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Structural homologies among type I restriction-modification systems

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Structural homologies among different restriction systems of *Escherichia coli* and several *Salmonella* species have been investigated by immunological methods using antibodies prepared against two subunits of the *E. coli* K12 restriction enzyme, and by DNA hybridization experiments using different fragments of the *E. coli* K12 *hsd* genes as probes. The results with both techniques show a strong homology between the *E. coli* K12 and B restriction-modification systems, weaker but nevertheless marked homology between *E. coli* K12 and the *Salmonella* systems SB, SP, and SQ and, surprisingly, no homology between the *E. coli* K12 and A systems.
Key words: DNA-DNA hybridization/immunological cross-reaction/restriction endonuclease genes

Introduction

Three basically different types of restriction and modification systems have been distinguished. The simplest are the so-called type II systems (Boyer, 1971) in which the endonuclease contains a single subunit and the modification methylase is a separate enzyme. Type II restriction enzymes require no cofactor other than Mg^{2+} and they cleave DNA within, or close to, the sequences that they recognise.

In contrast to these are the more complex type I and type III systems, in which the same enzyme catalyses both restriction and modification (for recent reviews, see Modrich, 1979; Yuan, 1981). Type I enzymes contain three different subunits, require ATP, S-adenosylmethionine, and Mg^{2+} for restriction, and in addition to their activities as restriction endonucleases and modification methylases are also DNA-dependent ATPases. Type III enzymes comprise only two subunits, require ATP and Mg^{2+} for restriction, but are not ATPases. While the type III enzymes cleave DNA some 24–27 bases distant from their recognition sequence, type I enzymes cleave DNA several thousands of base pairs away.

Early genetic analyses showed that three chromosomally located genes (*hsd* for *h*ost *s*pecificity *D*NA) encoded the type I restriction and modification systems of *Escherichia coli* K12 and *E. coli* B. Complementation studies led to the hypothesis that the product of one gene, *hsdS*, is necessary for recognition of the DNA sequence specific to the system, that of a second gene, *hsdM*, together with *hsdS*, is required for modification, while the product of the third gene, *hsdR*, together with the other two, is essential for restriction (Boyer and Roulland-Doussoix, 1969; Glover and Colson, 1969; Hubacek and Glover, 1970). More recently the *hsd* genes from *E. coli* K12 have been cloned in phage λ and the study of these λ*hsd* phages entirely corroborates the earlier interpretation. The order of the three closely linked genes is *hsdR, hsdM, hsdS*, and the genes, though organised into two transcriptional units, are transcribed in the same direction (Sain and Murray, 1980). The λ*hsd* phages have provided genetically characterized fragments of the *hsd* genes, and have permitted amplification of their gene products and the consequent production of antisera specific to the subunits of the K restriction enzyme.

Only two type I restriction enzymes have been extensively purified and studied. These are the enzymes *Eco*K and *Eco*B from the *E. coli* strains K12 and B. As expected from the fact that mutational defects in one of these systems can be complemented by wild-type alleles of the other, the enzymes have very similar subunit structures and reaction mechanisms. Both contain three non-identical subunits of mol. wts. ~135 000, 60 000, and 50 000 (Eskin and Linn, 1972; Meselson *et al.*, 1972), which are the products of genes *hsdR, hsdM*, and *hsdS*, respectively (Eskin and Linn, 1972; Sain and Murray, 1980). DNA hybridization experiments confirmed the similarity between the *E. coli* K and B systems (Sain and Murray, 1980). The *hsd* genes for the *E. coli* K and B systems as well as those for the *E. coli* A, *Salmonella typhimurium* SB, and *Salmonella potsdam* SP systems map close to, and counter-clockwise to, *serB* (Boyer, 1964; Glover and Colson, 1969; Arber and Wauters-Willems, 1970; Colson and Van Pel, 1974; Bullas and Colson, 1975). Evidence from complementation tests suggests that the *hsd*SB genes of *S. typhimurium* and the *hsd*SP genes of *S. potsdam* are related to the *hsd*K genes of *E. coli* K12 (Van Pel and Colson, 1974; Bullas *et al.*, 1976).

