Cloning of Chromosomal DNA from *Haemophilus influenzae*

Its Use for Studying the Expression of Type b Capsule and Virulence

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Abstract. *Haemophilus influenzae* may make any one of six chemically distinct capsular polysaccharides, but only strains of capsular serotype b commonly cause systemic infection (e.g., meningitis) in humans. Molecular cloning of DNA was used to investigate the expression of type b capsule and its association with *H. influenzae* virulence. A virulent *H. influenzae* type b strain was used to construct a lambda library of chromosomal DNA in Charon 4. Two independently isolated recombinant phage were isolated from the library and were found to possess DNA necessary for expression of type b capsule. Using a well-characterized rat model of *H. influenzae* systemic infection, we showed that type b transformants elicited by the cloned DNA were pathogenic, causing bacteremia and meningitis, whereas the untransformed capsule-deficient *H. influenzae* organisms were not. A 4.4-kb *Eco*RI fragment, common to both DNA clones, was used to characterize clinical isolates representing all six encapsulated serotypes as well as several capsule-deficient *H. influenzae* by Southern hybridization analysis. The probe hybridized to an identical sized (4.4 kb) fragment of *Eco*RI-digested chromosomal DNA from eight independently isolated type b strains. Single bands of homology to the probe were also found in *Eco*RI fragments of chromosomal DNA obtained from 33 encapsulated, nontype b *H. influenzae*. However, the size of these *Eco*RI fragments proved to be characteristic for each of the different capsular serotypes. These studies provide a basis for pursuing the molecular analysis of the epidemiology and virulence of pathogenic *H. influenzae*.

Introduction

Polysaccharide capsules are considered important determinants of bacterial pathogenicity. (For a recent review, see reference 1.) For example, the majority of cases of bacterial meningitis are caused by encapsulated bacteria, most commonly *Haemophilus influenzae*. Most cerebral spinal fluid (CSF)[1] isolates of *H. influenzae* elaborate a characteristic and biochemically distinct polysaccharide capsule, a linear polymer of ribosyl-ribitol phosphate (PRP) (2, 3). The proclivity of type b strains to cause meningitis as well as several other life-threatening systemic infections (septic arthritis, epiglottitis, cellulitis, septicemia) is remarkable, since *H. influenzae* isolated from the respiratory tract of humans may make any one of six biochemically distinct capsular polysaccharides (designated a–f) (4), yet encapsulated strains, other than type b, only rarely cause systemic infections (5, 6). One possibility is that the type b capsule itself has unique virulence properties which confer a measure of invasive potential denied to the other capsule serotypes. Since *H. influenzae* can be transformed to each of the different capsular serotypes using chromosomal DNA (7–9), we were able to compare these genetically similar strains using a well characterized and biologically relevant rat model of systemic *H. influenzae* infection (10). Type b transformants were always strikingly more virulent than other capsular types (11, 12), thus directly implicating the type b capsule itself as a major determinant of *H. influenzae* pathogenicity. However, these transformants were elicited using high molecular weight donor DNA (derived from representative serotypes), and might have resulted from a recombinant event affecting an uncertain but possibly large (~40 kb) region of the chromosome. If type b strains are clonal descendents of strains exhibiting enhanced pathogenicity, then the type b capsule might be merely one of several linked determinants mediating this

greater virulence. Thus, in comparing the virulence of the different capsular transformants, co-transformation of genes distinct from those determining capsule could not be excluded. If this latter hypothesis were to be partly or wholly correct, it would be very important to identify and characterize the contribution of these putative virulence genes.

To clarify the role of PRP in determining *H. influenzae* virulence, we have used molecular cloning techniques to isolate chromosomal DNA necessary for the expression of type b capsule and virulence. We have isolated two independent recombinant phage from a Charon 4 library and shown that part or all of a gene necessary for expression of type b capsule and virulence is located within a 4.4-kb *Eco*RI fragment of this cloned chromosomal DNA. Using this 4.4-kb *Eco*RI fragment as a probe we have shown, by Southern hybridization, that this probe hybridizes to an *Eco*RI fragment of identical size in the chromosomes of several type b strains and also to *Eco*RI fragments of different, but characteristic, size found in the chromosomes of the five other serotypes of *H. influenzae.*

