Genet. Res., Camb. (1978), **31**, *pp.* 1–12
With 3 text-figures
Printed in Great Britain

Enrichment and screening of heat-sensitive mutants of *Physarum polycephalum*

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(*Received* 29 *November* 1976)

SUMMARY

A new method for the isolation of temperature-sensitive mutants of *Physarum polycephalum* is described. It involves enrichment and pre-screening of mutagenized amoebae followed by screening at both the plasmodial and amoebal stage. A total of 74 temperature-sensitive strains were recovered of which 26 were temperature-sensitive only as plasmodia, 35 only as amoebae and 13 in both stages. After a shift to the non-permissive temperature, DNA and protein synthesis were followed in temperature-sensitive plasmodia to discover if the lesion affected functions of the nuclear cycle.

1. INTRODUCTION

The synchronous nuclear division cycle of *Physarum polycephalum* makes this organism attractive for the study of such cellular functions as the control of nuclear division and DNA-synthesis (Rusch, 1970; Sudbery & Grant, 1975; Funderud & Haugli, 1975). While biochemical and cytological approaches have been successful, much would seem to be gained for further progress could the organism be studied by genetic means. Progress in genetic analysis in *Physarum* has been significant, but has been slowed by lack of markers and certain technical difficulties.

Mutants affecting the plasmodial stage are required to study the processes of the nuclear cycle. However, the ability of plasmodia to migrate and fuse with other plasmodia would allow recessive mutations to be masked by fusion with plasmodia wild type at the locus. Thus only one plasmodium may be cultivated per plate. This considerably reduces the number of plasmodia which may be screened.

Attempts at isolating conditional temperature-sensitive mutants of nuclear cycle functions were first reported by Haugli & Dove (1972). Temperature-sensitive mutants were isolated from amoebal populations using BudR substitution followed by UV irradiation in a suicide selection regime. Plasmodia homozygous for a temperature-sensitive mutation were constructed but they failed to express the mutant characteristic. This work was hampered by the crossing needed before the mutant could be tested in the plasmodium. A solution to this problem was

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offered by the development of the *CL* strains in which plasmodia arise from one single amoebal clone and are thus haploid and homogeneous for any mutant induced in the amoebal stage.

Wheals, Grant & Jockusch (1976) screened *CL* amoebal populations for temperature-sensitive mutants. Such temperature-sensitive amoebal mutants were allowed to form plasmodia but again it was found that these were not temperature-sensitive.

As an alternative to first isolating temperature-sensitive amoebae Gingold, Grant, Wheals & Wren (1976) and Wright *et al.* (1976) screened for temperature-sensitive mutations directly using plasmodia derived from mutagenized amoebae. Temperature-sensitive plasmodia were isolated and some did indeed show defects in functions of the nuclear cycle. However, a severe limitation is placed on the number of plasmodia which may be screened.

In this paper we report attempts to combine both methods of isolating temperature-sensitive mutants. Mutagenized *CL* amoebal populations were enriched for temperature-sensitive cells by the suicide selection scheme of Haugli & Dove (1972) together with a selection based on netropsin as the suicide agent (Gorman & Dove, 1974). The survivors were pre-screened for amoebal temperature-sensitivity by plating at 25 °C and allowing small clones to form before shifting to 30·5 °C, the non-permissive temperature. Those clones which failed to increase in size were picked as potential mutants. These were then both tested directly for temperature sensitivity at the amoebal stage and also allowed to form plasmodia and tested for temperature sensitivity at this stage as well.

The rationale behind this scheme is that the amoebae and plasmodia may share common functions but that defects of these functions may be expressed to different degrees in the two stages of the life-cycle, thus mutants expressed to a limited degree in amoebae but strongly in plasmodia may be selected by the enrichment procedure but would be rejected if only strongly expressed mutants were chosen before testing in the plasmodia.

In addition the scheme allows a direct test of the usefulness of mutant enrichment in amoebal populations when mutant plasmodia are required. This is provided by screening for temperature-sensitivity in plasmodia after every stage of the procedure.

