Modification Enhancement by the Restriction Alleviation Protein (Ral) of Bacteriophage λ

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The product of the λ *ral* gene alleviates restriction and enhances modification by the *Escherichia coli* K-12 restriction and modification system. An open reading frame (*orf*) located between genes *N* and *Ea*10 has been assigned to the *ral* gene. We have cloned this *orf* in a plasmid where its transcription is controlled by a thermolabile λ repressor. Inactivation of the λ repressor caused a 1000-fold reduction in K-specific restriction of unmodified λ phage and a 100-fold increase in modification. In minicells transformed with ral^+ plasmids, derepression resulted in the appearance of a polypeptide with a lower mobility than that predicted for a protein encoded by the *orf* attributed to *ral*; in a transcription and translation system *in vitro* DNA from a ral^+ plasmid encoded a polypeptide with the same mobility. This polypeptide was absent when the plasmid DNA carried a mutant *ral* gene. The nucleotide sequence of this mutant gene defined two base changes, one of which inactivates the initiation codon of the *orf*.

The K restriction endonuclease, which is also a K-specific methylase, is encoded by three genes designated *hsdR*, *hsdM* and *hsdS*, although the *hsdR* polypeptide is not essential for the methylase activity. We show that Ral enhances modification in a host strain lacking the entire *hsdR* gene, and lambda phages carrying the *hsdM* and *S* genes modify their own DNA inefficiently in the absence of Ral, despite the fact that derivatives of these phages provide efficient amplification of the K-specific methylase. Our data support a model in which, as a consequence of the interaction of Ral with either the *hsdM* or the *hsdS* polypeptide, the conformation of the enzyme is changed and the efficiency of methylation of unmodified target sites is enhanced. It has been postulated that Ral counteracts Rho, but in our experiments Ral did not relieve transcriptional polarity.

1. Introduction

Many phages can protect themselves against restriction systems present in their hosts. The *Escherichia coli* K-12 restriction and modification system (*Eco*K) can be affected by phages T3, T7 and λ. T3 and T7 each encode a protein that blocks the *Eco*K enzyme activity completely (Spoerel *et al.*, 1979). Ral, the *r*estriction *al*leviation function of λ, differs from these in that it does not block the *Eco*K activity but changes it; not only is restriction severely reduced but modification is strongly enhanced (Zabeau *et al.*, 1980). Expression of Ral by K-modified λ^+ leads to increased survival of superinfecting unmodified phage. The increased survival is not due to recombination between λ^+ and the second phage, since it also happens if the latter phage is unrelated to λ. Unmodified λ^+ is restricted by *Eco*K as efficiently as λral^- because it does not produce Ral in time to protect its own DNA. However, the few λ^+ phage that survive restriction will be fully modified after one round of infection, whereas λral^- phage need multiple cycles to become fully methylated.

The chromosomally encoded *hsd* systems of *E. coli* K-12 and B are allelic and have functionally interchangeable subunits. Complementation analysis identified three *hsd* genes, designated *R*, *M* and *S* (Hubacek & Glover, 1970), the order of which was established as *R*, *M*, *S* after cloning the *hsd* region in λ (Sain & Murray, 1980). The genes are transcribed from two promoters, one upstream from *hsdR* (p_{res}), the other (p_{mod}) between genes *hsdR* and

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M, though it is not clear at present whether transcription of *M* and *S* can occur from p_{res}. The *hsdS* gene codes for the specificity polypeptide, so-called because it determines the specificity of recognition. The products of *M* and *S* together are capable of modification, while the complete *Eco*K complex encoded by *R*, *M* and *S* is needed for restriction. *Eco*K may restrict or modify depending on the state of methylation of the target site (Vovis *et al.*, 1974; Burckhardt *et al.*, 1981). Thus, *Eco*K will modify the second strand of hemimethylated DNA, but usually cuts DNA that includes an unmethylated site.

The role of Ral in the λ life cycle remains unknown, but the *ral* gene has been mapped to a region in the early leftward operon of λ (Debrouwere *et al.*, 1980*a*), and an *orf* of 201 bases between the *N* and *Ea*10 genes has been assigned to it (Ineichen *et al.*, 1981; Sanger *et al.*, 1982). In a strain continuously expressing *ral* from an induced defective λ prophage, generalized recombination was reduced and transcriptional polarity was relieved (Debrouwere *et al.*, 1980*b*). The implication that Ral causes this relief of polarity together with the observation that *rho* strains of *E. coli* show increased modification and decreased restriction led to the suggestion that Ral counteracts Rho and in this way may affect *Eco*K.

Changes in *Eco*K activity have also been reported in *sbc*A strains of *E. coli*. In this case the change is attributed to derepression of a lambdoid cryptic prophage, *rac* (Low, 1973; Kaiser & Murray, 1979), which expresses a *ral*-like gene, *lar* (Toothman, 1981).

We have cloned the *ral* gene in a plasmid expression vector. This plasmid has been used to identify the Ral protein and to elucidate the effect of Ral on *Eco*K and the possible interaction between Rho and Ral.

2. Materials and Methods

(a) *Bacterial and plasmid strains*

Bacterial strains are listed in Table 1, plasmid strains in Table 2. ED8654 was used as host for the preparation of K-modified lysates (λ.K), NM555 for that of unmodified lysates (λ.O). C600 and NM555 were used as standard restriction-proficient (r_K^+) and restriction-deficient (r_K^-) hosts.

(b) *Phage strains*

The vectors, λ*hsd* phages and all the derivatives used are listed in Table 3.

(c) *Media, microbial and general biochemical methods*

Media and general methods have been described by Murray *et al.* (1977). DNA was prepared, analysed and cloned as described by Sain & Murray (1980). β-Galactosidase was assayed as described by Miller (1972).

(d) *Analysis of polypeptides*

Minicells were prepared essentially as described by Reeve (1979). Cells were grown for only 12 to 16 h at 30°C, since prolonged incubation reduced the viability of the cells. Most large cells were removed by a short initial centrifugation step (5 min at 2000 revs/min in a Sorvall GSA rotor) prior to purification of the minicells in sucrose gradients. Initially cells were frozen in liquid N_2 and stored at −70°C, but fresh cells were found to give less background and were used in some experiments. Cells

