Ethidium Bromide Resistance: A Selective Marker Located on Linkage Group IV of *Dictyostelium discoideum*

By MALCOLM D. WRIGHT, KEITH L. WILLIAMS*
AND PETER C. NEWELL

Department of Biochemistry, University of Oxford, South Parks Road,
Oxford OX1 3QU

(Received 2 May 1977)

INTRODUCTION

The cellular slime mould *Dictyostelium discoideum* is a simple eukaryote that is eminently suitable for studies on differentiation and in which a parasexual system of genetic analysis is currently being developed. In this system two growth temperature-sensitive haploid strains are fused and diploids are selected at the restrictive temperature at a frequency of about 10^{-5} (Loomis, 1969; Katz & Sussman, 1972). Recently an alternative diploid selection system using *Bacillus subtilis* sensitivity has been reported (Newell *et al.*, 1977). The diploids so produced revert at low frequency to haploids through a process of transient aneuploidy (Sinha & Ashworth, 1969; Brody & Williams, 1974). Linkage analysis is possible since chromosome loss during haploidization is random and the frequency of mitotic crossing-over is low (Mosses, Williams & Newell, 1975). For parasexual genetics to be practicable in *D. discoideum* some means of selection is needed to isolate haploids and mitotic cross-over diploids from parental diploids (Katz & Sussman, 1972). Distal selective markers are required on each chromosome, all of which probably have terminal centromeres (Robson & Williams, 1977). So far selective markers have been reported on linkage groups I (*cycA* and *acrB*, Katz & Sussman, 1972; Williams, Kessin & Newell, 1974a), II (*acrA*, Williams *et al.*, 1974a) and III (*acrC*, Rothman & Alexander, 1975). These loci all involve recessive resistance to noxious compounds. In this report resistance to the intercalating dye ethidium bromide is described and the locus (designated *ebrA*) is assigned to linkage group IV.

METHODS

Chemicals. Acriflavin (neutral), ethidium bromide and cycloheximide were obtained from Sigma. Other chemicals, of the purest grade available, were purchased from BDH or Fisons.

Media and growth conditions. Amoebae or spores of *D. discoideum* were diluted in sterile SS salt solution (Sussman, 1966). The nutrient SM agar plates (Mosses *et al.*, 1975) were prepared with Oxoid agar no. 3 (15 g l^{-1}), Difco Bacto-agar (15 g l^{-1}) or Calbiochem agar (11 g l^{-1}).

Initially, ethidium bromide was added to SM Difco agar, after autoclaving, from a filter-sterilized stock solution. Latterly, unsterilized ethidium bromide has been added as a solid, or as a stock solution (3 mg ml^{-1}) to the agar after autoclaving. Media containing different brands of agar appeared to affect the toxicity of ethidium bromide. For this reason the final concentration of ethidium bromide used was $35 \text{ } \mu\text{g ml}^{-1}$ in SM Difco agar and $30 \text{ } \mu\text{g ml}^{-1}$ in SM Calbiochem agar. (Medium containing Oxoid agar no. 3 appeared to be considerably less toxic and was not used for these studies.) As far as possible, the ethidium bromide plates were used under conditions of low light intensity, i.e. exposure to sunlight or germicidal lamps was avoided.

SM agar plates containing methanol (2 %, v/v), acriflavin ($100 \text{ } \mu\text{g ml}^{-1}$) or cycloheximide ($500 \text{ } \mu\text{g ml}^{-1}$) were prepared as described previously (Mosses *et al.*, 1975). All agar plates were stored at 4°C in the dark until required.

* Present address: Genetics Department, Research School of Biological Sciences, The Australian National University, Canberra City, A.C.T. Australia.

