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Fragments Produced by Cleavage of λ Deoxyribonucleic Acid with the *Hemophilus parainfluenzae* Restriction Enzyme Hpa II†

Bernard Allet

ABSTRACT: One of the restricting enzymes extracted from *Hemophilus parainfluenzae*, Hpa II, is shown to cleave λ DNA at more than 50 sites. The resulting DNA fragments have been prepared from a variety of deletion or deletion–substitution mutants of λ, and analyzed by polyacrylamide gel electrophoresis. The major DNA segments (larger than 0.3 Mdalton) produced by digestion of λ DNA have been mapped and many of the cleavage sites in the immunity region, in the *b*2 region, and to the right of the immunity region have been identified.

Endonucleases are known that cleave DNA at specific sites, chiefly the so-called restriction enzymes from bacteria (Arber, 1971). Since it was realized that they can be used *in vitro* to prepare specific DNA fragments (Danna and Nathans, 1971), many investigators have devoted much work to characterizing these enzymes and to discovering new ones. When cleaved in favorable substrates, the fragments can be identified genetically with considerable precision (Danna *et al.*, 1973) and should therefore be useful for analysis of DNA functions.

In a previous report we described the cleavage of linear λ DNA with the endonuclease R·RI and the isolation and characterization of the six DNA fragments that were produced (Allet *et al.*, 1973a). Because one of these DNA fragments contained the early promoters, p_R and p_L, as well as the binding sites for repressor, the operators o_R and o_L, it seems that endonuclease R·RI does not cut the immunity region. In this paper I report the analysis of fragments produced by cleavage of λ DNA with one of the restriction enzymes isolated from *Hemophilus parainfluenzae* (Hpa II, which introduces one cut into SV40 DNA) (Sharp *et al.*, 1973). The *Hemophilus* enzyme cuts the immunity region in at least two places, permitting separation of p_L and o_L from p_R and o_R.

To map the DNA fragments that were produced, I compared by gel electrophoresis the pattern of the pieces cleaved from λ (λ*c*I857, S7) with those from a variety of deletion and substitution derivatives of λ. It is assumed throughout this work that all phage strains used were isogenic except for the indicated mutations. This implies that if a DNA fragment from a mutant has the same electrophoretic mobility as a fragment from the control λ, then both fragments are derived from an identical region of the λ genome. In the case of deletion–substitution mutants, it is further assumed that electrophoretic mobility alone is sufficient to identify a fragment. It is clear that if either assumption is invalid, then analysis of a number of mutants should quickly lead to an inconsistent interpretation. In fact, the internal consistency of the data presented in this paper entirely justifies both assumptions. Although the technique and the mutants used permit one to analyze many different regions of λ DNA, in this paper I have focused attention mainly on the immunity region, the *b*2 region, and the late promoter (p'_R) region.

Materials and Methods

Propagation of Phages and Extraction of DNA. The phage strains λ*b*511, λ*b*519, λ*bio*16A, λ*bio*7-20, λ*bio*69, λ*bio*10, λ*bio*256, λ*bio*3h-1 *nin*5, λ*bio*24-5 *nin*5, λ*bio*30-7 *nin*5, λ*bio*t124 *nin*5, λ*bio dc*30-7 *nin*5, λ*b*2 P4, φ80-λi^{λ}, φ80λi^{434}, and φ80λi^{21} were a kind gift from F. Blattner and W. Szybalski. The phages were propagated and the DNA was extracted by standard methods (Bovre and Szybalski, 1971) with minor modifications described previously (Allet *et al.*, 1973b).

Digestion of DNA with Hpa II (and R·RI) Endonucleases. The endonuclease Hpa II was prepared from *H. parainfluenzae* by Joe Sambrook (Sharp *et al.*, 1973). Samples of DNA (12–15 μg) were digested with the enzyme (50 μl) in a 400-μl reaction mixture containing 6.6 mM Tris (pH 7.5), 6.6 mM $MgCl_2$, and 6.6 mM β-mercaptoethanol (Calbiochem) for 10–15 hr at 37°. Addition of more enzyme or extension of the incubation time did not alter the pattern of cleavage, an indication that the reaction goes to completion. The DNA fragments were precipitated with 4–5 ml of ethanol (−20° for 3–4 hr) and collected by centrifugation for 20 min at 45,000 rpm in a Spinco SW50.1 rotor. The pellet was dried under vacuum and

