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Thymineless elimination of N group plasmids is Res⁻(RII)-dependent and determined by a different gene than the Uvp plasmid phenotype

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SUMMARY

The wild-type N group plasmid RN3, which is phenotypically Res⁺ Mod⁺(RII) is not eliminated by thymine starvation of its bacterial host. Derivatives of RN3 selected for the Res⁻ phenotype are eliminated. The presence or absence of the RII modification specificity does not affect thymineless elimination of RN3 Res⁻ plasmids. A Res⁻Mod(am) RN3 mutant is not eliminated by thymine starvation from either amber suppressing or non-suppressing hosts, suggesting that it carries a cryptic mutation in a novel genetic locus required for elimination. Thymineless elimination is shown to be *recA*⁺-dependent and the presence of the X group plasmid R6K significantly inhibits elimination of RN3 Res⁻Mod⁺. However, since R6K has no effect on two other plasmid-mediated functions of UV protection and UV-induced mutagenesis, which are also *recA*⁺-dependent, it would appear that elimination is determined by a separate plasmid gene than that encoding the UV functions. This is confirmed using derivatives of another N group plasmid R390, which eliminate but which have lost the ability to increase UV-induced mutagenesis in their host.

1. INTRODUCTION

The presence of plasmid pKM101 in strains of *Salmonella typhimurium* used in the Ames test for the detection of carcinogens as mutagens significantly increases the sensitivity of the test (McCann *et al.* 1975). Known carcinogens such as the previously used Japanese food preservative furylfuramide, which did not show positive in the original strains, give a mutagenic response in the plasmid-carrying strains (McCann *et al.* 1975). pKM101 is a derivative of the N incompatibility group plasmid R46, which also increases ultraviolet (UV)-induced mutagenesis in host strains (Mortelmans & Stocker, 1976), and protects host cells against UV irradiation (Drabble & Stocker, 1968; Tweats *et al.*, 1976). The UV-protective and UV-mutator effects of pKM101 and R46 are inducible (Tweats *et al.* 1976; Walker, 1978; Mortelmans & Stocker, 1979) and dependent upon a functional host *recA*-gene (Mortelmans & Stocker, 1976; Tweats *et al.* 1976; Walker, 1977). They are believed to be encoded by a plasmid-borne gene (*uvp*), which increases the host's capacity for error-prone repair (Mortelmans & Stocker, 1979).

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Another phenotypic trait of R46 is that it is 'eliminated' from thymine-requiring host cells starved of thymine (Pinney & Smith, 1971). This 'thymineless elimination' is dependent upon protein synthesis during the period of thymine starvation, and is thus inducible (Pinney & Smith, 1972); it is also *recA*$^+$-dependent (this paper). This suggests that it may be determined by the same plasmid gene product as the *uvp* functions.

Birks & Pinney (1975) tested a total of 25 plasmids from 12 incompatibility groups and found that only N group plasmids lacking the RII (previously *hsp*II) restriction specificity were eliminated by thymine starvation. The wild-type N group plasmid RN3 is phenotypically proficient in both restriction and modification and is not eliminated (Birks & Pinney, 1975). Schlagman & Hattman (1974) have isolated mutants of RN3 that are deficient in the RII restriction and modification specificities. We have used their mutants, and our own Res$^-$Mod$^+$(RII) and Res$^-$Mod$^-$(RII) derivatives (designations as recommended by Novick *et al.* 1976) to demonstrate that the absence of restriction, but not the modification specificity, is indeed a prerequisite for thymineless elimination. We also show that thymineless elimination is encoded by a plasmid gene separate from that responsible for the Uvp phenotype.

2. MATERIALS AND METHODS

(i) *Bacterial strains*

The strains of *Escherichia coli* used are listed in Table 1.

(ii) *Plasmids*

The designation and relevant phenotypes of the plasmids used are listed in Table 2.

(iii) *Phages*

Phage λ was obtained from J. T. Smith, λvir (Jacob & Wollman, 1954) from R. W. Hedges and $\lambda CI857$ (Sussman & Jacob, 1962) from B. Williams.

(iv) *Media*

Davis and Mingioli liquid and solid minimal media (DM) (Davis & Mingioli, 1950) were prepared and supplemented as described by Smith (1967). DM basal salts solution (termed DM base) was used as a diluent for viable counts, washing cultures and preparing suspensions for UV irradiation. MacConkey agar was Oxoid CM7, and Nutrient agar was Oxoid blood agar base (Code CM55). Unless specified in the text, cultures were routinely grown in Oxoid nutrient broth No. 2 (Code CM67). Soft agar overlays for the propagation of phage were nutrient broth solidified with 0·6 % lab M agar.

