulans*

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1. INTRODUCTION

This study was initiated in the knowledge that *Aspergillus nidulans* was a suitable organism not only for detecting but also for analysing genetic instability (Käfer, 1961; Arlett, Grindle & Jinks, 1962; Ball & Roper, 1966; Bainbridge & Roper, 1966). It was considered desirable to look in the first instance for systems bearing analogy with types of genetic instability already described in both higher organisms (e.g. McClintock, 1951) and bacteria (e.g. Dawson & Smith-Keary, 1963). *A. nidulans* is a chromosomal organism while at the same time having the general advantages of a microorganism from the point of view of genetic analysis and manipulation.
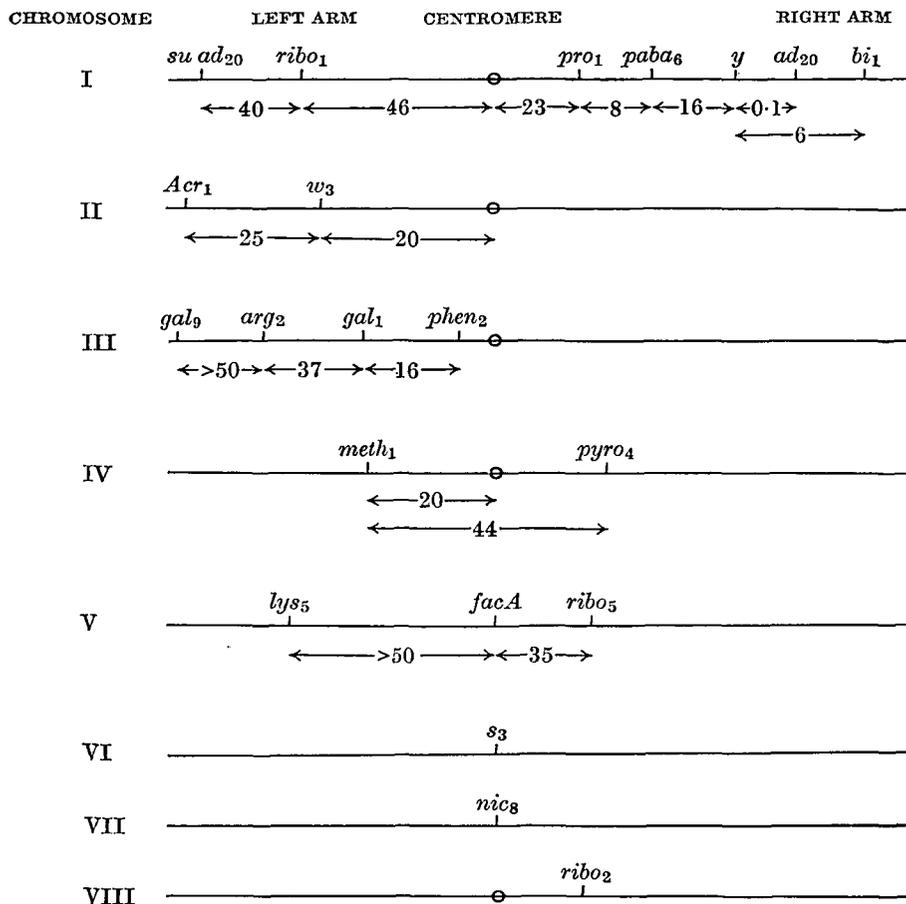
2. METHODS

General techniques and methods of genetic analysis were those of Pontecorvo, Hemmons, MacDonald & Bufton (1953). Allocation of a mutant allele to its linkage group via mitotic haploidization (Forbes, 1959) was facilitated by the parafluoro-phenylalanine (PFA) technique of Morpurgo (1961).