Table 1
Bacterial strains

Strain	Relevant features	Source, reference or origin†
C600	$r_K^+ m_K^+$	Appleyard (1954)
NM555	*hsd*Δ	Fuller-Pace *et al.* (1985)
ED8654	$r_K^- m_K^+$	Murray *et al.* (1977)
*psu*0	*sup*° *trpE*9777fs	Korn & Yanofsky (1976)
*psu*3	*rho* derivative of *psu*0	Korn & Yanofsky (1976)
W205	*sup*° *trp*/*lacZ* fusion *lac*ΔX174 *trpR*	Mitchell *et al.* (1975)
WL518	*trpE*9777fs derivative of W205	*trpE* transferred *via* WL249 (see Table 3)
MP926	*rho*112 *ilv* (Tn*10*)	S. Garges, for *rho*112 see Inoko *et al.* (1977)
WL537	*rho* 112 derivative of WL518	*rho*112 transferred from MP926 *via* P1
*hdf*4008	Rho⁻ strain	Stitt *et al.* (1980)
*hdf*026	Rho⁻ strain	Stitt *et al.* (1980)
SKB178	Parent of *hdf*026 and *hdf*4008	Stitt *et al.* (1980)
WL581	*hsdR*Δ	NM555 (λWL270)
WA2899	$r_A^+ m_A^+$	Arber & Wauters-Willems (1970)
B251	$r_B^+ m_B^+$	Arber & Dussoix (1962)
C3000*hsd*R	$r_B^- m_B^+$	Zabeau *et al.* (1980)
5K	$r_K^- m_K^+$	Hubacek & Glover (1970)
J53 (R124)	Plasmid R124 encodes *Eco*RIII	Hedges & Datta (1972)
NM144	*Eco*RIII, r_K^-	5K carrying R124 plasmid
WL542	*minA minB*	
NM522	*hsd*Δ(F')	Gough & Murray (1983)
C600 (λ*imm*21)	r_K^+ *imm*21	
NM555 (λ*imm*21)	r_K^- *imm*21	

† No reference or source indicates that the strains were constructed in this laboratory.

Table 2
Plasmids

Plasmid	Relevant features	Source or reference
pCQV$_2$	ColE1 replicon carrying p_R and cI857 of λ	Queen (1983)
pUC13	ColE1 replicon carrying *lacZ* with multiple cloning sites	Messing (1983)
*pral*3	4·2 kb (*bio*214 *ral*$^+$) *Bam*HI fragment in pCQV$_2$ (−) orientation	See Fig. 1
*pral*5	4·2 kb (*bio*214 *ral*$^+$) *Bam*HI fragment in pCQV$_2$ (+) orientation	See Fig. 1
*pral*12	*Bam*HI–*Bgl*II deletion derivative of *pral*5	See Fig. 1
*pral*18	4·2 kb (*bio*214 *ral*18) *Bam*HI fragment in pCQV$_2$ (+) orientation	See Fig. 1
*pral*19	4·2 kb (*bio*214 *ral*18) *Bam*HI fragment in pCQV$_2$ (−) orientation	See Fig. 1
pUC *ral*	400 bp *Hae*III–*Bam*HI *ral*$^+$ fragment in pUC13	See Fig. 1
pUC *ral*18	400 bp *Hae*III–*Bam*HI *ral*18 fragment in pUC13	See Fig. 1

were labelled with [^{35}S]-methionine (~800 Ci/mmol), either for 1 h or during a 2-min pulse followed by a 2-min chase. They were then concentrated by centrifugation, lysed in Tris buffer containing sodium dodecyl sulphate and 1 mM-dithiothreitol, and boiled for 5 min before analysis.

E. coli extracts for transcription–translation experiments *in vitro* (Zubay, 1973, 1980) were obtained from Amersham, U.K. (batch no. 380) and used according to the manufacturer's protocol.

Polypeptides were separated on either 20% or 15% (w/v) polyacrylamide gels (50:1 crosslinking: Laemmli, 1970) in the presence of sodium dodecyl sulphate.

(e) *Restriction alleviation test of* pral *plasmids*

Cells were grown at 30°C until mid-log phase in L broth supplemented with 10 mM-$MgSO_4$ and 50 μg ampicillin/ml. Samples (0·2 ml) were incubated for 25 min at 30°C or 42°C, and infected with phage at a multiplicity of infection of <1. The infected cells were spread in top layer and infective centres counted after overnight incubation at 30°C or 42°C. In all tests λv.K was used as control. Since strains carrying plasmid pCQV$_2$, or its derivatives, are λ-immune at 30°C, λv.O or λ*imm*434.0 was used instead of λ$^+$.O.

(f) *Modification enhancement test of* pral *plasmids*

Cells were grown and infected as described in (e), above. The unmodified *ral*$^-$ phage, λ*nin*L63 *imm*434.0, was used at a multiplicity of infection of 1. The *nin*L63 mutation deletes *ral* (see Fig. 1) and *imm*434 allows growth in λ-immune cells. After phage adsorption at 30°C or 42°C, cells were diluted 100-fold and grown for 70 min at 37°C, or 90 min at 30°C, to complete one cycle of growth. The proportion of progeny phage modified was estimated from the titre on C600 relative to that on NM555. Modification enhancement by the lysogenic strain WL581 was quantified using homoimmune derivatives of C600 and NM555.

Table 3
Phage strains

Strain	Relevant features†	Source or reference‡
λv	Virulent	J. S. Parkinson
λ*bio*214	λ genes *int* to *Ea*10 deleted	Zabeau *et al.* (1980)
λbio214 *ral*18	*ral*$^-$ derivative of λ*bio*214	
λ*bio*7.20 *nin*L63 *imm*434	*ral*$^-$ *imm*434	Salstrom *et al.* (1979) *via* M. Matfield
λNM459	*att*$^+$ vector for λWL263	
λ*ral*$^+$ cI60	*ral*$^+$	Debrouwere *et al.* (1980*a*)
λ*ral*18 cI60	*ral*$^-$	Debrouwere *et al.* (1980*a*)
λNM1091	*hsd*^{r} *ral*18	
λNM1092	*hsd*^{r} *ral*$^+$	
λNM1097	*hsd*^{l} *ral*18	
λNM1098	*hsd*^{l} *ral*$^+$	
λWL263	*hsdR*Δ^r *att*$^+$ *ral*$^+$ cI857	
λWL267	*hsdR*Δ^r *att*$^+$ *ral*18 cI60	
λWL270	*imm*21 derivative of λWL263	
λNM1013	*hsd*B^r *ral*$^+$	Sain & Murray (1980)
λNM1100	*ral*18 derivative of λNM1013	
λNM742	λ*trpE* (*att-red*)Δ *imm*21	Murray *et al.* (1977)
λWL244	cI857 derivative of λNM742	
λWL249	*trpE*9777 derivative of λWL244	
M13mp10	ss DNA phage used for sequencing	Messing (1983)
M13mp11	ss DNA phage used for sequencing	Messing (1983)
T7	*ocr*$^+$	Studier (1969)
T7 *ocr*	*ocr*$^-$	Studier (1969)

† In *hsd*^{l} phages the *hsd* genes are oriented so that the sense strand can be transcribed from p_L; in *hsd*^{r} phages the *hsd* genes are in the alternative orientation. ss, single-stranded.

‡ No source or reference indicates that the strain was constructed in this laboratory.