(v) *Thymine starvation and R plasmid elimination*

This was done as described by Pinney & Smith (1971). Exponential phase cultures were grown in fully supplemented DM liquid medium at 37 °C to about

Table 1. Escherichia coli *strains*

Strain	Genotype	Source	Reference
AB1157	*thr⁻, leu⁻, pro⁻, thi⁻ his⁻, arg⁻, lac⁻, gal⁻, ara⁻, xyl⁻, mtl⁻, tsx⁻, str, Su⁺*	N. Willetts	Howard-Flanders, Boyce & Theriot (1966)
AB2463	As AB1157, but also *recA⁻*	N. Willetts	Howard-Flanders, Boyce & Theriot (1966)
AB2470	As AB1157, but also *recB⁻*	N. Willetts	Howard-Flanders, Boyce & Theriot (1966)
JC5495	As AB1157, but also *recA⁻, recB⁻*	N. Willetts	Howard-Flanders, Boyce & Theriot (1966)
1100	*Su⁺, rgl⁻, endA⁻, thi⁻*	S. Hattman	Hattman, Schlagman & Cousens (1973)
1100 *dcm⁻*	As 1100, but also *dcm⁻*	S. Hattman	Hattman, Schlagman & Cousens (1973)
K12	Strain 58-161/*sp, met⁻, Su⁻*	J. T. Smith	Bachmann (1972)
343/113 *lys⁻*	*lys⁻, arg⁻, thi⁻, nad⁻, Su⁺*	D. J. Tweats	Mohn, Ellenberger & McGregor (1974)

Thymine-requiring derivatives of these strains were obtained by the trimethoprim selection technique of Stacey & Simson (1965).

Table 2. *Plasmids*

Designation	Relevant phenotype	Source	Reference
R46	IncN Ap Sm Su Tc Res⁻ Mod⁻ (RII)	N. Datta	Datta & Hedges (1971)
RN3 Res⁺ Mod⁺	IncN Sm Su Tc Res⁺ Mod⁺ (RII)	N. Datta	Datta & Hedges (1971)
RN3 Res⁻ Mod⁺	IncN Sm Su Tc Res⁻ Mod⁺ (RII)	S. Hattman	Schlagman & Hattman (1974)
RN3 Res⁻ Mod (am)	IncN Sm Su Tc Res⁻ Mod (am) (RII)	S. Hattman	Schlagman & Hattman (1974)
pMT1, pMT2	as RN3, but Res⁻ Mod⁺ (RII)		This paper
PMT3, pMT4	as RN3, but Res⁻ Mod⁻ (RII)		This paper
R390	IncN Ap Cm Sm Su Tc Res⁺ Mod⁺ (RII)	R. W. Hedges	Coetzee, Datta & Hedges (1972)
R390-3, -6, -7	IncN Ap Res⁻ Mod⁻ (RII)	R. W. Hedges	Coetzee, Datta & Hedges (1972)
R390-4, -5	IncN Ap Res⁻ Mod⁺ (RII)	R. W. Hedges	Coetzee, Datta & Hedges (1972)
R6K	IncX Am Sm	N. Datta	Datta & Kontamichalou (1965)

10^8 organisms/ml. They were washed with DM base and suspended at a concentration of about 10^6 organisms/ml in DM liquid medium, which contained all the required supplements except thymine and had been prewarmed to 37 °C. Samples were taken at hourly intervals, diluted in nutrient broth and plated on to MacConkey agar, supplemented with 60 μg/ml thymine. After overnight incubation the R plasmid content of the resulting clones was analysed by replica plating on to supplemented DM agar, with or without either 10 μg/ml tetracycline to test for loss of RN3 and R46, or 10 μg/ml ampicillin for R6K loss. Selected clones that had lost these phenotypes were streaked on to suitably supplemented DM to check that all the resistance markers carried by the plasmid were lost.

(vi) *UV irradiation*

Dose–response curves were obtained using the method described by Tweats *et al.* (1976). Washed exponential phase cultures were resuspended to 10^8 organisms/ml in chilled (4 °C) DM base. 2 ml of the suspension were transferred to a glass Petri dish (5 cm diameter) and irradiated whilst being stirred. The UV-source was a Hanovia model 12 low pressure mercury lamp emitting light at 254 nm. Samples were diluted in nutrient broth, plated on nutrient agar and incubated overnight at 37 °C. Exposure to ambient light was minimized to prevent photoreactivation of UV-induced damage (Marsh & Smith, 1969).