Strains. The previously described haploid strain NP14 is a cycloheximide- and methanol-resistant, white-spored and growth temperature-sensitive mutant containing the alleles *cycA1*, *acrA1*, *whiA*, *tsgD12*. Strain x2 is a brown pigmented and growth temperature-sensitive mutant containing the alleles *bwnA* and *tsgA1* (Williams *et al.*, 1974a). Strain NP81 is a spontaneous ethidium bromide-resistant mutant of x2 containing the allele *ebrA1*. Strain x22 is a methanol-resistant, white- and round-spored, growth temperature-sensitive mutant containing the alleles *acrA1*, *whiA*, *sprA1*, *tsgE13* and *tsgD12* (Williams & Newell, 1976), and strain HU26 is a spontaneous mutant of x22 resistant to cobaltous chloride and carries the allele *cob-353*. All strains were maintained as spores collected in horse serum, dried on to a silica gel and stored at 4 °C. During experiments, clones were maintained on SM agar plates with the bacterium *Klebsiella aerogenes*.

Parasexual genetic techniques. Heterozygous diploid strains were constructed between haploids NP14 and NP81 (diploid DP219) and between haploids HU26 and NP81 (diploid DU133). In each case the haploid strains carried non-allelic growth temperature-sensitive mutations and diploids were selected at the restrictive temperature as described previously (Williams, Kessin & Newell, 1974b). Haploid segregants were obtained from these diploids by plating approximately 10^4 amoebae or spores on to SM agar containing either cycloheximide, methanol or ethidium bromide. These plates were spread at the same time with 0.1 ml of a thick suspension of *K. aerogenes*. In some experiments only haploids of certain independent origin were chosen (see Mosses *et al.*, 1975). Haploids and diploids were distinguished by spore size.

RESULTS AND DISCUSSION

Isolation of ethidium bromide-resistant mutants

Spontaneous mutants resistant to ethidium bromide were isolated in strain x2 at a frequency of 10^{-6} by plating on SM agar containing ethidium bromide at $25 \mu\text{g ml}^{-1}$. Of several such mutants isolated, strain NP81 containing the mutation *ebrA1* was chosen for further study. Strains containing the *ebrA1* mutation plated with about 50 % efficiency on SM agar and SM agar containing ethidium bromide at up to $50 \mu\text{g ml}^{-1}$, although at this concentration growth was considerably slower than on SM agar. SM agar containing 30 or $35 \mu\text{g}$ ethidium bromide ml^{-1} was generally used as this produced good growth on ethidium bromide and avoided leakiness in sensitive strains. At 30 to $35 \mu\text{g}$ ethidium bromide ml^{-1} , resistant clones appeared after 5 days compared with 3 days on SM agar.

Dominance and assignment to a linkage group

The *ebrA1* allele is recessive to wild type since diploids heterozygous for this marker, e.g. DP219, DU133, are sensitive to ethidium bromide but segregate resistant haploid and diploid clones at a frequency (haploids at 1×10^{-4} ; diploids at 1.5×10^{-4}) similar to that found for other recessive resistance markers, e.g. *cycA*, *acrA* (Williams *et al.*, 1974a; Mosses *et al.*, 1975).

The *ebrA1* locus has been located on linkage group IV by analysing haploid segregants of diploids DP219 and DU133 (Table 1). Both diploids allowed examination of linkage groups I to IV, with DP219 allowing selection for linkage groups I (*cycA*), II (*acrA*) and IV (*ebrA*) and DU133 allowing selection for linkage groups II (*acrA*) and IV (*ebrA*). Of 947 haploid segregants examined 946 co-segregated ethidium bromide resistance with brown pigment (*bwnA*) on linkage group IV. Linkage groups I, II and III segregated independently of ethidium bromide resistance in both diploids (Table 1), consistent with *ebrA1* being on linkage group IV. In DU133 ethidium bromide resistance segregated independently of *cob-353* which has recently been located on linkage group VII (Williams & Newell, 1976; D. I. Ratner & P. C. Newell, unpublished observations; K. L. Williams, unpublished observations).