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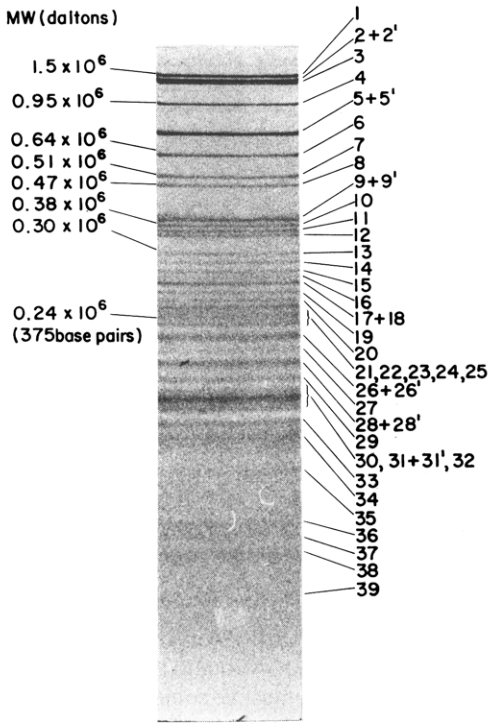

FIGURE 1: Acrylamide gel electrophoresis of the products cleaved from λ DNA with Hpa II endonuclease. Results are shown for a 15-μg sample of λ DNA (λcI857, S7). Bands were numbered as shown on the right; when stain intensity suggested that a band contained more than one DNA species, two numbers were assigned (*e.g.*, 2 + 2′). The molecular weights (numbers to the left) were estimated by electron microscopy, except that the value for fragment 24 was a personal communication from T. Maniatis.

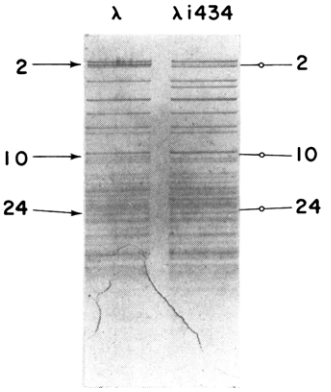

FIGURE 2: Fragments derived from the λ-immunity region. The DNAs from λ and from λi^{434} (12 μg each) were analyzed as described in Figure 1. The arrows point to bands that are present, and the circles indicate bands that are missing. Although λi^{434} does not produce fragment 2, it shows at this position a DNA species (2′) with lesser stain intensity than the smaller fragment 3. A possible origin of this nonstoichiometric fragment is discussed in the text.

dissolved in 15 μl of buffer containing 4 mM Tris-acetate, 2 mM sodium acetate, 10% sucrose, and 0.02% Bromophenol Blue (pH 7.9).

The R·RI endonuclease was purified according to the method of Yoshimori (1971). When R·RI and Hpa II were used together, the reaction was carried out as described above except that the digested DNA was extracted with phenol equilibrated at 4° with a solution containing 10 mM Tris-HCl (pH 8)–1 mM EDTA before ethanol precipitation.

Polyacrylamide Gel Electrophoresis of the DNA Fragments. The gelling conditions have been worked out by P. G. N. Jeppesen (manuscript in preparation). The lower gel was a 0.4 × 14 × 9 cm slab containing a linear gradient of acrylamide (Eastman) from 2.5% at the origin to a final concentration of 7.5%, of *N*,*N*′-methylenebisacrylamide (Eastman) from 0.125 to 0.185%, and of sucrose (Schwarz/Mann) from 10 to 25% in E buffer (40 mM Tris-acetate–20 mM sodium acetate, pH adjusted to 7.9).