(vii) *UV-induced mutation frequencies*

The method described by Mortelmans & Stocker (1976) was employed using *E. coli* strain 343/113 *lys*$^-$ (Mohn, Ellenberger & McGregor, 1974). The UV-induced reversion frequencies to lysine independence of this strain and of plasmid-containing derivatives were determined. Stationary phase overnight cultures, grown in nutrient broth, were washed and resuspended in DM base to give about 10^8 organisms/ml. Samples were plated on to fully supplemented DM containing 1 % nutrient broth and on to similar medium lacking lysine. The former plates were used to obtain survival levels after exposure to the mutagenic UV dose of 18 J/M^2 and the latter medium for the determination of spontaneous and UV-induced reversion levels. Mutation frequencies were calculated as *lys*$^+$ revertants per 10^7 survivors, corrected for the level of spontaneous reversion.

(viii) *The isolation of Res*$^-$*(RII) mutants of plasmid RN3*

The method used for this isolation procedure was similar to that described by Bannister & Glover (1970). 10 ml of an exponential phase culture of *E. coli* strain 1100 *dcm*$^-$ (*λCI857*) (RN3) were washed and the cells resuspended in 1 ml of 0·1 M-ethyl methane sulphonate (EMS) in 0·85 % saline. This was incubated at 25 °C for 30 min and then 10 ml of saline containing 5 % sodium thiosulphate were added. After centrifugation, the pellet was resuspended in 50 ml nutrient broth and incubated in a 250 ml conical flask overnight at 25 °C. 0·5 ml of this culture was then added to 0·5 ml of a λ suspension, that had been propagated on *E. coli* strain 1100 *dcm*$^-$, to give a multiplicity of infection of unity. After 40 min

incubation at 25 °C to allow lysogenesis, the culture was transferred to 42 °C and left until lysis occurred. Cells carrying RN3 Res⁻ mutants will be more readily lysogenized than those harbouring the restricting wild-type RN3 Res⁺ plasmid. Since lysogenized cells will be protected from *λCI857* heat induction by the presence of the heat-stable wild-type λ repressor, survivors will be enriched for RN3 Res⁻ mutants. A sample of lysed culture was then diluted in nutrient broth, plated on to nutrient agar and incubated overnight at 25 °C to obtain discrete colonies.

Overnight cultures of 50 isolated colonies were grown in nutrient broth at 25 °C and tested for the presence of RN3 Res⁻ plasmids. 20 μl of each culture were placed on to segments of nutrient agar and incubated at 25 °C for 2 h. A suspension of *λvir*, grown on strain 1100 *dcm*⁻, was diluted to give 2×10^4 pfu/ml and a loopful containing about 10^2 pfu was placed into the middle of each patch of culture. After overnight incubation, sectors showing confluent lysis indicated the absence of RII restriction. The plasmid was then transferred from these clones through an intermediate host back to *E. coli* strain 1100 *dcm*⁻ and its restriction and modification characteristics checked by full plate assays of the relative efficiency of plating of λ. Two independent RN3 Res⁻Mod⁺ plasmids were obtained and designated pMT1 and pMT2.

(ix) *The isolation of Mod⁻(RII) plasmids*

Exponential phase cultures, grown in nutrient broth at 25 °C, of *E. coli* strains 1100 *dcm*⁻ (pMT1) and 1100 *dcm*⁻ (pMT2) were washed in saline and resuspended in 0·3 M-EMS in saline. After incubation at 25 °C for 40 min these cultures were washed in saline containing 5% sodium thiosulphate and resuspended in 4·5 ml of nutrient broth. 0·1 ml of this culture was added to 9·9 ml of nutrient broth and incubated overnight. These cultures were plated on to nutrient agar and isolated colonies were checked for R plasmid retention by streaking on to supplemented DM agar containing 10 μg/ml tetracycline.

Fifty isolates from each mutagenized strain were then examined for their RII modification characteristic by propagating a *λvir* phage suspension on each and diluting this to obtain about 2×10^4 pfu/ml. Two sets of nutrient agar plates were prepared, one set overlayed with soft agar seeded with *E. coli* strain 1100 *dcm*⁻ (RN3 Res⁺Mod⁺), the other with *E. coli* 1100 *dcm*⁻ (RN3 Res⁻Mod⁺). A loopful of each *λvir* suspension, containing about 10^2 pfu, was spotted on to a segment of each of the tester plates which were then incubated overnight at 37 °C. Modified phage produced confluent lysis on both plates, unmodified only on the plate seeded with *E. coli* 1100 *dcm*⁻ (RN3 Res⁻Mod⁺). Plasmids whose RII specificity was apparently Res⁻Mod⁻ were transferred through an intermediate host back to the original *E. coli* 1100 *dcm*⁻ strain. *λvir* suspensions prepared from growth on these strains were used in full plate assays to check the Res⁻Mod⁻(RII) phenotype. Two plasmids were isolated that were Res⁻Mod⁻(RII) and were designated pMT3 (isolated from pMT1) and pMT4 (isolated from pMT2).

