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Genetic evidence for restriction targets in the DNA of phages λ and φ80

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1. INTRODUCTION

The phenomenon of restriction, recently reviewed in depth by Arber (1968) and by Arber & Linn (1969), is observed as the inactivation of a genome following transfer from one host to another. Thus, phages propagated on one host may form plaques on a second host with an efficiency lower than that on the first. Those progeny phages which do emerge from the second host generally will have become modified so that they are no longer restricted in that host. This modification is not replicated, and is diluted out upon further propagation of the phages in the first host. The ability of a bacterium to restrict or to modify depends upon three linked bacterial cistrons: one required for restriction, the second for modification and the third required for both functions and determining the specificity of each (Glover, Schell, Symonds & Stacey, 1963; Wood, 1966; Boyer & Roulland-Dussoix, 1969; Glover & Colson, 1969).

Restriction of a phage by a bacterium was shown some years ago to be exerted directly upon the DNA of the phage after it is injected into the bacterial cell (Dussoix & Arber, 1962). Restricting enzymes have now been purified and found to act at only a limited number of sites in the target DNA molecule, making double-strand breaks (Meselson & Yuan, 1968; Linn & Arber, 1968; Roulland-Dussoix & Boyer, 1969). Subsequent degradation *in vivo* presumably occurs by non-specific nuclease action on these fragments.

Comparison of the restriction properties of several phages has now led to observations of a genetic nature which confirm the conclusion, based on biochemical evidence, that restriction is directed at target sites localized within the phage genome. The restriction system of *E. coli* K 12 is far more active on phage λ than on the related phage φ 80. The restriction properties of these phages are unaffected by each other in a *trans* complementation test. Rather, the restriction properties can be exchanged only by genetic recombination, behaving as a small set of mappable restriction targets. Sensitivity of λ to K restriction can be lost by genetic deletion.

2. MATERIALS AND METHODS

The strains of *Escherichia coli* and the phages used are listed in Table 1. Bacterial strains possessing different alleles of the B, K 12 and P 1 restriction systems were isolated by Wood (1966) and kindly given to us by Dr Daisy Roulland-Dussoix. These bacteria will be referred to by their restriction class: *E. coli* C and 803 as O, *E. coli* B as B, *E. coli* K 12 as K, and K lysogenic for phage P 1 as K-P 1.

Table 1. *Bacterial and phage strains*

Bacterial strain	Restriction-modification type	Other characters	Reference
C	O	.	.
W 1485	K	F^+ prototroph	.
CR 63	K	λ-resistant	Appleyard *et al* (1956).
B 707	B	gal$^-$met$^-$	Wood (1966)
B 834	O	Mutant of B 707	
K 704	K	gal$^-$met$^-$	
803	O	Mutant of K 704	
K 704 (P 1)	K–P 1	Lysogen of K 704	

Phage strain	Source	Reference
λ PaPa	S. Brenner	Kaiser (1957)
21	S. Brenner	Jacob & Wollman (1961)
ϕ 80	A. Matsushiro	Matsushiro (1963)
ϕ 81	S. Brenner	Yamagishi *et al.* (1965)
82	A. D. Kaiser	Jacob & Wollman (1961)
299	R. L. Baldwin	Jacob & Wollman (1961)
T 5	I. R. Lehman	Adams (1959)
T 7	W. Studier	Adams (1959)
$i^{\lambda}h^{\phi 80}$ = hybrid of λ and ϕ 80		Franklin *et al.* (1965)
$i^{\phi 80}h^{\lambda}$ = hybrid of λ and ϕ 80		Franklin *et al.* (1965)

For measurements of restriction, bacteria were grown in broth (10 g Bactotryptone + 5 g NaCl per litre H_2O). Log phase cultures were centrifuged, washed and resuspended in 10^{-2} M-$MgSO_4$, or preferably in 10^{-3} M-$MgSO_4$ for phages with ϕ 80 host range. Phages were adsorbed to 4×10^8 bacteria/ml. for 15 min at 37 °C and plated at 37 °C.

Phage lysates were prepared by two sequential passages on a given host, using the soft agar layer method. The propagating host is shown following the phage symbol: e.g. ϕ 80.K designates ϕ 80 grown on K.

The titre of the plaques on the propagating host divided by the titre on the test host gives the restriction ratio *R*.

3. RESULTS

Several phages of *E. coli* have been compared in their sensitivity to restriction by strains B, K and K-P 1 (Table 2). Virulent phages T 5 and T 7 are restricted in K-P 1 but not in B or K. The other phages tested, all temperate, are uniformly

restricted in B and in K-P 1, but differ in their sensitivity to restriction in K. Comparable results were obtained in a more extensive survey involving some of the same phages (Eskridge, Weinfeld & Paigen, 1967).

The low restriction of ϕ 80 by K stands in contrast to the strong restriction of λ, although the two phages are closely related in organization and functions (Franklin, Dove & Yanofsky, 1965; Sato *et al.* 1968), in cohesive ends of their DNA molecules (Yamagishi, Nakamura & Ozeki, 1965), and in constituent DNA bases, if not in DNA base distribution (Skalka, 1969). It was therefore of interest to examine the basis of ϕ 80's resistance to restriction in K.