Before the gel was poured from a gradient maker, 50 μl of 10% ammonium persulfate and 150 μl of 10% *N*,*N*,*N*′,*N*′′-tetramethylethylenediamine (Sigma Chemical Co.) were added to the solution of 2.5% acrylamide, and 50 μl of ammonium persulfate and 30 μl of tetramethylethylenediamine were added to the 7.5% acrylamide solution. The time for complete polymerization was 40–60 min. The upper gel (20 ml) contained 2.5% acrylamide, 0.125% bisacrylamide, 200 μl of 10% ammonium persulfate, and 200 μl of 10% tetramethylethylenediamine in E buffer diluted 1:5. Electrophoresis was carried out at room temperature in E buffer with a current of 50 mA (6–7 V/cm) until the Bromophenol Blue dye reached the bottom of the gel (5–6 hr). The DNA was stained by soaking the gel in a 0.02% solution of Methylene Blue for about 2–3 hr, and excess dye was removed with several changes of deionized water.

Fragments were electrophoretically eluted from the gel as described previously (Allet *et al.*, 1973a,b), and in collaboration with H. Delius samples were prepared for electron microscopy length measurements by means of a modification of the Kleinschmidt spreading procedure (Davis *et al.*, 1971; Delius *et al.*, 1972). DNA from PM2 phage (mol wt 6.40 × 10^6 ± 2%) was used as internal standard.

Results

Figure 1 shows the numerous bands separated by electrophoresis and gives molecular weights of some of the isolated fragments. The technique allows one to estimate the molecular weight values for DNA fragments larger than 3 × 10^5 daltons.

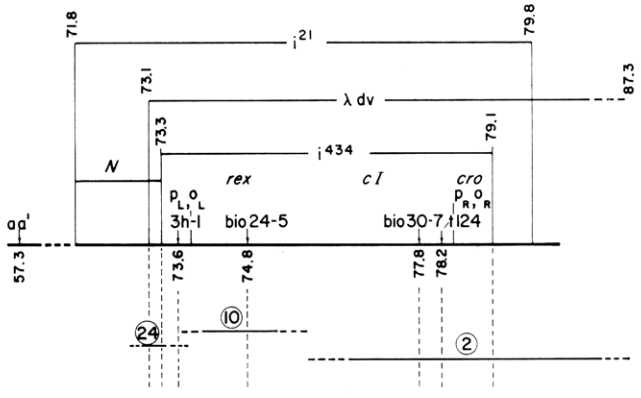

FIGURE 3: Mapping of fragments from the immunity region. The drawing shows segments deleted, etc. (Davidson and Szybalski, 1971). The different sites are specified by numbers that represent percentage distances in units of molecular length measured from the left end of the λ DNA molecule. The bars labeled i^{21}, λ*dv*, i^{434} indicate segments deleted in the corresponding mutants. The arrows labeled 3*h*-1, *bio*24-5, *bio*30-7, and t124 indicate the right ends of deletions extending to aa′. N, *rex*, *c*I, and *cro* are genes. The cleaved DNA fragments are represented in the lower part of the figure (circled numbers) by heavy lines whose lengths are approximately at the same scale as the map. The dashed lines indicate roughly the uncertainty of origin of the fragments.

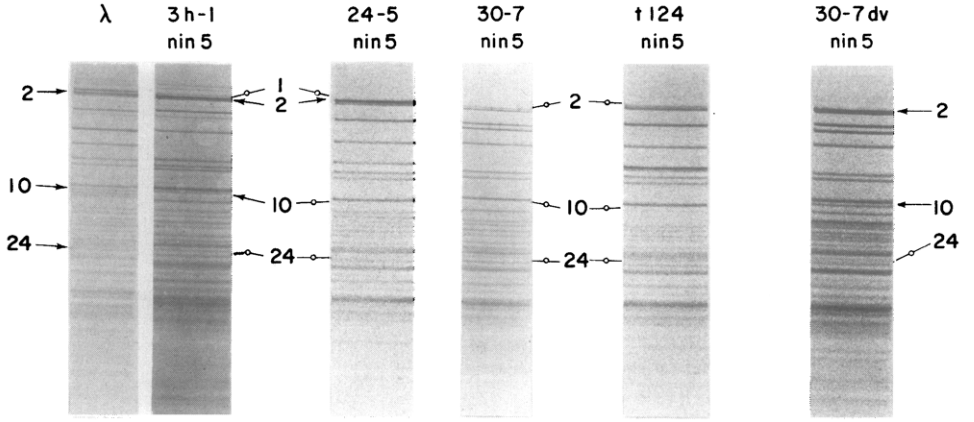

FIGURE 4: Fragments derived from mutants with altered immunity regions. The mutants are explained in Figure 3. The arrows point to bands that are present, and the open circles to bands that are missing. In all the mutants band 1 is missing for the reason discussed in the text.