Media. Minimal medium (MM). Czapek-Dox with 2% glucose. Complete medium (CM) a complex medium with yeast extract, hydrolysed casein, hydrolysed nucleic acids, vitamins, etc. Solid media contained 2% agar. 'Selective medium' is MM supplemented with biotin and other appropriate growth factors but lacking methionine. Streptomycin sulphate (BP) was a Glaxo product and was used at a concentration of 2·5 g./l.

Organisms. Initially strains were taken from laboratory stocks maintained on CM. Certain strains were obtained from the Fungal Genetics Stock Center, Dartmouth College, Hanover, New Hampshire, U.S.A. Mutant alleles used in this work are described by Pontecorvo *et al.* (1953), Käfer (1958), Roper & Käfer (1957), Apirion (1962) and Roberts (1963). Those of main importance were: y, yellow conidia; w_3, white conidia; bi_1, $meth_1$, pro_1, $paba_1$, $ribo_1$, $ribo_2$, $ribo_3$ and $ribo_5$, nic_8, ad_{20}, $phen_2$, $pyro_4$, lys_5, s_3, arg_2, growth requirements respectively for biotin, methionine, proline, para-aminobenzoic acid, riboflavin, nicotinic acid, adenine, phenylalanine, pyridoxine, lysine, thiosulphate and arginine; $su\ ad_{20}$ suppressor of ad_{20}; Acr_1 (semi-dominant) mutant conferring resistance to acriflavine; $facA303$ inability to grow on acetate medium; gal_1 and gal_9 inability to grow vigorously on galactose medium. The respective location of these mutants on the linkage map of *A. nidulans* is as follows:

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CHROMOSOME   LEFT ARM   CENTROMERE   RIGHT ARM

I $su\ ad_{20}$ $ribo_1$ pro_1 $paba_6$ y ad_{20} bi_1
    ←40→ ←—46——→ ←23→←8→←16→←0·1→
    ←—6—→

II Acr_1 w_3
    ←—25—→ ←—20—→

III gal_9 arg_2 gal_1 $phen_2$
    ←>50→←—37—→←16—→

IV $meth_1$ $pyro_4$
    ←—20—→
    ←——44——→

V lys_5 $facA$ $ribo_5$
    ←——>50——→←—35—→

VI s_3

VII nic_8

VIII $ribo_2$

Relative to master strains MSE and MSD (McCully & Forbes, 1965), all strains used initially were untranslocated. Classification of nutritional requirements of meiotic segregants was based on 100–200 segregants from most crosses, an exception being where the number of segregants was less than 100 in which case all segregants were tested.
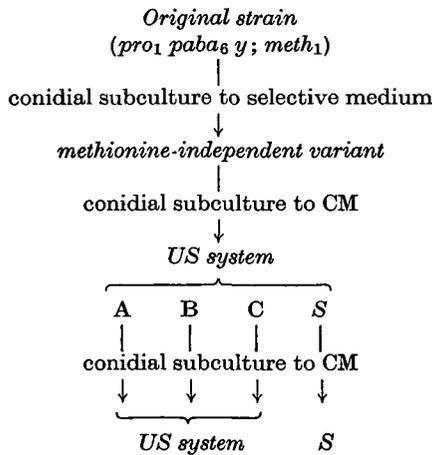
3. RESULTS

The experimental procedure involved:
- (i) Isolation of an unstable system and identification of several components through behaviour on vegetative subculture.
- (ii) Genetic analysis of these components.

(i) *Isolation and subculture*

The technique employed for isolation involved selecting morphological variants from among slow-growing colonies on selective medium, after plating conidia of a strain $pro_1\ paba_6\ y$; $meth_1$ at high density (10^4/dish) and after incubation for 10 days at 37°C. Such variants were termed 'methionine independent', if they retained the