In this paper we use two independent molecular approaches to ask whether the type I restriction systems of the *Enterobacteriaceae* are related to the *E. coli* K12 system. One technique was again DNA-DNA hybridization using fragments derived from the *hsd*K genes as probes. This method gives information concerning the presence or absence of the structural genes for the enzyme, but does not say whether such sequences are transcribed and translated. The second method was an immunological approach using antibodies directed against two of the three subunits of *Eco*K, the *hsdM* and *hsdR* gene products.

Results

Properties of antibodies prepared against the subunits of EcoK

*Eco*K was purified as described in Materials and methods. Its three subunits were fractionated in amounts sufficient to raise antibodies in rabbits by preparative electrophoresis in polyacrylamide gels containing SDS. The purity of these fractions is shown in Figure 1. Two of the subunits, the *hsdM* and *hsdR* gene products, induced good antibody preparations. The third subunit, the *hsdS* gene product, has so far failed to stimulate antibody production in rabbits, even when the animals were immunised with the intact enzyme.

The reaction of these antibodies with *Eco*K is shown in Figure 2. At high enzyme concentrations a weak cross-reaction is seen between the anti-*hsdM* serum and the *hsdR* gene product. This may indicate that the *hsdM* and *R* subunits have shared determinants, but the more trivial alternative of cross-contamination has not been precluded.

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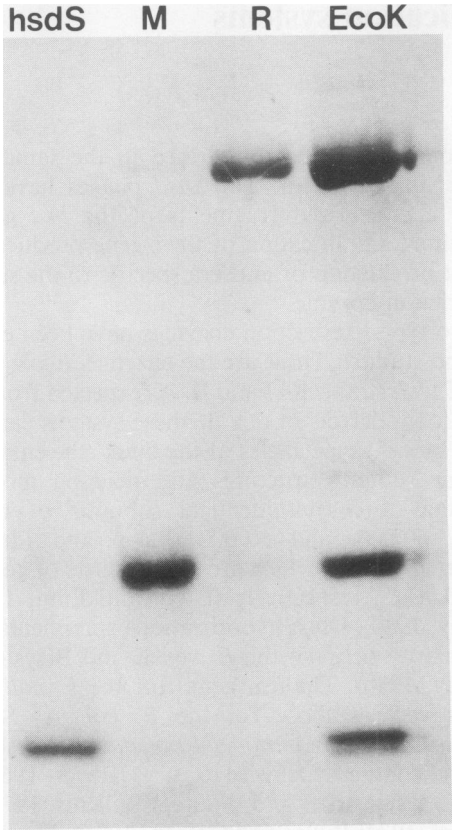

Fig. 1. Isolated subunits of *Eco*K. The three subunits coded by the *hsdS, M,* and *R* genes were purified from *Eco*K as described in Materials and methods and separated by electrophoresis through a polyacrylamide gel containing SDS. The figure shows the gel stained with Coomassie blue. The right-hand lane shows the *Eco*K preparation used for the purification.

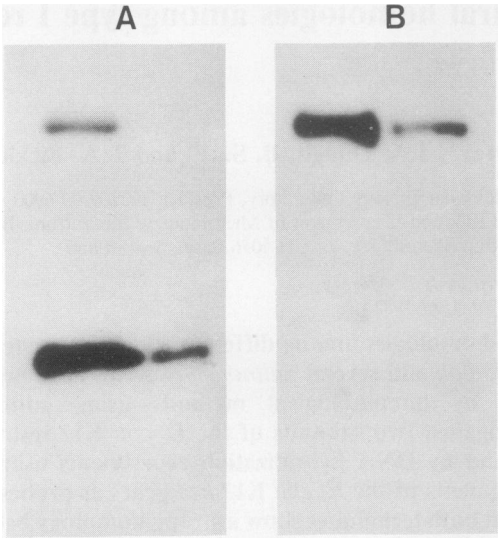

Fig. 2. Cross reaction between the *Eco*K gene products and antibodies prepared against the *hsdM* and the *hsdR* subunits. Either 500 ng (left lanes) or 125 ng (right lanes) of *Eco*K were separated by electrophoresis in an SDS-polyacrylamide gel and analysed by the blotting technique of Towbin *et al.* (1979) with antisera prepared against *hsdM* subunits (panel A) and *hsdR* subunits (panel B).