As can be seen, there is no piece of DNA larger than 1.5×10^6 daltons (about 5% of intact λ DNA); the smallest pieces migrate with the Bromophenol Blue marker. In the upper part of the gel (mol wt $>3 \times 10^5$ daltons), the intensity of the bands after staining with Methylene Blue generally allows one to predict whether they arise from single or multiple kinds of fragments. In the lower part of the gel, however, such estimation is much harder. Therefore, the numbering in Figure 1 certainly does not correspond to a correct number of different species, but rather characterizes fractions that are amenable to analysis.

Fragments Derived from the Immunity Region. Fragments 2, 10, and 24 are present in digests of λ but absent from digests of i^{434} (Figure 2). These fragments therefore must come in whole or part from the immunity region of λ. To determine precisely the relative locations of these fragments, the DNAs from a number of deletion–substitution mutants were cleaved with Hpa II and analyzed for the presence or the absence of these three species. The deletions used (λ*bio* derivatives) all start from the same point (aa′) to the left of the immunity region, and their extent increases progressively for λ*bio*3*h*-1, λ*bio*24-5, λ*bio*30-7, and λ*bio*t124 (Figure 3).

The cleaved DNA from λ*bio*3*h*-1 lacks fragment 24 (in addition to others which are discussed below) but contains fragments 2 and 10 (see Figure 4). In the DNA of λ*bio*24-5, fragments 10 and 24 are missing, but fragment 2 is present. Neither λ*bio*30-7 nor λ*bio*t124 contains any of these fragments. Therefore, the relative order of the fragments, from left to right, is 24, 10, 2. Furthermore, the left end of fragment 10 must lie to the left of *bio*24-5 but to the right of *bio*3*h*-1, and the left end of fragment 2 must lie somewhere between *bio*24-5 and *bio*30-7. It is possible that some of the smaller DNA pieces generated by Hpa II digestion of λ DNA also belong to the immunity region, but are not detected in the test situation used. Nevertheless, it is clear for two reasons that fragments 2 and 24 contain DNA sequences that flank the immunity region: (i) they direct *in vitro* the synthesis of RNA species that correspond to those directed by the early promoters p_R and p_L, respectively (manuscript in preparation); (ii) they specifically bind the λ repressor (T. Maniatis, personal communication). Moreover, this pattern of cleavage in the λ-immunity region is supported by analysis of the digestion products from λ*db*30-7 *dv nin*5 DNA; this strain is derived from λ*db*30-7 *nin*5 by insertion of λ*dv* DNA which includes the immunity region in addition to short sequences of the two early operons (see Figure 3). Interestingly, the cleaved DNA from this mutant (Figure 4) yields both fragment 2 and fragment

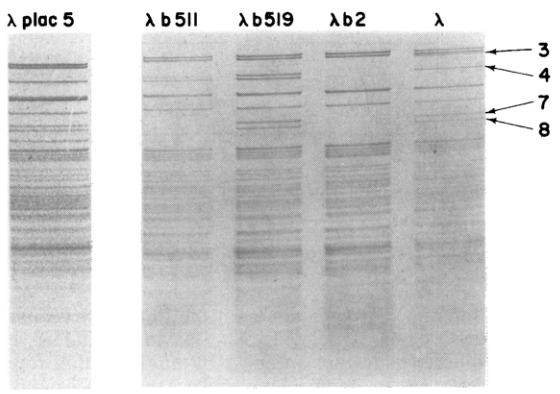

FIGURE 5: Fragments from mutants altered in the *b*2 region. The arrows point to bands for which presence or absence is examined in the various digests. The significance of two nonstoichiometric bands in the pattern of λ*b*511 (one close to the position of band 4, the other just above band 6) and of an apparently stoichiometric species migrating below band 4 in the patterns of λ*b*511 nad λ*b*519 has not been investigated.

