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Physical Mapping of β-Converting and γ-Nonconverting Corynebacteriophage Genomes

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Deoxyribonucleic acids (DNAs) from wild-type and mutant strains of β-converting and γ-nonconverting corynebacteriophages were isolated and physically characterized. The data obtained from DNA heteroduplexes, restriction enzyme banding profiles, and restriction maps reinforce the conclusion that β and γ phages are very closely related. The major physical differences seen in the DNA heteroduplexes are a small substitution bubble and one or two insertions which are present on the γ phage genome. The insertions account for the differences in the genome sizes of β and γ phages, and with the substitution they are responsible for most of the differences in the restriction endonuclease profiles and maps of these corynebacteriophages. In the course of developing restriction maps of the corynebacteriophage genomes, two special sites and the DNA fragments carrying them were identified. These were the cohesive (*cos*) sites and the specific attachment (*att*P) site of the vegetative phage genome. The behavior of these sites indicated that the transition of phage DNA from the vegetative to the prophage state involves the circularization of vegetative DNA through the *cos* sites and its integration into the bacterial chromosome via the *att*P site. The mechanism of corynebacteriophage integration was similar to that employed by *Escherichia coli* phage λ. From the data assembled the physical and genetic maps of β and γ phage were oriented with respect to one another. The extensive similarity in their maps provides additional confirmation of a close evolutionary relationship.

The ability of corynebacteriophages to convert nontoxinogenic *Corynebacterium diphtheriae* to toxinogeny is phage specific. The β-converting phage described by Freeman (10) and the γ-nonconverting phage described by workers in this laboratory (11) have been studied comparatively for many years. The two phages are serologically related (17), exhibit similar one-step growth characteristics and patterns of inducibility by UV light (12), and are morphologically similar, though differing slightly in head and tail measurements (17). They recombine vegetatively, though at a frequency lower than in homoimmune β matings (16) and also recombine as prophages when in tandem in a lysogen (18). It has been shown through genetic crosses (18) that the γ-nonconverting phage carries a portion of or perhaps the entire gene for diphtheria toxin (*tox*) and obviously does so cryptically.

The major differences exhibited by the β and γ phages, besides their relationship to conversion, is that they are heteroimmune, i.e., each is able to superinfect lysogens of the other (11). Whereas the immune repressors of each appear to be distinct, superinfection is dependent on the synthesis of an antirepressor which enables the superinfecting phage to overcome exclusion by the resident prophage (14). However, both phages appear to use the same antirepressor in so doing. This finding indicates that the regulatory functions of β and γ phages are also interrelated. Finally in a preliminary study (3) it was shown by means of DNA heteroduplexes that approximately 99% of the β DNA sequences were homologous to corresponding γ DNA sequences under conditions in which at least 70% base pairing was required for duplex formation.

The present study was undertaken with two purposes in mind. One was to extend the comparative study of β and γ corynebacteriophages; the second was to provide a basis for isolating a DNA fragment carrying the gene for diphtheria toxin. To this end, physical maps of β and γ phages have been constructed by analysis of their restriction enzyme banding profiles, and genetic markers have been located on specific restriction fragments. In addition, anomalies in the DNA structures of γ phage relative to β phage have been identified and mapped by het-

eroduplex analysis. Finally, the restriction and heteroduplex maps of these phages have been oriented with their genetic maps. In accompanying papers (1, 2), DNA restriction enzyme fragments bearing the *tox* gene are identified, and the deletion-insertion loops visualized in the two γ-β DNA heteroduplexes are further characterized.

MATERIALS AND METHODS

Strains of bacteria and phage. *C. diphtheriae* strains C7(−)$^{tox-}$, C7(β)$^{tox+}$, C7(γ)$^{tox-}$, C7(β-*tsr*-3)$^{tox+}$, C7(γ-*tsr*-1)$^{tox-}$, and C7(γ-*tsr*-2)$^{tox-}$ were taken from the stock culture collection of this laboratory. Phage β-*h tox*$^-$(CRM45) was produced by selecting a host range (*h*) mutant of phage β-*tox*$^-$(CMR45) (31) on bacterial strain C7/β^{wr}(14), and its lysogen C7(β-*h*)$^{tox-}$(CRM45) was isolated. This lysogen produces a protein (45,000 daltons) that cross-reacts serologically with diphtheria toxin.

Media and phage methodology. The procedures for phage activity assays, isolation of lysogens, tests for lysogeny, and production of phage stocks by UV induction were previously described (18), as was the tryptose yeast extract (TYE) medium employed.

Production of phage stocks. Stocks of *tsr* (temperature-sensitive repressor) phage mutants were produced by heat induction of lysogens (13). Lysogens were streaked for confluent growth onto fresh TYE agar plates and incubated overnight at 30°C. Two or three plates were streaked for each 100 ml of phage stock required. The bacteria were washed into TYE broth preequilibrated to 38.5°C and supplemented to 0.2% Tween 80. The cultures (100 to 500 ml) were gently shaken for 4 to 4.5 h until maximum lysis, after which the bacterial debris was removed by centrifugation (10 min, 10,000 × *g*). The phage lysates were again subjected to centrifugation (20 min, 10,000 × *g*) and decanted. Lysate titers varied with the phage strain, but generally ranged from 5×10^9 to 50×10^9/ml.

Stocks of other phages were produced by UV induction followed by two successive cycles of lytic infection during which the volumes were increased from 5 ml to 20 ml to 200 ml. The TYE medium was supplemented to 0.002 M $CaCl_2$ and 1.5% maltose. In the lytic infections which were carried out at 35°C, the cultures were stationary during a 15-min adsorption period, but then were vigorously shaken until lysis began. At this time, the cultures were supplemented to 0.07 M sodium citrate to prevent adsorption of free phages to cell debris, and shaking was slowed until lysis was completed. In the final lytic cycle, 0.2% Tween 80 was added to the lysates in place of sodium citrate to prevent phage adsorption. When the cultures were fully lysed, bacterial debris was removed by centrifugation as above. Final phage titers generally ranged from 1×10^9 to 10×10^9/ml.

Isolation of bacteriophage DNA. Phage stocks produced by UV or heat induction were concentrated directly from lysate supernatants by high-speed centrifugation (60 min, 100,000 × *g*). The pellets were allowed to resuspend overnight in 0.2 M NaCl–0.02 M putrescine (Sigma Chemical Co., St. Louis, Mo.)–0.005 M disodium EDTA–0.01 M Tris (pH 7.8); after two low-speed centrifugation steps (10 min, 8,000 × *g*) to remove debris, the DNA was extracted with sodium dodecyl sulfate by the procedure of Murphy et al. (21). The released phage DNA was further purified by equilibrium centrifugation in CsCl-ethidium bromide density gradients (23). The DNA was precipitated with ethanol from the CsCl solution (30) and resuspended in 0.05 M NaCl–0.005 M disodium EDTA–0.03 M Tris (pH 8.0). The DNA was reprecipitated with isopropanol as described previously (24) and resuspended in 0.005 M Tris (pH 7.0)–0.0005 M disodium EDTA.