Deviations from the 1:1 segregation of unlinked markers

The different segregant classes were recovered with unequal frequencies (Table 1). When linkage group II was selected by plating on SM agar containing methanol, an excess of the haploid segregants were sensitive to ethidium bromide. In experiment 1, there were 78 *ebrA*⁺ haploids and 9 *ebrA*. Similarly, in experiment 5, 110 of the 126 haploids selected on

Table 1. Assignment of ethidium bromide resistance (*ebrA1*) to linkage group IV by haploidization of diploids DP219 and DU133

Linkage group I was selected by plating clones on cycloheximide plates; linkage group II was selected on methanol plates; linkage group IV was selected on ethidium bromide plates. Temperature resistance was determined by replica-plating at 22 °C and at 27 °C. Experiments 1 to 6 involved 20, 20, 32, 16, 21 and 27 independent clones respectively.

Expt no.	Linkage group selected	Ratio of haploid segregants in linkage group							
		I	II			III	IV		
Diploid DP219		<u>+</u> <i>cycA1</i>	<u>+</u> <i>whiA</i>	<u>+</u> <i>acrA1</i>	<u>+</u> <i>tsgD12</i>	<i>tsgA1</i> +	<i>ebrA1</i> +	<i>bwnA</i> +	
1	I	$\frac{0}{41}$		$\frac{34}{7}$		$\frac{2}{37}$	$\frac{1}{40}$	$\frac{1}{40}$	
		$\frac{37}{50}$		$\frac{0}{87}$		ND	$\frac{9}{78}$	$\frac{9}{78}$	
	IV	$\frac{43}{62}$		$\frac{47}{58}$		$\frac{43}{11}$	$\frac{105}{0}$	$\frac{105}{0}$	
	II	$\frac{0}{17}$		$\frac{8}{9}$		ND	$\frac{15}{2}$	$\frac{15}{2}$	
		$\frac{17}{13}$		$\frac{0}{30}$		ND	$\frac{20}{10}$	$\frac{19^*}{11}$	
2	IV	$\frac{3}{15}$		$\frac{8}{10}$		ND	$\frac{18}{0}$	$\frac{18}{0}$	
	IV	$\frac{141}{200}$		$\frac{177}{164}$		$\frac{154}{43}$	$\frac{341}{0}$	$\frac{341}{0}$	
3	IV	$\frac{25}{84}$		$\frac{54}{55}$		$\frac{42}{20}$	$\frac{109}{0}$	$\frac{109}{0}$	
Diploid DU133		<u>+</u> <i>sprA1</i>	<u>+</u> <i>tsgE13</i>	<u>+</u> <i>whiA</i>	<u>+</u> <i>acrA1</i>	<u>+</u> <i>tsgD12</i>	<i>tsgA1</i> +	<i>ebrA1</i> +	<i>bwnA</i> +
5	II	$\frac{78}{48}$		$\frac{0}{126}$		ND	$\frac{16}{110}$	$\frac{16}{110}$	
6	IV	$\frac{51}{22}$		$\frac{37}{36}$		$\frac{24}{5}$	$\frac{73}{0}$	$\frac{73}{0}$	

ND, Not determined.

* Includes an *ebrA1 bwnA*⁺ segregant which presumably arose by a cross-over prior to haploidization.

methanol carried *ebrA*⁺. Ethidium bromide-resistant colonies grew more slowly than ethidium bromide-sensitive colonies on all types of plates (clones taking 5 rather than 3 days to achieve a given size).

When linkage group IV was selected by plating on SM agar containing ethidium bromide, both genotypes of linkage group II segregated equally (experiment 3; 177 *whi*⁺, 164 *whi*) and all the colonies grew slowly. These results suggest that the *ebrA* mutation, or a mutation(s) linked to *ebrA* in strain NP81, causes slow growth and possibly a lower plating efficiency than the wild-type allele(s). Hence when *ebrA1* is not selected, the chromosome carrying this mutation is found at a lower frequency than the wild-type chromosome. This differential recovery must be borne in mind when using *ebrA* with other selectors for linkage analysis.

Resistance to ethidium bromide establishes the first selective marker on linkage group IV. This marker should be of considerable use for assigning markers to linkage groups and for mitotic mapping within linkage group IV.

M.D.W. was in receipt of a S.R.C. postgraduate studentship during the course of this work. P.C.N. thanks the S.R.C. for financial support.

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