Lastly, by comparing the proportion of mutants which are expressed in both stage as opposed to those which are only expressed at one or the other stage it will be possible to estimate the degree to which genetic functions are shared by plasmodia and amoebae.

All the temperature-sensitive plasmodia obtained were screened for defects of the mitotic cycle, for this protein and DNA synthesis were measured by incorporation of radioactive precursors after a shift to the non-permissive temperature. It was reasoned that a block in the nuclear cycle would cause a depression of DNA synthesis before protein synthesis was affected.

2. METHODS

(i) *Strains and culture techniques*

Throughout this analysis the Colonia strain (*CL*) of *Physarum polycephalum* was used. Here the occurrence of heat-sensitive mutants in the plasmodia as well as in the amoebae can be studied (Wheals, 1970; Cooke & Dee, 1974).

Myxamoebae were maintained with live *E. coli* on 2% agar plates with 0·05% liver infusion. Plasmodia formed on 1·5% agar plates made up with 10–20% semidefined medium (Dee & Poulter, 1970). They were maintained on agar plates with 50% semidefined medium or in liquid shake culture on 15–20 ml semidefined medium in 250 ml baffled Erlenmeyer flasks, rotating at 160 rev/min. Permissive temperature is 25 °C for both plasmodia and myxamoebae. 30·5 °C was chosen as non-permissive temperature for the myxamoebae, while 32 °C was used for the plasmodia. This difference was chosen because wild-type plasmodia appear to grow at somewhat higher temperature than wild-type myxamoebae (Fig. 1).

(ii) *Mutagenesis*

Cells were treated in the following way; *CL* amoebae growing logarithmically were flooded on the plate with 4 ml of 0·05 M phosphate buffer (pH 7) containing NG at 100 μg/ml (freshly diluted from stock solution stored at -20 °C). Cells were treated for 60 min and then harvested, washed in water and plated on liver infusion plates for recovery and fixation of putative mutations. Viability was usually 50%. Cells were allowed to grow for 24–48 h after mutagenesis before being harvested, washed and resuspended in water.

(iii) *Enrichment*

(*a*) *Bromodeoxyuridine – suicide enrichment*

Mutagenized cells were plated at 30·5 °C with bromodeoxyuridine and allowed to grow at this non-permissive temperature until most cells had divided 2–3 times, as judged by inspection in the dissecting microscope. They were then irradiated with long-wave UV light (Westinghouse, FS20) under two plastic petri-dish lids. (For details of methods, see Haugli & Dove, 1972.) Cells were harvested and replated to recover for 24–28 h at 25 °C.

(*b*) *Netrospin suicide enrichment*

Mutagenized cells were plated on Millipore membranes resting on stainless-steel grids in glass petri dishes filled with 15 ml of 0·05% liver infusion. They were allowed to enter growth phase (24 h) at 25 °C and were then shifted for 6–8 h to 30·5 °C. Next, Netropsin was added to the medium at 20 μg/ml and incubation continued at 30·5 °C for 24 h (Gorman & Dove, 1974).

After bromodeoxyuridine or netropsin enrichment, surviving cells either underwent repeated cycles of enrichment or were plated 100 at a time on a large number of liver infusion plates for colony formation and screening for mutants.

(iv) *Pre-screening of amoebae*

For pre-screening of potential heat-sensitive mutants, the colonies were allowed to develop at 25 °C until the diameter was about 0·5 mm. The plates were then shifted to 30·5 °C and incubation continued for 3 days. At this time a fraction of the colonies were still small, while the majority had continued to grow to diameters of 2–3 mm. Amoebal cells which failed to grow further on transfer to 30·5 °C are referred to as 'pre-screened'.

(v) *Screening*

Prescreened amoebae were then screened both as amoebae and plasmodia for temperature sensitivity.

(*a*) *Amoebae*

Amoebae from each clone were spread on duplicate plates. One was incubated at 30·5 °C and the other at 25 °C. The growth on the two plates was then compared.