Isolation of bacterial DNA. Bacterial DNA was isolated as described by Schiller et al. (24), except that after cell lysis with sodium dodecyl sulfate, the centrifugation step was omitted, and the released DNA was sheared by repeated pipetting (15 to 20 times) of the suspension through a 10-ml pipette.

Molecular mass determination. The molecular masses of both intact phage DNA and isolated restriction fragment DNA were determined by electron microscopy and gel electrophoresis. For electron microscopic determinations the DNAs were spread by either the standard Kleinschmidt technique (8) or a modified Kleinschmidt procedure (6). In the modified procedure approximately 0.01 μg of the DNA sample was suspended in 0.5 M ammonium acetate (pH 8.0)–100 μg of cytochrome *c* (Sigma) per ml in a final volume of 50 μl. This 50-μl hyperphase solution was placed in drop form on parafilm, as was 50 μl of hypophase–0.25 M ammonium acetate (pH 8.0). The hyperphase droplet was allowed to stand for 60 s at which time a Parlodion coated microscope grid prepared as described previously (8) was gently inverted over it for 5 s and removed to the hypophase drop for 10 s. The grids were stained and rotary shadowed as described previously (8). RSF2124, an 11.2-kilobase (kb) (7.4×10^6-dalton) derivative of the *Escherichia coli* plasmid ColE1 with an inserted transposon, Tn*3*, was spread with the phage DNA as a standard. The mean contour length of ca. 20 molecules of each type of DNA was determined, and the relative molecular masses were calculated. All electron microscopy was done with a JEOL model 100B electron microscope.

Molecular masses were also calculated by electrophoresing restriction products of the DNAs in parallel with standard restriction fragments of known mass. A plot of the logarithms of the mobilities of the standard fragments versus the logarithms of their known molecular masses generated a curve from which the approximate masses of the phage restriction fragments were determined. The sum of the masses of the fragments from each restriction digest provided an independent estimate of the molecular mass of the intact DNA.

Heteroduplex analysis. Heteroduplexes were prepared between whole phage DNAs by mixing approximately 0.1 μg of each DNA in 0.25 ml of an aqueous solution of 0.1 M NaOH–0.01 M disodium EDTA (pH 12.4 to 12.6) for 10 min, at which time the pH was reduced to 8.4 to 8.6 by adding 2.0 M Tris (pH 7.0). Formamide (0.25 ml) was added, and the mixture was allowed to reanneal at room temperature for 2 to 4 h, at which time the DNA was spread and rotary shadowed (8).

Restriction enzyme digestions. Restriction endonucleases were obtained from Bethesda Research Laboratories, Inc. (Rockville, Md.) and used as prescribed. Reactions were stopped with 0.25 volumes of 20% Ficoll–0.2% Sarkosyl–0.05% bromophenol blue.

Double digestion of phage DNA. Phage DNA was cleaved with *Hpa*I restriction endonuclease, and the reaction was stopped by a 10-min, 60°C incubation. The salts were removed from the reaction mixture by passage over a miniature column by the procedures of Neal and Florini (22). Bio-Gel P2, 100-200 mesh (Bio-Rad Laboratories, Richmond, Calif.) was used as the gel matrix. The small volume eluate (100 μl) was divided into equal aliquots, supplemented to the appropriate reaction conditions, and cleaved with the second endonuclease.

Agarose gel electrophoresis. Samples of restriction enzyme-treated DNA were loaded into wells (1 by 6 by 5 mm) in horizontal 0.5 to 0.7% agarose slab gels (20) (0.5 by 16 by 25 cm) prepared in TENA gel buffer (0.04 M Tris, 0.02 M sodium acetate, 0.018 M NaCl, 0.002 M disodium EDTA, pH 8.15). Electrophoresis was carried out at 50 V for 12 to 15 h, at which time the gels were stained in 1 μg of ethidium bromide per ml in water for approximately 1 h. The restriction profiles were photographed during illumination with shortwave UV light on a Chromato-vue transilluminator (Ultra-Violet Products, Inc., San Gabriel, Calif.).

Vertical gels (28) of 0.7 to 1.2% agarose were run in TB gel buffer consisting of 0.09 M Tris–0.09 M boric acid–0.0025 M disodium EDTA (pH 8.3). DNA samples were loaded into various-sized wells, electrophoresed for 2 h at 100 V, stained, and photographed as described above.

Isolation of restriction fragments from agarose gels. Specific restriction fragments were isolated from agarose gels by the procedures of Tabak and Flavell (29). Restriction fragments visualized by ethidium bromide staining and UV illumination were cut out of preparative agarose gels. Each slice of the gel containing a specific restriction fragment was placed into a section (6 cm long) of a 10-ml plastic disposable pipette (Falcon Plastics, Oxnard, Calif.). The disposable pipette section had been prepared with one of its ends sealed by dialysis tubing and packed with 0.1 to 0.2 ml of DNA-grade hydroxylapatite (Bio-Rad Laboratories) held in place with glass wool. The pipette sections containing the gel slices were immersed in TENA gel buffer, and the fragments were electrophoresed into the hydroxylapatite matrix at 50 V for 12 to 18 h. The hydroxylapatite was removed from the pipette sections to a 1.5-ml plastic disposable microtest tube (West Coast Scientific, Inc., Seattle, Wash.) and pelleted by centrifugation in a Centrifuge 5412 (Eppendorf, Hamburg, Germany) for 30 s. The hydroxylapatite was washed three times by centrifugation as above in 0.5 ml of 0.005 M Tris (pH 7.5)–0.005 M disodium EDTA. The DNA fragments were eluted from the matrix by three similar washes with 0.1 ml of 1 M phosphate buffer (pH 7.0), and the three supernatants were pooled. The pooled supernatants were filtered through 3 MM filter paper (Whatman Ltd., Clifton, N.J.) and dialyzed exhaustively against 0.005 M Tris (pH 7.5)–0.0005 M disodium EDTA. The solution was brought to 0.4 M sodium acetate and 40% isopropanol and held at −20°C for a minimum of 2 h. The precipitate was collected by centrifugation (4 min) in a Centrifuge 5412, dried, and resuspended in 0.1 ml of 0.005 M Tris (pH 7.5)–0.0005 M disodium EDTA.

In vitro labeling of DNA. Whole phage and isolated restriction fragment DNA were labeled by nick translation with ^{32}P-deoxyribonucleotides as described by Thomashow et al. (30).

Preparation of nitrocellulose filters. DNA was transferred from agarose gels to nitrocellulose filters by established techniques (27, 30). After the gels were photographed, they were soaked for 2 h in 0.5 M NaOH–0.8 M NaCl, followed by 4 h in 0.5 M Tris (pH 7.0)–1.5 M NaCl with continuous gentle agitation. DNA was transferred to presoaked nitrocellulose sheets (Schleicher & Schuell Co., Keene, N.H.) by the procedure outlined by Southern (27) with 10× SSC (1× SSC is 0.15 M NaCl–0.015 M sodium citrate) as the transfer buffer. The gels were blotted for 16 to 24 h, after which they were restained with 1 μg of ethidium bromide per ml and showed almost no trace of remaining DNA. The filters were then cut to size, rinsed briefly in 2× SSC, air dried, and baked 2 to 3 h at 80°C in vacuo.

