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Common Organization of Chromosomal Loci for Production of Different Capsular Polysaccharides in *Haemophilus influenzae*

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Cloned *Haemophilus influenzae* type b capsulation genes were used as hybridization probes to isolate DNA from the capsulation loci (*cap*) of other serotypes of *H. influenzae*. Mapping of the resulting clones and Southern hybridization analysis of chromosomal DNAs from type a, b, c, and d strains showed that in each strain *cap* was organized in the same way: a central DNA segment specific to each serotype flanked by DNA segments of common structure. We infer that enzymes necessary for the synthesis of specific capsular polysaccharide are encoded in the central segment of *cap*, while proteins involved in a more general way in the process of capsulation are encoded in the flanking segments. Studies of the function of the DNA in one of these non-serotype-specific flanking segments (J. S. Kroll, I. Hopkins, and E. R. Moxon, Cell 53:347–356, 1988) have previously identified a gene encoding a protein necessary for polysaccharide export, an event now deduced to proceed by a mechanism independent of the nature of the disaccharide subunit in the polysaccharide. The near-total duplication of *cap* that has been found in most type b strains was not found at the analogous locus in the other serotypes. This reinforces our previous hypothesis, based on study of type b strains alone, that while such a duplication is unnecessary for capsulation, it confers some unexplained survival advantage on the widely prevalent strains with this clinically important serotype.

Haemophilus influenzae is an important human pathogen, with type b strains among the commonest causes of nonepidemic bacterial meningitis in children, and the type-specific polysaccharide capsule is a well-characterized determinant of pathogenicity. While the capsulation locus (*cap*) in a prototypic virulent type b strain has been identified and partially characterized (6, 9, 10), *cap* loci in other serotypes have not been studied. Hybridization studies have demonstrated, however, that DNA located in the type b *cap* locus is also to be found in the chromosomes of other serotypes (5, 9), although its physical relationship to other *cap* sequences in such strains has not been defined.

Two sets of findings suggest that there are likely to be close relationships between the *cap* loci of different serotype strains of *H. influenzae*. (i) We have shown that a type b strain can be transformed to the other five serotypes with donor chromosomal DNA, producing chimeras in which the only phenotypic change detected is the capsular serotype (A. Zwahlen, J. S. Kroll, L. G. Rubin, and E. R. Moxon, unpublished data). In chromosomal hybridization studies of these strains using DNA probes cloned from the type b *cap* locus, while different patterns of hybridization characterized each serotype, the DNA flanking the *cap* loci appeared to be unaltered compared with that of the recipient type b strain, suggesting that changes within *cap* could suffice to change the serotype. (ii) Multilocus enzyme electrophoresis has been used to study genetic relationships among 2,209 encapsulated clinical isolates of *H. influenzae* (13). Analysis of electrophoretically demonstrable allelic variation at 17 chromosomal enzyme loci revealed that there were two primary phylogenetic divisions, I and II, in this bacterial population, and a review of the results obtained on *cap* probe hybridization to chromosomal DNAs prepared from several hundred clinical isolates of capsulate *H. influenzae* of all serotypes suggested that the greatest differences in probing patterns were to be found between strains in different divisions, while similarities existed between strains studied from one division, regardless of serotype.

In this work, we showed that the *cap* locus in strains segregating to phylogenetic division I has a physical organization independent of serotype and that the capsular phenotype expressed appears to depend on the nature of a cassette of DNA inserted into common flanking sequences in *cap*.

MATERIALS AND METHODS

Media and culture conditions. *H. influenzae* strains were grown in brain heart infusion broth supplemented with 2 μg of NAD and 10 μg of hemin per ml. Translucent brain heart infusion plates were prepared with 1% agar and supplemented with 10% Levinthal base (1). Colony phenotypes were assessed by viewing with obliquely transmitted light as previously described (5). Strains were stored at −70°C in supplemented brain heart infusion broth containing 20% glycerol.