Cross reactions between anti-hsdK antibodies and other restriction-modification systems

S. typhimurium and *S. potsdam* carry the SB and SP restriction specificities. The SQ specificity originated by recombination between the SB and SP genes (Bullas *et al.*, 1976). *E. coli* K12 strains in which the *hsd*K genes have been replaced by the determinants for SB, SP, or SQ (see Table I) were screened for the presence of material cross reacting with the *Eco*K antibodies. The following *E. coli* strains were also examined: *E. coli* B, known to be allelic in the *hsd* region with *E. coli* K12 (Boyer and Roulland-Dussoix, 1969; Glover and Colson, 1969; Hubacek and Glover, 1970), *E. coli* C which has no known restriction system (Arber and Dussoix, 1962) and whose DNA does not hybridize with *hsd*K probes (Sain and Murray, 1980), and *E. coli* 15T$^-$ which codes for the *Eco*A restriction system (Arber and Wauters-Willems, 1970). An *E. coli* K12 derivative transduced to the A specificity was also used.

Figure 3 shows the results using antisera induced by the *hsdM* gene product, although similar results were obtained using antisera against the *hsdR* polypeptide. Two classes of restriction systems are revealed. One class consists of enzymes with subunits of the same mol. wts. as the *hsdM* and *hsdR* gene products of *E. coli* K12, and which cross react with antibodies prepared against these subunits. Of these, the system found in *E. coli* B gave bands of equal intensity when equal numbers of *E. coli* B and *E. coli* K12 cells were analysed, while the *Salmonella* systems SB, SP, and SQ gave weaker reactions. This last result may indicate that these enzymes are more distantly related to *Eco*K than is *Eco*B. However, the same result would have been obtained if these *Salmonella* enzymes had simply been made in smaller quantities. The second class of enzyme has, so far, only been found in strains expressing the *Eco*A restriction specificity. This system behaves in physiological tests as though it were type I (Lark

Table I.

Species	Strain no.	Specificity systems	Reference
E. coli K12	C600	K	Appleyard (1954)
E. coli K12	NM477(C600*hsd*V)	–	
E. coli C		–	Bertani and Weigle (1953)
E. coli B		B	Arber and Dussoix (1962)
E. coli 15T$^-$		A;P15	Arber and Wauters-Willems (1970)
E. coli	629*	B	
E. coli	E166*	D	
S. typhimurium LT2	4247	SA;SB;LT2	Bullas *et al.* (1976)
S. typhimurium/ *S. potsdam*	L3008	SA;SP;LT2	see L3003 in Bullas *et al.* (1976)
S. typhimurium/ *S. potsdam*	L3004	SA;SQ;LT2	Bullas *et al.* (1976)
E. coli K12	WA960	B	Wood (1966)
E. coli K12	WA2899 and WA2379	A	Arber and Wauters-Willems (1970)
E. coli K12	L4001	SB	Bullas *et al.* (1976)
E. coli K12	L4002	SP	Bullas *et al.* (1976)
E. coli K12	L4003	SP;SA	Bullas *et al.* (1976)
E. coli K12	L4004	SQ;SA	Bullas *et al.* (1976)

*These strains were isolated by K. Cartwright in the Western General Infirmary, Edinburgh; their DNA was the generous gift of K. Kaiser. 629 probably has the same specificity as *E. coli* B while E166 has a novel specificity system (J.A. Gough and N.E. Murray, unpublished data).