Methods

Bacterial strains. Strain KW 20b, possessing one-step, independent mutations for streptomycin and erythromycin resistance, was used to construct the Charon 4 gene library and has been previously described (11). Strain Eag, previously described (13), and its one-step streptomycin-resistant mutant (E-1) were gifts of Ms. Lynn Harding, Harvard University, Boston, MA. Strain S_2 (donated by Dr. Porter Anderson, University of Rochester, New York) was a one-step, capsule-deficient mutant of strain E-1 selected on the basis of its noniridescent colonial appearance. It has been shown to elaborate detectable PRP, but in greatly reduced amounts (<1/1,000) compared with its parent (14). Strain Santo ab is a transformant isolated by G. Leidy, Columbia University, New York, which elaborates both type a and b capsular polysaccharides (15). Clinical isolates of *H. influenzae* were obtained from several sources: type b strains; Rut (CSF), Gast (blood) from New York; Burchart (CSF), Sterm (trachea) from Maryland (1982–1983); and A02-07 (CSF), A19-51 (nasopharynx), A89 (blood) from Arizona (1981–1982). Type a strains ATCC 9006; Fin-7, Fin-18, Fin-31, Fin-35 from Finland (1974–1976); St-a from Massachusetts; Morg from Tennessee; and A09-49 (CSF), A15-49 (CSF), A14-09 (nasopharynx) from Arizona (1982). Type c strains ATCC 9007; 1647A from Missouri; St-c from Massachusetts; and Ruggiero (CSF) from New York (1958). Type d strains ATCC 9008; St-d from Massachusetts; Fin-28 from Finland; and D-45 (Centers for Disease Control, Atlanta). Type e strains ATCC 8142; Fann from Tennessee; 1698K, 1207, and 3174A from Missouri; and Hernandez (CSF) and Anderson (CSF) from New York. Type f strains ATCC 9833; Fisk from Tennessee; 1271 and 3028 from Missouri; Nossal (nasopharynx) and Conroy (blood) from New York (1976); and BD-257 (blood) and BD 332 from Louisiana. Nontypable strains Cuesta (CSF) and Kaseman (CSF) from New York (1982); C-296133 (trachea) from Maryland (1981); A29-13, A16-23, A26-23, A10-14, A08-34, A29-22, A04-22 nasopharyngeal isolates from Arizona (1981–1983); Foos (cellulitis) from Maryland; Fin-4 and Fin-19 from Finland; and BD-108 (nasopharynx), BD-125 (middle-ear), BD-263 (sputum), and BD-355 (eye) from Louisiana.

Serotyping and quantitation of PRP elaboration. H. influenzae strains were serotyped by slide agglutination using type a–f antisera (Biologic

Products Division, Centers for Disease Control). The amount of PRP elaborated into broth supernatants of overnight cultures was measured using enzyme-linked immunosorbent assay (ELISA), as previously described (16). For some strains the surface distribution of PRP was characterized by staining with fluorescein-conjugated (17) type b antisera (a generous gift of Dr. John Robbins and Dr. Rachael Schneerson, National Institutes of Health, Bethesda, MD). Suspensions of *H. influenzae* were air-dried on glass slides and incubated (30 min at 22°C) with 10 μl of the antibody-conjugate diluted 1:4 in neutral phosphate-buffered saline (PBS). After thorough rinsing in PBS, the preparation was mounted in an equal mixture of glycerol and PBS under a coverslip. Photographs were obtained by Dr. Andre Zwahlen with a Zeiss fluorescence microscope (Ektachrome 400 ASA [Eastman Kodak Co., Rochester, NY]; exposure time 60 s). Final magnification was × 6,000.

Animal studies. Virulence studies were performed using Sprague-Dawley albino rats (strain COBS-CD) aged 5 d challenged by intranasal inoculation (11). Blood and CSF cultures were performed using previously described techniques (10, 11). To detect the presence of small numbers of virulent, encapsulated *H. influenzae* within a population of predominantly capsule-deficient organisms, rats aged 40 d were inoculated intraperitoneally with 10^8 organisms and blood cultures were obtained after 48 h.

Preparation and analysis of DNA. Large molecular weight chromosomal DNA was prepared from liquid cultures (3 ml). The pellet (10^9 cells) was lysed in 0.1 M NaCl, 10 mM Tris-HCl, and 10 mM EDTA (pH 8.0) containing 1% SDS and heated to 60°C for 10 min. The lysate was digested at 37°C for 60 min with the addition of Proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN) to 1 mg/ml. The mixture was extracted twice with an equal volume of phenol followed by water-saturated butanol before precipitation with cold ethanol (95%). The DNA was resuspended in 10 mM Tris, 1 mM EDTA, pH 8, containing RNase (Worthington Biochemical Corp., Freehold, NJ) at 40 μg/ml. Phage DNA was purified by precipitation with polyethylene glycol and was banded in a CsCl step-gradient as described by Yomanoto et al. (18).

DNA was digested to completion using *Eco*RI (New England Biolabs, Beverly, MA) and fractionated on 1% agarose gels (horizontal) using a glycine buffer (200 mM glycine, 15 mM NaOH, 0.1 mM Na$_2$ EDTA, and 0.002% sodium azide, pH 8). DNA was recovered from gels by the method of Danner (19).

DNA transformation. H. influenzae was grown to early log phase in supplemented heart-infusion broth before transfer of the washed cells to Herriott's MIV medium (20) and incubation at 37°C for 100 min. Large molecular weight chromosomal DNA or CsCl-purified DNA from Charon 4 phage was incubated with the competent cells for 30 min at 37°C. After uptake of DNA, cells were plated on supplemented brain-heart-infusion medium solidified with 1.5% agar. Type b transformants were identified by their characteristic iridescence, which was optimally visualized by viewing colonies with obliquely transmitted light.