Bacterial strains. *H. influenzae* RM135, RM127, and RM128 are laboratory transformants of the capsule-deficient host $Rd^-/b^-/02$ to types b, c, and d (18; unpublished results). All of the other *H. influenzae* strains used were clinical isolates from our collection as follows: type a, RM7190; type b, RM8055 and RM153; type c, RM8032 and RM7422; type d, RM6137 and RM7033; capsule-deficient mutants of type d strains, RM118 (Rd) and RM7051.

Escherichia coli P2-392, a P2 lysogen obtained from Northumbria Biologicals Ltd., was used to propagate bacteriophage lambda. Constructions in plasmid pUC13 were propagated in *E. coli* DH5α.

Preparation and analysis of genomic DNA. Total cellular DNA was prepared from 3-ml broth cultures as previously described (5). The products of digestion of this DNA with different restriction endonucleases were separated electrophoretically on 0.7% agarose-Tris acetate gels, transferred to nitrocellulose filters (17), and hybridized at high stringency

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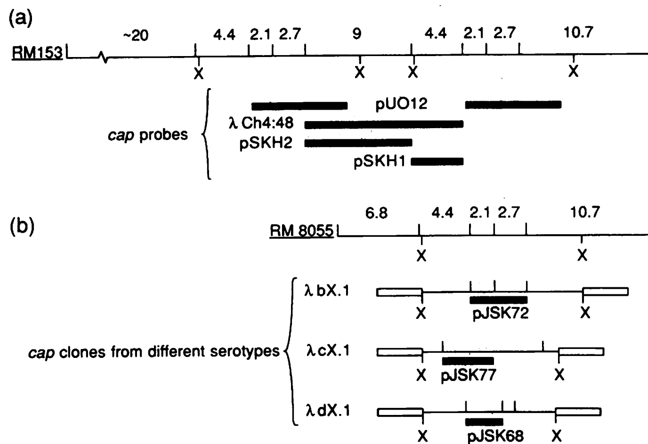

FIG. 1. (a) Physical map of *cap* in type b strain RM153 and DNA cloned from the locus. *Eco*RI sites are represented by unlabeled vertical lines above the chromosome, and the sizes of *Eco*RI fragments are given in kilobases. *Xho*I sites (X) are shown below the chromosome. Bars indicate DNAs cloned from *cap* in various constructions. Both copies of the insert of pUO12 in the locus are shown to indicate the overlap of this probe with the insert of pSKH2. (b) Physical map of *cap* in type b strain RM8055, showing *Eco*RI and *Xho*I sites and *Eco*RI fragment sizes as in RM153. The *Xho*I fragment in this locus was cloned in λbX.1, shown below the RM8055 chromosome. Below this, aligned by the left-hand *Xho*I site, are the cloned *Xho*I fragments of λcX.1 and λdX.1, from the type c and d *cap* loci, respectively. Hollow bars represent the lambda arms, and solid bars represent the parts of each lambda clone subcloned in various constructions.

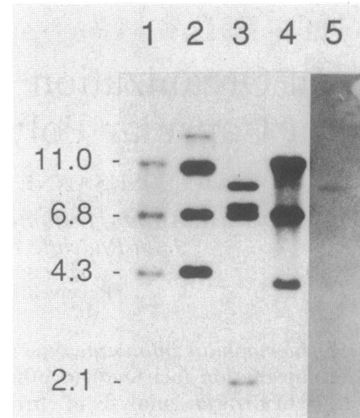

FIG. 2. Southern blot of *Eco*RI-digested chromosomal DNAs from different strains of *H. influenzae* probed with λCh4:48. The DNAs in lanes 1 to 5 were from the following strains (serotypes): 1, RM7190 [a(K)]; 2, RM8055 (b); 3, RM8032 (c); 4, RM6137 (d); 5, RM118 (d⁻). To show the faintly hybridizing 8.5-kb *Eco*RI fragment in RM118, this part of the autoradiograph was overexposed. The sizes of fragments in lane 1 and the smallest fragment in lane 3 are shown on the left in kilobases.

to nick-translated (14) [α-^{32}P]dCTP-labeled DNA probes derived from *Haemophilus cap* loci as follows (Fig. 1).