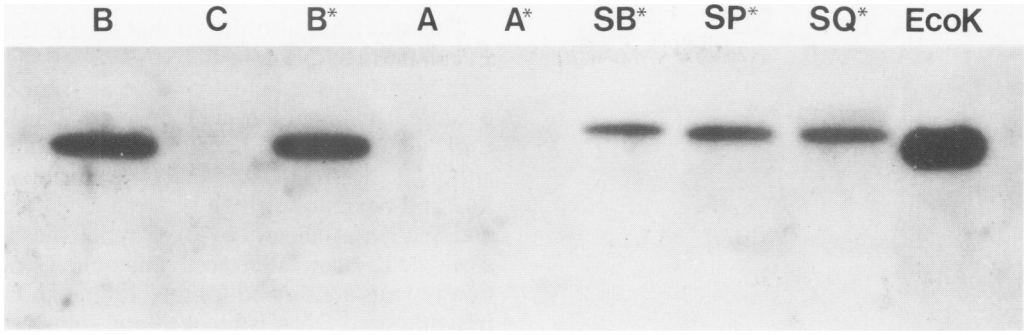

Fig. 3. Analysis of material cross reacting with anti-*hsdM* antiserum. For each strain, 2 x 10^8 cells were analysed for polypeptides cross reacting with antisera to the *hsdM* gene product. The blotting technique of Towbin *et al.* (1979) was used with the modifications described in Materials and methods. The strains are described in Table I. An asterisk indicates that the *hsd* genes have been transduced from the original strain into *E. coli* K12. B is *E. coli* B; C is *E. coli* C; B* is WA960; A is *E. coli* 15T$^-$ and A* is WA2379. SB*, SP*, and SQ* are L4001, L4002, and L4004, respectively.

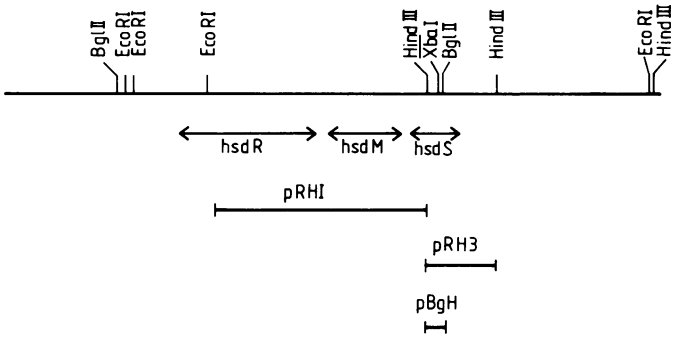

Fig. 4. The *hsd* region of *E. coli* K12. The extent and genetic content of the plasmid derivatives is indicated. Genetic (Sain and Murray, 1980) and DNA sequence (J.A. Gough and N.E. Murray, unpublished data) analyses indicate that the chromosomal DNA fragment within pBgH lies entirely within the *hsdS* gne.

and Arber, 1970), and yet it gave no cross reactivity with any of the *hsd*K antibodies.

Hybridization of the hsdK genes with DNA from other strains

Defined fragments of the *hsd* genes of *E. coli* K12 subcloned in the plasmid pBR322 (Figure 4) were used as probes. These probes were found to share homology with discrete fragments of DNA not only from *E. coli* B, but also from *S. typhimurium, S. potsdam*, and some natural isolates of *E. coli*.

An experiment in which *Eco*RI digests of bacterial DNAs were screened for homologous sequences using pRH1 as a probe illustrates three findings of particular relevance (Figure 5). Firstly, pRH1, which carries all of the *hsdM* gene, >3 kb of *hsdR*, and part of *hsdS*, shares homology with fragments of DNA from *S. typhimurium* and *S. potsdam*. Secondly, the hybridization pattern for the DNA from an *E. coli* K12 transductant having only the relevant *Salmonella* specificity (SB or SP) was identical to that found for the DNA of the *Salmonella* donor strain (e.g., Figure 5: compare tracks for K, SB*, and SA + SB). Clearly, the hybridizing fragments are associated with the *Salmonella hsd* genes. On the basis of many experiments, the intensity of the signal in the hybrid bands is weaker when the *hsd* genes are from *Salmonella* rather than *E. coli* B. Thirdly, in agreement with the immunochemical results, no homologous DNA sequence was detected in a strain expressing the A specificity.