ability to grow on selective medium after repeated subculture on CM. One variant behaving in this manner was also unstable in that on conidial subculture a number of differing morphological variants were recovered. Further subculture of certain of these yielded the same spectrum of types again, this being repeated in all further subcultures from one generation to the next. In all, four differing morphological categories A, B, C and S were classified in this unstable system which was designated *US*. Types A and B occurred in equal frequency and were the majority class, while types C and S represented only 1% to 5% of the morphologies of the system. The different types were distinguished on the basis of density of conidiation, intensity of mycelial pigmentation and linear growth rate. C and S represented the opposite extremes in that C types were almost aconidial with poor linear growth and had intense dark-brown mycelial pigmentation, while S types were relatively vigorous, having normal density of conidiation, near normal linear growth and less intense

$$
\begin{array}{c}
\textit{Original strain} \\
(pro_1\ paba_6\ y\ ;\ meth_1) \\
| \\
\text{conidial subculture to selective medium} \\
\downarrow \\
\textit{methionine-independent variant} \\
| \\
\text{conidial subculture to CM} \\
\downarrow \\
\textit{US system}
\end{array}
$$

$$
\begin{array}{cccc}
\text{A} & \text{B} & \text{C} & S \\
| & | & | & | \\
\multicolumn{4}{c}{\text{conidial subculture to CM}} \\
\downarrow & \downarrow & \downarrow & \downarrow \\
\multicolumn{3}{c}{\textit{US system}} & S
\end{array}
$$

mycelial pigmentation. A and B were distinguishable by various combinations of properties that were intermediate in effect compared with those of C and S. The S types arose mainly as sectors although a small proportion were detected as whole colonies. These vigorous types were designated S types because they were relatively stable on conidial subculture, producing only S types again, which had the same characteristics as the S type of origin. The following scheme summarizes the isolation and subculture experiments described above:

   A number of S types designated s_1, s_2, etc. were isolated and tested for degree of methionine independence by their relative ability to produce growth on selective medium. Table 1 shows how a sample of these could be distinguished not only in this way, but also by a number of other criteria. Outstanding amongst these criteria was the ability of certain types (e.g. s_2) to produce more growth on selective medium when streptomycin was present. Also there was variable ability to produce G types, which were morphologies possessing grey spore pigmentation and considerably reduced linear growth, appearing as whole colonies or as apparent sectors on selective medium. Subculture of G types revealed that they were unstable on

CM in that all such types produced sectors with normal conidiation and with a degree of methionine independence similar to that of the S types from which they were derived, (e.g. s_{1GSEC} in Table 1). The G types showed enhanced methionine independence in all cases tested in that on selective medium G type colonies were produced by most plated G type spores.

Table 1. *Characterization of several* S *types*

		Approximate percentage colony growth/plated spore†		
		Selective medium		Selective medium + streptomycin
S type	Mycelial pigmentation	Total	G types	
S_1	Dark	50	<1	100
$S_{1GSEC}*$	Dark	50	<1	100
S_2	Pale	1	1	100
S_3	Pale	100	10	100
S_4	Pale	<1	<1	<1
Normal control ($pro_1\ paba_6\ y$; $meth_1$	Pale	0	0	0

　* Sector produced by G type derived from s_1.
　† 200 conidia/dish; 4 days' incubation at 37°C

In addition to the criteria listed in Table 1 it was found that at 25°C. *US* and *S* types exhibited a greater degree of methionine independence. Also *US*, *S* and *G* types were susceptible to thiosulphate inhibition on selective medium. This was found during genetic analysis involving MSE and MSD strains, which require thiosulphate for growth. This limited the information obtainable from these crosses. However, an explanation of the inhibition may be forthcoming when data are available on the methionine biosynthetic pathyway in *A. nidulans*. Indeed, Spencer and co-workers (personal communication) have found thiosulphate repression during studies on choline sulphate synthesis.

(ii) *Genetic analysis*

Meiotic and mitotic haploidization analysis of S types was undertaken, while *US* and *G* types were analysed meiotically.