Derivation of type b capsule-deficient mutants. We isolated several independent mutants deficient in type b capsule elaboration (Fig. 1). S_2 was transformed using donor DNA from strain Eag. We observed a single colony that displayed both iridescent and noniridescent sectors. This sectored colony of S_2b transformants was subcultured and five noniridescent colonies were isolated and designated Sec 1–5. Sec 1 had characteristics that distinguished it from Sec 2–5; these features were: spontaneous reversion to full type b capsule elaboration at a frequency of 10^{-7} as judged by animal inoculations; elaboration of ~20 times more PRP in cell-free broth supernatants, as assessed by ELISA, compared with S_2; and a higher frequency of transformation by high molecular
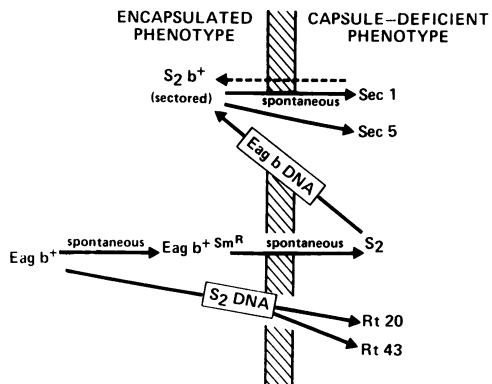
Figure 1. Scheme depicting the independent derivation of type b capsule-deficient mutants from strain Eag.

weight chromosomal DNA to full type b capsule elaboration as compared with strain S_2. Sec 2–5 did not spontaneously revert and elaborated greatly reduced amounts of PRP in quantities intermediate between Sec 1 and S_2. Strains Rt 20 and Rt 43 were derived by selecting capsule-deficient transformants elicited by incubating strain Eag in the presence of DNA extracted from strain S_2; these capsule-deficient transformants occurred at a frequency of 0.24% in the presence of DNA, whereas in the absence of DNA no capsule-deficient colonies were detected. Capsule-deficient mutants KW20b M 34, which elaborated reduced amounts of PRP, and M 38, which made no detectable PRP, were obtained by mutagenesis with 0.4 mg/ml N-methyl-N-nitrosoguanidine.
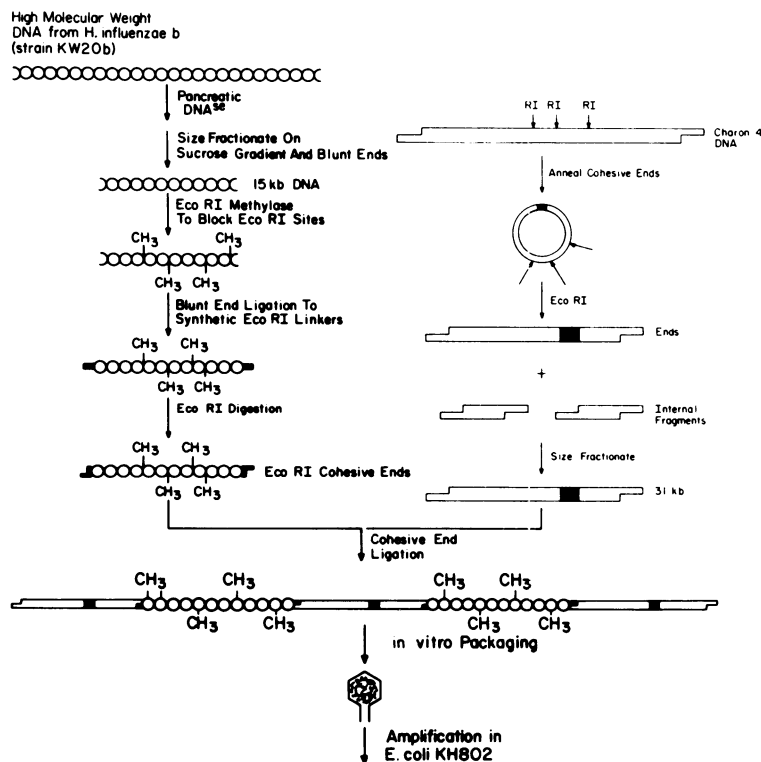
Construction of Charon 4 library. The general approach (Fig. 2) was based on the procedures described by Blattner (21). Chromosomal DNA from strain KW20 b was partially digested with pancreatic DNase to yield fragments averaging 10–20 kb long and sized by sucrose-gradient sedimentation. The purified fragments were blunt-ended with T4 polymerase (New England Biolabs), modified with *Eco*RI methylase, and ligated to *Eco*RI linkers (Collaborative Research Inc., Lexington, MA). After *Eco*RI cleavage and the removal of the free-linker DNA by agarose gel electrophoresis, the fragments were ligated to the free arms of the vector Charon 4 (21) and packaged using an in vitro lambda packaging mix. Approximately 24,000 independent recombinant phage were obtained and amplified in *Escherichia coli*, strain K802 (22), yielding a library suspension (5 ml) containing $\sim 10^9$ phage/ml. The library suspension was kept at 4°C after the addition of a few drops of chloroform.