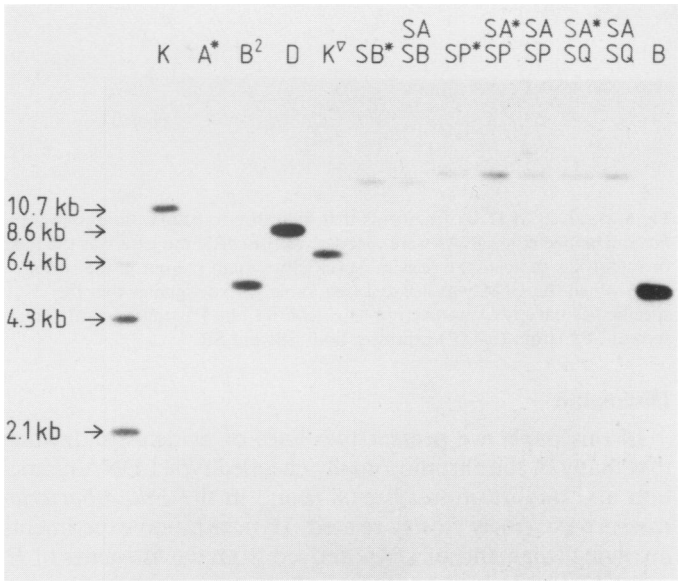

Fig. 5. Analysis of DNA fragments that cross-hybridize to pRH1, the probe for the *hsdR* and *hsdM* genes. The left-hand track contains plasmid (pK13) markers; monomers and oligomers of 2.15-kb unit size (Bouché *et al.*, 1982). The bacterial DNAs were digested with *Eco*RI; the letters at the head of each track indicate the specificity system, or systems, expressed in the strain from which the DNA was isolated; * designates that the specificities were transferred to *E. coli* K12 by P1 transduction (for further details of strains see Table I). K is C600; A*, WA2899; B², 629; D, E166; K▽, NM477 a derivative of C600 in which *hsdM, hsdS,* and part of *hsdR* are deleted; SB*, L4001; SA SB, 4247; SP*, L4002; SA SP*, L4003; SA SP, L3008; SA SQ*, L4004; SA SQ, L3004 and B is *E. coli* B. For the *Salmonella* spp. the hybridizing fragments are clearly characteristic of the *Salmonella* SB and SP systems, and independent of the *Salmonella* SA system present in only some of the strains.

The bacterial DNAs were then hybridized to pBgH, a plasmid carrying the neighbouring sequence from within the *hsdS* gene (see Figure 4). This experiment was particularly important since we had been unable to prepare antibodies against the *hsdS* gene product. All of the DNAs that share homology with the extensive segment of the *hsd* genes present in pRH1 (Figure 5) showed homology with this sequence from the *hsdS* gene (Figure 6), thereby extending to the *hsdS* gene itself evidence for relatedness between the *E. coli* K12 specificity, the *E. coli* B specificity, and the *Salmonella* specificities SB, SP, and SQ. No homology was detected in the DNA of the *Eco*A strain.