pSKH1 and pSKH2, cloned from the *cap* locus of RM153 via lambda clone λCh4:48, have been described previously (5, 12). pUO12, kindly provided by S. Ely, contains *cap* DNA from a different gene bank of this strain, subcloned into plasmid vector pBR322. pUO12 overlaps with pSKH2 and hybridizes to chromosomal loci corresponding to the left part of the insert of the latter probe (Fig. 1a). In RM8055, the 10.7-kilobase (kb) *Eco*RI fragment, but not the 6.8-kb *Eco*RI fragment, hybridizes to pUO12. pAD2, subcloned from a gene bank of a type a strain, was kindly provided by A. Dhir. It consists of a 1.4-kb type a-specific *Eco*RI fragment (see Fig. 3) isolated from the *cap* locus of a type a strain from phylogenetic division II subcloned into pUC13 (J. S. Kroll, A. Dhir, and E. R. Moxon, unpublished data). pJSK72, pJSK68, and pJSK77 are described in this work.

Construction of lambda gene banks. Total cellular *Haemophilus* DNA was digested with restriction endonuclease *Xho*I. The resulting fragments were size fractionated by centrifugation through a sucrose density gradient, and the fractions containing 15-kb fragments were selected. These were ligated to *Xho*I-digested arms of bacteriophage lambda 2001 (8), and the resulting concatemers were packaged to form infectious particles by using a packaging kit purchased from Amersham International. The resulting phage banks were designated λbX, λcX, and λdX on the basis of the serotype of the *Haemophilus* transformant used.

Plasmid constructions. Portions of each λ *cap* clone were subcloned into plasmid vector pUC13. For *Eco*RI fragments from λbX.1 and λdX.1, the products of digestion were ligated into the polylinker *Eco*RI site of the vector, giving pJSK72 and pJSK68 (Fig. 1b). pJSK77 (Fig. 1b) contains an *Eco*RI-*Cla*I fragment from λcX.1 which was subcloned into pUC13 modified by conversion of the polylinker *Bam*HI site to a *Cla*I site (achieved by religating blunted *Bam*HI ends filled in by using the Klenow fragment of *E. coli* polymerase I). In each case, recombinants were distinguished from nonrecombinants by failure to confer on DH5α the ability to generate a blue colony in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and isopropyl-β-D-thiogalactopyranoside. Restriction endonuclease cleavage maps of these constructions derived by multiple endonuclease digestion matched the maps of the corresponding chromosomal loci.

pSZ1 was isolated from a gene bank of *Eco*RI fragments of RM118 cloned into pBR328. *E. coli* harboring the desired recombinant clone was identified by colony hybridization by using λCh4:48 as a probe with no homology to vector sequences.

RESULTS

Diversity of *cap* in clinical isolates of type a, c, and d *H. influenzae*. λCh4:48 (Fig. 1a) was used as a hybridization probe to assess the diversity of *cap* loci of different serotypes (Fig. 2). As in our previous studies of type a and b strains (2, 3), *Eco*RI was chosen as the reporter enzyme for mapping. Twenty-seven type d strains collected from all over the world over several decades gave identical probing patterns, consistent with their putative clonal evolutionary origin (13); *Eco*RI fragments of approximately 11.3, 6.8, and 3.95 kb hybridized to the probe. Thirteen type c strains of similarly diverse origins were examined. All but two strains showed *Eco*RI fragments of 8.7, 7.2, 6.8, and 2.1 kb hybridizing to the probe; two differed in lacking the 7.2-kb band (Southern blot not shown). This restriction fragment length polymorphism correlated exactly with the segregation of type c strains into two evolutionary lineages within phylogenetic division I as previously described (13). The situation with type a strains was found to be complex. Three restriction fragment length polymorphisms were found among type a strains from phylogenetic division I. Two of these, types a(T) and a(N), have been described previously (2), and on

being probed with λCh4:48 they showed *Eco*RI fragments of approximately 11.0, 9.5, and 4.3 kb in common while differing in possession of 6.8 (T)- or 5.4 (N)-kb fragments (Southern blots not shown). We identified a third type, a(K) (Fig. 2). This is of worldwide distribution, like a(T), and is clonally indistinguishable from a(T) by electrophoretic typing (13) but lacks the 9.5-kb *Eco*RI fragment that hybridizes to the probe in the a(T) chromosome. Strains with the a(K) pattern thus have *Eco*RI fragments of approximately 11.0, 6.8, and 4.3 kb hybridizing to λCh4:48 (Fig. 2).