Southern analysis of genomic DNA. DNA (~ 2 μg) was digested with *Eco*RI and run on 1% agarose-glycinate gels at 60 V overnight. The DNA from these gels was hydrolyzed with 0.25 M HCl, denatured with 0.5 M NaOH, 1.5 M NaCl, and neutralized with 1 M NH_4 acetate, 0.02 M NaOH, then transferred bidirectionally to nitrocellulose filters as described by Smith and Summers (23). Nick-translated hybridization probes were prepared by the method of Rigby et al. (24) using ^{32}P dCTP (3000 Ci/m mol, Amersham Corp., Arlington Heights, IL) and *Micrococcus luteus* DNA polymerase (Miles Laboratories Inc., Elkhart, IN). The filter-bound DNA was hybridized to Nick-translated probes at 37°C in 50% formamide and 10% dextran sulphate for 16 h according to the method of Scott et al. (25) as modified by Wahl et al. (26). The filters were washed at 45°C in 0.015 M NaCl, 0.0015 M sodium citrate for 2 h with three changes of buffer. Autoradiograms were obtained using Kodak XAR-5 film (Eastman Kodak Co.) and a Cronex quanta III (DuPont Laboratories, Wilmington, DE) intensifying screen.
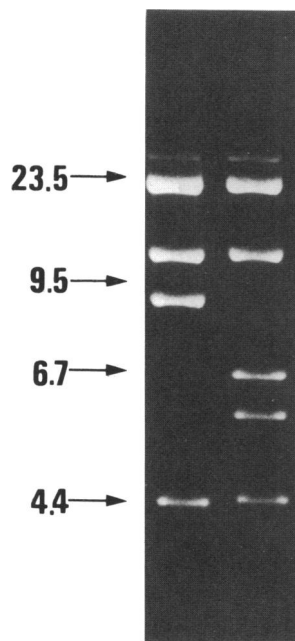

Figure 2. Construction of *H. influenzae* type b gene library in Charon 4.

Figure 3. Agarose gel electrophoresis of caesium-chloride-purified DNA from Charon 4:48 (*left*) and Charon 4:60 (*right*) after digestion with *Eco*RI and staining with ethidium bromide. A Hind III digest of phage lambda (not shown) was performed to provide molecular sizing markers (indicated by arrows; kilobase pairs). In each lane, the bands corresponding to 19.8 and 10.9 kb are the Charon 4 arms.

Results

Cloning of DNA necessary for type b capsule expression. To isolate individual clones from the library, ten dilutions from the complete library, calculated to yield ~1,000 phage, were amplified to complete lysis in *E. coli,* strain K802. Phage DNA present in the supernatant of phage lysates was tested for its ability to elicit type b transformants of Sec 1. Transformants were identified by visual inspection of colonies (several thousand per plate) for the iridescent phenotype characteristic of type b organisms. In this manner, cloned DNA sequences necessary for expression of type b capsule were identified using a method that was independent of virulence expression by the transformants.

We found that DNA from three of the original ten lysates could elicit type b transformants. One of these was diluted so as to yield a few hundred plaques on a plate. Phage were scraped from individual plaques, amplified, and assayed for ability to elicit type b transformants. Using this approach we selected two Charon 4 phage (Ch.4:48 and Ch.4:60). DNA from Ch.4:48 and Ch.4:60 were not identical, as indicated by the different ethidium bromide stained bands obtained on agarose-gel electrophoresis following *Eco*RI digestion (Fig. 3). In addition to the Charon 4 arms, Ch.4:48 had two fragments of 9 and 4.4 kb whereas Ch.4:60 yielded three fragments of 6.7, 5.6, and 4.4 kb. By Southern hybridization analysis (using the ^{32}P-labeled 4.4- and 9-kb gel-purified *Eco*RI fragments of Ch.4:48 as probes), we showed that there was homology between the 4.4-kb fragment of Ch.4:48 and 60 and between the 9.0-kb fragment of Ch.4:48 and the 5.6-kb fragment of Ch.4:60. This suggested that we had cloned overlapping fragments of chromosomal DNA.

Use of cloned DNA to characterize capsule-deficient mutants. Either Ch.4:48 or 60 could elicit type b transformants in some, but not all, of a series of capsule-deficient mutants. Five mutants, Sec 1, Sec 5, S_2, M 34, and Rt 43, which elaborated reduced amounts of PRP, could be transformed to type b; in contrast, mutants Rt 20 and M 38 (which made no PRP) could not be transformed. These findings suggested that Ch.4:48 and 60 contained some but not all genes necessary for type b capsule elaboration.

The capsule-deficient mutants that elaborated reduced amounts of PRP were distinctive in appearance; when stained with fluorescein-congugated antibodies (Fig. 4) they had an irregular patchy distribution of PRP on the bacterial cell surface. This appearance contrasted with the regular, plump rings of brilliant fluorescence exhibited by type b clinical isolates or type b transformants. Thus, the mutants elaborating reduced amounts of PRP apparently have mutations in one or more genes affecting either the quantity and/or the assembly of PRP on the bacterial surface; furthermore, there were DNA sequences capable of restoring the full type b capsular phenotype present in both Ch.4:48 and 60. In an attempt to localize the relevant sequences more precisely, the 4.4- and 5.6-kb *Eco*RI fragments of Ch.4:48 were gel purified and tested to determine whether one, the other, or both fragments could restore full type b capsule production to each of the mutants. Sec 1 was transformed by the 4.4-kb fragment alone, but the other mutants (S_2, Sec 5, Rt 43, and M 34) required both fragments. Since Sec 1 spontaneously reverts to type b, it appears likely that a point-mutation that maps within the 4.4-kb fragment is responsible for its leaky phenotype. The genetic changes resulting in capsule-deficiency among the other mutants must be more complex, since restoration of type b capsule required DNA from both the 4.4- and 9.0-kb fragments.