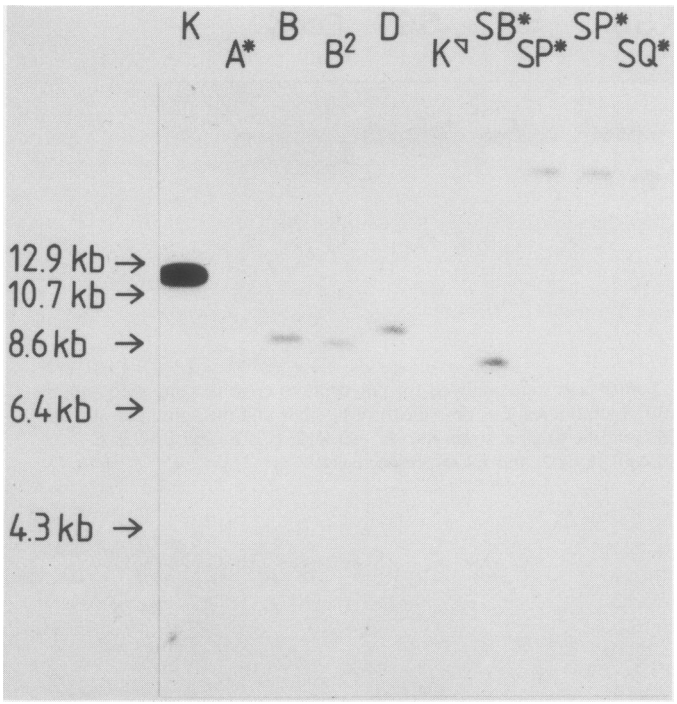

Fig 6. Analysis of DNA fragments that hybridize to pBgH, the probe for *hdsS*. The bacterial DNAs were digested with *Eco*RI; the letters at the head of each track indicate the relevant specificity system present in the strain from which the DNA was isolated (see Table I); * designates that the specificities have been transferred to *E. coli* K12 by P1 transduction. The second SP* strain (L4003) expresses both SA and SP.

Discussion

In this paper we present two lines of evidence indicating that many of the chromosomally encoded type I DNA restriction and modification systems found in the *Enterobacteriacaea* are extremely closely related. Hybridization experiments involving fragments of DNA derived from the *hsd* genes of *E. coli* K12 and restriction digests of chromosomal DNA from the other species revealed cross-hybridizing fragments of different sizes. The intensity of the signal in these cross-hybridizing bands varied. The signals were consistently stronger for fragments of *E. coli* DNA than for *Salmonella* genes when the probe spanned the *hsdR* and *hsdM* genes (see Figure 5). However, when the probe was confined to the *hsdS* gene alone (see Figure 6), the intensities of the bands indicated that even the homology between different *E. coli* strains is far from perfect. Strains whose DNA cross hybridized with *hsd*K probes contained proteins that cross reacted with anti-*Eco*K antibodies. Moreover, the mol. wts. of the cross-hybridizing material were the same as those of the *Eco*K subunits, a result that makes us confident that the material was derived from the restriction enzymes in these strains.

The restriction systems coded by the *hsd*K and *hsd*B genes of *E. coli* strains K12 and B together with the *hsd*SB and *hsd*SP systems of *S. typhimurium* and *S. potsdam*, represent an allelic family of proteins that recognise different DNA sequences. The only other such family known includes the type III restriction enzymes *Eco*P1 and *Eco*P15 (Yuan, 1981). A limited search for homologies among type II restriction enzymes revealed no immunologically detectable relatedness (Imber and Bickle, 1981). The availability of structurally similar proteins that nevertheless bind to different DNA sequences has many possibilities for the study of protein-nucleic acid interactions.

This study has also shown that the restriction system from *E. coli* A, *Eco*A, is unrelated to *Eco*K on the basis of the tests used. *hsd*A was anticipated to be a type I restriction system because it maps in the same region of the *E. coli* genome as *hsd*K and, like *hsd*B, is cotransducible with *serB* and *deoA*, although the frequencies of cotransduction between *E. coli* K12 and A are lower than between *E. coli* K12 and B (Arber and Wauters-Willems, 1970). Physiologically, *hsd*A behaves as a type I system. When cells containing type II or III restriction systems are starved for methionine, modification of newly synthesized DNA is blocked and the DNA is degraded by the restriction enzymes. In contrast, strains having only type I restriction enzymes do not degrade their DNA under these conditions since the activity of type I restriction enzymes has an absolute requirement for S-adenosylmethionine. By this test, the *hsd*A system is type I (Lark and Arber, 1970). However, the enzyme, or enzymes, mediating the A-specificity have never been studied and the possibility exists that *hsd*A is the first member of a new class of restriction systems.