(*b*) *Plasmodia*

Clones of myxamoebae were transferred by toothpick to 4 ml wells of the Linbro no. FB-16-24 plastic culture tray, filled with 2 ml freshly poured 25% semidefined medium solidified with 1·5% Bactoagar and containing a 7·5 μl droplet of bacterial suspension. Here plasmodia formed uniformly in puddles after 3–4 days at 25 °C. The trays, each carrying 24 clones, were then shifted to 32 °C, incubated for 3 days and plasmodial growth scored. Those clones where plasmodia had not developed further after the temperature shift were rescued and retested for heat sensitivity.

A reduction in growth rate at the non-permissive temperature compared to a wild-type control in three successive tests after the initial isolation was taken as the criterion of temperature sensitivity. During re-testing it was generally observed that a large number of clones which initially appeared temperature-sensitive would generally show wild type growth in one or more of the retests. Such clones were discarded. The exact figures would vary from experiment to experiment but the final number of temperature sensitive retained was always less than one-fifth of the initial number tested. These clones were then further tested to ensure that progeny clones showed the temperature sensitive characteristic. No failures to inherit the characteristic were observed. Clones tested in this fashion were designated as temperature-sensitive mutants.

(vi) *Characterization of heat-sensitive plasmodial mutants*

A preliminary characterization of heat-sensitive plasmodia was done using double labelling with [^{3}H]thymidine (Amersham, Code TRK 120) and [^{14}C]-methionine (Amersham, Code CFA-433) added to shake cultures in semidefined medium at radioactive concentrations 0·5 and 0·2 μCi/ml respectively. Labelling was for 15 min in young cultures, growing at permissive or non-permissive

temperature, and was stopped by adding TCA to 5%. The samples were washed twice in ice-cold 2·5 ml 5% TCA in acetone and twice in 2·5 ml 0·25 M PCA. Finally samples were dissolved in 0·4 N-NaOH, counted in Diluene (Packard) in a Packard liquid scintillation counter to yield [^{3}H] and [^{14}C]acid insoluble counts to construct growth curves or to calculate the ratio of ^{3}H to ^{14}C counts at any one time point.

3. RESULTS

(i) *Observations on permissive and non-permissive temperatures*

Choosing the limit-temperature for obtaining heat-sensitive mutants is a question of importance for such an undertaking. In *Physarum* this is a problem because the useful temperature interval is rather narrow, because the wild-type may behave

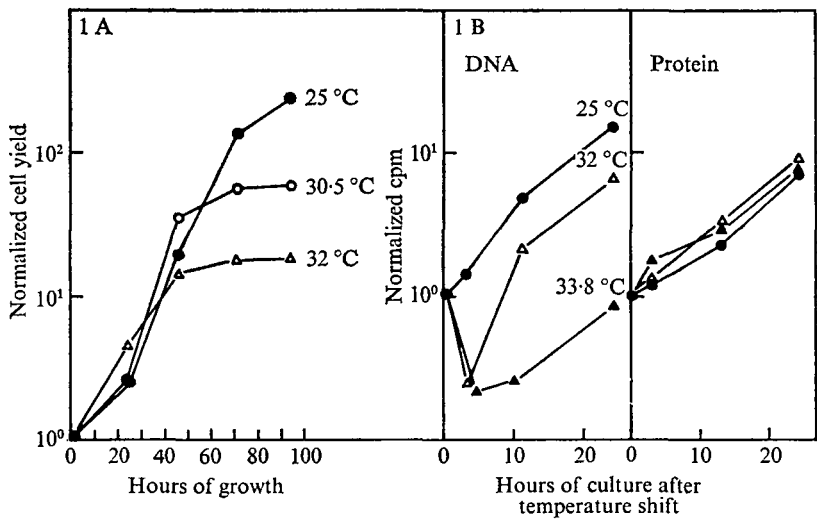

Fig. 1. (A) Amoebae growth curves. Myxamoebae of the Colonia strain were plated 5×10^4 at a time on liver infusion plates with live *E. coli*. At time points indicated duplicate cultures were harvested at each temperature and the total cell number determined by haemacytometer counting. ●——●, 25 °C; ○——○, 30.5 °C; △——△, 32 °C.