Hybridization of in vitro-labeled DNA to nitrocellulose-bound DNA. The prepared nitrocellulose filters were placed in polyethylene bags to which was added a prehybridization mixture consisting of 5× SSC, 5× Denhardt solution (1× Denhardt solution is 0.02% each of Ficoll 400, polyvinylpyrrolidone, and bovine serum albumin [9]), 0.1% sodium dodecyl sulfate, 0.005 M disodium EDTA, 20 μg of sheared and denatured calf thymus DNA per ml, and 0.02 M Tris (pH 7.6). The bags were sealed and incubated at 68°C for 12 to 16 h. The prehybridization mix was replaced with a similar hybridization mix which contained 15 to 25 ng of denatured (100°C for 10 min) radioactive probe per ml. The bags were resealed and incubated at 68°C for 18 to 24 h. The filters were washed (four times for 30 min each, two times for 15 min each) in 2× SSC–0.1% sodium dodecyl sulfate–0.005 M disodium EDTA–0.01 M Tris (pH 7.6) at 68°C, followed by two additional washes (once for 30 min, once for 15 min) in 0.3× SSC–0.015% sodium dodecyl sulfate–0.75 mM disodium EDTA–1.5 mM Tris (pH 7.6), also at 68°C. After two brief rinses in 2× SSC at room temperature, the filters were allowed to dry. They were mounted between two sheets of cellulose acetate, sandwiched between Lightning Plus intensification screens (Du Pont Co., Wilmington, Del.) with Kodak RP-X-Omat film, and exposed for 3 h to 5 days at −70°C.

RESULTS

Restriction enzyme analysis of wild-type and mutant phage DNAs. Almost all of this study was done with phage DNA derived from heat-inducible (*tsr*) mutants. The DNA genomes of these mutant phages were compared with those of the appropriate wild-type phage by restriction enzyme analysis. The results (Fig. 1) showed that the DNA restriction profiles of wild-type γ and γ-*tsr*-2 phages were identical, but that the restriction profiles of mutant γ-*tsr*-1 phage contained unique fragments, called "het-

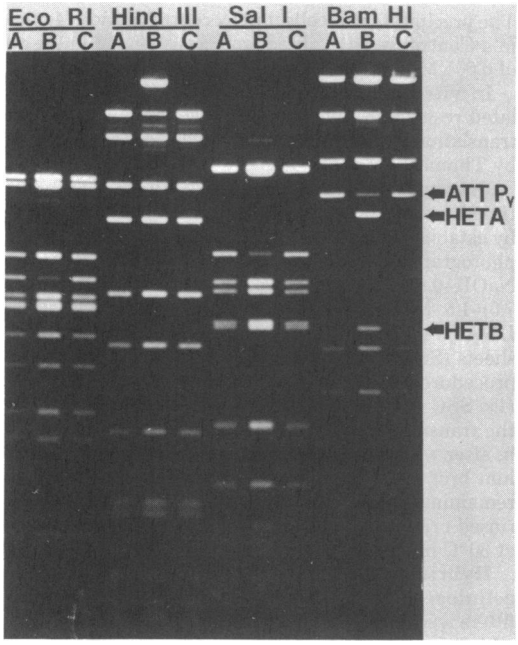

FIG. 1. *Restriction enzyme banding profiles of DNA from wild-type and mutant corynebacteriophage γ. Phage DNA was digested with the appropriate enzyme and electrophoresed horizontally for 12 h through a 0.7% agarose gel at 50 V in TENA gel buffer: (A) γ wild-type DNA, (B) γ-tsr-1 DNA, (C) γ-tsr-2 DNA. ATTP$_\gamma$ is the restriction fragment in the BamHI digests of all γ phages that is shown elsewhere (1) to carry the vegetative phage attachment site (attP$_\gamma$). HETA and HETB are the heterogeneous restriction fragments that appear in BamHI restriction profiles of DNA from lysates of γ-tsr-1 phage produced by heat induction of a lysogen. HET fragments of similar intensity are also evident in the profiles of γ-tsr-1 DNA cleaved by other restriction endonucleases (Table 1). Minor bands that appear in the EcoRI, HindIII, and SalI restriction profiles of all of these γ phages are the terminal fragments of vegetative phage DNA that are joined by the annealed cohesive (cos) sites (Fig. 2).*

erogeneous" (HET) fragments, in addition to the "normal" fragments exhibited by wild-type γ. The concentrations of the HET fragments varied in different preparations of phage DNA. This phenomenon of heterogeneity, noted previously (3), is described in detail in an accompanying paper (1). The banding patterns of the DNAs of β wild-type phage and mutants *β-tsr*-3 and *β-h tox*$^-$ (CRM45) were compared by using the same four restriction enzymes (data not shown). All β phage patterns were identical and differed only slightly from the γ wild-type pattern (see below and Fig. 7).

Genome sizes of β and γ phages. The sizes of the restriction products of DNA from *β-tsr*-3 and *γ-tsr*-1 mutant phages (Table 1) were estimated by digesting their genomes with restriction endonucleases and electrophoresing the fragments in agarose gels in parallel with molecular weight standards (data not shown). The sizes of the restriction products of *β-tsr*-3 phage DNA were summed to obtain an estimate of the genome size of β phage. The approximate size of the γ wild-type phage genome was determined by summing the sizes of all of the normal fragments (i.e., the heterogeneous fragments were not included) in the digests of DNA from *γ-tsr*-1 phage. The genome sizes of *β-tsr*-3, *γ-tsr*-1, and *γ-tsr*-2 phages were also determined by direct contour length measurement in the electron microscope (Table 2). The results from both methods were similar. The sizes of *β-tsr*-3, *γ-tsr*-1, and *γ-tsr*-2 phage genomes were 34.7, 37.7, and 35.9 kb, respectively.

Of particular interest to later studies was the observation previously reported for *β-tsr*-3 and *γ-tsr*-1 (3), and now also confirmed for *γ-tsr*-2, that Kleinschmidt spread DNA preparations of these phages contained relaxed circular DNA strands of the same contour length as the intact linear phage genomes. This finding indicated that the DNAs of vegetative corynebacteriophages have complementary single-stranded ends, i.e., cohesive ends (*cos* sites) which spontaneously anneal in solution. It also suggested that corynebacteriophage DNA circularizes after penetration but before replication or integration. This model is analogous to that proposed for the lambdoid phages of *E. coli* (4, 5). Its applicability to corynebacteriophages was previously suggested on the basis of the permuted relationship of vegetative and prophage genetic maps (18, 19).