Restoration of virulence to capsule-deficient mutants by cloned DNA. We found that all type b transformants elicited by the cloned DNA were highly virulent for rats. In typical experiments, 10 independent type b transformants (e.g., of strain S_2) elicited by Charon 4:48 DNA consistently caused bacteremia and meningitis in rats following intraperitoneal inocula of 10^3 organisms. Indeed, as few as two transformed organisms resulted in bacteremia. In contrast, none of the untransformed, capsule-deficient mutants resulted in bacteremia. When an inoculum of 10^7 type b transformants of S_2 was inoculated intranasally, bacteremia and meningitis occurred in 8 of 10 rats, whereas a similar inoculum of untransformed S_2 organisms colonized the nasopharynx, but did not cause invasive infection. (All blood and CSF cultures were sterile.)

In summary, all avirulent bacterial strains capable of being transformed by the cloned DNA (including Sec 1 transformants elicited by the isolated 4.4-kb *Eco*RI fragment) became as virulent as fully encapsulated type b clinical isolates.

Use of cloned DNA as probe for Southern hybridization analysis. We have used the 4.4-kb internal *Eco*RI fragment of Ch.4:48 as a probe in Southern hybridization analysis of *Eco*RI digests of chromosomal DNA from different *H. influenzae.* Fig. 5 shows that the probe hybridized to a 4.4-kb *Eco*RI fragment present
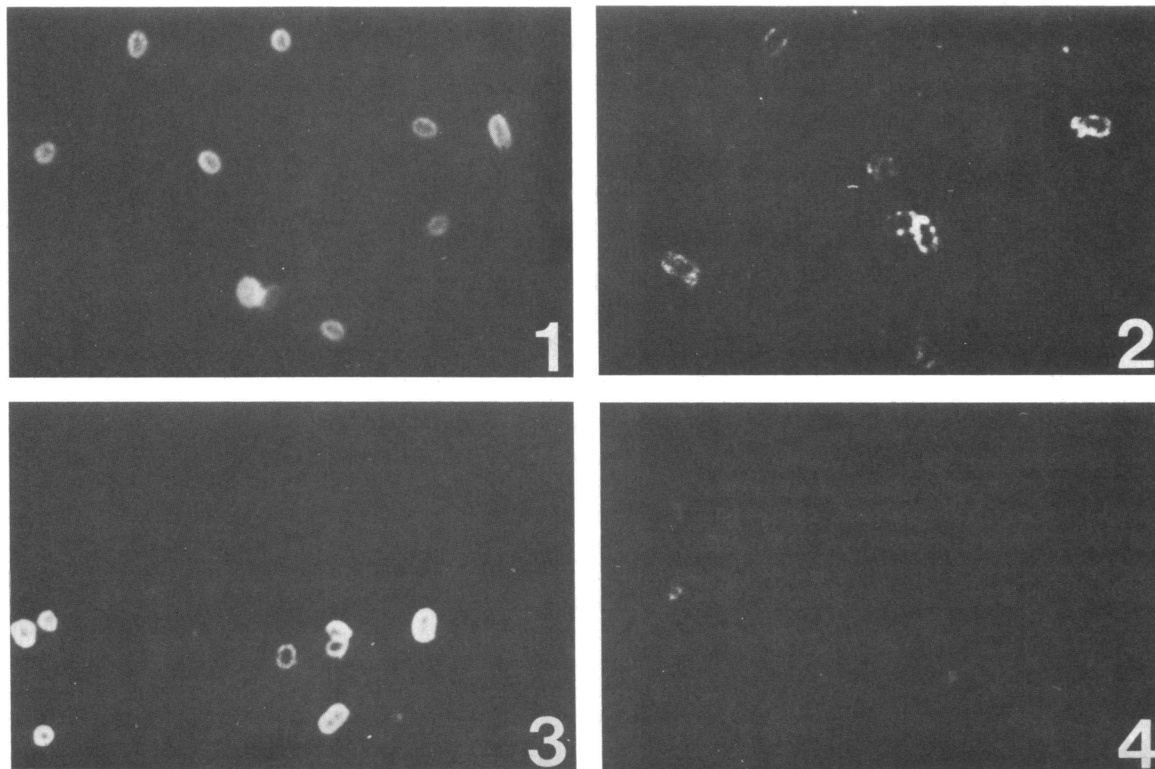
Figure 4. Morphology of *H. influenzae* stained with fluorescein-conjugated rabbit antiserum to type b capsule antigen. Panel 1: strain Eag. Panel 2: S_2. Panel 3: type b transformant of KW20. Panel 4: KW20. Note the patchy, irregular distribution of fluorescence-positive material associated with the leaky mutant S_2 (panel 2) as compared with Eag and KW20b. Sec 1, Sec 5, Rt 43, and M 34 were similar in appearance to S_2. KW20 (panel 4) elaborates undetectable PRP.