Analysis of US

Crosses were attempted between *US* and several normal strains. Initial screening of segregants revealed that *US* passed through meiosis and that there were more *US* than normal phenotypes among the progeny. The results of more precise colony counts are shown in Table 2. Classification of the nutrient requirements of segregants produced evidence for the following:

　1. Suspected segregation of a genetic determinant that suppresses $meth_1$ in that methionine-requiring segregants were recovered. These composed one-half of the normal segregants from the cross of *US* with $ribo_1\ y$; nic_8.

2. The genetic determinants for suppression of $meth_1$, and for US, were closely linked, if not identical, since no methionine-requiring US types were recovered in the cross of US with $ribo_1 y$; nic_8.

Table 2. *Crosses of* US *with normal*

| Normal strain | Perithecium | Segregant morphologies | | χ^{2*} |
		US†	Normal	
$ribo_1 y$; nic_8	1	131	83	0·08
MSD	1	120	65	0·6
	2	78	56	0·038
MSE	1	84	54	0·15
	2	36	27	0·1

* Test of goodness of fit (D.F. = 1) of ratio 2:1 for US:normal.

† Segregant morphologies that resembled any of the components A, B, C or S of the US system.

3. The centres and sectors (S type) of a number of US segregants were classified for nutritional requirement. The genotypes of any one centre corresponded with that of its sectors except for classification of one particular allele, gal_1. This was detected in the cross of US with MSE. Here it was found that centres that could grow vigorously on galactose medium could produce, in certain instances, sectors which would not produce vigorous growth on galactose medium. The opposite case was not detected, although US types that could not grow on galactose medium were found.

The most likely reason for the segregation described and the apparent 2:1 ratio of US to normal morphologies observed (Table 2), was formulated after analysis of S types derived from the original US type.

Analysis of S *types*

S_1 was the most thoroughly analysed S type. Crosses to several strains (Table 3) were carried out. Segregants from certain of these crosses were later outcrossed (Table 4). In all meiotic analyses, certain general features emerged:

1. Complete absence of US types among the segregants. The transition of US to S types, is, therefore, not due to a modifier mutation distant from the US locus and would rather seem to represent a change at the same locus.

2. A morphological type, 'compact', could be detected among the segegants. Table 3 shows that this type composed about one-third of all segregants in the crosses in which it was classified.

3. Certain 'compacts' were found to sector faster growing, more normal types. The nutritional requirements of centres and sectors showed that, in the cross to MSE, certain compact types which grew well on galactose medium produced sectors which did not. Furthermore, in other crosses a pattern of sectoring analogous to this was obtained with markers gal_9 and arg_2 but not $phen_2$.

Table 3. *Crosses of* S *types with normal*

Cross	S type	Normal strain	Perithecium	Abnormal S type	Abnormal Compact	Normal	χ^{2*}
1	s_1	$ribo_1\, y\,;\, nic_8$	1	54	21	37	0·001
2	s_1	$y\,;\, pyro_4\,;\, ribo_3$	1	19	32	29	0·15
3	s_1	$y\,;\, pyro_4\,;\, ribo_5$	1	33	19	22	0·09
4	s_1	MSE	1	61	73	50	0·12
5	s_1	MSD	1	49	39	51	0·4
6	s_1	$bi_1\,;\, arg_2$	1	248		159	0·015
6	s_1	$bi_1\,;\, arg_2$	2	132		64	0·9
7	s_1	$bi_1\,;\, w_3\,;\, gal_9$	1	104		62	0·25
8	$s_{1\mathrm{GSEC}}$	$ribo_1\, y\,;\, nic_8$	1	39	17	29	0·015
9	s_2	$ribo_1\, y\,;\, nic_8$	1		13	41	0·15
9	s_2	$ribo_1\, y\,;\, nic_8$	2		45	149	0·003
9	s_2	$ribo_1\, y\,;\, nic_8$	3		22	54	0·4

* Test of goodness of fit (D.F. = 2) of 1:1:1 ratio for the three categories shown in crosses 1–5 and cross 8.