Some members of the *Enterobacteriaceae* have a chromosomally encoded restriction-modification system allelic to that of *E. coli* K12, while others may have host-specificity determinants related to *hsd*A, or no host specificity system whatsoever. The *hsd*A genes have now been cloned and this permits a screen for *hsd*A-related specificities. Currently, it is not known whether most *E. coli* strains have a host specificity system. The *hsd*K genes are not essential since a K12 strain (NM477, see Table I) in which *hsdS, hsdM*, and part of *hsdR* are deleted remains quite viable in the laboratory (H. Senior and N.E. Murray, unpublished data). Presumably, the present variety of *hsd*K-related restriction specificities found in the *Enterobacteriaceae* has a common ancestor. The evolutionary pressures in natural populations that have allowed diversification while conserving the basic genetic organisation and enzyme structure are unknown. Present evidence would suggest that the *hsdM* and *R* genes are relatively conserved, in keeping with the finding that a different *hsdS* polypeptide can confer a new specificity.

Materials and methods

Bacterial and phage strains

The bacterial strains analysed in this work are listed in Table I.

A λ phage carrying the *hsdK* genes has been described previously (Sain and Murray, 1980). In this phage the genes are oriented such that they are transcribed from the strong leftwards λ promoter p_L. With the aim of optimising production of *Eco*K from the cloned *hsd* genes, a *cI*857 *cro*ts *Q*am73 *S*am7 derivative of this phage was made and used to lysogenize a suppressor-free host, 594 (Weigle, 1966). At high temperature both the *cI* and *cro* repressors are inactivated and the induced phage should provide derepressed transcription of the *hsd* genes from p_L. The amber mutation in gene *Q* blocks transcription of late genes and lysis is prevented by the mutations in both genes *Q* and *S*. In practice, a *cro*$^-$ phage does not use the early λ promoters optimally since the derepressed transcription is associated with poor DNA replication (Folkmanis *et al.*, 1977); nevertheless, a useful amplification of gene products can be obtained (Davison *et al.*, 1974; Moir and Brammar, 1976). The *hsdS*$^-$ *recA*$^-$ strain HB101 (Boyer and Roulland-Dussoix, 1969) was used as a transformation host.

Enzyme and antibody preparations

*Eco*K was purified from the λ-*hsdK* lysogen described in the last section. Cells were grown at 30°C in L broth to an OD_{600} of 0.6 (~ 2 x 10^8 cells/ml), induced by heating them to 42°C and then grown for a further 3 h at 40°C (OD_{600} of 1.5). The cells were harvested by centrifugation and washed in a buffer containing 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 7 mM 2-mercaptoethanol.

The cells were disrupted by sonication and *Eco*K was purified from the sonicate by a combination of precipitation steps with polyethyleneimine and

$(NH_4)_2SO_4$, and chromatography on phosphocellulose, DEAE-cellulose, and heparin-agarose as described elsewhere (Meselson and Yuan, 1968; Bickle *et al.*, 1977). The subunits from ~ 500 μg of *Eco*K were separated on a 7% polyacrylamide gel containing SDS (Laemmli, 1970). The bands containing the purified subunits were excised from the gel using stained strips as a guide, the gel slices were cut into ~ 1-mm cubes and the proteins were eluted by three extractions (5 h each) with 25 mM Tris-HCl pH 8.0, 0.2 mM EDTA. The solutions were then concentrated by dialysis against dry Sephadex G-50.

Antibodies were raised against the purified subunits in rabbits. 25 – 70 μg of protein in Freund's complete adjuvant was injected intradermally at 8 – 10 sites and the animals were boosted 3 weeks later with half the amount of protein in Freund's incomplete adjuvant.