3. RESULTS

(i) *Thymineless elimination is recA⁺-dependent*

Plasmid R46 and its derivative, pKM101, increase UV-induced mutagenesis and protect against the lethal effects of UV irradiation in host strains that are *recA*⁺, but not in homologous strains that are *recA*⁻ (Monti-Bragadin, Babudri & Samer, 1976; Tweats *et al.* 1976; Walker, 1977). To test whether thymineless elimination of R46 is also dependent upon a functional host *recA* gene, the plasmid was transferred into thymine-requiring derivatives of strain AB1157 *rec*⁺ and its *recA*⁻, *recB*⁻ and *recA*⁻*recB*⁻ mutants.

Table 3. *Elimination frequencies (per cent) of plasmid R46 from* rec⁻thy⁻ *strains of* E. coli *starved of thymine*

Time of thymine starvation (h)	*E. coli* strain			
	AB1157 *rec⁺ thy⁻*	AB2463 *recA⁻ thy⁻*	AB2470 *recB⁻ thy⁻*	JC5495 *recA⁻ recB⁻ thy⁻*
0	0·30 (100)	0·40 (100)	0·30 (100)	0·30 (100)
3	11 (0·48)	0·81 (14·2)	13 (3·9)	0·59 (7·2)
5	0·96 (0·08)	0·33 (4·4)	11 (2·1)	0·68 (3·6)

Figures in parentheses are per cent survival at each time of sampling.

All the thymine-requiring *rec*⁻ mutants were less sensitive to death by thymine starvation than the AB1157 *rec*⁺*thy*⁻ parent strain (Table 3), but after 5 h starvation the viability of all *rec*⁻ strains was reduced to levels that give satisfactory plasmid elimination from *rec*⁺*thy*⁻ strains (Pinney & Smith, 1971, 1972). Optimal plasmid elimination occurred from strain AB1157 *rec*⁺*thy*⁻ after 3 h thymine starvation (Table 3) and after the same period of thymineless death R46 was also eliminated from the *recB*⁻*thy*⁻ strain, even though its survival level was nearly tenfold greater than strain AB1157 *rec*⁺*thy*⁻. In contrast, neither the *recA*⁻*thy*⁻ strain nor the *recA*⁻*recB*⁻*thy*⁻ strain showed plasmid elimination after 3 or 5 h thymine starvation (Table 3). The survival levels of the *recA*⁻*thy*⁻ and *recA*⁻*recB*⁻*thy*⁻ strains after 5 h starvation were similar to those of the *recB*⁻*thy*⁻ strain after 3 h where elimination was observed.

(ii) *The Res⁻(RII) phenotype is required for thymineless elimination of N group plasmids*

Birks & Pinney (1975) tested representative plasmids from 12 incompatibility groups and showed that only N group plasmids were eliminated by thymine starvation of their host. All six N group plasmids that were eliminated lacked the RII restriction specificity, whereas Res⁺ N group plasmids were resistant to thymineless elimination. In addition, all plasmids that eliminated were also Mod⁻(RII). However, since every plasmid tested was wild-type it was not possible to ascribe a definite causal relationship between elimination and the Res⁻Mod⁻ phenotype.

Schlagman & Hattman (1974) have isolated a restrictionless mutant of the N group plasmid RN3. From this they derived a plasmid that is not only Res⁻ but also carries an amber mutation in its modification methylase gene. The phenotype of this latter plasmid will be designated RN3 Res⁻Mod⁺(am) when in an amber suppressing host and RN3 Res⁻Mod⁻(am) when in an amber non-suppressing host. These plasmids provided an opportunity to examine whether the Res⁻ state is indeed a prerequisite for elimination, and also whether the Mod⁻ phenotype is

Table 4. *Restriction by plasmid RN3 Res⁺Mod⁺* of λvir *grown on* E. coli *strains 1100 and K12 harbouring derivatives of RN3 Res⁺Mod⁺*

E. coli strain on which λ*vir* grown	Relative plating efficiency of λ*vir* on *E. coli* strains	
	1100 *dcm*⁻ (RN3 Res⁻ Mod⁺)	1100 *dcm*⁻ (RN3 Res⁺ Mod⁺)
1100 *dcm*⁺	1	$2{\cdot}1 \times 10^{-2}$
1100 *dcm*⁻	1	$3{\cdot}7 \times 10^{-5}$
K12 (R46 Res⁻Mod⁻)	1	$2{\cdot}8 \times 10^{-2}$
1100 *dcm*⁺ (RN3 Res⁻Mod⁺)	1	1
1100 *dcm*⁺ (RN3 Res⁻Mod⁺(am))	1	1
K12 (RN3 Res⁻ Mod⁻(am))	1	$3{\cdot}4 \times 10^{-2}$
1100 *dcm*⁻ (pMT1 Res⁻ Mod⁺)	—	1
1100 *dcm*⁻ (pMT2 Res⁻ Mod⁺)	—	1
1100 *dcm*⁻ (pMT3 Res⁻ Mod⁻)	—	$1{\cdot}2 \times 10^{-5}$
1100 *dcm*⁻ (pMT4 Res⁻ Mod⁻)	—	$0{\cdot}9 \times 10^{-5}$