We therefore identified a 6.8-kb *Eco*RI fragment hybridizing to λCh4:48 in strains of serotypes a, c, and d, a feature they have in common with type b strain RM8055 (Fig. 2), in which the *cap* locus has been mapped (10). In RM8055, the 6.8-kb *Eco*RI segment has been identified as one containing DNA encoding non-serotype-specific functions and present in all capsulate *H. influenzae* strains (9). Examples of type a(K), c, and d strains were therefore chosen for structural analysis of *cap* and comparison with the locus in type b strain RM8055.

Cloning of DNA from the *cap* loci from type b, c, and d transformants of Rd⁻/b⁻/02. Laboratory transformants of Rd⁻/b⁻/02 to all six serotypes (Zwahlen et al., unpublished data) were used as a convenient source of *cap* DNA from well-characterized strains of each serotype in the genetic background of phylogenetic division I. Southern blots of electrophoretically separated *Xho*I-digested chromosomal DNA were hybridized to *cap* probe pUO12 (Fig. 1a, map). Type a, b, c, and d transformants showed single strongly hybridizing fragments, and in types b, c, and d these were of appropriate size for cloning into λ2001. The resulting banks of phage, designated λbX, λcX, and λdX, were used to infect *E. coli* P2-392, and the resulting plaques were blotted to nitrocellulose and probed with [α-^{32}P]dCTP-labeled pUO12. Phage DNA was prepared from plaques hybridizing to the probe, and the *Eco*RI sites in the inserts were mapped in one clone derived from each serotype (Fig. 1b).

Chromosomal mapping in wild-type strains by using DNA probes subcloned from the *cap* loci. DNAs prepared from *cap* clones λcX.1 and λdX.1 were subcloned into pUC13, and these constructions, together with intact λ clone DNA and pSKH1 and pSKH2 (Fig. 1a), were used as hybridization probes to map in detail the chromosomal *cap* loci in RM8055 (serotype b), RM8032 (serotype c), and RM6137 (serotype d). In addition, pAD2 was used to map *cap* in RM7190 [serotype a(K)]. Maps of the *cap* loci were constructed by using a panel of seven restriction endonucleases—*Eco*RI, *Sca*I, *Nco*I, *Xho*I, *Pst*I, *Cla*I, and *Xba*I—which located 22 to 26 sites over a span of 23.4 (serotype a), 26.7 (serotype b), 25.0 (serotype c), and 25.8 (serotype d) kb (Fig. 3). The internal consistency of the chromosomal maps was confirmed by probing Southern blots of doubly digested chromosomal DNA.

The possibility of duplication at *cap* in type a, c, and d strains, as a direct repeat analogous to the situation found in many type b strains (6), was excluded by identifying restriction sites in DNA flanking the locus in each case, thus confirming that the entire locus was mapped and accounting for all hybridizing fragments in Southern blots. In the presence of a duplication, extra bridging fragments hybridize to the probe. Instead, in each case the *cap* locus superficially resembled that previously described in type b strains as single copy (10), and when the maps were aligned by the left-most *Eco*RI site, striking similarities were immediately obvious.