Table 2. *Sensitivity of various phages to B, K and K-P 1 restriction**

	Restriction ratio (R) on:		
Phage	B	K	K-P 1
T 5	−	−	+
T 7	−	−	+
21	+	+	+
ϕ 80	+	10	10^4
ϕ 81	+		+
82	+	+	+
299	+	−	+
λ	+	2000	+

* Phage lysates were prepared by two successive passages on the non-modifying host: O for testing against B or K, and K for testing against K-P 1. Generally R has been roughly estimated by streaking the lysate on the different hosts: + indicates R greater than 100, − indicates R less than 3.

(i) *Nature of the difference in restriction sensitivity between ϕ 80 and λ*

Neither the resistance of ϕ 80 nor the sensitivity of λ to restriction could be attributed to a physiological event.

(1) ϕ 80 is equally restricted by K whether or not it is allowed to function after infection. This was shown by adsorbing ϕ 80.O at low multiplicity to O($i^{\phi 80}h^{\lambda}$) or to K($i^{\phi 80}h^{\lambda}$). The superinfected lysogens were aerated at 37 °C for 30 min, induced with u.v. to derepress both phages, and infective centres plated. Infective centres containing ϕ 80 could be selectively scored since ϕ 80 can plate on CR 63, a K bacterium which is resistant to phage $i^{\phi 80}h^{\lambda}$. The yield of ϕ 80 from the O lysogen was three times greater than the yield from the K lysogen. Thus, the restriction ratio is similar whether ϕ 80 infects an immune or a sensitive host. Since ϕ 80 does not become more sensitive to restriction when it infects an immune cell, rather than a non-immune cell, the resistance of ϕ 80 to K restriction cannot be dependent upon a function of the infecting phage, nor upon the rate at which development of that phage proceeds.

(2) The possibility of a protective modifying function of ϕ 80 was tested by growing λ simultaneously with ϕ 80 in the same bacterial cells. A double lysogen O(λ) (ϕ 80) was induced and allowed to lyse. Progeny phages were found to be restricted by K to the degree characteristic of each phage grown individually,

R = 2000 for λ, R = 10 for ϕ 80. Therefore if ϕ 80 produces a modification function which protects itself against K restriction, that function is not transferable to λ.

(3) No evidence was found for the possibility that ϕ 80 directly antagonizes the K restriction system. This was tested by infecting K($i^{\phi 80}h^{\lambda}$) with ϕ 80 at a multiplicity of 5 as well as with λ.O at a low multiplicity. The λ phages were restricted by K to the same extent as when ϕ 80 was absent.

(ii) *Location of K restriction targets in ϕ 80 and λ*

Because the low sensitivity of ϕ 80 to K restriction was found not to be transmissible to λ nor dependent upon the functioning of the ϕ 80 genome, it became reasonable to postulate that the degree of sensitivity to restriction depends upon a structural feature of the DNA itself. Genetic crosses between λ and ϕ 80 showed that those phages differed by more than a single determinant of sensitivity to restriction. By selecting for the assortment of the immunity (i) and host range (h) characters peculiar to λ or ϕ 80 (Franklin *et al.* 1965), the following recombinant classes were found: $i^{\phi 80}h^{\lambda}$, R = 2000; $i^{\lambda}h^{\phi 80}$, R = 1000; $i^{\lambda}h^{\phi 80}$, R = 30. These crosses between λ and ϕ 80 are complicated by the small degree of genetic homology between the two phages, reflected in the low frequency of recombination. The available evidence indicates that λ and ϕ 80 differ in at least three genetic determinants affecting restriction sensitivity.

Table 3. *Restriction by K of complete or centre-deleted hybrid phages*

Phage	Restriction ratio
$i^{\phi 80}h^{\lambda}$.O	2900, 2200
$i^{\phi 80}h^{\lambda}$ deletion.O	1700, 1600
$i^{\lambda}h^{\phi 80}$.O	1300, 840
$i^{\lambda}h^{\phi 80}$ deletion.O	13, 31

In addition to the recombination evidence for DNA determinants of susceptibility to restriction, there is direct genetic evidence that particular regions of the phage genome are targets for the restriction process. This evidence is found in the loss of restriction sensitivity which accompanies certain deletions of non-essential genetic material from the phage genome. Such deletions can be generated from a temperate phage by abnormal excision of the prophage from its lysogenic state. If one of the two prophage termini is lost, induction of the prophage leads to rare and erratic excision of phage genomes. These may retain all functions needed for vegetative development, and yet lack various amounts of non-essential genome from the region adjacent to the locus of attachment (*att*) to the bacterial chromosome (Franklin, 1967). Deletions of non-essential genome have been obtained from two λ–ϕ 80 hybrid phages, $i^{\lambda}h^{\phi 80}$ (R = 1000) and $i\phi^{80}h^{\lambda}$ (R = 2000) (deletions from the former hybrid were isolated by W. G. Spiegelman). From each hybrid a deletion mutant was examined which had lost about 30 % of the phage genome, or almost all of the non-essential centre region which lies between h and gene N in