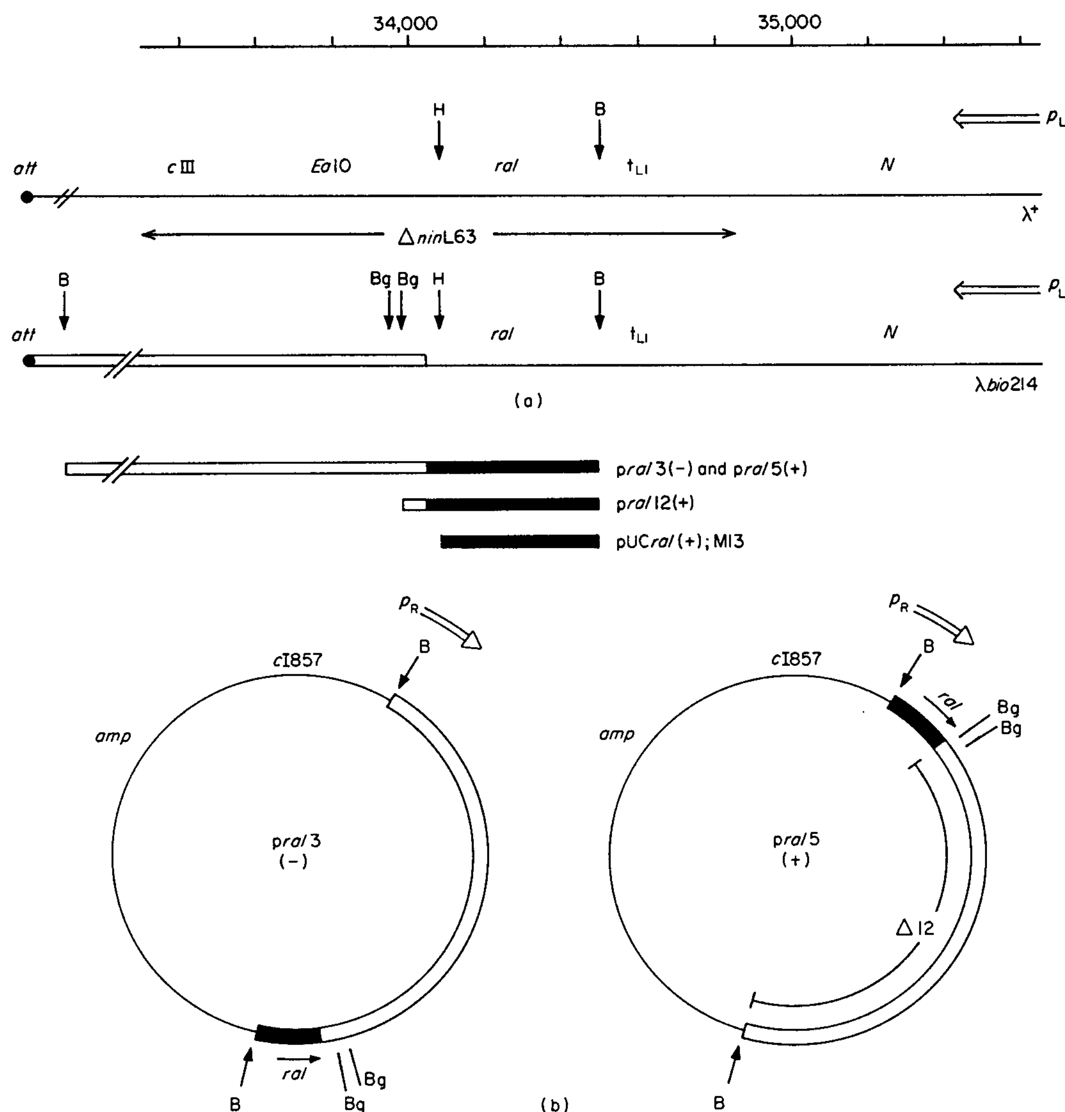


Figure 1. Maps of the relevant regions of λ^+, $\lambda bio214$ and the *ral* plasmids. (a) Partial maps of λ^+ and $\lambda bio214$ are shown. The large arrows identify the direction of transcription from the λ promoter p_L. The *orf* assigned to *ral* is downstream from the transcription terminator, t_{L1}, between genes *N* and *Ea*10. The boundaries of the Ral$^-$ deletion λninL63 are shown below the λ map; the scale above indicates the position of the λ genome in base-pairs. The *bio* substitution in $\lambda bio214$ replaces the λ DNA from *att* (attachment site) to gene *Ea*10. B, H and Bg indicate the targets for *Bam*HI, *Hae*III and *Bgl*II, respectively. The *ral* fragments cloned in plasmids and M13 are drawn below the λ genomes. Open bars indicate *bio* DNA in both λ phages and plasmids; filled bars identify λ DNA cloned in plasmids or M13 vectors. (b) Plasmids p*ral*3 and p*ral*5. The cloned fragment has asymmetrically located *Bgl*II sites. An analysis of *Bgl*II digests indicated that p*ral*5 has the *orf* for *ral* in the clockwise orientation (+), allowing expression; p*ral*3 has the fragment in the alternative orientation (−). The extent of the deletion in p*ral*12 is indicated as Δ12. The open arrow identifies the direction of transcription from p_R at 42°C, the filled bar λ DNA including the *orf* for *ral*, filled arrows the direction in which this *orf* is read and the open bar identifies *bio* DNA.

(g) *DNA sequencing*

The sequence of the *ral*18 mutation was determined using the dideoxy chain termination method described by Sanger *et al.* (1980). The 400 base-pair *Hae*III–*Bam*HI fragments covering *ral* were cloned in M13mp10 and 11.

3. Results

(a) *Cloning the* ral *gene*

The *orf* assigned to *ral* is in the leftward operon of λ between genes *N* and *Ea*10 (Fig. 1). In $\lambda bio214$, the *bio* substitution starts at the phage attachment site and ends just to the left of the putative *ral* gene (Zabeau *et al.*, 1980). A 4·2 kb† DNA fragment, from the *Bam*HI site present in the *bio* DNA to the *Bam*HI site 200 bp upstream from the *orf* (Sanger *et al.*, 1982) (Fig. 1), was cloned in the expression vector pCQV$_2$ (Queen, 1983). This plasmid vector

† Abbreviations used: kb, 10^3 bases or base-pairs; bp, base-pair(s); e.o.p., efficiency of plating; AdoMet, *S*-adenosylmethionine.

Table 4
Restriction alleviation by p*ral5*

Host	Plasmid	Restriction alleviation†			
		λv.O		T7*ocr*.O	
		30°C	42°C	30°C	42°C
C600 ($r_K^+ m_K^+$)	p*ral3*(−)	<2	<2	<2	<2
C600	p*ral5*(+)	~5	3×10^3	$4{\cdot}5 \times 10^1$	4×10^2
C600	pCQV$_2$	None	None	<2	None

† Restriction alleviation is estimated as the ratio of the titre on C600 carrying the designated plasmid to that on C600 itself. At 42°C p_R is derepressed and, in the case of p*ral5*, restriction is alleviated. The r_K^- strain used was NM555; λv.O plated with an e.o.p. of 10^{-4} on C600, T7 *ocr*.O with an e.o.p. of 2×10^{-3}. λv.K plated with equal efficiency on all strains.

allows transcription of cloned genes from the p_R promoter following heat inactivation of the *c*I857 repressor. The plasmid DNAs were digested with *Bgl*II and an analysis of the products showed that p*ral5* includes the fragment in the orientation appropriate for transcription of *ral* from p_R (+), while p*ral3* has the fragment in the opposite orientation (−) (Fig. 1).