Relative plating efficiency is the ratio of the titre on the R⁺ strain to that on strain 1100 *dcm*⁻ R⁻.

necessary. To test this, RN3 Res⁺Mod⁺, RN3 Res⁻Mod(am) and R46 Res⁻Mod⁻ were transferred into *E. coli* strains 1100 *dcm⁺thy⁻Su⁺* (Hattman, Schlagman & Cousens, 1973) and *E. coli* K12 *thy⁻Su⁻* (Bachmann, 1972). The restriction and modification phenotypes of the plasmids in these strains were confirmed by propagating λ*vir* on them and titring the resulting phage crops on *E. coli* strains 1100 *dcm*⁻, 1100 *dcm*⁻(RN3 Res⁺Mod⁺), 1100 *dcm*⁻ (RN3 Res⁻Mod⁺) and 1100 *dcm*⁻(RN3 Res⁻Mod⁺(am)). Control λ*vir* suspensions grown on *E. coli* 1100 *dcm*⁺ and *E. coli* 1100 *dcm*⁻ were also plated and it can be seen (Table 4) that the cytosine methylase encoded by the chromosomal *dcm* gene gave some protection against RII restriction. This has previously been demonstrated by Hattman *et al.* (1973). All λ*vir* preparations plaqued on strains 1100 *dcm*⁻ (RN3 Res⁻Mod⁺) (Table 4) and 1100 *dcm*⁻ (RN3 Res⁻Mod⁺(am)) (results not shown) with an efficiency of unity, confirming that both plasmids are indeed Res⁻. The *E. coli* strains 1100 *dcm*⁺(RN3 Res⁻Mod⁺) and 1100 *dcm*⁺(RN3 Res⁻Mod⁺(am)) gave rise to λ*vir* suspensions that were fully protected against RII restriction, whereas phage λ*vir* grown on strains K12 *dcm*⁺(RN3 Res⁻Mod⁺(am)) and K12 *dcm*⁺(R46 Res⁻Mod⁻) were restricted to the same extent as λ*vir* grown on strain 1100 *dcm*⁺R⁻ (Table 4). These results confirm the restriction and modification phenotypes of the plasmids including the amber-suppressible nature of the RN3 Res⁻Mod (am) modification gene.

Plasmid elimination was tested from the *E. coli* 1100 *dcm*$^+$*thy*$^-$ and *E. coli* K12 *dcm*$^+$*thy*$^-$ strains listed in Table 5. The percentage survival and plasmid loss after 3 h thymine starvation is also given in Table 5. As expected, the wild-type RN3 Res$^+$Mod$^+$ plasmid did not eliminate from either host strain, whereas plasmid R46 Res$^-$Mod$^-$ and the mutant plasmid RN3 Res$^-$Mod$^+$ were eliminated from both strains. Thus the selection of a restrictionless derivative of the non-eliminating RN3 Res$^+$Mod$^+$ plasmid results in its thymineless elimination. However, the RN3 Res$^-$Mod(am) plasmid was not eliminated from either *E. coli* 1100 *dcm*$^+$*thy*$^-$, where it is phenotypically Res$^-$Mod$^+$ and might be expected to behave as the RN3 Res$^-$Mod$^+$ mutant, or from *E. coli* K12 *dcm*$^+$*thy*$^-$, where it is phenotypically Res$^-$Mod$^-$ and should eliminate as R46 Res$^-$Mod$^-$.

Table 5. *Thymineless elimination (per cent) of N group plasmids from amber suppressor and non-suppressor hosts*

	Time of thymine starvation (h)	
E. coli thy$^-$ strain	0	3
1100 *dcm*$^+$ *Su*$^+$ (RN3 Res$^+$ Mod$^+$)	0·52 (100)	0·51 (7·6)
1100 *dcm*$^+$ *Su*$^+$ (RN3 Res$^-$ Mod$^+$)	0·16 (100)	11 (11)
1100 *dcm*$^+$ *Su*$^+$ (RN3 Res$^-$ Mod$^+$(am))	0·63 (100)	0·72 (6·1)
1100 *dcm*$^+$ *Su*$^+$ (R46 Res$^-$ Mod$^-$)	0·72 (100)	20 (3·7)
K12 *Su*$^-$ (RN3 Res$^+$ Mod$^+$)	0·21 (100)	0·20 (0·41)
K12 *Su*$^-$ (RN3 Res$^-$ Mod$^+$)	0·28 (100)	10 (1·3)
K12 *Su*$^-$ (RN3 Res$^-$ Mod$^-$ (am))	$<$0·60 (100)	0·30 (0·8)
K12 *Su*$^-$ (R46 Res$^-$ Mod$^-$)	0·32 (100)	15 (1·3)

Figures in parentheses are the per cent survival at each time of sampling.