(*b*) Test of goodness of fit (D.F. = 1) of 2:1 ratio for abnormal:normal shown in crosses 6 and 7.

(*c*) Test of goodness of fit (D.F. = 1) of 2:1 ratio for normal:compact in cross 9.

4. All abnormal segregants, i.e. S type and 'compact', were methionine independent; while one-half of normal segregants were methionine requiring, with the exception of MSE and MSD crosses.

Inspection of the chromosome map of *A. nidulans* (see Methods) reveals that the markers gal_1, gal_9 and arg_2 are on the left arm of chromosome III. However, the marker $phen_2$ (MSD strain) which is also on this arm, did not behave in an analogous manner. These facts, coupled with the fact that compact types composed one-third of all segregants, suggested that 'compact', might be compared with 'crinkled' previously described by Bainbridge & Roper (1966). In the latter case, 'crinkled' was produced by crossing to certain non-translocated strains, a strain carrying a non-reciprocal translocation of part of the right arm of chromosome III to chromosome VIII. In the case of *US* and *S* types this would mean that part of the left arm of chromosome III distal to the $phen_2$ locus had been translocated to another chromosome. Crossing such a strain to normal would result in an inviable class of segregants due to chromosome deficiency and hence segregation of the chromosome types: translocated, duplicated and normal in the ratio 1:1:1 (see Table 3). On this basis the most economical explanation of the facts would be that the point of translocation determined both abnormal morphology and ability to suppress methionine requirement. If this were so, it might be expected that sectoring of duplicated 'compact' types would be accompanied by change in the degree of methionine independence as well as the change in morphology observed. One case of unambiguous change in degree of methionine independence has so far been detected.

This was a reversion to complete methionine dependence. Here, a possible explanation would be the complete loss of the translocated section of chromosome III. Where the mechanism of chromosome loss in duplicated strains is concerned, however, there is considerable evidence that it may not always be simple. Indeed Nga & Roper (1966) have made a detailed study of instability of another duplicated type in *A. nidulans*, and interstitial loss and gain of genetic material seem to be possibilities. Comparison of the behaviour of this duplicated strain with the compact types described in this paper has shown numerous points of similarity, including the production of a very extreme morphological type, designated 'brown' with considerably delayed conidiation.

Further support for the presence of a translocation in S types came from mitotic haploidization analysis of diploids s_1 with MSE and s_2 with MSE. The expected pattern of segregation, in the case of chromosome translocation, has been fully

Table 4. *Crosses of segregants to normal*

| | | | | Segregant morphologies | | | |
| | | | | Abnormal | | | |
Cross	Normal parent	Abnormal parent†	Cross of origin	S type	Compact	Normal	χ^{2}*
1	$ribo_1\,y$; nic_8	Non-compact$_1$	$s_1 \times ribo_1\,y$; nic_8	39	57	44	0·15
2	$ribo_1\,y$; nic_8	Non-compact$_2$	$s_1 \times ribo_1\,y$; nic_8	51	32	37	0·08
3	$ribo_1\,y$; nic_8	Compact$_1$	$s_1 \times ribo_1\,y$; nic_8		71	87	0·2
4	$ribo_1\,y$; nic_8	Compact$_2$	$s_1 \times ribo_1\,y$; nic_8		25	111	$< 0\cdot0001$
5	$ribo_1\,y$; nic_8	Non-compact$_3$	$s_1 \times bi_1\,arg_2$	43	34	58	0·04
6	MSE	Non-compact$_3$	$s_1 \times bi_1\,arg_2$	47	34	43	0·35
7	$ribo_1\,y$; nic_8	Non-compact$_4$	$s_1 \times bi_1\,arg_2$	22	34	30	0·3

* (a) Test of goodness of fit (D.F. = 2) of 1:1:1 ratio for three categories in crosses 1, 2, 5, 6, and 7.

(b) Test of goodness of fit (D.F. = 1) of 1:1 ratio for two categories shown in crosses 3 and 4.