(B) Plasmodia growth characteristics. Microplasmodia of the Colonia strain were inoculated at 25 °C. After 16 h cultures were shifted to temperatures shown and pulsed for 15 min with [^{3}H]thymidine and [^{14}]methionine at time points indicated (see Methods section for details). Cultures were split in two halves, and incorporated thymidine and methionine determined by liquid scintillation counting. ●——●, 25 °C; △, 32 °C; ▲, 33·8 °C.

erratically at the upper limit and because limits are higher for plasmodia than for amoebae. Fig. 1A shows growth curves for *CL* myxamoebae and Fig. 1B gives growth characteristics of *CL* microplasmodia at selected temperatures. We conclude that 25 °C is optimal as the permissive temperature for both amoebae and plasmodia. Non-permissive temperature is more problematic to select, but after several trials we concluded that 30·5 °C for the myxamoebae and 32 °C for

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plasmodia are optimal temperatures. When agar plates are used at the high temperature it is essential that they be freshly prepared, that the humidity in the incubator is kept high and that air circulation to each plate is unimpeded.

(ii) *Observations on mutagenesis and mutant frequencies*

Quantitative studies on mutagenesis in *Physarum* to date are limited to those with ultraviolet irradiation and caffeine (Haugli & Dove, 1972). We have used

Table 1. *Yields of heat-sensitive mutants*

(Myxamoebae of the Colonia strain were submitted to various mutagenic and selective regimes shown in the table. NG = nitrosoguanidine treatment. BU = bromodeoxyuridine enrichment. NE = netropsin enrichment. X1 = once, X2 = twice. Am = anoebae. Pl = plasmodia. Am/Pl = both amoebae and plasmodia. – = not tested. 0 = no mutants recovered. Each Roman numeral denotes a population of separately mutagenised amoebae. All amoebal clones which failed to grow at the non-permissive temperature during pre-screening were screened.)

Population code and treatment				Clones pre-screened	Clones screened		Number of mutants			
					Am.	Pl.	Am.	Pl.	Am/Pl.	Total
IB	NG×1	BU×1	—	7150	170	170	12	0	6	18
IIB	NG×2	BU×1	—	3750	90	90	15	4	3	22
IIE	NG×2	BU×2	NE×1	10000	800	800	8	21	4	33
IIC	NG×2	BU×1	NE×1	—	—	1450	—	1	—	1
IA	NG×1	BU×1	—	—	—	1600	—	0	—	0
V	NG×2	—	—	3000	89	89	0	0	0	0
VIA	NG×2	—	—	5000	214	214	0	0	0	0
III	NG×2	—	—	—	—	1000	—	0	—	0
IV	NG×2	—	—	—	—	2100	—	0	—	0
VIB	NG×2	—	—	—	—	1000	—	0	—	0

nitrosoguanidine in the present investigation. Nitrosoguanidine has not, in our hands, increased the frequency of bromodeoxyuridine resistance above the background level of 10^{-5} to 10^{-6} (Lunn, Cooke & Haugli, 1976), but the frequency of cycloheximide resistance was observed to increase from the background level of 10^{-8} (Haugli, Dove & Jiminez, 1972) to a high frequency of 10^{-6} after treatment with NG as described in Methods (Sudbery, unpublished). Also, the frequency of colony size mutants (Haugli & Dove, 1972) has been observed to increase after nitrosoguanidine treatment. Although the information is incomplete, we suggest on available evidence that nitrosoguanidine does increase mutant frequencies in *Physarum*, and so it was used throughout the present investigation.