Identification of restriction fragments bearing the *cos* sites. The first fragments identified in vegetative β phage DNA were those carrying the terminal *cos* sites. Cohesive sites already annealed are expected to remain annealed through restriction enzyme cleavage and gel electrophoresis. However, when the restriction enzyme-digested DNA preparation is heated to 75°C for 10 min and subsequently immersed in ice water, the *cos* sites will disassociate and remain so during electrophoresis. Thus, by electrophoresing heated and unheated DNA restriction digests in parallel lanes, it is theoretically possible to identify the terminal *cos* fragments of linear DNA and the annealed *cos* fragment of circular DNA. With this approach the annealed and separated *cos*-bearing fragments of the β phage genome were clearly identified in *Hpa*I digests (Fig. 2) as well as in *Bam*HI, *Sal*I, and *Hin*dIII digests of vegetative β phage DNA (data not shown). Although the

TABLE 1. *Size of restriction enzyme cleavage products of β and γ corynebacteriophages as determined by agarose gel electrophoresis*

Fragment	Fragment sizes (kb) after digestion with:									
	*Bam*HI		*Sal*I		*Hin*dIII		*Eco*RI		*Hpa*I	*Sma*I
	β[a]	γ[b]	β	γ	β	γ	β	γ	β	β
A	11.0	11.0	6.2	6.2[d,e]	15.0	10.8[e]	6.8	5.8	13.0	26.0
B	8.5	8.5	5.7	3.9	4.7	8.8	5.8	5.4[c,d]	12.0	9.2
C	3.9	6.4	3.4	3.4	3.7	7.5	3.8	3.8	9.2	
D	3.4	5.3	3.2	3.2	3.2	5.6	3.4	3.4	0.7	
E	3.0	4.8[e]	2.7	2.8	2.5	4.7	3.1	3.1		
F	2.4	2.7[e]	1.2[c]	2.7	2.0	3.2	3.0[c]	3.0[c]		
G	1.9	2.4	1.0[c]	1.2[c]	1.5	2.5	2.2	2.6		
H	1.0	1.9	0.7	1.0[c]	0.8	1.5	1.6	2.2		
I		1.0	0.6	0.7	0.8	0.8	1.4	1.6		
J			0.5	0.6	0.4	0.8	0.6	1.4		
K			0.3	0.5	0.3	0.4	0.25	0.6		
L				0.4	0.25	0.3		0.25		
M				0.3		0.25				
Totals[f]	35.1	36.5	33.9	35.3	35.2	36.4	35.0	36.2	34.9	35.2

[a] Digestion products from β-*tsr*-3 DNA.
[b] Digestion products from γ-*tsr*-1 DNA.
[c] Doublet.
[d] Triplet.
[e] HET fragment, present in γ-*tsr*-1 but not in γ-*tsr*-2 digests. Where these bands are doublets or triplets, only one is unique to γ-*tsr*-1.
[f] Only fragments present in both γ-*tsr*-2 and γ-*tsr*-1 digests were summed for the γ totals.

TABLE 2. *Molecular masses of corynebacteriophage DNA as determined by electron microscopic measurement of contour lengths*

Source of DNA	Megadaltons[a]	kb[b]
β-*tsr*-3	22.9 ± 0.08	34.7
γ-*tsr*-2	23.7 ± 0.15	35.9
γ-*tsr*-1	24.9 ± 0.07	37.7

[a] Measurements are given ± the standard error.
[b] Molecular masses were converted into kb by dividing by 660, the approximate average molecular mass of a single base pair.

cos fragments were never 100% annealed, the intensity differences between the annealed and separated bands were sufficient for identification. In each digest, the sum of the molecular sizes of the two disassociated fragments equaled that of the annealed fragment. *Hpa*I restriction endonuclease cut β-*tsr*-3 DNA into only three major fragments. Since the two which carried *cos* sites were identifiable as the terminal fragments, a rudimentary restriction map of the vegetative phage genome could be constructed.

Identification of the β phage DNA restriction fragment bearing *att*P. As discussed previously, the integration of β-converting phage probably proceeds by a mechanism similar to that used by the lambdoid phages of *E. coli*. In this process (Fig. 3), linear vegetative-phage DNA is circularized through the *cos* sites, and integration is accomplished by recombination between specific phage (*att*P) and bacterial (*att*B) attachment sites. In recombination, *att*P is split, one half going to each end of the inserted prophage genome. This transition of phage DNA from linear to circular vegetative and then to prophage produces some predictable alterations in restriction enzyme digest-banding patterns. In particular, it predicts that a single *att*P-bearing fragment (ATTP) in vegetative phage DNA will be replaced by two fragments (ATTL and ATTR) in the prophage digest (Fig. 3). Since it has been shown (18, 19) that the *tox* gene is closely linked to *att*P, identification of the ATTP fragment in vegetative-phage DNA restriction digests would locate either the *tox*-bearing fragment or one closely linked to it.

ATTP fragments were identified by comparing the restriction profiles of prophage and vegetative phage DNA. The prophage restriction profiles were visualized by hybridizing vegetative β-*tsr*-3 DNA, labeled with ^{32}P-deoxyribonucleotides by nick translation, to nitrocellulose blotted agarose gels of restriction enzyme-digested genomic DNA isolated from C7(β). When linear vegetative β-*tsr*-3 phage DNA and genomic C7(β) DNA digested by the same enzyme were electrophoresed, blotted, and hybridized in parallel, the predicted differences in their restriction profiles were observed (Fig. 4, columns A and B). With each enzyme, fragments present

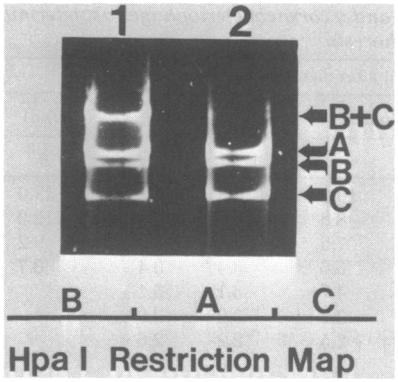

FIG. 2. *Identification of the restriction fragments containing the cohesive ends of β phage DNA. β-tsr-3 phage DNA was digested with HpaI restriction enzyme and electrophoresed vertically for 2.5 h on a 0.7% agarose gel at 100 V in TB gel buffer. Lane 1 contains normal untreated restriction fragments in which a significant fraction of the cos sites remain annealed. Lane 2 contains a sample that was incubated at 75°C for 10 min and subsequently immersed in ice water before loading the gel. The cos sites are denatured by this treatment. Band B+C (i.e., fragments B and C joined by the annealed cos sites) disappears, and bands B and C (containing the separated cos L and cos R sites, respectively) increase in concentration, i.e., brighten. The HpaI restriction map provided by these data is also shown. Recently, an additional small (0.7-kb) HpaI fragment was found and located between fragments A and C in this restriction map.*

in vegetative DNA restriction patterns disappeared and were replaced by fragments of different mobility in the prophage profiles. The vegetative fragments that disappeared in the prophage profiles included those carrying the *cos* sites which are covalently joined in the prophage and the ATTP fragments which are split in the prophage. Since the *cos* fragments were independently located (see above), the ATTP fragment in a particular restriction digest was identified as the only fragment that disappeared in the prophage restriction pattern that was not *cos* bearing. By this technique the ATTP fragments were identified as the *Bam*HI-C, *Sal*I-B and HindIII-A restriction fragments.