† Non-compact refers to S type segregant.

discussed by Käfer (1962) and in essence involves linked segregation of markers on the chromosomes involved in the translocation. From PFA breakdown of the diploids mentioned, it was possible to recover haploid segregants which exhibited random association of chromosomes as judged by marker segregation except for the case of chromosomes III and V. In these cases, haploids had retained the parental association of markers. Only four instances of recombinant association were detected in a total of 111 haploids tested from the two diploids. Furthermore, a diploid of a 'compact' meiotic segregant with MSE, when analysed, showed that the compact morphological determinant could be allocated to chromosome V.

Where duplicated types are concerned, problems arise in certain instances that can be explained in terms of the instability of these strains. Thus in Table 4, cross 4, the considerable deviation from a 1:1 ratio of 'compact':normal can be explained in terms of instability of the 'compact' genome in ascogenous hyphae or in the ascospore. Similar deviations from an expected 1:1 segregation were observed by Bainbridge and Roper in their analogous study with 'crinkleds'.

The purpose of the crosses shown in Table 4 was not only to examine predicted morphological segregation but also to check segregation of the suppressor of methionine requirement (su^-). A method had been devised whereby the supposed genotype $su^- meth_1$ could be detected. This was based on both slow growth rate and conidiation and thiosulphate inhibition of growth of such types on selective medium. This was supported by data from the crosses shown in Table 4 since such phenotypes, which had composed one-half of abnormal segregants of previous crosses, yielded the predicted $meth_1$ segregants on outcrossing to methionine-independent strains, while abnormal strains without this phenotype did not yield $meth_1$ segregants in equivalent crosses.

Additional points of interest relevant to the suppressor problem emerged, in that in the crosses of s_1 to b_1; arg_2 (Table 3) and subsequent outcrossing of segregants (Table 4), no arginine-requiring dark pigmented S type and compact segregants were recovered. But on outcrossing certain selected dark pigmented s type segregants (presumed translocated), arginine-requiring segregants of normal morphology were recovered (Crosses 5, 6 and 7, Table 4). It appeared, therefore, that the point of translocation was not only associated with determination of morphological abnormality in certain cases and suppression of $meth_1$, but with suppression of arg_2 as well.

Attempts to detect meiotic linkage of the translocation point to any of several chromosome V markers were unsuccessful. Hence it is not yet possible to say which arm of chromosome V is involved.

Analysis of G types

These types posed a challenge to analysis, due to their considerable instability on medium containing methionine. Therefore a selective approach to crossing was adopted. A G type (s_{2G}) derived from s_2 was chosen since s_2 itself and s_{2GSEC} showed a low degree of methionine independence on selective medium (Table 1). Consequently the media on which the heterokaryons were initiated and grown were kept free of exogenous methionine. Very few areas of the supposed heterokaryon of s_{2G} with MSD were found to be heterokaryotic for s_{2GSEC} with MSD. Perithecia, although small, were selected from the most abnormal areas. Results of screening a number of such perithecia are shown in Table 5. It can be seen that the G type passes through meiosis and that ascospore viability is considerably reduced. Classification for nutritional requirements was carried out on segregants from several perithecia, with pooling of the consequent data. Classification of centres and sectors of G types showed identity between any one centre and its sector except for certain instances where a phenylalanine-independent centre produced a phenylalanine-dependent sector. Indeed all G type segregants had phenylalanine-independent centres and could produce either independent or dependent sectors, but never both. Instances of the latter, however, were obtained together with the other types mentioned when a G type sectoring phenylalanine-dependent types was outcrossed to $ribo_1 y$; nic_8.

For a number of reasons, such as the relatively low numbers tested (twenty G types in each cross), the analysis is in many ways qualitative. However, a reasonable

explanation of the observed facts is that G types are aneuploids due to duplication of the centromere-containing fragment of chromosome III.