(b) *Restriction alleviation by* p*ral5*

To test the effect of Ral on restriction, K restriction-proficient ($r_K^+ m_K^+$) cells carrying p*ral3* (−), p*ral5* (+), or the parental vector, were grown until mid-log phase when samples were induced at 42°C. Both induced and uninduced cells were infected with unmodified phage (λv.O) and infective centres were assayed. The presence of pCQV$_2$, or p*ral3* (−), had no significant effect on the restriction activity (Table 4). In contrast, for cells carrying p*ral5* (+) a shift to 42°C, which was predicted to activate transcription of the putative *ral* gene from p_R, produced a dramatic increase in the efficiency of plating of λv.O. This alleviation of restriction was more than 1000-fold. At 30°C, at which temperature p_R was repressed, there was a slight increase in the e.o.p. of λv.O, suggesting some expression of *ral* at this temperature. A similar alleviation of restriction by p*ral5* was obtained when the λ*ral*⁻ phage, λ*nin*L63 *imm*434.0, was used (data not shown).

The restriction of phage T7 was also tested. Since the product of the *0·3* gene (also called *ocr*) blocks both restriction and modification activity of *Eco*K (Spoerel *et al.*, 1979), T7 *ocr* was used. T7 *ocr*.O had an e.o.p. of approximately 10^{-3} on all control strains, but as with λv.O restriction was alleviated in the strain carrying p*ral5* (Table 4).

(c) *Modification enhancement by* p*ral5*

In the absence of the *ral* gene, unmodified λ is only partially modified by *Eco*K after one cycle of growth in a restriction-deficient, K modification-proficient host ($r_K^- m_K^+$) (Zabeau *et al.*, 1980). The effect of p*ral5* on modification levels was tested by growing unmodified λ*ral*⁻ phage, for one cycle in an $r_K^- m_K^+$ strain carrying p*ral3*, p*ral5*, or pCQV$_2$, and determining the e.o.p. of the progeny phage on an r_K^+ host. The e.o.p. of the progeny phage was 10^{-2} in the absence of Ral (p*ral3* and pCQV$_2$) (Table 5) and approached one when the *ral* gene of p*ral5* was derepressed. Similar results were obtained with T7 *ocr*.O, although the enhancement of modification was less. This may reflect the faster replication rate of T7 compared to that of λ.

Table 5
Modification enhancement by p*ral5*

Phage	Host on which phage were grown	e.o.p. of phage	
		Grown at 30°C	Grown at 42°C
λ*ral*⁻	ED8654 (pCQV$_2$)	4×10^{-3}	1×10^{-2}
	ED8654 (p*ral3*)	8×10^{-3}	2×10^{-3}
	ED8654 (p*ral5*)	6×10^{-2}	9×10^{-1}
T7 *ocr*⁻	ED8654 (pCQV$_2$)	2×10^{-3}	2×10^{-3}
	ED8654 (p*ral3*)	2×10^{-3}	1×10^{-2}
	ED8654 (p*ral5*)	1×10^{-1}	2×10^{-1}

The λ*ral*⁻.O (λ*nin*L63*imm*434) and T7 *ocr*.O phages were grown for one cycle of growth in the $r_K^- m_K^+$ host ED8654 at 30°C or 42°C. The e.o.p. of the phage on C600 (r_K^+) relative to NM555 (r_K^-) is an indication of the modification acquired during the previous single cycle of growth. p*ral5* enhances the modification of λ.O approximately 10-fold at 30°C and 100-fold at 42°C when the *ral* gene is derepressed. The modification of T7 DNA is also enhanced, but less well.

(d) *Time course of Ral induction*

The alleviation of restriction by Ral was checked at intervals following derepression of the *ral* gene. Restriction alleviation was estimated as the ratio of the titre of λv.O on the $r_K^+m_K^+$ strain carrying plasmid p*ral*5 (+) relative to that on the strain carrying p*ral*3 (−) (Fig. 2(a)). Both strains were grown until mid-log phase at 30°C and incubated for various periods at 42°C before infection with λv.O at 30°C. Within one minute of induction nearly a 1000-fold difference in e.o.p. was detected, suggesting a very rapid action of Ral on *Eco*K.

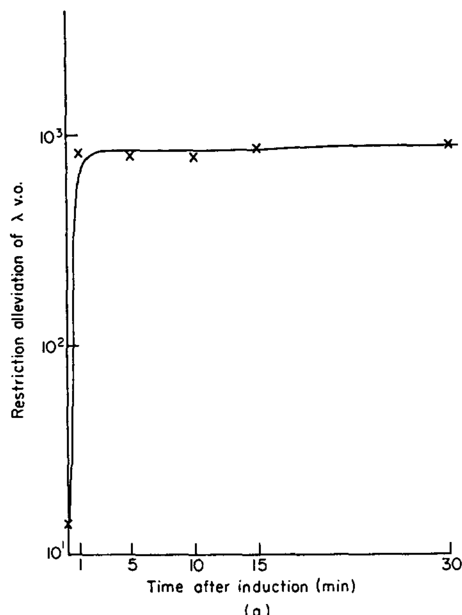
(e) *Stability of the Ral activity*

Ral activity was induced at 42°C in the strain carrying the p*ral*5 plasmid and restriction alleviation was tested at intervals following transfer to 30°C. The Ral effect decreased exponentially during the first 20 minutes (Fig. 2(b)), but even after one hour a tenfold alleviation of restriction was detected (data not shown). This suggests that either the Ral protein is very stable, or that Ral changes *Eco*K and this altered *Eco*K is replaced slowly by new enzyme.

(f) *The sequence of a mutant* ral *gene*

To confirm that the *orf* between *Ea*10 and *N* encodes Ral, this *orf* was cloned from the λ*ral*18 mutant isolated by Debrouwere *et al.* (1980*a*). A *ral*18 derivative of λ*bio*214 was isolated and the 4·2 kb *Bam*HI fragment including the *orf* (see Fig. 1) was cloned in $pCQV_2$. The resulting plasmids, p*ral*18 and p*ral*19, should differ from p*ral*5 and p*ral*3, respectively, only in the *ral*18 mutation. As predicted, irrespective of the orientation of the fragment, no Ral activity was detected (data not shown). The *orf* presumed to be *ral* is within a 400 bp *Hae*III–*Bam*HI fragment (Sanger *et al.*, 1982); this fragment was cloned in M13 and in the plasmid vector pUC13, using both λ*ral*$^+$ *c*I60 and λ*ral*18 *c*I60 as a source of DNA (see Fig. 1). In this plasmid, the *orf* gene is oriented so that it can be transcribed from the *lac* promoter. pUC*ral*, carrying the fragment from λ*ral*$^+$, conferred the Ral$^+$ phenotype to an $r_K^+m_K^+$ host while pUC*ral*18, carrying the fragment from λ*ral*18, did not (data not shown).