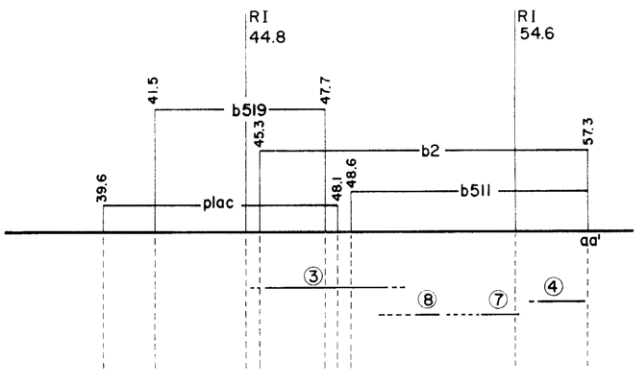

FIGURE 6: Mapping of fragments derived from the *b*2 region. The bars labeled *b*2, *b*511, *b*519, *plac* indicate segments deleted in the corresponding mutants (Davidson and Szybalski, 1971). λ*b*2 has lost the DNA segment from map position 45.3 to 57.3, λ*b*519 from 41.5 to 47.7, and λ*b*511 from 48.6 to 57.3. In λ*plac*5, a piece of DNA from the *lac* region of the *E. coli* chromosome has been substituted for the λ segment between map positions 39.6 and 48.1 (Malamy *et al.*, 1972). The RI markers indicate the sites of cleavage by the R·RI restriction enzyme (Allet *et al.*, 1973b). The cleaved DNA fragments, indicated by circled numbers, are represented as in Figure 3.

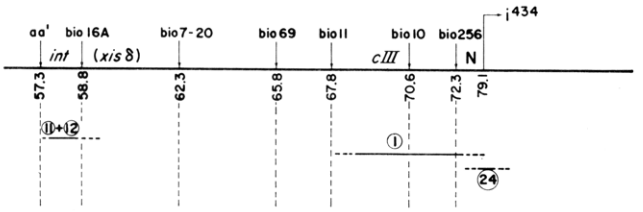

FIGURE 7: Mapping of fragments derived from the segment lying between the attachment site and the immunity region. The arrows labeled *bio*16A, *bio*7-20, *bio*69, *bio*11, *bio*10, and *bio*256 indicate the right ends of deletions extending to aa′. N, *c*III, (xis δ), and *int* are genes. The cleaved fragments, indicated by circled numbers, are represented as in Figure 3.

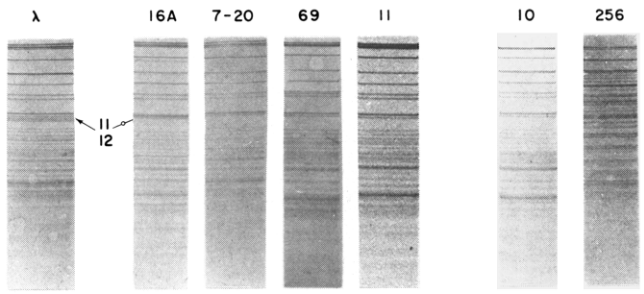

FIGURE 8: Fragments from mutants altered between the attachment site and the immunity region. The mutants are explained in Figure 7. The arrows point to bands that are present, and open circles indicate bands that are missing.

10, an indication that these fragments are comprised within λ*dv* DNA. Fragment 24, however, is not produced. Because λ*dv* DNA extends about 120 base pairs into the N gene (Blattner and Dahlberg, 1972) the left end of fragment 24 must be to the left of this point.

Fragments Derived from the b2 Region. In Figure 5, the pattern of fragments from λ is compared with those from four different λ derivatives with deletions in the *b*2 region (Figure 6). The λ*b*2 and λ*b*511 DNAs fail to produce fragments 3, 4, 7 and 8. From λ*b*519 and λ*plac*5, fragment 3 is the only one missing. Fragment 3 therefore originates on the left of the other three, and its right end must lie to the right of map position 48.6, where the *b*511 deletion terminates. Fragment 4, but not the other three, is a digestion product of the R·RI fragment that includes DNA between map positions 54.6 and 66.2 (data not shown). Fragment 4 therefore originates on the right of the other three. As will be shown below, fragment 7 is not made from λ when endonucleases R·RI and Hpa II act together, an indication that segment 7 overlaps the R·RI cleavage site at map position 54.6. The location of the R·RI cleavage site in the *b*2 region (indicated in Figure 6) and the molecular weights of the fragments dictate that fragment 8 must lie to the left of fragment 7 and that therefore the order of the fragments in the *b*2 region, from left to right, is 3, 8, 7, 4.