The two prophage restriction fragments (ATTL and ATTR) containing the prophage attachment sites (*att*L and *att*R, respectively) were also identified in this process. The new fragments that appeared in each of the prophage restriction profiles (Fig. 4, column B) included ATTL, ATTR, and a fragment formed by the covalent linkage of the *cos* sites of the terminal vegetative fragments. Since the *cos*-bearing fragments were previously identified, the remaining new fragments in the prophage profiles contained *att*L and *att*R sites and were designated ATTL and ATTR.

To verify and extend these results, the ATTP

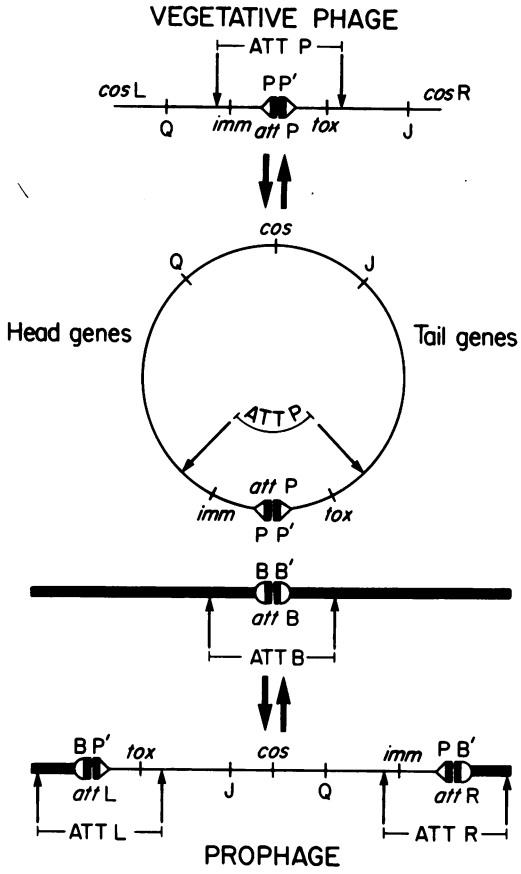

FIG. 3. *Restriction fragment rearrangement caused by the integration of vegetative phage DNA into the bacterial chromosome. The figure shows the model for the integration of vegetative phage DNA into the bacterial chromosome and the resultant restriction alterations. Hypothetical restriction cleavage sites are indicated with arrows, and the restriction fragments are labeled in capital letters according to the markers they carry, i.e., ATTP carries the vegetative phage attachment site (attP), ATTB carries the bacterial attachment site (attB), and ATTL and ATTR carry the prophage attachment sites (attL and attR, respectively). Since the ATTP fragments from different restriction digests carry different segments of DNA, the tox gene and the phage immunity genes (imm) are included in this fragment for convenience only. The relative locations of the J and Q cistrons, representing phage tail and head synthesis genes, respectively, and the cos sites, cos L and cos R of linear vegetative DNA and cos which represents the ligated cohesive sites of circular vegetative and prophage DNA, are given. Bacterial chromosome (■), phage chromosome (——).*

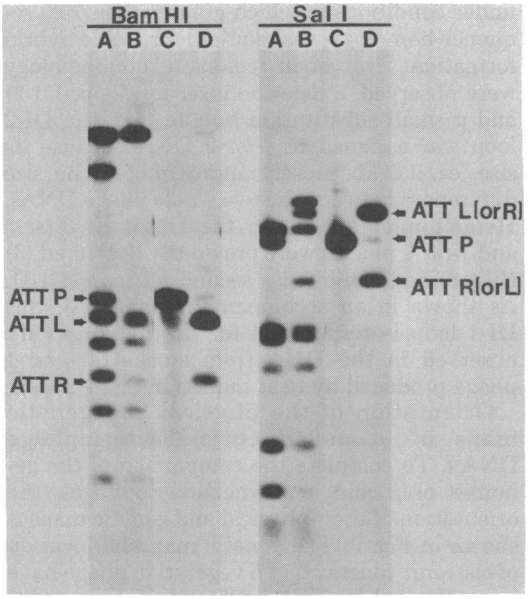

FIG. 4. *Identification of attachment site-bearing fragments in restriction digests of vegetative and prophage β DNA. The DNA samples from linear β-tsr-3 phage and lysogenic C7(β) were digested with BamHI or SalI restriction endonuclease and electrophoresed horizontally for 14 h in a 0.5% agarose gel at 50 V in TENA gel buffer. The gels were blotted to nitrocellulose filter paper and hybridized with nick-translated, ^{32}P-deoxyribonucleotide-labeled probes. The BamHI ATTP fragment was isolated by electroelution from agarose gels. (A) Whole β-tsr-3 DNA probe hybridized to blots of vegetative β-tsr-3 DNA; (B) whole β-tsr-3 DNA probe hybridized to blots of genomic DNA from C7(β); (C) BamHI ATTP fragment probe hybridized to blots of vegetative β-tsr-3 DNA; and (D) BamHI ATTP probe hybridized to blots of genomic DNA from C7(β). For the BamHI digests, ATTP designates the fragment in columns A and C. ATTL and ATTR designate fragments in columns B and D which run just slightly faster than the vegetative bands in column A. The differences in intensity of the ATT bands in the prophage digests could be due to a difference in the amount of phage DNA carried by these hybrid phage-bacterial DNA fragments. The second and sixth bands (numbering from the top) in column A are the two vegetative cos-bearing fragments (not labeled). The single prophage cos-bearing fragment comigrates with the topmost band in column B. For the SalI digests, ATTP designates the fragment in the second band, column A, and is clearly identified in column C. The ATTL and ATTR fragments are easily identified in columns B and D. The two vegetative cos-bearing fragments (not labeled) are in the first and seventh bands, column A, both of which are doublets. The single prophage cos-bearing fragment is the topmost band, column B. We know from evidence presented elsewhere (1, 2), that the ATTL fragment in the BamHI digest of C7(β) DNA carries the end of the prophage DNA containing the tox gene (Fig. 3). However, it is not known which fragment of the BamHI digest (band C) was isolated and hybridized to BamHI and SalI restriction digests of vegetative β-tsr-3 DNA and genomic C7(β) DNA (Fig. 4, columns C and D). As expected, on each blot the probe hybridized to the appropriate vegetative fragment identified above as ATTP (Fig. 4, column C) and to the two fragments in each prophage digest identified above as carrying the prophage attachment sites (column D). Hybridization to a single fragment in the vegetative phage digest and to two fragments in the prophage digest could only have occurred if the fragment used as probe was indeed ATTP. These results confirmed the identification of the ATTP fragments as BamHI-C, SalI-B and HindIII-A in restriction enzyme digests of vegetative β-tsr-3 DNA. As expected, these fragments overlap in the restriction maps of β phage (see Fig. 6). Since the attP site and the tox gene are very closely linked in the vegetative phage genome, the tox gene is probably within these or immediately adjacent fragments.*

Restriction enzyme maps of β phage DNA. As noted above, the identification of the terminal *cos* site-bearing fragments provided a simple three-fragment HpaI restriction map of the β phage DNA. This simple map was further defined by double digestion of β phage DNA with HpaI and other restriction endonucleases. In the initial experiments the DNA was digested with HpaI and then with a second enzyme, and the products were electrophoresed in agarose gels in parallel with DNA digested by only the second enzyme (Fig. 5). Fragments carrying HpaI cleavage sites were identified as those in the second enzyme digest that disappeared on double digestion.