(iii) *Both Res$^-$Mod$^+$(RII) and Res$^-$Mod$^-$(RII) RN3 plasmids are eliminated*

The RN3 Res$^-$Mod(am) plasmid of Schlagman & Hattman (1974) is not eliminated from either amber suppressing or non-suppressing hosts (Table 5) whereas its Res$^-$Mod$^+$ parent is eliminated from both host backgrounds. The absence of RN3 Res$^-$Mod(am) elimination is not therefore due to a function of its bacterial host strain but may reflect a second cryptic mutation in the plasmid genome at a position distinct to that of the plasmid-encoded modification methylase gene. Therefore to determine whether the amber mutation in the plasmid modification gene was indeed the direct cause of this loss of elimination, or whether a cryptic locus had been affected by the EMS treatment used in deriving this plasmid (Schlagman & Hattman, 1974), four further derivatives of the wild-type RN3 Res$^+$Mod$^+$ plasmid were isolated. These were designated pMT1 and pMT2, which were phenotypically Res$^-$Mod$^+$, and pMT3 and pMT4, which had the Res$^-$Mod$^-$ phenotype and were derived from pMT1 and pMT2 respectively. Completely unmodified phage *λvir*, obtained from growth on *E. coli* 1100 *dcm*$^-$, was not restricted by strain 1100 *dcm*$^-$ carrying pMT1 or pMT2 (results not shown) but was by the strain carrying RN3 Res$^+$Mod$^+$. *λvir* obtained by growth on strains carrying

pMT1 and pMT2 was protected against RN3 Res+Mod+-specified restriction, but pMT3 and pMT4 afforded no such protection (Table 4).

The four pMT plasmids were then transferred into *E. coli* 1100 *dcm*+*thy*⁻ for thymineless elimination testing. Table 6 gives the percentage survival and the percentage R⁻ clones after 3 h thymine starvation of each strain. At this time all four plasmids were efficiently eliminated. Thus, as was previously shown with the RN3 Res⁻Mod+ plasmid, selection of the restrictionless characteristic results in plasmids that are eliminated by thymine starvation. The results in Table 6 also show that a further mutation in the modification gene does not affect plasmid elimination.

Table 6. *Thymineless elimination (per cent) of restriction- and modification-deficient derivatives of plasmid RN3 Res+Mod+ from* E. coli *strain 1100* dcm+thy⁻

	Time of thymine starvation (h)	
E. coli thy⁻ strain	0	3
1100 *dcm*+ (RN3 Res+ Mod+)	0·52 (100)	0·51 (7·6)
1100 *dcm*+ (pMT1 Res⁻ Mod+)	0·21 (100)	18 (5·8)
1100 *dcm*+ (pMT2 Res⁻ Mod+)	0·18 (100)	16 (8·2)
1100 *dcm*+ (pMT3 Res⁻ Mod⁻)	0·23 (100)	21 (4·9)
1100 *dcm*+ (pMT4 Res⁻ Mod⁻)	0·40 (100)	15 (7·5)

The figures in parentheses represent per cent survival.

(iv) *The Res⁻(RII) phenotype is not a requirement for the expression of UV protection and UV mutagenesis mediated by N group plasmids*

Plasmid-mediated UV protection and mutagenesis are *recA*+-dependent (Mortelmans & Stocker, 1976; Tweats *et al.* 1976; Babudri & Monti-Bragadin, 1977; Walker, 1977) and at least partly inducible (Walker & Dobson, 1979). Thymineless elimination is also *recA*+-dependent (Table 3) and inducible (Pinney & Smith, 1972). All three effects involve damage to DNA and may therefore result from the interaction of plasmid gene product(s) with the host's error-prone DNA repair pathway (Witkin, 1976). The expression of thymineless elimination is dependent on the absence of the plasmid-mediated RII restriction specificity (Birks & Pinney, 1975; Tables 5 and 6). However, it was found that the wild-type RN3 Res+Mod+ plasmid and all the Res⁻ (Table 7) and Mod⁻ (results not shown) derivatives of RN3 Res+Mod+ gave a significant increase in UV-induced mutation frequency and a low, but reproducible increase in post UV-survival. Whereas the control plasmid R6K, which is known not to confer UV protection or increase host cell mutagenesis (Molina *et al.* 1979), showed no significant effect (Table 7).