Area between aa′ and cI. As pointed out earlier, a series of deletion mutants of λ share a common left-hand point, called aa′. These mutants carry variable amounts of DNA from the biotin operon of the host (Davis and Davidson, 1968; Davis and Parkinson, 1971; Parkinson and Davis, 1971; Westmoreland *et al.*, 1969; Hradecna and Szybalski, 1969, 1970; Fiandt *et al.*, 1971; Davidson and Szybalski, 1971). The DNA from some of these mutants, whose map positions are shown in Figure 7, has been cleaved with Hpa II, and the pattern of the resulting fragments is shown in Figure 8. Although only 1.5% of the λ DNA molecule is deleted in λ*bio*16A, two DNA species, 11 and 12, are not seen in the pattern of the mutant. Therefore, fragments 11 or 12, or both, contain DNA sequences for *int* and perhaps part of the *xis* gene. As expected bands 11 and 12 are also missing in the pattern of the other λ*bio* mutants that have lost larger amounts of DNA. Moreover, some bands of lower molecular weights are progressively eliminated when λ*bio*7-20, λ*bio*69, λ*bio*11, λ*bio*10, and λ*bio*256, respectively, are examined, but these differences have not been studied in detail. However, an examination of the occurrence of fragment 1 (about 1.5×10^6 daltons) led to an interesting finding. This fragment is not seen in the digests of λ*bio*10 and of mutants with deletions ending farther to the right; it is present, however, in the digests of λ*bio*11 and of mutants with deletions ending to the left of map position 67.8. Therefore, the left end of fragment 1 is located somewhere between the right ends of *bio*10 and *bio*11 and, as the fragment contains about 5% of the total DNA, its right end point must lie in gene N (see in Figure 7), possibly adjacent to fragment 24 discussed above. This means that fragment 1 must contain the complete *c*III gene.

Fragments Originating on the Right of the Immunity Region. Two mutants with deletions to the right of the immunity region were studied (see Figure 9). In the *nin*5 mutant, DNA between map positions 83.8 and 89.2 has been deleted (Court and Sato, 1969; Kayajanian, 1968, 1970). In P4, the deletion extends from 83.8 to 95.0, and DNA from an uncharacterized lambdoid phage has been inserted in its place (Fiandt *et al.*, 1971). In Figure 10, the digestion products from λ, λ*b*2 P4, and a *nin*5 derivative (λ*bio*3*h*-1 *nin*5) are compared. Bands 3, 4, 7, and 8 are missing from λ*b*2 P4 as a consequence of the *b*2 deletion (see above); however, segments 6, 9 (or 9′), 15, 26 (or 26′), and 27 are absent also, and must therefore originate from a DNA region that is contained in, or overlaps, the P4 deletion. In this series only segment 6 is missing from the *nin*5 derivative. As segment 6 is not a digestion product of φ80-λi^{λ} (Figure 13), its left end must be to the left of map position 89.2 (right end of *nin*5 deletion) and its right end to the right of map position 90.6 (φ80-λ junction). As expected from these assignments, fragment 6 is not a digestion product of λ*dv* DNA (Figure 3). Furthermore, fragments 9 (or 9′), 15, 26 (or 26′), and 27 must lie to the right of map position 90.6. Frag-