In the orientation of Fig. 3, the left-most (6.8-kb) *Eco*RI

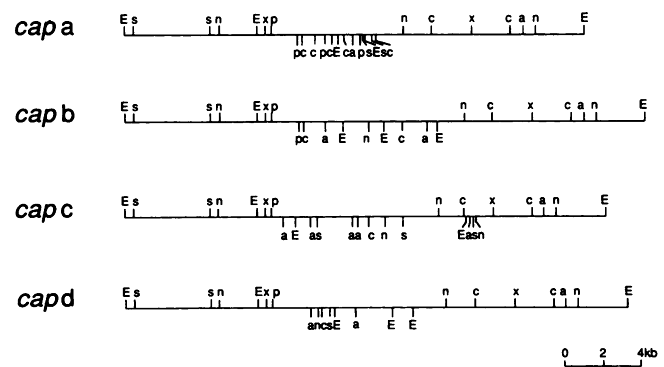

FIG. 3. Physical maps of the chromosomal capsulation loci *cap*a, *cap*b, *cap*c, and *cap*d from strains RM7190, RM8055, RM8032, and RM6137, respectively, aligned by their left ends. Restriction sites common to all serotypes are shown above the line of the chromosome, and those found only in one serotype are below the line. Abbreviations: E, *Eco*RI; s, *Sca*I; n, *Nco*I; x, *Xho*I; p, *Pst*I; c, *Cla*I; a, *Xba*I.

fragments have the same map in all four serotypes, and the similarity extends at least 1 kb into pSKH1-hybridizing DNA, to a *Pst*I site found in all four *cap* loci. The *cap*c and *cap*d maps diverge from each other and from those of *cap*a and *cap*b after this site, but the latter maps remain the same for a further 1.4 kb before diverging in turn.

In each *cap* locus, there follows a stretch of DNA with an endonuclease cleavage map unique to each serotype. Alignment of the maps allowed the sizes of these regions to be estimated at 3.2 to 5.4 (serotype a), 5.8 to 8.2 (serotype b), 5.3 to 7.7 (serotype c), and 5.3 to 8.7 (serotype d) kb; the lower and upper limits represent the span between unique and common restriction sites, respectively. In type a and b *cap* loci, this region contains DNA known to be serotype specific (A. Dhir, personal communication; S. Ely, J. Tippett, and E. R. Moxon, unpublished data). The hypothesis that this region in *cap*c and *cap*d would similarly contain serotype-specific DNA was tested by using subclones pJSK77 and pJSK68 (Fig. 1b) together with pAD2 and pJSK72 (Fig. 1b) as hybridization probes to Southern blots of chromosomal digests. These probes hybridized uniquely to the c, d, a, and b chromosomes, respectively (Fig. 4). The c and d probes were similarly tested against chromosomal DNAs from type e and f strains, and no hybridization was found.

Beyond this serotype-specific region, the maps for the loci in all four strains converge on a common set of restriction sites. In *cap*a, *cap*b, and *cap*d, the similarity persists to the limit of the mapped DNA, but in *cap*c a short stretch of DNA bears unexpected *Eco*RI, *Sca*I, *Nco*I, and *Xba*I restriction sites, after which the map reverts to the common pattern. The capsulation locus was also mapped in RM7422, one of the two type c strains with an anomalous probing pattern. The locus had a map identical to that found in RM8032, with the exception of the location of the right-most *Eco*RI and *Nco*I sites; the *Eco*RI site was displaced 1.5 kb further rightward, giving rise to a second 8.7-kb fragment rather than a 7.2-kb fragment hybridizing to λCh4:48 and so explaining the probing result.

cap was mapped in lesser detail in a selection of other type a(K), c, and d strains by using a subset of the panel of restriction enzymes, and in each case the pattern conformed to the detailed map.