The same fragments cloned in M13 were sequenced using the dideoxy chain termination method (Sanger *et al.*, 1980). The sequence of the *ral*$^+$ fragment was identical to the published sequence (Ineichen *et al.*, 1981; Sanger *et al.*, 1982), while that from λ*ral*18 had two changes in the *orf* assigned to *ral*. A transversion from G to T changes the start codon from ATG to ATT, while at base 72 there is a change from G to A. The mutation in the start codon suggests that the λ*ral*18 mutant will lack the Ral polypeptide.



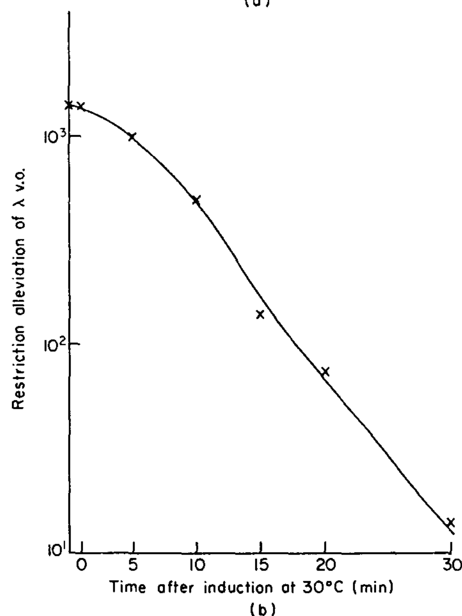


Figure 2. (a) Restriction alleviation following derepression of p*ral*5. The expression of Ral was estimated by the alleviation of restriction of λv.O at various time intervals following derepression of p*ral*5 at 42°C. Restriction alleviation was quantitated at 30°C as the ratio of infective centres following infection of the r_K^+ host, C600, carrying p*ral*5 (+) to those on infection of C600 carrying p*ral*3 (−). Both cultures were treated identically. (b) Stability of Ral activity. Restriction-proficient cells were induced at 42°C, incubated at 30°C and infected with λv.O at increasing time intervals. The restriction alleviation resulting from derepression of p*ral*5 is estimated (at 30°C) as the ratio of infective centres on infection of the r_K^+ host carrying p*ral*5 (+) to those on infection of the r_K^+ host itself.

(g) *Identification of the Ral polypeptide*

A minicell strain (WL542) was used to facilitate the identification of the Ral polypeptide. This strain was transformed with p*ral*5, p*ral*3 and the

parent vector $pCQV_2$. The polypeptides encoded by the plasmids were labelled with [^{35}S]methionine and separated by electrophoresis through sodium dodecyl sulphate/polyacrylamide gels (Fig. 3). A polypeptide of low molecular weight was identified following thermal induction of the strain carrying the *ral*$^+$ plasmid p*ral*5 (Fig. 3(a), lane 6). This polypeptide was not detected following induction of strains carrying either the vector plasmid (Fig. 3(a), lane 2) or the plasmid, p*ral*3, in which the *ral* gene is in the alternative orientation (Fig. 3(a), lane 4). The low molecular weight polypeptide, however, was usually difficult to resolve from a diffuse zone of other products. Additional plasmids were made; p*ral*18 differs from p*ral*5 in that the source of the *ral* gene was the λ*ral*18 mutant, while p*ral*12 is a deletion derivative of p*ral*5 that retains the *orf* for Ral but lacks all but 100 bp of the *bio* DNA (see Fig. 1). Derepression of p*ral*12 induced a polypeptide of the same mobility as that encoded by p*ral*5 (Fig. 3(b), lane 4), while p*ral*18, as expected from the DNA sequence, did not (Fig. 3(b), lane 2).

DNAs from the same plasmids were used in a transcription–translation system *in vitro* (Zubay, 1973. 1980). Those from p*ral*5 and p*ral*12 (Fig. 3(c), lanes 3 and 5) encoded a polypeptide that migrated parallel to the one found *in vivo*, while p*ral*3, p*ral*18 and $pCQV_2$ DNAs, all of which lack a functional *ral* gene, did not (lanes 1, 2 and 4).

The low molecular weight polypeptide that migrates just behind cytochrome *c*-552 (molecular weight 8500) correlates with the expression of a functional *ral* gene, although the mobility of this polypeptide is lower than that expected for the molecular weight of 7600 deduced from the coding sequence.

(h) *The effect of Ral on different type I restriction and modification systems*

Ral affects the closely related type I restriction systems of *E. coli* K-12 (*Eco*K) and *E. coli* B (*Eco*B), but does not affect either *Eco*RI (type II) or *Eco*PI (type III) (Zabeau *et al.*, 1980). Type I restriction systems that may be unrelated to *Eco*K and *Eco*B have been identified in other *E. coli* strains. These include *Eco*A, a chromosomally encoded restriction system (Arber & Wauters-Willems, 1970; Fuller-Pace *et al.*, 1985; Suri & Bickle, 1985), and *Eco*RIII, encoded by plasmid R124 (Firman *et al.*, 1985). Ral did not alleviate the restriction activity of either of these systems although, as expected, restriction by both *Eco*K and *Eco*B was alleviated (Table 6). Failure to affect *Eco*A or *Eco*RIII was not due to a defective p*ral*5, since in each case when the plasmid was transferred to an $r_K^+m_K^+$ strain the Ral$^+$ phenotype was confirmed.

It has been suggested that Ral antagonizes ATPases (Debrouwere *et al.*, 1980*b*). Type I restriction enzymes are ATPases but our data show that not all are affected by Ral.

(i) *Modification enhancement in the absence of the* hsdR *polypeptide*

Ral strongly enhances the modification activity when the $r_K^-m_K^+$ strain carries a mutant *hsdR* gene (Table 5). The *hsdR* mutation used could be a missense mutation and the possibility remains that a restriction polypeptide, even when defective in restriction activity, is essential for Ral to enhance modification. The effect of Ral on modification in a strain deleted for the entire *hsdR* gene was determined. An appropriate r_K^- host was made by lysogenizing NM555, a strain lacking the *hsd* region, with an integration-proficient λ*hsd* phage carrying only the *hsdM* and *S* genes. These *hsd* genes were cloned as a *Sma*I–*Eco*RI fragment (Fig. 4), since the *Sma*I site is only 20 bases upstream from the promoter for the modification genes (Loenen *et al.*, unpublished results). The resulting lysogen, designated WL581, has wild-type modification activity. WL581 was transformed with p*ral*5 and induction of Ral enhanced modification 100-fold (Table 7), a level of enhancement similar to that shown by the *hsdR* mutant strain (see Table 5). A restriction polypeptide is therefore not necessary for the effect of Ral on modification.