(iii) *Enrichment and screening for heat-sensitive mutants: analysis of alternative selection and screening regimes*

Having established optimal temperature ranges for both amoebae and plasmodia and efficient mutagenic techniques, a systematic study was undertaken to determine the most efficient combinations of the enrichment, prescreening and screening techniques described in Methods.

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All amoebae were mutagenized by two rounds of NG treatment. They were then subjected to different combinations of enrichment, pre-screening and screening as shown in Table 1. Amoebal populations denoted by the same Roman numeral were derived from survivors of the same mutagenesis.

Populations IB and IIB received a single round of BudR enrichment, while IIE received two rounds of BudR enrichment separated by a single round of netropsin enrichment. All three populations were then pre-screened and screened. Temperature-sensitive mutations acting at the amoebal and/or plasmodial stage were recovered as indicated.

Amoebal population IIC was subjected to a single round of both BudR and netropsin enrichment but the pre-screening stage omitted. As shown the numbers of plasmodial mutants recovered was greatly reduced. The number of amoebal mutants was not tested in this experiment.

It is possible that among those amoebae which grow at 30.5 °C at the pre-screening stage are mutants which are expressed only at the plasmodial stage. To check this possibility a sample of the clones which grew at 30.5 °C during pre-screening and thus normally discarded were screened at the plasmodial stage. No mutants were recovered from this sample (IA).

Lastly, the enrichment stage was omitted (V, VIA) or both the enrichment and pre-screening (III, IV, VIB).

The following conclusions can be drawn from the results:

(1) Some form of suicide enrichment increases the yield of both amobeal and plasmodial mutants. No mutants were obtained in those experiments where amoebal enrichment was omitted. (Sets III, IV, V, VI.)

(2) Pre-screening increases the frequency of recovery of both amobeal and plasmodial mutants. The frequency of recovery of plasmodially expressed mutants among the clones screened rose from 1 in 1500 (set IIC) to approximately 1 in 28 (Sets IB, IIB, IIE) following pre-screening. Furthermore it is clear that there are few plasmodial mutants among the amoebal colonies which grow at 30.5 °C during pre-screening (set IA).

(iv) *Characterization of temperature-sensitive plasmodial mutants*

One of the major objectives in this work was to obtain temperature-sensitive mutants defective in functions of the mitotic cycle. As the suicide agents used are selective against cells in DNA synthesis it was hoped that the mutants obtained would have either a primary block in DNA synthesis or a secondary inhibition due to a block elsewhere in the mitotic cycle. A characteristic of such mutants is that after a shift to the non-permissive temperature, DNA synthesis should stop before protein synthesis.

Mutants were tested for this property by double labelling experiments using shaken liquid cultures of microplasmodia. DNA was labelled with [^{3}H]thymidine and protein with [^{14}C]methionine, one of the essential amino acids for *P. polycephalum*. The experiments were carried out either constructing a growth curve in which increase of protein and DNA were followed at 25 and 32 °C (Fig. 2) or by deter-

mining the ratio of ^{3}H to ^{14}C incorporation at a fixed time after the temperature shift and comparing it with that obtained at 25 °C (Fig. 3). The former approach has the advantage that such ratios can be readily calculated from the growth curves so that more information is obtained. However, such growth curves depend on the use of replicate cultures, all of which must initially contain the same amount of