In a second series of double-digestion experiments, two-dimensional agarose gels, as described by Villems et al. (32), were used to identify second-enzyme fragments contained within each HpaI fragment (data not shown). Relatively complete maps of BamHI, SalI, and HindIII restriction endonuclease cleavage sites on the β phage genome were constructed in this manner. To clear up some of the remaining ambiguity in the SalI and HindIII restriction maps, all of the fragments generated by BamHI digestion of β-tsr-3 DNA were isolated from agarose gels. Appropriate groups of these fragments were subjected to cleavage with HindIII and SalI endonucleases and electrophoresed in agarose gels in parallel with whole phage DNA digested with the same enzyme. SalI and HindIII fragments encompassed by particular BamHI fragments were identified and placed in

of the prophage fragments in the SalI digest of C7(β) is ATTL and which is ATTR.

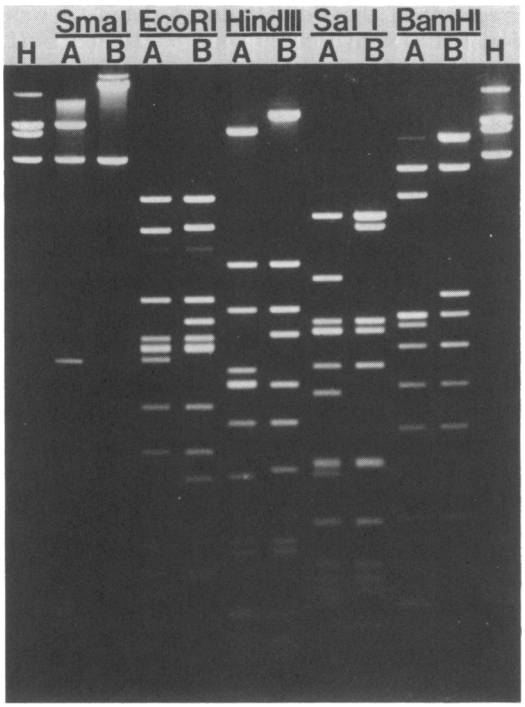

FIG. 5. *Restriction profiles of β-tsr-3 DNA cleaved with two restriction endonucleases. Phage β-tsr-3 DNA was cleaved with HpaI restriction endonuclease. The reaction mixture was passed over a miniature Biogel P2 column (see text) to remove the salts, and the DNA was redigested with a second restriction enzyme. The restriction products were electrophoresed horizontally for 13 h in a 0.7% agarose gel at 50 V in TENA gel buffer. Samples digested with the second enzyme only were run in parallel with the double-digested DNA. (H) DNA digested with HpaI alone; (A) DNA digested with both HpaI and the second enzyme; and (B) DNA digested with the second enzyme only. Less intense bands, e.g., the uppermost band in the HpaI digests, in some of the restriction profiles are the terminal fragments of the phage genomes joined by their annealed cos sites.*

sequence by analysis of partial digestion products. The restriction maps (Fig. 6) were derived from all of these data.

Comparison of β and γ phage DNAs. DNA from *β-tsr*-3 phage was compared with that of *γ-tsr*-2 phage. As noted above, the restriction patterns of these mutants were identical to those of their respective wild-type progenitor strains. The restriction profiles of their genomes were very similar, although a few minor yet very distinct differences were observed (Fig. 7).

The physical relatedness of the DNAs of these β and γ phages was also examined by heteroduplex analysis (Fig. 8). Over 99% of the *β-tsr*-3 DNA hybridized with sequences of *γ-tsr*-2 DNA under conditions in which greater than 70% sequence homology was required for stable hybrid formation. Two small regions of nonhomology were observed, a deletion insertion loop (DI-2) and a small substitution bubble (S). The DI-2 loop was assigned to *γ-tsr*-2 DNA because its size, ca. 1.2 kb, closely approximated the size differences between the two whole phage DNAs. Heteroduplexes between the DNAs of *β-tsr*-3 and *γ-tsr*-1 phages were previously described (3) (Fig. 8) and exhibited a second DI loop (DI-1). As shown in an accompanying paper (1), this DI-1 loop is responsible for the heterogeneity observed in the DNA from stocks of *γ-tsr*-1 phage produced by heat induction of a lysogen.

Orientation of the physical and genetic maps of γ and β corynebacteriophage DNAs. To complete the comparison of the genomes of β and γ corynebacteriophages, the orientation of their physical and genetic maps is shown in Fig. 9. The genetic map and location of cistronic functions of β vegetative phage have been detailed by Singer (25, 26). We have previously established that the *att*P site in β vegetative phage DNA is located between the genes for immunity (*imm*) and *tox* (18, 19). In accompanying papers we have shown that the *tox* gene is closely associated with the DI-2 loop of the β/γ DNA heteroduplexes (2), and that the DI-1 loop of the β/*γ-tsr*-1 heteroduplex identifies the location of the *att*P site (1). Since *att*P is located between *imm* and *tox* on their genetic maps, the heteroduplex maps of these phages were oriented with their genetic maps as shown.

The *Bam*HI restriction map of the genome of γ phage was deduced from that of β phage by comparing the *Bam*HI restriction profiles of the DNAs from *γ-tsr*-2 and *β-tsr*-3 phages (Fig. 7). The DNA restriction profiles of these two phages were similar, except that three fragments, bands C, D, and E, in the *β-tsr*-3 phage DNA digest were replaced by two fragments of different mobility, bands C and D, in the *γ-tsr*-2 DNA digest. Because of the high degree of homology between the DNAs of these phages, we assumed that fragments common to the *Bam*HI restriction profiles of DNA from both phages were located in corresponding regions of their genomes. In addition, we have demonstrated by heteroduplex analysis that band C of the *Bam*HI digest of *β-tsr*-3 DNA and band D of the *γ-tsr*-2 DNA digest carry corresponding sequences (1, 2). With these data, all of the restriction fragments produced by *Bam*HI digestion of the genomes of these two phages, with the exception of band C in the *γ-tsr*-2 digest and bands D and E in the *β-tsr*-3 digest, were aligned as shown. Because all of the other fragments had been aligned, we assumed that band C in the γ-