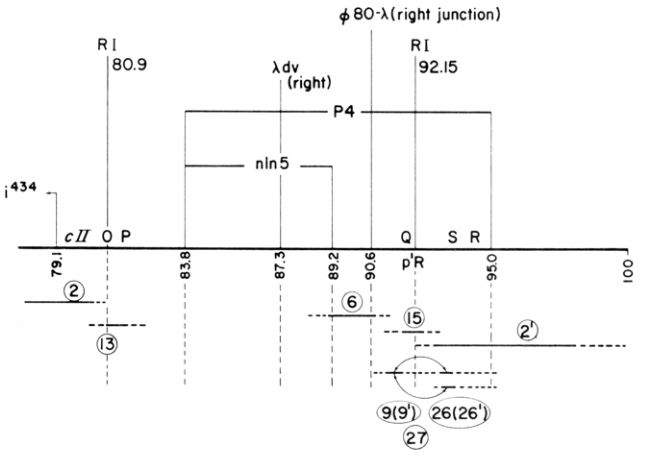

FIGURE 9: Mapping of fragments to the right of the immunity region. The bars labeled *nin*5 and P4 indicate segments deleted in the corresponding mutants. The RI markers indicate the sites of cleavage by the R·RI restriction enzyme. The right end of λ*dv* and the right junction of the λ and φ80 DNAs are indicated by vertical bars. *c*II, O, P, Q, S, and R are genes. The cleaved fragments are designated by circled numbers, and they are represented as in Figure 3.

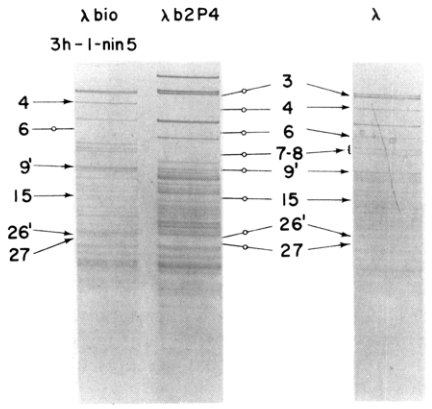

FIGURE 10: Fragments from mutants altered to the right of the immunity region. The mutants are explained in Figue 9. The arrows point to bands that are present, and the open circles indicate bands that are mising.

ment 15 is not produced when λ is digested by a mixture of R·RI and Hpa II (Figure 11), an indication that it overlaps the R·RI cleavage site at map position 92.15. Two other fragments, 7 and 13, are not found when R·RI and Hpa II act jointly (Figure 11). As discussed above, fragment 7 is derived from the *b*2 region and must contain the R·RI cleavage site at map position 54.6. Fragment 13 is present in all the mutants with deletions in the *b*2 region or in the region between aa′ and *c*I, and therefore it must contain the R·RI cleavage site at map position 80.9 (Figure 9).

Analysis of Hybrid DNAs. Examination of the digestion products from φ80-λ hybrids with various immunity regions (Figure 12) confirms the map positions established above. Thus, as found before, fragments 2, 10, and 24 are found only when the λ-immunity region is present (Figure 13). As expected, none of the hybrids produces any DNA piece that was previously assigned to the left of map position 64.5 (φ80-λ left junction) or to the right of map position 90.6 (φ80-λ right junction). Conversely, the φ80-λi^{λ} hybrid does yield fragments assigned between map positions 64.5 and 90.6. An interesting feature is that fragment 2′, which migrates with fragment 2 when λ is used as substrate, is not a digestion product of the φ80-λ hybrids. Fragment 2, therefore, which contains the early rightward promoter, can be isolated as a pure DNA species from φ80-λ hybrid DNA. Although the DNA segment corresponding to fragment 2′ is not present in the R·RI fragment (data not shown) that includes λ genes from map positions 0 and 44.80 (Allet *et al.*, 1973a), it was recovered from the mutants carrying deletions in the *b*2, aa′-*c*I, and *nin*5 regions. Thus, the left end of fragment 2′ must be to the right of the R·RI cleavage site at map position 92.15 (Figure 9). Analysis of mutants that fail to produce fragment 2, such as λi^{434} and λ*db*30-7 (see Figures 2 and 4), makes it clear that fragment 2′ is not yielded in stoichiometric amounts. Instead, there is a DNA fragment in position 5 that (i) is produced in more than stoichiometric amounts for a single DNA species, as indicated by the stain intensity of the band; (ii) has a molecular weight one-half that of fragment 2′; and (iii) is not attributable to any DNA region covered by the analysis presented. I suggest that Hpa II cleaves pieces of almost the same length (0.7×10^6 daltons) from each extremity of the λ DNA molecule, and that the two are occasionally bound together (as fragment 2′) through the complementary single-stranded regions located at the molecular ends. This view is compatible with the fact that both the purified R·RI fragment 1 (which contains the left molecular end) and a mixture of the other fragments (containing the right end) yield a band that migrates at position 5 after digestion with Hpa II (data not shown).