A residual capsulation locus in capsule-deficient mutants of

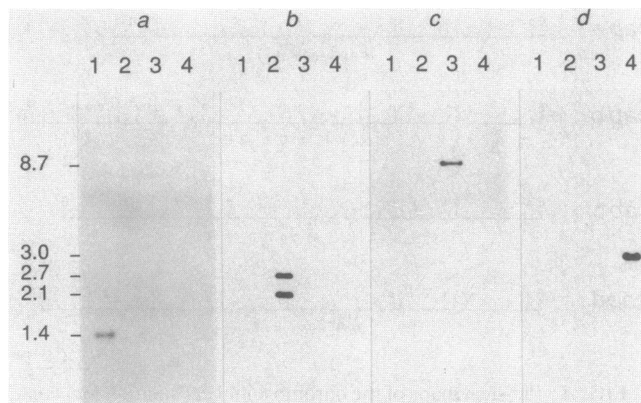

FIG. 4. Southern blots of *Eco*RI-digested chromosomal DNAs from serotype a(K), b, c, and d *H. influenzae*. Duplicate filters a to d contained DNAs from the following strains (serotypes): lane 1, RM7190 [a(K)]; lane 2, RM8055 (b); lane 3, RM8032 (c); lane 4, RM6137 (d). Nick-translated DNA probes (serotype of probe origin) were used as follows: filter a, pAD2 (a); filter b, pJSK72 (b); filter c, pJSK77 (c); filter d, pJSK68 (d). Fragment sizes in kilobases are shown on the left.

type d strains. In RM8055 and other type b strains with a single-copy *cap* locus, the capsulate phenotype appears to be stable (10), but capsule-deficient mutants of type d strains, established here as having single-copy *cap* loci also, are well recognized, and one of these, strain Rd, has become a widely used laboratory strain. The capsulate parent of Rd has unfortunately not been preserved, but in the studies reported here, a pair of strains was recovered, allowing the genetic basis for this loss of capsule to be examined.

Type d strain RM7033 was recovered from the blood of a patient with pneumonia, but on propagation of the culture in vitro, occasional colonies of nontypable organisms were noted and these mutants were studied separately. Biotype and outer membrane protein profiles of the capsulate parent and a noncapsulate daughter strain (RM7051) were identical, confirming that they were indeed related (data not shown). Chromosomal DNA was prepared from both strains and from strain Rd and probed as described above. Rd and RM7051 each had a single *Eco*RI fragment of 8.5 kb hybridizing to λCh4:48 (Fig. 2)—subsequently shown to correspond to a part of the probe cloned in pSKH2 (data not shown)—while the hybridization pattern of RM7033 was indistinguishable from that of any other type d strain.

A gene bank of *Eco*RI fragments from strain Rd cloned into pBR328 was screened for hybridization to λCh4:48, a positive clone (pSZ1) was recovered, and the cleavage map of this construction was derived by multiple-endonuclease digestion (Fig. 5). At each end of the cloned DNA, the same restriction sites were found as in the flanks of intact *cap*d, including *Pvu*II sites mapped in the corresponding positions in *cap*d and *cap*b (data for *cap*b not shown). A Southern blot of *Pvu*II-digested pSZ1 DNA hybridized to radiolabeled pSKH2 showed that only the two indicated *Pvu*II fragments in the insert bound to the probe.

DISCUSSION

The *H. influenzae* capsular polysaccharides we have considered here are chemically diverse (J. S. Kroll and E. R. Moxon, *in* K. Jann and B. Jann, ed., *Bacterial Capsules and Adhesins, Facts and Principles*, in press), but the chromosomal loci for capsulation in all four types are very similar.

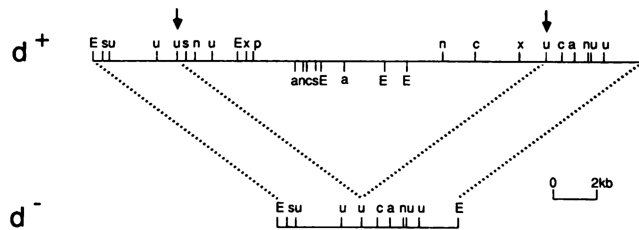

FIG. 5. Physical maps of *cap*d and the residual *cap* locus of d$^-$ strain Rd (RM118) subcloned as an 8.5-kb *Eco*RI fragment in pSZ1. Restriction sites are marked as in Fig. 3, with the addition of *Pvu*II (u) sites. The dotted lines indicate the regions of identity of the two loci and thus the extent of the segment lost from the d$^+$ *cap* locus in its reduction. The *Pvu*II sites indicated in *cap*d by arrows lie in small segments of presumptively directly repeated DNA at the ends of the locus; the *Pvu*II fragments in pSZ1 indicated by bars span the homology of DNA in this clone to the insert of pSKH2.