the vegetative configuration. Deletion of this centre region from $i^{\phi 80}h^{\lambda}$ had essentially no effect upon its susceptibility to K restriction. Deletion of a similar region from $i^{\lambda}h^{\phi 80}$, however, resulted in a marked decrease in the sensitivity of this phage to K restriction (Table 3). The loss of restriction-sensitivity by deletion of the centre segment of $i^{\lambda}h^{\phi 80}$ is evidence that a target for restriction exists in the DNA near the centre of that (vegetative) genome. Apparently no such target exists in the centre segment of $i^{\phi 80}h^{\lambda}$. The formulation shown in Table 4 could account for the known characteristics of λ and ϕ 80 with respect to restriction by K.

Table 4. *Postulated loci of restriction targets in λ, ϕ 80 and their hybrids*

		General genetic configuration of λ and ϕ 80					
		Loci:	*A* *h*	*att*	*N*	*i*	*R*
Phage	*R*	Segments:	Left	Centre	Right		
λ	2000		+	+	+		
ϕ 80	20		−	−	+		
$i^{\lambda}h^{\phi 80}$	30		−	−	+		
$i^{\lambda}h^{\phi 80}$	1000		−	+	+		
$i^{\lambda}h^{\phi 80}$ deletion	20		−	−	+		
$i^{\phi 80}h^{\lambda}$	2000		+	−	+		
$i^{\phi 80}h^{\lambda}$ deletion	2000		+	−	+		

4. DISCUSSION

The difference between λ and ϕ 80 in susceptibility to restriction by K is seen to depend upon three (or more) reassortable genetic factors. Further, sensitivity to restriction is markedly reduced in a phage whose genome has been partly deleted. Therefore, sensitivity is a positive attribute of phage DNA, and is interpreted as depending upon specific target loci which can be attacked by the specific enzymes of a restriction system.

The characterization of restriction sensitivity as target sites in the genome subject to nuclease attack accounts for observations of the *cis*-dominance of restriction. As reported above, when λ and ϕ 80 grow simultaneously in the same bacterium, each retains its own restriction sensitivity. The restriction character of transducing phages derived from λ and ϕ 80 is similarly pertinent. The transduction of galactose genes to K by λ d*gal*.O is restriction-sensitive (Arber, 1964). On the other hand, the transduction of tryptophan genes by transducing derivatives of a ϕ 80-like phage can be as resistant to K restriction as ϕ 80 itself (Franklin, unpublished). If the *gal* region itself does not include a K restriction target, the presence of restriction targets in the phage portions of λ d*gal* could suffice to impart restriction-sensitivity to the measure of *gal* transduction.

Other evidence for mappable phage loci affecting restriction of λ is emerging. Arber & Linn (1969) place a locus for *E. coli* A restriction between genes cII and O. A site affecting K restriction, corresponding to that covered by the deletion reported above, maps between *red* and *int* (Franklin, unpublished). A second site for K restriction maps close to gene N (N. Murray, pers. comm.), as had been

suggested by Terzi (1968) on the basis of indirect evidence. Mutation of these loci has also been observed (Arber, 1968; N. Murray, pers. comm.). In order to characterize these genetic factors as restriction targets it will be necessary to demonstrate their *cis*-dominance.

Some estimate of the length of restriction targets can be attempted by considering the frequency of targets in a variety of relatively small genomes. If there is no selective pressure maintaining sensitive sites, they may arise simply as random occurrences of certain base sequences. It was observed here and by Eskridge *et al.* (1967) that several phage genomes of size similar to λ (5×10^4 nucleotide pairs) lack sensitivity to K restriction. The K target may therefore be a sequence of eight nucleotides ($4^8 = 6 \times 10^4$). In contrast, few genomes of size as low as 5×10^3 nucleotides lack sensitivity to B or P 1 restriction, so that B and P 1 targets may be the size of a hexanucleotide.

Our analysis of the number of K restriction sites in λ should be compared with the estimates of nucleotide methylation in λ. Arber and Linn (1969) have reviewed the evidence and arguments suggesting that modification may involve the production of N-methyladenine, and that a restriction site may carry a receptor adenine on each DNA strand. Therefore, we would expect that λ would carry six N-methyladenine residues due to K modification (three on each strand). In fact, both λ.K and λ.O carry 100 ± 10 such residues (Gough & Lederberg, 1966), many more than are required for host-induced modifications.

SUMMARY

Temperate phages ϕ 80 and λ grown in *E. coli* strain C differ markedly in the degree to which they are restricted by *E. coli* strain K. The basis of this difference is subject to genetic recombination and deletion. The loss of restriction sensitivity by deletion shows that sensitivity is determined by loci in the phage genome. These loci are interpreted to be targets of the restriction mechanism, in agreement with previous biochemical evidence.

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