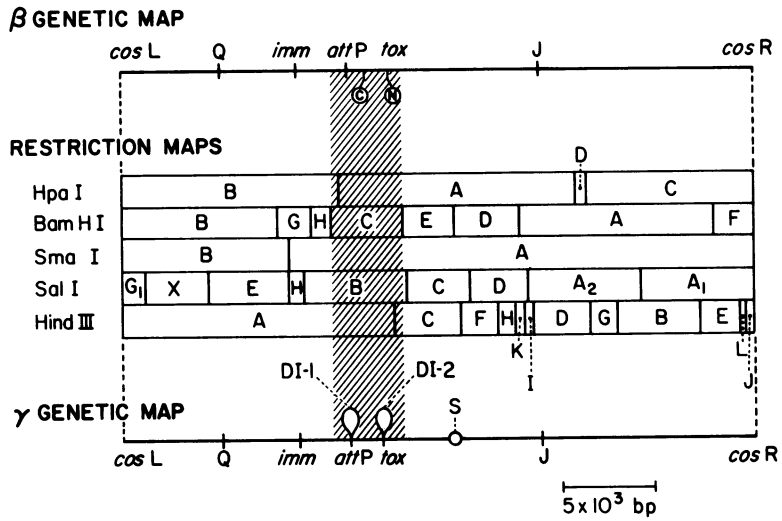

FIG. 6. *Restriction enzyme maps of β-converting corynebacteriophage. The restriction maps of β phage are shown oriented with the genetic maps of β and γ phages (see Fig. 3 for an explanation of the genetic markers and symbols). The amino-terminal (N) and carboxy-terminal (C) coding regions of the tox gene are shown on the genetic map of β phage. The loops and bubble on the genetic map of the γ phage were visualized in heteroduplexes between the DNAs of β and γ phage (Fig. 8). The loops represent the DI-1 and DI-2 insertions which have been shown in accompanying papers (1, 2) to be associated with attP and tox, respectively. The shaded regions of the maps show the overlap between the ATTP fragment in the BamHI digest (band C) and fragments in the other restriction maps. BamHI fragment C carries attP and, as is shown elsewhere (2), carries tox. Fragments with subscripts, e.g., SalI fragments A_1 and A_2, comigrate in doublets or triplets on agarose gels. Some small restriction fragments (F, G_2, I-K) in the band X region of the SalI map have not been ordered unequivocally. Hind III fragments H, I, K, and L have not been ordered unequivocally.*

tsr-2 digest and bands D and E from the β-*tsr*-3 digest carried corresponding regions of the genomes of these phages. This assumption was reinforced because the size of γ-*tsr*-2 band C (6.4 kb) equaled the sum of the sizes of β-*tsr*-3 bands D (3.4 kb) and E (3.0 kb). From these data, the *Bam*HI restriction map of the genome of γ phage was derived (Fig. 9).

Finally, to orient the restriction maps with the genetic maps of β and γ phage DNA, we compared the *Bam*HI restriction profiles of the genomes of these phages to that of a previously isolated β-γ recombinant phage which resulted from a crossover within the *tox* gene (2). The recombinant carried the left arm (from *tox* through *imm* to *cos*L) of γ phage DNA and the right arm (from *tox* through *h* to *cos*R) of β phage DNA. The *Bam*HI restriction profiles of the genomes of these recombinants were identical to those of the parental β phage DNA. Thus, the left arms of the γ and β phage DNAs have identical *Bam*HI restriction profiles, and any differences in the profiles of these phages are in the right arms of their genomes. *Bam*HI fragment C of β-*tsr*-3 DNA and fragment D of γ-*tsr*-2 DNA were shown elsewhere (2) to carry *tox*; therefore, they are the focal fragments of this analysis. However, as discussed above, fragments D and E in the *Bam*HI digest of β-*tsr*-3 DNA are replaced by fragment C in the *Bam*HI digest of γ-*tsr*-2 DNA. Therefore, these *Bam*HI fragments, D and E of the β-*tsr*-3 digest and C of the γ-*tsr*-2 digest, are on the right arm (*tox* through *h* to *cos*R) of the genetic map. This information permitted the orientation of the restriction enzyme cleavage maps of these phage DNAs with their genetic and heteroduplex maps (Fig. 9).

DISCUSSION

The DNAs from wild-type and mutant strains of β-converting and γ-nonconverting corynebacteriophages were isolated and physically characterized. The data obtained from heteroduplexes, restriction enzyme profiles, and restriction enzyme cleavage maps reinforce the conclusion (see above) that β and γ phages are very closely related. The heteroduplexes of the DNAs of these phages exhibit virtually 99% homology, although there are small regions of nonhomology. The nonhomologous regions include a small substitution and a small insertion (DI-2 loop) which is assigned to γ DNA. The substitution is located among phage tail synthesis and assembly genes. It is unknown whether this represents a

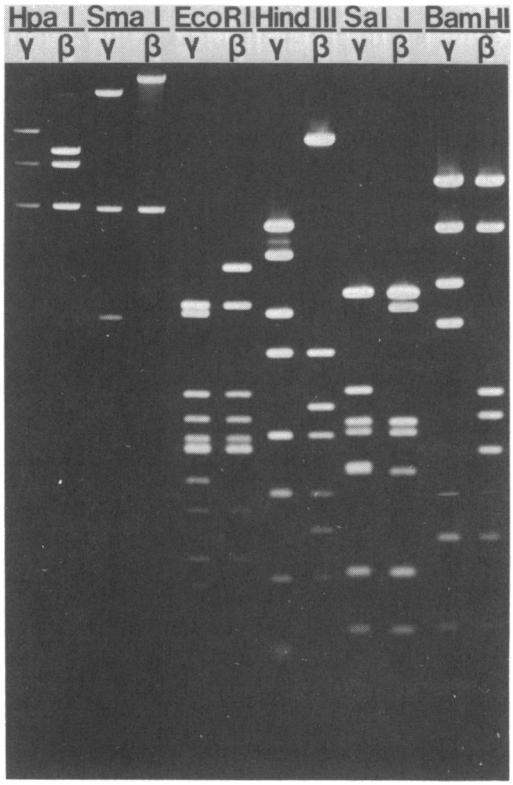

FIG. 7. *Restriction enzyme banding profiles of β-tsr-3 and γ-tsr-2 corynebacteriophage DNAs. Phage DNA was digested with the appropriate enzyme and electrophoresed horizontally for 14 h in a 0.7% agarose gel at 50 V in TENA gel buffer. (β) β-tsr-3 DNA; (γ) γ-tsr-2 DNA. Less intense bands in some of the restriction profiles are the terminal fragments of the phage genomes joined by their annealed cos sites.*

change in the coding sequence of a specific gene or a nonexpressed region in the phage genome. The DI-2 loop insertion accounts for the difference in the sizes of the β and γ genomes and also, along with the substitution, accounts for most of the differences in their restriction profiles (Fig. 9). We have shown in accompanying papers that the DI-2 loop insertion is apparently a transposable sequence carried on the bacterial genome (1), and that it is inserted into the *tox* gene of γ phage (2). This implies that γ was originally a converting corynebacteriophage. Finally, because β and γ are heteroimmune phages, it is surprising that their genomes exhibit such a high degree of homology. In fact, their *imm* genes are located on the phage genomes in regions of heteroduplex homology (Fig. 9) and therefore must only differ by relatively minor changes in base sequence. All of this evidence attests to the close evolutionary relationship between β and γ corynebacteriophages and suggests that they are very recent derivatives of a common ancestral phage.