Table 5. *Cross of* s_{2G} *to MSD**

Hybrid perithecium	Segregant morphologies	
	G type	Others
1	12	18
2	3	7
3	3	17
4	6	5
5	3	7
6	6	13
7	3	4
8	5	1
9	4	15
10	—	10^3

* Approximately 10^3–10^4 ascospores plated in each case.

4. DISCUSSION

Analogy with the behaviour of other duplicated strains of $A.$ *nidulans* has been described, but only as part of a complex system of genetic instability. The US variant (Ball, 1966) arose as a result of a spontaneous translocation event; part of chromosome III being translocated to chromosome V; the region of attachment of the translocated fragment being the genetic determinant of both morphological instability and the suppression of $meth_1$. One explanation of this might be that the instability and suppression arose due to the breakage and fusion of two cistrons, one terminal to chromosome V and the other part of chromosome III. The new cistron formed might determine the structure of a protein which conveyed ability to suppress methionine requirement. However, if this protein were labile, it is possible that some conidia might have higher concentrations than others. The initial concentration in the conidium might determine the phenotype of the resulting colony. The colonies produced, would again produce conidia with differing concentrations of the protein in question. The change of US to S types could be explained by postulating spontaneous mutation, in the new cistron, to give a stable protein product. Pigmentation differences in S types could be explained as pleiotropic effects of such mutations. Such stable types, because of their faster growth rate, might be selected for on CM. However, selective forces other than growth rate would have to be invoked to explain the occurrence of the relatively aconidial, dark pigmented types with poor linear growth that occurred in the US system. Nevertheless, subsequent observations of US, S and G types can be generally accommodated by the hypothesis, a prevalent theme of which is spontaneous mutation plus selection.

Since selection has to be postulated, it is worth while entertaining other explanations which would minimize this necessity. Hence US could be due to causes

similar to those producing V-type position effects in certain higher organisms (Lewis, 1950). Variable chromosome inactivation in a haploid genome would probably have to apply to the hyphal stage of development and not the conidial, otherwise one might expect a discrepancy to be observed between conidial survival of US on CM and selective medium, due to the occurrence of conditional lethals. However, it is possible to construct other postulates that would remove this assumption. For example, during the translocation process part of chromosome III may have been duplicated (e.g. by unequal sister chromatid break). Such a region would be available for variable inactivation in the conidium with no detrimental effects on overall viability. Such duplication might be the cause of dark pigmentation. Mutation to S types might take place such that part of one of these regions is permanently inactivated (i.e. pale pigmented S type) or activated (i.e. dark pigmented S type). Furthermore, if the region duplicated consisted of part attached to chromosome V and part remaining on chromosome III, then any tendency towards mitotic pairing of these homologous regions might induce duplication of chromosome III. This could explain the high frequency of G types and their enhanced methionine independence since G types might have a locus in triplicate. Such a locus would convey no methionine independence when single, but would convey independence when duplicated (US and S types).

The question of the mechanism of suppression of mth_1 and arg_2 by the region of the genome associated with the point of translocation, has also general relevance, in that the mechanism of suppression of $meth_1$ may be of the 'informational type' (Gorini & Beckwith, 1966). This conclusion is based on the effect of streptomycin and simultaneous suppression of two unlinked loci, arg_2 and $meth_1$. Suppression of arg_2 raises a problem in that arg_2 is located on the translocated fragment and its suppression may be related to a position effect type of event. However, one would expect any inactivation to go undetected since arg_2 is recessive. Alternatively, activation of arg_2 may be taking place; this being covered generally by present concepts of 'informational suppression'.

SUMMARY

A complicated system of chromosome instability related to gene suppression has been analysed. In addition, unstable genetic events analogous to those previously described in *A. nidulans* and in other organisms have been detected and one type of unstable variant recovered may be determined by V-type position effect. Furthermore, the selective systems used, clearly offer scope for analysis of genetic instability in general.

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