The expression of N group plasmid-mediated UV protection and mutagenesis is therefore not dependent upon the absence of the RII restriction specificity. Indeed the results in Table 7 suggest that the expression of both functions is reduced in its absence. It therefore appears that the Res⁻-dependent property of thymineless

elimination is determined by a separate plasmid gene than that coding for the UV-protective and UV-mutator functions. This is confirmed in the next section.

(v) *The separate expression of plasmid elimination and UV-induced functions*

(*a*) *Plasmid R6K inhibits thymineless elimination but not the expression of the UV-protective or UV-mutator phenotypes*

Pinney & Smith (1971, 1974) have shown that the X-group plasmid R6K not only prevents the transfer of the N group plasmid R46 from strains carrying both plasmids, but also inhibits the thymineless elimination of R46. Strains were therefore constructed carrying plasmids R6K and RN3 Res$^-$Mod$^+$, either singly or

Table 7. *The UV-protective and UV-mutagenic effects mediated by R6K, R46 Res$^-$Mod$^-$, RN3 Res$^+$Mod$^+$ and its Res$^-$ derivatives*

	R6K	R46 (Res$^-$ Mod$^-$)	RN3 (Res$^+$ Mod$^+$)	RN3 (Res$^-$ Mod$^+$)	pMT1 (Res$^-$ Mod$^+$)	PMT2 (Res$^-$ Mod$^+$)
Fold increase in survival after 100 J/M^2 UV compared with the R$^-$ strain*	0·95	88	25	3·2	3·0	5·0
Fold increase in induced mutagenesis after 18 J/M^2 UV compared with the R$^-$ strain†	0·85	9·1	5·0	2·5	2·6	2·4

* Determined in *E. coli* strain 1100 *dcm*$^+$.

† Determined as frequency of reversion to lysine independence in *E. coli* strain 343/113 *lys*$^-$.

together, to test whether R6K also inhibited the expression of the Uvp phenotype carried on RN3 Res$^-$Mod$^+$. Plasmid R6K inhibited transfer (results not shown) and thymineless elimination (Table 8) of RN3 Res$^-$Mod$^+$ but had no significant effect on UV-protection and UV-induced mutagenesis mediated by RN3 Res$^-$Mod$^+$ (Table 8). Therefore even though R6K phenotypically represses N group plasmid transfer and elimination, it has no effect on the expression of plasmid-mediated UV protection or mutagenesis. Hence it would appear that the UV-mutator and protective phenotypes are determined by a different plasmid gene than *elm* (for elimination).

(*b*) *Deletion mutants of R390*

Coetzee, Datta & Hedges (1972) have isolated 12 deletion mutants of another N group plasmid R390. All of these were tested for Res(RII) phenotype and five (R390–3, R390–4, R390–5, R390–6 and R390–7) were found to be Res$^-$. Every Res$^-$ R390 derivative eliminated whereas the parent plasmid R390 Res$^+$ and all

the remaining Res+ derivatives did not. This further underlines the requirement for the Res⁻ phenotype in plasmid elimination. Plasmid R390 has an unusual phenotype in that although conferring UV-induced mutagenesis, it does not protect against the lethal effect of UV (R.J.P., accepted for publication). However, two Res⁻ R390 derivatives, R390–6 and R390–7, were found to have lost the UV-induced mutator activity conferred by R390 (results not shown) which confirms that the *elm* and *uvp* genes are separate.

Table 8. *The influence of R6K on selected phenotypic traits of RN3 Res⁻Mod⁺ in* E. coli *strain 343/113* thy⁻lys⁻

Plasmid content	R+/R⁻ UV protection*	R+/R⁻ UV-induced mutagenesis*	Frequency (per cent) of RN3 Res⁻ Mod+ elimination after 4 h thymine starvation
R6K	1·0	0·85	—
RN3 Res⁻ Mod+	16	2·1	31 (3·5)
RN3 Res⁻ Mod+/R6K	12	1·9	1·7 (2·9)
R6K/RN3 Res⁻ Mod+	10	1·9	1·0 (2·6)

* Obtained as described in Table 7.
The figures in parentheses represent the per cent survival at the time of sampling.