in strain Eag. Similar results were obtained from capsule-deficient mutants making reduced amounts of PRP (as exemplified in Fig. 5 by S_2 and M 34). In contrast, strain's Rd-KW20 and M 38, mutants making undetectable PRP, had no homology to the probe. Their type b transformants, exemplified by Rd-KW20b (Fig. 5, lane 4), acquired a 4.4-kb *Eco*RI fragment that was homologous with the probe. Thus, type b transformation using donor DNA from strain Eag was associated with the acquisition of a 4.4-kb *Eco*RI fragment of DNA that shared sequence homology with the cloned 4.4-kb *Eco*RI fragment of Ch.4:48 and 60. Fig. 5 (lanes 7, 8, and 9) also shows three representative examples of the results obtained when we probed eight independently isolated type b strains cultured from children with systemic *H. influenzae* infections who lived in different regions of the USA. Thirty encapsulated, non–type b isolates (representing all serotypes) also showed single bands of homology, but the probe hybridized to *Eco*RI fragments of different sizes which, nonetheless, proved to be highly characteristic of the particular capsular serotypes (Fig. 6). These results can be summarized as follows: the probe hybridized to *Eco*RI fragments of 5.4 kb in seven type a strains, 2.3 kb in four type c strains, 4.0 kb in four type d strains, 1.6 kb in seven type e strains, and 2.3 kb in eight type f strains. After these studies were completed,

three additional type a isolates from Apache Indian children with systemic *H. influenzae* infection (two cases of meningitis and one of pneumonitis) were studied and found to exhibit an exceptional pattern of hybridization with the 4.4-kb *Eco*RI probe. Instead of the typical homology to a 5.4-kb fragment characteristic of type a strains, the probe hybridized to a 4.4-kb *Eco*RI fragment typical of type b strains. Additional studies by Dr. G. Losonsky and Dr. A. Zwahlen of this laboratory have shown that these isolates elaborate no detectable PRP (ELISA), have an identical Kilian biotype II, as well as similar outer membrane protein and lipopolysaccharide profiles. Thus, it is likely that these isolates represent the same or a closely related strain.

A series of encapsulated transformants of Rd-KW20 (type a–f) prepared by Dr. Zwahlen also showed the characteristic hybridization to the probes. A type ab transformant (which exhibited simultaneous production of type a and type b polysaccharides) obtained from Grace Leidy, Department of Pediatrics of this institution, displayed the two characteristic bands of hybridization of 5.4- and 4.4-kb, respectively (Fig. 6). Since all encapsulated strains (41 clinical isolates) showed hybridization to the probe, we examined 17 capsule-deficient (nontypable) clinical isolates. We were particularly interested to determine whether or not nontypable *H. influenzae* causing systemic in-
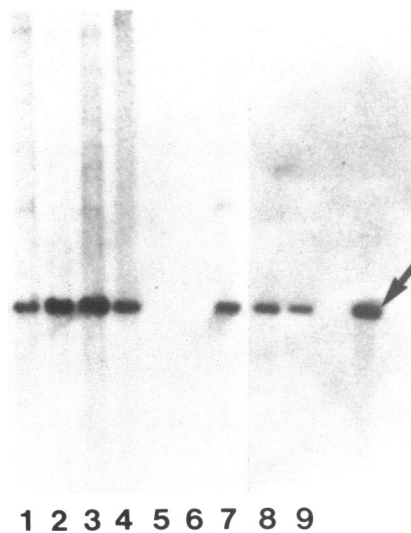
1 2 3 4 5 6 7 8 9

Figure 5. Southern hybridization analyses of chromosomal DNA from different *H. influenzae* strains after digestion with *Eco*RI. The probe was the 4.4-kb *Eco*RI fragment of CH.4:48 labeled by nick-translation with ^{32}P dCTP. Lanes 1–6 show (in order): Eag, S$_2$, type b transformant of S$_2$, type b transformant of KW20, KW20, and M 38. Lanes 7–9 are CSF isolates (strains Rut, Burchart, AO2-07) of children with *H. influenzae* type b meningitis. The arrow indicates the gel-purified 4.4-kb fragment of Ch.4:48.

fections showed homology to the probe. None of three CSF isolates showed homology to the probe. Of the remaining 14 isolates, 3 possessed chromosomal *Eco*RI fragments (6.6, 6.1, and 6.0 kb, respectively) with homology to the probe. These isolates were obtained from a child with otitis media (BD-125), and from two individuals with asymptomatic colonization who were contacts of cases with systemic *H. influenzae* b infection (A16-023, A08-34).