The physical data assembled here confirm the suggestion that in many ways the corynebacteriophages are remarkably similar to the lambdoid phages of *E. coli*. The changes observed in the restriction profiles of the vegetative and prophage forms of β phage DNA conform to the model proposed for the integration lambdoid phages into the *E. coli* chromosome (5). It appears that after penetration, the terminal cohesive (*cos*) sites of vegetative corynebacteriophage DNA anneal and ligate to circularize the phage genome. The phage DNA is then integrated into the bacterial chromosome by a site-specific recombination which results in the splitting of the specific phage (*att*P) and bacterial (*att*B) attachment sites to form the prophage attachment sites, *att*L and *att*R.

Mutant phage γ-*tsr*-1 is unusual in that its genome contains a second insertion (DI-1 loop) that is not present on the genome of γ wild-type phage. Furthermore, the restriction enzyme cleavage profile of γ-*tsr*-1 DNA contains heterogeneous fragments in addition to all of the normal fragments found in restriction digests of other γ phages. This heterogeneity is a consequence of a change in specificity in the prophage excision process and results in the formation of specialized transducing γ-*tsr*-1 elements. This phenomenon is described in detail in an accompanying paper (1).

ACKNOWLEDGMENTS

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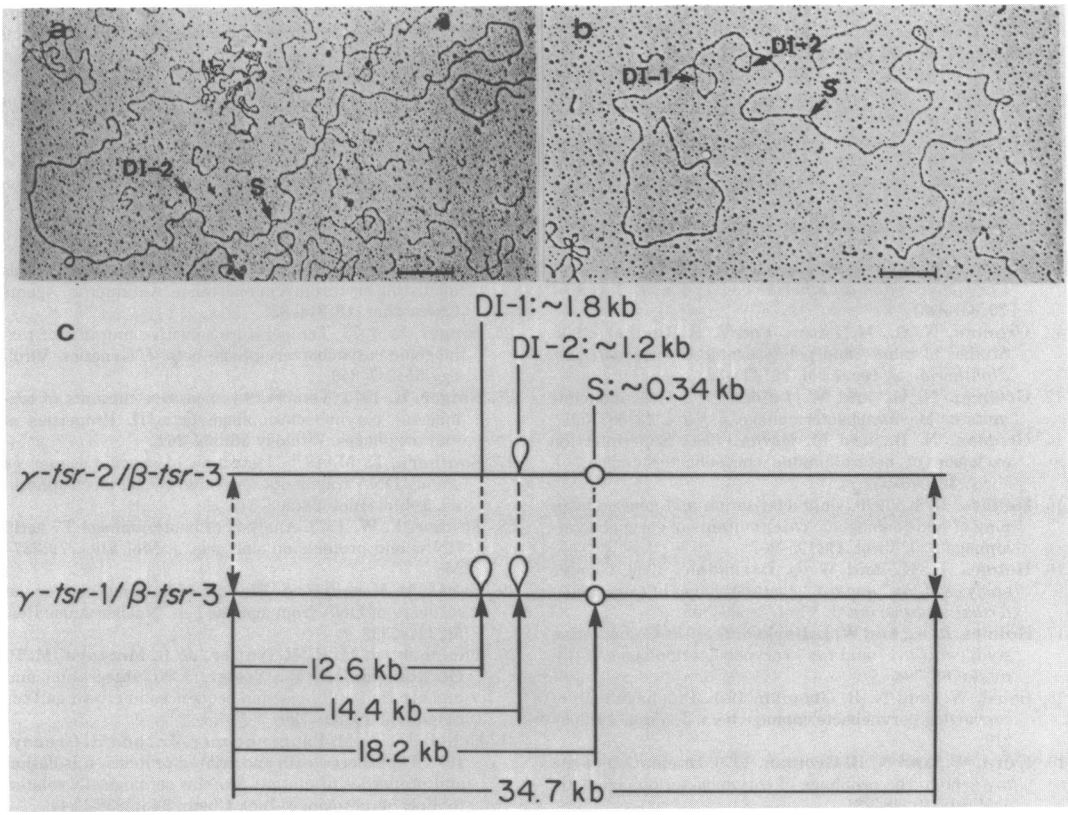

FIG. 8. *Heteroduplexes between the genomes of β and γ corynebacteriophages. (a) Heteroduplex between γ-tsr-2 and β-tsr-3 DNA; (b) heteroduplex between γ-tsr-1 and β-tsr-3 DNA; and (c) graphical representations of the heteroduplexes. Bars, 0.5 μm. The two deletion-insertion loops (DI-1 and DI-2) and the substitution (S) seen in these heteroduplexes are noted.*

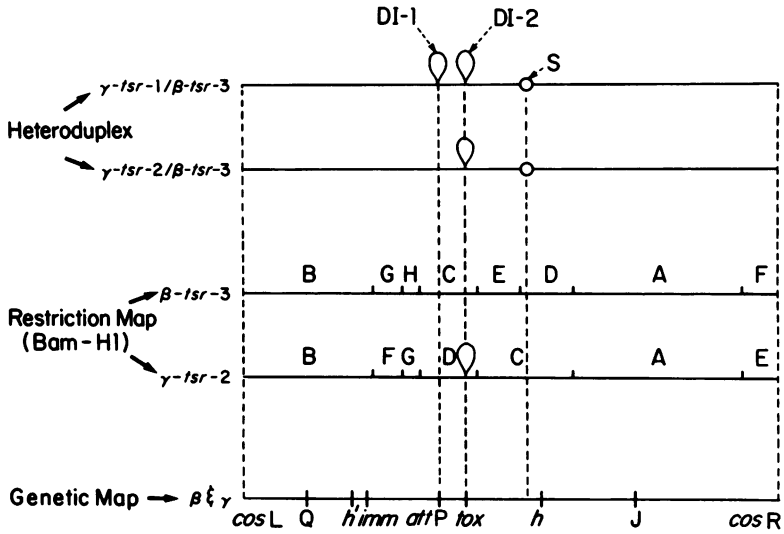

FIG. 9. *Orientation of physical and genetic maps of the genomes of β and γ corynebacteriophages. The heteroduplexes of β-tsr-3 DNA with the DNAs of γ-tsr-1 and γ-tsr-2 phages and the BamHI restriction enzyme cleavage maps of the genomes of β-tsr-3 and γ-tsr-2 phages are oriented with the known genetic map of β and γ phages. The relative positions of the selective host range markers, h and h', are given. Other genetic loci and symbols are explained in Fig. 3 and 8.*

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