4. DISCUSSION

Plasmid RN3 Res+Mod+ is not eliminated during thymine starvation of its bacterial host (Table 5). However, derivatives of RN3 with the Res⁻Mod+ or Res⁻Mod⁻ phenotypes are eliminated (Tables 5 and 6). This supports the hypothesis proposed by Birks & Pinney (1975) that the restrictionless phenotype is a prerequisite for thymineless elimination. It was therefore perhaps surprising that plasmid RN3 Res⁻Mod(am) was not eliminated from either amber suppressing or non-suppressing hosts (Table 5). To explain this non-elimination, we propose that a further cryptic mutation has occurred within the genome of RN3 Res⁻Mod(am) at a locus (*elm*) responsible for thymineless elimination. The occurrence of single strand breaks in plasmid DNA during thymine starvation (Freifelder, 1969; Hill & Fangman, 1973; Pinney, Bremer & Smith, 1974) and the reduction in burst size of ligase-defective T4 phage in a thymine-starved host carrying plasmid R46 (Tweats, Pinney & Smith, 1974) lead us to suggest that *elm* codes for an endonuclease.

The RII modification system protects against restriction by methylating the 5-C site of specific cytosine residues in the recognition sequence of endonuclease *Eco* RII (Boyer *et al.* 1973; May & Hattman, 1975). If the *Eco* RII endonuclease recognizes uracil as unmethylated cytosine, the misincorporation of uracil could result in the production of pseudo-RII cleavage sites, which, since they are unmethylated, would initiate double strand breakage of plasmid DNA. The plasmid would thus be effectively 'eliminated'. However, since such pseudo-RII cleavage sites would also occur as the result of uracil misincorporation into chromosomal

DNA, the chromosomes of cells carrying 'eliminated' plasmids would also sustain lethal damage. These cells would therefore not be capable of replication into clones suitable for plasmid elimination testing. This may explain why Res+ N group plasmids are not eliminated.

Protection against DNA damage at pseudo-RII restriction sites would be conferred by a plasmid-encoded endonuclease that specifically initiated the removal of uracil groupings misincorporated at such sites. Thus the *elm* gene may code for an endonuclease that is site-specific and acts to initiate the excision repair of misincorporated uracil residues. If specific sites occur at a greater frequency in the plasmid than in the chromosome, the activity of this nuclease may initiate the production of overlapping single strand gaps in plasmid DNA. Since these are effectively double strand breaks, this would lead to demonstration of plasmid elimination.

This model to account for thymineless elimination depends upon the misincorporation of uracil into DNA, and there is now considerable evidence that uracil is indeed misincorporated under thymineless conditions. Pauling & Hanawalt (1965) demonstrated that although semi-conservative DNA replication ceases during thymine starvation, non-conservative replication continues. This has been suggested (Nakayama & Hanawalt, 1975) to be due to replicative repair of single strand breaks introduced into DNA during transcription. The cellular pool of deoxyuridylate increases markedly during thymine starvation (Goulian & Beck, 1966) and Breitman, Maury & Toal (1972) have suggested that under thymineless conditions uracil is misincorporated into DNA during the repair replication process. Misincorporated uracil groups are removed by excision repair, which is initiated by the activity of a uracil-DNA glycosylase (Lindahl, 1974). As a consequence, during thymine starvation a continuous cycle of uracil misincorporation and excision occurs resulting in the net persistence of both DNA-uracil and single strand gaps. Garrett *et al.* (1978) have recently shown that a uracil-DNA glycosylase-deficient *E. coli* strain is resistant to thymineless death, presumably because the occurrence of potentially lethal single strand breaks is reduced in this strain.

Lackey *et al.* (1977) have described an endonuclease associated with plasmid pKM101, a derivative of R46, which requires only Mg^{2+} for activity and produces 3′-hydroxy and 5′-phosphate termini. They claimed that it did not show site specificity, but did not test its activity against uracil-containing DNA. Gates & Linn (1977) have characterized an enzyme from *E. coli* which has similar properties to the pKM101-associated enzyme, but which also shows preferential activity against uracil-containing DNA. This latter enzyme is constitutively expressed, whereas the endonucleolytic activity described by Tweats *et al.* (1974) was inferred to be inducible by thymineless conditions. However, a constitutive enzyme active against uracil-containing DNA would only be effectively observed under the conditions employed by Tweats *et al.* (1974) when uracil has been incorporated: that is, as a result of thymine starvation.

Tweats *et al.* (1976) suggested that R46-mediated UV protection was determined

by the same plasmid-encoded gene that was responsible for thymineless elimination. However, results presented in this paper show that UV-protection and UV-mutagenesis are plasmid functions separable from elimination. The UV-protective and UV-mutator effects of pKM101 and R46 are *recA*⁺-dependent (McCann *et al.* 1975; Mortelmans & Stocker, 1976; Tweats *et al.* 1976; Walker, 1977) and results presented here show that thymineless elimination is also dependent upon a functional host *recA* gene (Table 3). Therefore, if as is suggested, the *elm* gene product preferentially introduces strand breaks in plasmid DNA during thymine starvation, these strand breaks must interact with a *recA*⁺-dependent pathway before plasmid inactivation occurs and elimination is observed.

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