Table 6
Alleviation of restriction by systems other than Eco*K*

Bacterial strain	Restriction system	e.o.p. of λv.O†	Restriction alleviation‡	
			30°C	42°C
C600	*Eco*K	10^{-4}	~5	3×10^3
B251	*Eco*B	10^{-5}	~5	9×10^4
WA2899	*Eco*A	5×10^{-3}	None	None
NM144	*Eco*RIII	10^{-2}	None	None

† This e.o.p. is the ratio of the titre of λv.O on the host in the absence of a plasmid to that on the r$^-$ strain NM555.

‡ The restriction alleviation is expressed as the ratio of the titre in the presence of p*ral*5 to that in the presence of p*ral*3. The ratio at 42°C reflects the effect of derepression of the *ral* gene. p*ral*3 has no effect on restriction by any of the strains.

Figure 3. The Ral polypeptide. (a) Polypeptides synthesized in minicells carrying pCQV$_2$, *pral*3(−) or *pral*5(+) were labelled with [^{35}S]methionine at either 30°C or 42°C for one hour. They were separated on a sodium dodecyl sulphate/20% polyacrylamide gel. A low molecular weight polypeptide was detected when the *ral*$^+$ gene was activated (lane 6). The bands corresponding to β-lactamase (processed and unprocessed) and λ repressor are indicated as amp and *c*I respectively. The high molecular weight polypeptide in lanes 5 and 6 relates to the *bio* DNA. cyt identifies the position of the unlabelled cytochrome *c*-552 marker (monomer (1) and dimer (2)) where the molecular weight of the monomer is 8500. The arrow above cyt(1) indicates the putative product of *ral*. (b) Polypeptides synthesized in minicells carrying *pral*12 and *pral*18 were pulse-labelled for 2 min, followed by a 2-min chase. The polypeptides were separated on a sodium dodecyl sulphate/15% polyacrylamide gel. Plasmid *pral*12, though deleted for most of the *bio* DNA, encodes the low molecular weight polypeptide (arrow), whereas *pral*18, the *ral*18 derivative of *pral*5, does not. (c) Polypeptides synthesized *in vitro*. Plasmid DNAs were incubated for 30 min at 37°C with *E. coli* cell-free extracts in the presence of [^{35}S]methionine. The polypeptides were separated through a sodium dodecyl sulphate/15% polyacrylamide gel. The amp polypeptide is not processed *in vitro* and migrates as 1 band. The Ral$^+$ plasmids, *pral*5 and *pral*12, but not the Ral$^-$ plasmids, produced a low molecular weight polypeptide (arrow) of the same mobility as that found *in vivo*. This polypeptide, migrating slightly slower than cyt(1), is Ral.

Table 7
Modification enhancement in the absence of the hsd*R polypeptide*

Bacterial host (*hsdR*Δ)	Plasmid	e.o.p. of phage: Grown at 30°C	Grown at 42°C
WL581	p*ral*3	1×10^{-2}	1×10^{-2}
WL581	p*ral*5	2×10^{-2}	8×10^{-1}
WL581	—	1×10^{-2}	1×10^{-2}

WL581 is an $r_K^- m_K^+$ host in which the entire *hsdR* gene is deleted (see Results, section (i)). The λ*ral*$^-$.O phage, λ*nin*L63*imm*434, was grown for 1 cycle, at either 30°C or 42°C. in WL581 and in derivatives carrying the p*ral* plasmids. The e.o.p. of the resulting phages on the r_K^+ strain, C600, relative to that on the r_K^- strain, NM555, was determined. Ral enhanced modification nearly 100-fold, the same extent as in ED8654, therefore the *hsdR* polypeptide is not involved in Ral-enhanced modification.

(j) *Increased synthesis of methylase does not substitute for Ral*

Incomplete modification of phage DNA could reflect insufficient methylase or that the rate of methylation is limiting. The levels of "self modification" of *ral*$^+$ and *ral*$^-$ phages carrying the *hsd* locus were determined. An *Eco*RI fragment carrying the *hsd* genes of *E. coli* K-12 has been cloned in λ (Borck *et al.*, 1976; Sain & Murray, 1980), and a derivative of this phage has been used to amplify the K-specific methylase (Murray *et al.*, 1982). Enzyme amplification could result simply from an increase in the number of copies of the *hsd* genes, but should be augmented by transcription from the λ promoters. When the *hsd* genes are oriented so that their transcription is leftward (λ*hsd*^{l}), transcription from the early promoter p_L is possible, while in the opposite, rightward, orientation (λ*hsd*^{r}) transcription can occur from the late promoter p'_R (Fig. 4). Transcription from p_L should increase the level of modification enzyme soon after infection by λ*hsd*^{l} phages. After p_L is switched off, transcription of *M* and *S* may continue from the modification promoter, p_{mod}, itself. Modification enzyme can therefore be made from an early stage onwards. In the case of λ*hsd*^{r} phages, convergent transcription from p_L initially blocks expression of *M* and *S* from p_{mod} until p_L is switched off (Ward & Murray, 1979). In this case no significant amount of modification enzyme can be expected until ten minutes after infection, and then transcription of *M* and *S* can occur from either p'_R or p_{mod}. The efficient, orientation-dependent synthesis of the modification enzyme following infection of cells with λ*hsd* phages has been demonstrated, and the levels of the *hsd* polypeptides produced by λ*hsd*^{l} phages were boosted when p_L was derepressed and DNA replication was blocked (Sain & Murray, 1980).

The self modification of isogenic λ*hsd ral*$^+$ and λ*hsd ral*$^-$ phages was assessed by their e.o.p. on an r_K^+ host. The orientation of the *hsd* genes relative to λ promoters had only a minor effect, while the presence of an active *ral* gene enhanced the e.o.p. as much as 100-fold (Table 8). Although infection with a λ*hsd*^{l} phage is presumed to lead to amplification of the methylase, Ral remains essential for efficient modification. Modification, therefore, may not be limited by the level of methylase but rather by the kinetics of methylation. A preferred role for Ral would be one of increasing the affinity of the methylase for its DNA substrate.