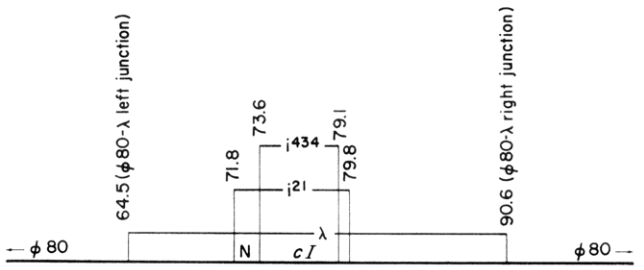

FIGURE 12: Genetic maps of φ80-λ hybrids with various immunity regions. The bars labeled λ, i^{21}, and i^{434} indicate the part of DNA of the corresponding phages that has been substituted for the corresponding part of φ80 (or φ80-λ) DNA. N and *c*I are genes.

Discussion

The Hemophilus restriction enzyme Hpa II cleaves λ DNA into variously sized fragments (Figure 1). Most of the fragments that can be identified (greater than 0.3 Mdalton) originate on the DNA part that is involved in the early functions of phage development, between the *b*2 region and the right end of the linear molecule (60% of total λ DNA). As the added molecular weights of these fragments account for about 40% of intact λ DNA, it follows that the larger fragments cover more than 50% of the early region of the DNA. A large

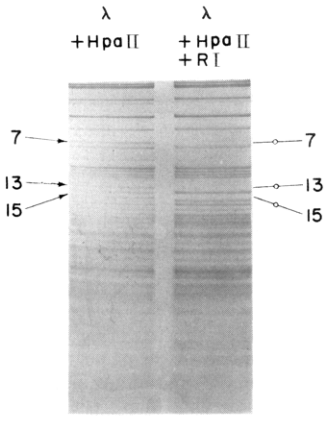

FIGURE 11: Fragments cleaved from λ DNA by joint action of Hpa II and R·RI. Open circles indicate components produced by Hpa II, but not by the mixed enzymes.

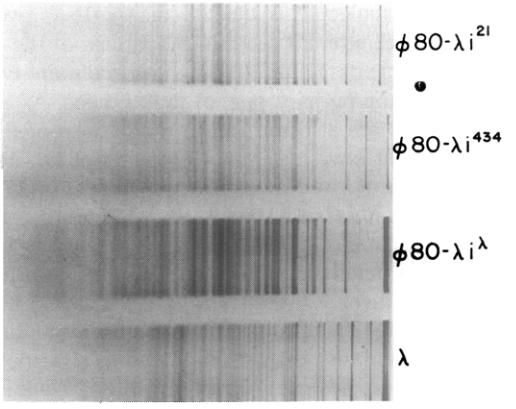

FIGURE 13: Fragments from φ80-λ hybrid DNAs. See Figure 12 for description of hybrids.

majority of the numerous smaller fragments originate on the DNA part that contains the genes for the late functions, and this has been tested directly by cleavage of the R·RI fragment that contains DNA from map positions 0 and 44.8 (data not shown).

A number of the fragments identified are of particular interest, because they contain DNA that is involved in the regulatory functions of the phage. Fragment 24, for example, which is of manageable size for DNA sequence analysis, contains the binding site for repressor, the operator o_L as well as a part of gene N; it is not clear whether it also contains the promotor p_L. On the other side of the immunity region, fragment 2 certainly contains both the promoter p_R and o_R, as well as a segment of DNA extending into gene *cro* and perhaps *c*II. Such fragments and those cleaved in the *b*2 and in the late promoter p'_R region are being used for *in vitro* transcription and coupled transcription/translation studies. This kind of analysis may help understand more intimately the functions of DNA.

In order to map the DNA fragments, I have taken advantage of well characterized deletion mutants of λ. With the data accumulated, however, the reverse process of characterizing unknown deletion, substitution, and insertion mutants can be easily achieved. Given the extreme simplicity of the technique, this direct method of analysis may be a tool of choice in many cases.

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