Alignment of the restriction enzyme cleavage maps suggests that production of different polysaccharide capsules proceeds in part via common biosynthetic steps and shows that each *cap* locus is organized into three regions: regions 1 and 3, apparently the same from serotype to serotype, flanking a serotype-specific region 2. This organization is the same as that found for the *kps* regulon in *E. coli* K1 (4, 16).

The functional organization of *cap* in *H. influenzae* seems to match these structural findings. In RM8055, one of the proteins encoded by a cluster of genes in region 1 has been shown to be involved in polysaccharide export (9), and the finding of apparently identical region 1 DNA in all four serotypes leads us to speculate that all of the proteins encoded there contribute to a function, such as export, in which any of the polysaccharides can be processed. There is no indication of the nature of the non-serotype-specific function(s) encoded in region 3, but in region 2 the finding of DNA unique to each serotype suggests that these genes encode enzymes necessary for the synthesis and polymerization of the serotype-specific polysaccharide. Such a functional organization matches that proposed for *kps* in *E. coli* strains expressing K1, K5, and K7 antigens (15). The extended similarity of *cap*a to *cap*b, into region 2 of the locus, compared with that of any other pair of *cap* loci may reflect a partial common path of polysaccharide biosynthesis. The disaccharide subunits of type a and b polysaccharides differ from all the rest in containing ribitol-5-phosphate, and it is tempting to speculate that genes in the shared part of region 2 are involved in ribitol modification. However, a caution against overinterpretation is provided by the lack of any extended map similarity between *cap*c and *cap*d, although the disaccharide subunits of these polysaccharides both contain a [−4]β-*N*-acetylglucosamine-[1-3]-unit.

Such a physical organization of *cap* seems tailor-made for exchange of region 2 DNA through transformation leading to alteration of serotype. This is easily achieved in vitro (19), and the *cap* loci of the resulting transformants have the anticipated physical maps (Fig. 1b; unpublished data), but there is no indication that such antigenic variation occurs readily in vivo; strains of different serotypes remain clonally distinct (13). Nonetheless, examination of *cap* in type a and b strains segregating to phylogenetic division II indicates that horizontal transfer of region 2 DNA has occurred between the divisions (13; J. S. Kroll, A. Dhir, and E. R. Moxon, unpublished data) and leaves open the question of the importance of such gene transfer events in the biology of capsulate *H. influenzae*. The detailed mapping of *cap* in

different serotypes presented here has allowed us to localize the region 1-region 2 and region 2-region 3 junctions quite closely, and inspection of the nucleotide sequences in these regions may allow us to identify DNA motifs involved in a recombination (7) or transposition event underlying such exchange of DNA in region 2.

A feature of the *H. influenzae cap* locus not shared with the *kps* locus of *E. coli* is its susceptibility to *rec*-mediated reduction (6, 9). This has been considered, in most type b strains, to be the consequence of their possessing a 17-kb direct repeat of DNA at *cap* (Fig. 1a), but our results show that the single-copy locus too is subject to spontaneous reduction. Comparison of the *Pvu*II maps of single-copy and directly repeated *cap*b loci has suggested that *Pvu*II sites corresponding to those indicated in Fig. 5 lie in a small direct repeat of DNA flanking the single-copy locus (9, 10). Reduction of *cap*d to the residual locus cloned in pSZ1 can be explained in a straightforward way by a process of homologous recombination between just such identical segments in the vicinity of these sites, bringing together the two *Pvu*II fragments indicated in Fig. 5 from wide separation at the flanks of *cap* into immediate proximity. The nature of this putative small repeat and the specific role that the DNA may play in capsulation or recombination-mediated reduction of *cap* are being investigated by nucleotide sequencing and site-directed mutagenesis.

Finally, the findings presented here should be considered in the context of the population biology of *H. influenzae*. Serotype b strains have been highly successful among capsulate *H. influenzae*, as reflected in their worldwide distribution as pathogens and as commensal flora of the nasopharynx in great excess over other serotypes (11). The finding that single-copy *cap* loci are commonplace in the non-b serotypes studied here emphasizes the unusual character of the duplicated locus common in type b strains and encourages speculation on its possible biological role in contributing a survival advantage through, for example, a gene-dosage effect (10).

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