(k) *The effect of Rho on* Eco*K*

Debrouwere *et al.* (1980*b*) reported a tenfold drop in restriction activity in the *rho* strain, *rho ts*15, upon a temperature shift to 42°C. We compared the restriction levels of several different *rho* strains with that of their parents and found only a two- or threefold reduction in restriction activity (data not shown). This reduction is small compared to the effect of Ral and could simply reflect the physiological state of *rho* strains. *rho* strains are less vigorous than their parent strains and restriction

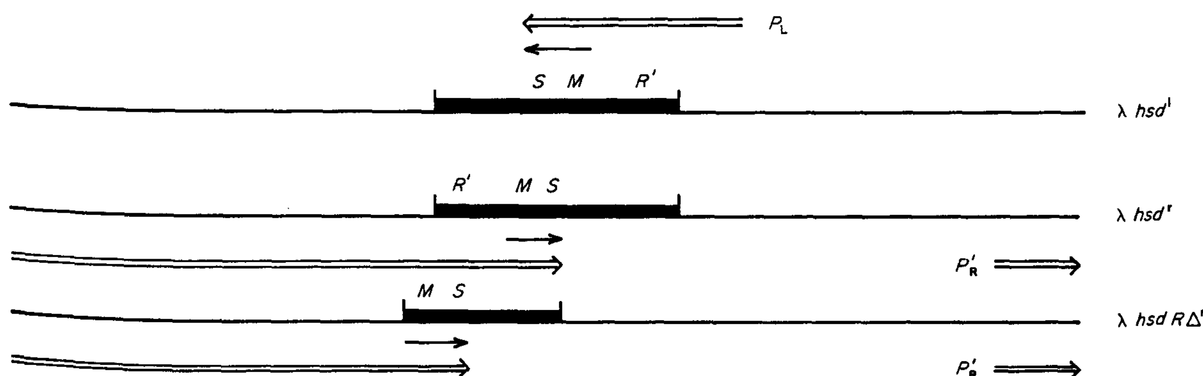


Figure 4. λ*hsd* phages. λ*hsd*^{l} (NM1097 and NM1098) and λ*hsd*^{r} phages (NM1091, NM1092, WL263 and WL270) have the *E. coli* K-12 *hsd* genes in the leftward and rightward orientation, respectively, allowing transcription of *hsdM* and *S* from the early leftward promoter p_L or late rightward promoter p'_R (⇒) as well as from their own promoter (→). *R'* indicates an incomplete *hsdR* gene. In *hsdR*Δ' (WL263 and WL270) *hsdR'* is deleted; a *Sma*I site 20 bp upstream from the *hsdM* gene links the *hsd* fragment to the left arm of the λ vector NM459 (see the text).

Table 8
The effect of Ral on self-modification of λhsd phages

Phage	Relevant characteristics	e.o.p. on C600
λNM1097.0	(*hsd*)^l *ral*18	8×10^{-3}
λNM1098.0	(*hsd*)^l *ral*$^+$	8×10^{-1}
λNM1091.0	(*hsd*)^r *ral*18	2×10^{-3}
λNM1092.0	(*hsd*)^r *ral*$^+$	1×10^{-1}
λWL267.0	(*hsdR*Δ)^r *ral*18	8×10^{-3}
λWL263.0	(*hsdR*Δ)^r *ral*$^+$	3×10^{-1}

Lysates of λ*hsd* K phages were made on the m_K^- host NM555. The e.o.p. of a lysate on the r_K^+ host C600 relative to that on the r_K^- host NM555 is an indication of the efficiency of K-specific self-modification. In λ*hsd*^{l} phages the *hsd* genes are oriented so that their sense strand can be transcribed from p_L, in *hsd*^{r} phage the *hsd* genes are in the alternative orientation. There is a minor difference in modification levels between λ*hsd*^{l} and λ*hsd*^{r} phages but a 40- to 100-fold difference between *ral*$^+$ and *ral*$^-$ phages.

levels are known to be reduced under adverse conditions.

Debrouwere *et al.* (1980*b*) also reported that the modification levels in *rho* strains were affected. The available *rho* strains are K restriction-proficient and therefore cannot be used for K-specific modification tests. Consequently, B-specific self-modification levels of λ*hsd*B phages in various Rho defective strains were checked, since *Eco*B and *Eco*K are similarly affected by Ral (see Table 8). λ*hsd*B *ral*$^-$.K modified its own DNA more efficiently in each of three different *rho* strains than in the isogenic wild-type *E. coli* strains (Table 9). The effect of Rho was only a fewfold for *ral*$^+$ phages, but in the absence of Ral Rho depressed the modification levels as much as 50-fold. In contrast to the minor change in restriction activity, modification is substantially enhanced in *rho* strains.

(l) *Ral and relief of polarity*

Debrouwere *et al.* (1980*b*) proposed that Ral interacts with Rho. The experiments that led to this suggestion used strains carrying defective prophages. Derepression of the *N*, *ral* and *Ea*10 genes, present in the defective prophage of strain M5219, resulted in some relief of transcriptional polarity. In a similar strain in which *Ea*10 and *ral* were deleted, no relief of polarity was found. It was concluded that the relief of polarity was due to Ral.

We have investigated the effect of Ral on the relief of polarity induced by the well-characterized frame-shift mutation *trpE*9777 in strain *psu*0 (Korn & Yanofsky, 1976). The *trpE* strain, *psu*0, was transformed with the p*ral* plasmids, but the resulting strains grew poorly at 42°C when the p_R promoter was activated. Therefore, the *trpE* frame-shift mutation was transferred from *psu*0 *via* a λ*trpE* transducing phage to the *trp*/*lac* fusion strain W205 (Mitchell *et al.*, 1975). In W205 the *lacZ* gene is fused to the distal end of the *trp* operon immediately downstream from *trpA*. Both the *trp* terminator and the *lacZ* promoter are deleted thereby rendering *lacZ* expression dependent on the *trp* promoter. The *trpE* polar mutation, when present in the *trp*/*lac* fusion strain, caused a tenfold reduction in β-galactosidase activity (Table 10).

Table 10
The effect of Ral on transcriptional polarity

Host: Genotype of *trp*/*lac* fusion strain	Plasmid	Enzyme activity† 30°C	Enzyme activity† 42°C
trpE$^+$	—	100	114
trpE fs	—	10	11
*trpE fs rho*112	—	24	29
trpE$^+$	p*ral*3 (−)	100	116
trpE$^+$	p*ral*5 (+)	90	100
trpE fs	p*ral*3 (−)	11	13
trpE fs	p*ral*5 (+)	11	13

† The levels of β-galactosidase were measured at 30°C and at 42°C, when the p_R promoter of the p*ral* plasmid was derepressed. The level in the parental fusion, 1100 units (using the units defined by Miller, 1972), is arbitrarily set as 100%, the other values are expressed relative to this figure. The values are the average from 2 experiments.

Table 9
The effect of Rho on the self-modification of λhsd*B* phage

Host on which λ*hsd*B phage was grown	*ral* genotype of λ*hsd*B phage	e.o.p. on $r_B^+m_B^+$ strain	Rho effect	*ral* genotype of λ*hsd*B phage	e.o.p. on $r_B^+m_B^+$ strain	Rho effect
Rho$^+$ strain WL518	−	2×10^{-3}	50	+	1×10^{-1}	3
*rho*112 derivative WL537	−	1×10^{-1}		+	3×10^{-1}	
Rho$^+$ strain SKB178	−	5×10^{-3}	20	+	2×10^{-1}	<2
Rho$^-$ derivative *hdf*4008	−	1×10^{-1}		+	3×10^{-1}	
Rho$^+$ strain SKB178	−	5×10^{-3}	10	+	2×10^{-1}	<2
Rho$^-$ derivative *hdf*026	−	5×10^{-2}		+	3×10^{-1}	
$r_B^-m_B^+$ (control)	−	8×10^{-1}		+	8×10^{-1}	

λ*hsd*B *ral*$^-$ and *ral*$^+$ phages grown in Rho$^+$ and isogenic Rho$^-$ bacteria were titred on r_B^+ and r_B^- strains; the efficiency of B-specific modification is reflected by the e.o.p. on the r_B^+ strain. Rho antagonizes modification and thus depresses the e.o.p. This is shown as the Rho effect. The phage lysates assayed in this experiment were derived by multiple rounds of infection; single rounds of infection gave the same results (data not shown). Three different *rho*112 strains have shown similar effects (data not shown).