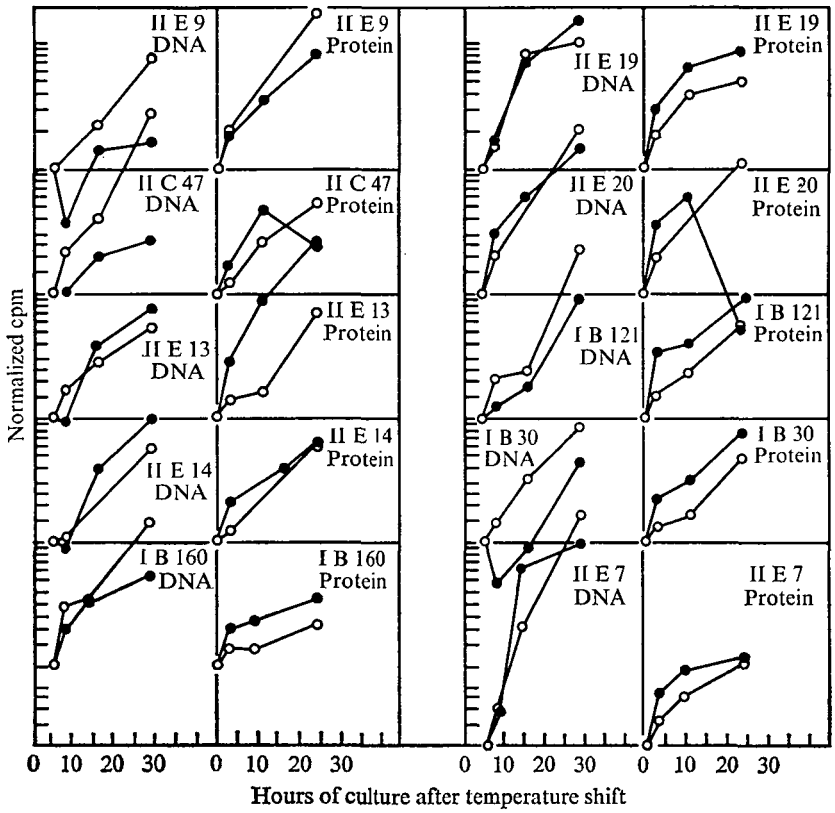

Fig. 2. Patterns of incorporation of [^{3}H]thymidine and [^{14}C]methionine into mutant plasmodia at permissive (25 °C) and non-permissive (32 °C) temperature. Cultures of microplasmodia were inoculated at 25 °C and shifted to permissive and non-permissive termperature after 16 h (time point 0 in the graphs). At 0, 3, 11 and 24 h thereafter, the cultures were pulsed with [^{3}H]thymidine and [^{14}C]methionine, and incorporation determined as described in the Methods section. Wild-type control is shown in Fig. 1 B. Mutant clone is written in each frame. ○——○, 25 °C; ●——●, 32 °C. Left panel: [^{3}H]thymidine (DNA). Right panel: [^{14}C]methionine (protein).

material. The use of ratios is independent of the exact amount of material, and if the ratio alone is determined a larger number of cultures can be handled in the same experiment. For each growth curve replicate 15 ml cultures were prepared and allowed to grow at 25 °C for 16 h to ensure exponential growth. Half the cultures were shifted to 32 °C, and 15 min pulses with [^{14}C]methionine and [^{3}H]thymidine were given at intervals. The reaction was stopped by the addition of 1 ml 100% (w/v) TCA, the culture was divided in two and each half was analysed separately.