Immunochemical techniques

The main technique used was a modification of immunoreplicate electrophoresis as described by Towbin *et al.* (1979). Between 10^8 and 10^9 bacterial cells were harvested by centrifugation and the cells were resuspended in sample buffer (Laemmli, 1970), sonicated briefly, boiled for 2 min, and applied to a polyacrylamide gel containing SDS (Laemmli, 1970). After fractionation, the proteins were transferred electrophoretically from the polyacrylamide gel to a sheet of nitrocellulose (Schleicher and Schull, 0.45 μm). The additional protein-binding capacity of the sheet was saturated by incubating it for 1 h at room temperature with gentle shaking in 0.2% polyvinyl-pyrrolidone (PVP) in phosphate buffered saline (PBS) buffer (1% NaCl, 0.8% Na_2HPO_4, 0.029% NaH_2PO_4). For a 8 x 15 cm sheet of nitrocellulose, a volume of 40 ml was used for this and all subsequent incubations. The sheet was then incubated with antiserum (10 – 15 μl depending on the titre) in PBS buffer containing 0.2% PVP for 2 h and washed for 30 – 60 min in six changes of PBS buffer. ^{125}I-labelled protein A was added in PBS buffer + 0.2% PVP (0.15 μg, ~ 1 μCi) for 45 min and the sheet was washed five times with PBS buffer, dried, and the radioactive bands were visualised by autoradiography for 12 – 48 h.

Preparation of DNA

Bacterial DNA was purified by a modified version of the Marmur procedure (Marmur, 1961) essentially as described by Kaiser and Murray (1979) except that in later preparations dialysis was used in preference to ethanol precipitation. Plasmid DNA was purified from cleared bacterial lysates by centrifugation to equilibrium in CsCl/ethidium bromide (Clewell and Helinski, 1969).

Restriction and ligation of DNA

DNAs were digested in a buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, and 5 mM dithiothreitol (DTT). Restriction endonucleases were either purchased from New England Biolabs or prepared by H. Cambier, T4 DNA ligase was a gift from E. Remaut. Ligations were performed at 15°C in a buffer containing 10 mM $MgCl_2$, 66 mM Tris-HCl, pH 7.2, 1 mM EDTA, 10 mM DTT, 1 mM ATP.

Construction of plasmid pBgH

The 414-bp *Bgl*II-*Hind*III fragment of pRH3 (see Figure 4) was cloned in pBR322 after digestion of the vector with *Bam*HI and *Hind*III. Following ligation, recombinant plasmids were recovered as Amp^R Tet^S transformants of HB101. Small-scale preparations of plasmids were made (Gough *et al.*, 1980) and the presence of the *Hind*III-*Bgl*II fragment was identified by digestion with *Xba*I, which cleaves within this sequence (see Figure 4) but not within pBR322 itself (Sutcliffe, 1978).

Hybridization of DNA to labelled probes

DNA samples (1 – 2 μg), loaded in 4% ficoll, 0.05% bromophenol blue, were separated by electrophoresis on horizontal 0.7% (w/v) agarose slab gels in 0.012 M Tris, 0.06 M NaH_2PO_4, 2 mM EDTA (pH 7.8). For transfer to nitrocellulose (Southern, 1975), gels were treated successively with two changes of 0.25 M HCl (Wahl *et al.*, 1979) once with 0.5 M NaOH, 1.5 M NaCl and with two changes of 3 M NaCl, 1 M Tris-HCl pH 7.5 and then transferred to nitrocellulose filters (Schleicher and Schull, 0.45 μm) in the presence of 20 x SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate). After overnight transfer, filters were rinsed briefly in 2 x SSC and baked at 80°C under vacuum for ~ 2 h.

Prior to hybridization, the baked nitrocellulose filters were soaked in 2 x SSC, 0.1% SDS, 0.2% ficoll, 0.2% PVP, 0.2% bovine serum albumin, 50 μg/ml denatured salmon sperm DNA at 65°C, for several hours. The filters were hybridized in the same buffer by overnight incubation at 65°C using probes that were radioactively labelled by nick translation (Maniatis *et al.*, 1975). After hybridization, filters were washed extensively in 2 x SSC, 0.1% SDS at 65°C and then briefly in 2 x SSC at room temperature.

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