This decrease in activity was relieved by the *rho*112 mutation, confirming the polar effect of the *trpE* frame-shift mutation. If Ral relieves polarity, derepression of p*ral*5 should similarly increase the β-galactosidase activity. No increase was found (Table 10), the β-galactosidase activity was unaffected by Ral, and therefore despite the sensitivity of this test we found no support for the idea that Ral relieves polarity.

4. Discussion

Debrouwere *et al.* (1980*b*), on the basis of the changed activity of *Eco*K in *rho* strains and a relief of polarity attributed to Ral, explained the effect of Ral as the result of a direct interaction between Ral and Rho. They suggested that Rho participates in both the endonuclease and methylase activities of *Eco*K and could be a component of the enzyme complex. If Rho acts by binding *Eco*K, it must in some way stimulate restriction but decrease modification. It is possible that Ral could bind Rho, thereby destabilizing the Rho–*Eco*K complex, or it could compete with Rho for a site in the *Eco*K complex, but there is no evidence for either hypothesis. Since Rho is a known factor for the termination of transcription, an alternative explanation is one in which Rho affects *Eco*K at the transcriptional level. Ral could antagonize the termination function of Rho leading to a Rho^- phenotype. Our experiments do not support this concept of Ral as an antagonist of Rho. Ral encoded by p*ral*5 does not relieve polarity in our *trp*/*lac* fusion strain, therefore Ral does not make a strain phenotypically Rho^-. We do confirm that *Eco*K activity is altered in *rho* strains, but this change is less dramatic than that caused by Ral and modification levels are further enhanced by Ral in Rho^- strains. In the experiments of Debrouwere *et al.* (1980*b*), from which it was concluded that Ral relieves polarity, both *ral* and *Ea*10 are derepressed. *Ea*10 codes for a single strand binding (ssb) protein of unknown function and the possibility remains that the observed relief of polarity was due to the *Ea*10 gene product rather than Ral. In phage T5, gene *D5* encodes a ssb protein that is known to affect both replication and late transcription (McCorquodale *et al.*, 1979).

Several alternative explanations for the effect of Ral on *Eco*K have been suggested (Toothman, 1981; Burckhardt *et al.*, 1981; Krueger & Bickle, 1983). These involve interaction of Ral either with the DNA recognition site or the *Eco*K enzyme itself. The steps in the *Eco*K restriction pathway have been analysed *in vitro* in detail (for a review, see Yuan, 1981), and are probably essentially the same for *Eco*B. AdoMet activates the enzyme (*Eco*K*) that binds the DNA specifically at the s_K site. If the site is fully methylated, the complex dissociates, if hemimethylated it modifies the second strand, and only if the site is unmodified does restriction occur. In the latter situation *Eco*K* undergoes an ATP-dependent conformational change to $EcoK^+$ during which it loses AdoMet. $EcoK^+$ remains bound to the target site while DNA is translocated past the bound enzyme, a process that requires ATP.

Ral is not a methylase with specificity for s_K and s_B sites (Toothman, 1981). Ral could bind to the s_K and s_B sites, but this would require Ral to recognize specifically at least two different DNA sequences. Ral cannot simply affect initial AdoMet binding since this is required for both restriction and modification. It could block activation of *Eco*K* to $EcoK^+$ by preventing either loss of AdoMet or the interaction of EcoK* with ATP. Such mechanisms fail to account for the dramatic effect on modification in the absence of the restriction polypeptide.

Ral, however, could change the affinity of the methylase for the recognition site in either the hemimethylated (m^+/m^-) or the unmethylated (m^-/m^-) state. The modification rate of *Eco*K and *Eco*B *in vitro* is 100-fold faster for m^+/m^- than m^-/m^- DNA (Vovis *et al.*, 1974), and that of the methylase $[(MS)_K]$ moiety of *Eco*K 35-fold faster (Suri *et al.*, 1984). The major effect of Ral on methylation of unmodified λ DNA by the $(MS)_K$ complex (see Table 7) would therefore suggest that Ral enhances the efficiency of methylation of m^-/m^- DNA. Our data require this to occur even in the absence of the restriction polypeptide, and are therefore consistent with a model in which the primary action of Ral is on modification. We predict that Ral interacts with either the $(MS)_K$ complex or one of the subunits, converting $(MS)_K$ to $(MS)'_K$, a methylase with increased affinity for unmodified DNA.

An additional role of Ral in antagonizing restriction is not ruled out. Ral could interfere with restriction in one of several ways: it could physically prevent the R polypeptide from binding to $(MS)'_K$, or even displace the R polypeptide from the *Eco*K complex. Alternatively, the conformational change from $(MS)_K$ to $(MS)'_K$ could prevent the transition from *Eco*K* to $EcoK^+$, or interfere with the ATP binding necessary for this transition.

If Ral affects restriction and modification *via* direct interaction with *Eco*K, we are left to explain the observed changes in *Eco*K activity in *rho* strains. The pleiotropic phenotype of *rho* strains could be due to the partial derepression of several different genes *via* transcriptional readthrough of Rho-dependent terminators. One such gene could be *lar*, the *ral* analogue expressed by the cryptic *rac* prophage in *sbcA* strains (Toothman, 1981). We, however, find evidence of Lar activity in *E. coli* K-12 hosts in the absence of *sbcA* mutations. LE451, a C600 derivative deleted for the *rac* prophage, and hence for the *lar* gene, showed a tenfold increase in restriction activity while the non-isogenic strain AB1157, which also lacks *rac*, restricts λ 30-fold more efficiently than C600 (our unpublished results). In *rho* strains, increased expression of *lar* could lead to further reduction in restriction and enhancement of modification. If the

effect of Rho on restriction is by partial derepression of *lar*, then a *rho* derivative of AB1157 should show the same *Eco*K activity as its Rho+ parent.

The T7 *0·3* protein, which blocks both restriction and modification activity of *Eco*K, has recently been shown to bind *Eco*K at or near the DNA binding site thereby competing with the DNA for the enzyme (Bandyopadhyay *et al.*, 1985). Ral differs from *0·3* protein in that it does not prevent sequence-specific methylation, therefore it must allow binding to the DNA. Identification of the Ral protein should allow its purification, and consequently analysis *in vitro* of the postulated interaction of Ral with the methylase.

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