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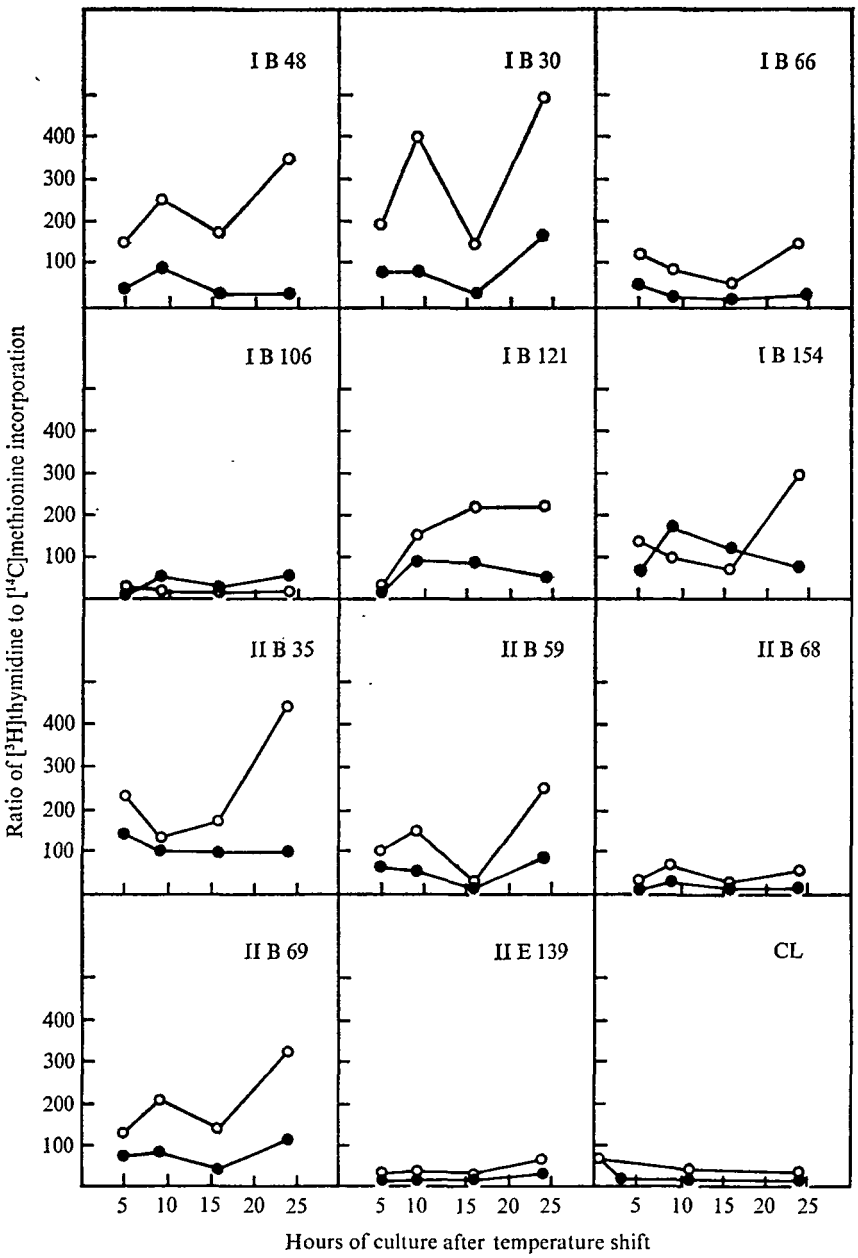

Fig. 3. Ratios of [^{3}H]thymidine to [^{14}C]methionine incorporation in mutant plasmodia at permissive (25 °C) and non-permissive (32 °C) temperature. Cultures of microplasmodia were inoculated at 25 °C and shifted to permissive and non-permissive temperature after 16–24 h (time point 0 in the graphs). At 5, 9, 16 and 24 h, cultures were pulsed with [^{3}H]thymidine and [^{14}C]methionine for 15 min and incorporation determined as described in Methods section. Mutant clone indicated in each frame. ○——○, 25 °C; ●——●, 32 °C.

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Pigment extraction, washings and countings were performed as described in Methods.

After the shift to the non-permissive temperature the wild-type *CL* showed an initial decrease in the rate of DNA synthesis which was not accompanied by a corresponding drop in protein synthesis. This initial drop increased in size as the temperature was raised to 32.0 and 33.8 °C (Fig. 1B). This initial decrease in the rate of [^{3}H]thymidine incorporation was often observed with mutant plasmodia also. In each case protein synthesis was unaffected. This initial decrease causes the ratio of ^{3}H/^{14}C incorporation to be lower at 32 °C compared to 25 °C, which complicated the interpretation of this ratio in mutant strains.