Discussion

The importance of the polysaccharide (PRP) capsule in virulence and, conversely, of serum anti-PRP antibodies in protection has been a logical and dominant feature of research into mechanisms of pathogenesis and prevention of systemic *H. influenzae* infections, especially meningitis (1). However, there have been no definitive studies on the genetics of type b capsule expression and virulence. Thus, details of the biosynthesis, transport, polymerization, and surface assembly of PRP are unknown. Progress has been thwarted since *H. influenzae* requires a complex defined media for its growth, and suitable vectors, such as transducing phages, have not been available. As a result, only limited chromosomal mapping (9) and a few defined mutants of *H. influenzae* are available. In the present studies, we used the techniques of molecular cloning and transformation to identify and isolate chromosomal DNA from one or more of the genes necessary for expression of type b capsule. In contrast to the

cloning of virulence determinants (usually proteins) in other bacterial pathogens, our task was further complicated since none of the genes involved in expression of the polysaccharide capsule was known. Earlier studies, however, had indicated that a chromosomal region, perhaps as large as 50 kb, might be required (9). Thus, we considered it unrealistic to attempt isolation of all the genes necessary to elaborate PRP using presently available cloning vectors. Our approach, therefore, was to obtain one or more clones from the Charon 4 library that could restore, by transformation, full type b expression to a mutant deficient in at least one of the genes necessary for PRP elaboration. The library was shown to contain cloned DNA sequences capable of restoring capsule elaboration to a capsule-deficient mutant by using the rat as a biological cell selector. This approach had the advantage of permitting the detection of extremely small numbers of type b transformants. Once it was established that the library contained DNA capable of restoring type b capsule expression, we proceeded to isolate individual clones that could restore capsule expression to Sec 1.

The successful isolation of Ch.4:48 and Ch.4:60 has facilitated the identification and preliminary characterization of one or more genes necessary, but not sufficient, for type b capsule elaboration and virulence. Four independently derived mutants, shown to make reduced amounts of PRP, could be restored to full capsule production and virulence by both Ch:4:48 or 60. In the case of Sec 1 (which spontaneously reverts), the transforming DNA has been further localized to the 4.4-kb *Eco*RI fragment common to both Ch.4:48 and 60. Additional studies are underway to characterize further this region. Since Sec 1 exhibited irregular, patchy distribution of PRP, it is possible that Sec 1 lacks expression of a gene that is involved in surface assembly of PRP. If so, the gene (product) might be a potential target for specific antibodies and therapeutic agents and offer a novel approach to attenuating virulence. Alternatively, Sec 1 may lack full expression of the gene involved in PRP biosynthesis, thus resulting in quantitatively altered PRP production. In subsequent studies, using *Eco*RI fragments from Ch.4:48 and 60 as probes, we have identified an additional 50 recombinant phage from the Charon 4 library that share overlapping homology with these probes. Using this approach, or by identifying larger pieces of DNA from a cosmid bank, we hope to identify additional sequences that will enable us to define the entire region involved in type b capsule expression.

We are impressed by the similarities between capsule expression in *H. influenzae* as compared with other encapsulated bacterial pathogens, particularly *Streptococcus pneumoniae* (27). The general phenomonology, known for many years but never explained, includes the following features: spontaneous loss of the capsular and virulence phenotype at variable and sometimes high frequencies; the occurrence of leaky or tight capsule-deficient mutants which may, but usually do not, revert spontaneously to full capsule production (28); strains of one type do not spontaneously change to another, but both intertype and intratype change can occur through DNA transformation. Thus, our studies in *H. influenzae* may prove to be a good model for
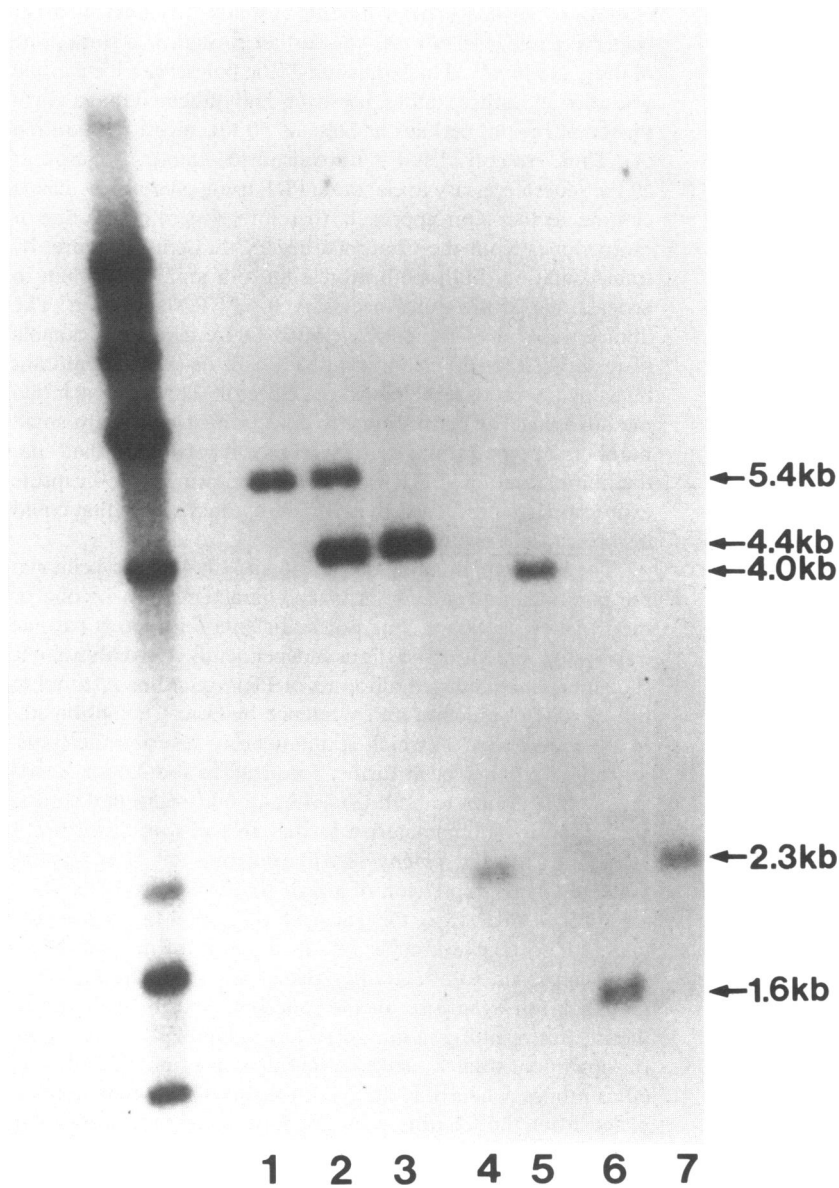
Figure 6. Southern hybridization performed as in Fig. 6. Lanes 1–7 show (in order): ATCC 9006 (type a), Santo ab (transformant), Gast (type b), Ruggiero (type c), St-d (type d), ATCC 8142 (type e), and Conroy (type f).