In these incorporation tests, most mutant strains showed no significant change in their pattern of incorporation of [^{3}H]thymidine and [^{14}C]methionine at 32 °C during the 24h duration of the growth curve, compared with that observed in CL wild type. This is presumably because the mutants take more than 2–3 generations to cease growing (assuming a generation time of 8–12 h). On agar plates, where the original classification of wild-type and heat-sensitive plasmodia occurred, considerably more than 2–3 doublings can be accommodated before the scoring of differences in growth takes place. We suggest, however, that useful mutants of the nuclear cycle should show their defect within two generations, and therefore assume that mutants not giving clear response within this time period are of limited value. It can be seen from Figs. 1B, 2 and 3 that the following mutants have features warranting a further study (now in progress). II E9 shows early drop in [^{3}H]thymidine incorporation, while protein synthesis is unaffected at 32 °C.

II C47 shows early drop in both [^{3}H]thymidine and [^{14}C]methionine incorporation at 32 °C.

II E20 shows early, pronounced drop in [^{14}C]methionine incorporation while DNA synthesis appears unperturbed (for the time period studied) at 32 °C.

I B160 shows some drop in the rate of [^{3}H]thymidine incorporation at 32 °C.

DISCUSSION

The objectives of this work were to develop improved methods for the isolation of temperature-sensitive mutants of the plasmodial stage and then to screen such mutants for lesions in the nuclear cycle.

A new technique for obtaining plasmodial temperature-sensitive mutants was developed which combined the advantages of methods used formerly. Amoebal populations were enriched and pre-screened (for definitions of enrichment and pre-screening see Methods), and then screened directly at the plasmodial stage as well as the amoebal stage. In previous work plasmodia were screened which were either derived from mutagenized but non-enriched amoebal populations or from well-defined temperature-sensitive amoebal strains.

From the results reported in Table 1 it is clear that enrichment and pre-screening of amoebae greatly increase the yield of plasmodial temperature-sensitive mutants.

Without enrichment no mutants were recovered from 4400 plasmodia screened but with both enrichment and pre-screening the yield increased to 1 in 28 screened.

Selecting well-defined amoebal temperature-sensitive strains is not profitable when plasmodial temperature-sensitives are required, and such attempts have failed previously (Haugli & Dove, 1972; Wheals, Grant & Jokusch, 1976). From the present study it can be seen that of 48 mutants clearly temperature-sensitive as amoebae only 13 were also temperature-sensitive as plasmodia (Table 1). Many potential mutants will be lost because they are only weakly temperature-sensitive as amoebae and so will be discarded during the amoebal screen. The most efficient overall selection is one that involves enrichment and pre-screening at the amoebal stage followed by large-scale screening at the plasmodial stage.

It should be noted that experiments where both enrichment and pre-screening are omitted are equivalent to the selection procedures of Gingold *et al.* (1976) and Wright (1976). While no mutants were recovered in the present experiments, Gingold *et al.* (1976) obtained yield of 18 temperature-sensitive mutants out of 6000 screened. The difference is almost certainly due to the severity of the mutagenic procedures employed. In the present study two rounds of NG mutagenesis were employed with a 50 % survival rate in each. It was hoped in this way to avoid the problems of double mutations frequently encountered when NG is used as a mutagen. In contrast, the conditions employed by Gingold *et al.* lead to a much lower survival rate of between 0·5 % and 5 % (personal communication), but some of the mutations induced were double mutations (Dee, Grant & Burland, 1976). Presumably stronger mutagenic treatment would have improved yields beyond the 1 in 28 recovered.

By screening both amoebae and plasmodia it is possible to get some idea of the fraction of genetically controlled functions common to both. A minimum estimate is given by the observation that 18 % of the mutations are expressed in both stages. The real value is probably higher than this because some mutations may be only weakly expressed in one stage compared to the other. Such mutations may have been included in those counted as being stage specific.

The temperature-sensitive plasmodial strains were screened for defects in the nuclear cycle by comparing DNA and protein synthesis after a shift to the non-permissive temperature. The results were disappointing. Most strains continued to increase DNA and protein for some time after the shift even though many of them were strongly temperature-sensitive on plates. Several did show changes in incorporation which warrant further study currently in progress.

P.S. was supported by European Science Exchange Post-Doctoral Fellowship.
K.H. was supported by the Norwegian Research Council for Science and the Humanities.

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