understanding the genetic mechanisms that underlie capsule expression in several pathogenic bacteria. Indeed, the availability of a well studied, biologically relevant model of *H. influenzae* systemic infection allows us to analyze the independent contribution of the type b capsule at each of several critical stages (e.g., nasopharyngeal colonization, bloodstream dissemination, meningeal invasion, etc.) of the pathogenic sequence. Furthermore, our Charon 4 library has also been used to isolate and perform preliminary characterization of a gene encoding an *H. influenzae* IgA I protease, a potentially important virulence determinant for both local and systemic *H. influenzae* infections (29).

The 4.4-kb *Eco*RI fragment has proved to be a useful probe for characterizing the chromosomal DNA of encapsulated and capsule-deficient strains. It is interesting that all encapsulated strains showed homology (using conditions of high stringency) to this 4.4-kb *Eco*RI fragment. This finding suggests that one or more genes within this region may be required for common steps in capsule elaboration (e.g., exporting the capsule from the cytoplasm to the bacterial cell surface). Owing to the distinctly different chemical composition of several of the *H. influenzae* capsular polysaccharides, at least some of the biosynthetic genes should prove to be unique for a particular serotype. It also seemed noteworthy to us that, with some interesting exceptions,

the 4.4-kb probe hybridized to chromosomal *Eco*RI fragments of a size consistently characteristic of the different serotypes. The interesting exceptions (among 41 independently isolated, encapsulated clinical isolates) were obtained from three young Apache Indians in Arizona. These type a *H. influenzae* (serotype was confirmed independently by G. Leidy) caused typical systemic infections (meningitis, pneumonia) and were collected as part of an ongoing prospective study of systemic infections occurring in this community.[2] All three isolates were biotype II and shared similar outer membrane protein and lipopolysaccharide profiles (Losonsky, G., and A. Zwahlen, personal communication), so we presume they are closely related. These type a isolates represented 20% (3/15) of all systemic infections occurring during a 14-mo period, an incidence of type a infection substantially higher than expected based on extensive experiences in the USA and elsewhere (30–32). The possession by these type a isolates of an *Eco*RI fragment similar to that found in type b strains is provocative. Exchange of capsule genes among type a and type b strains might be expected owing to the similarities in structure between the respective polysaccharides. Indeed, Leidy and Alexander have described an "ab" transformant that elaborated both polysaccharides simultaneously (15). We showed that this type ab transformant possessed two chromosomal *Eco*RI fragments, each of typical size (Fig. 6), whereas its isogenic type a and type b variants each had only one. Could the clinical isolates from Arizona represent a more virulent recombinant derived through exchange of genes from type b to type a strains? Such an hypothesis would require that the chromosome retained the structural genes essential for biosynthesis of type a polysaccharide, but acquired type b sequences involved in other aspects of capsule elaboration. In this case, transfer of DNA from a type b parent might also confer enhanced virulence mediated through expression of linked genes distinct from those involved in capsule expression.

In summary, these preliminary findings indicate the potential of molecular cloning techniques for analyzing *H. influenzae* virulence. An understanding of the genetic basis of capsule expression in *H. influenzae* should indicate some of the principles underlying virulence expression by encapsulated bacterial pathogens as well as provide a molecular explanation for the extraordinary propensity of type b strains to cause invasive infections of humans.

Acknowledgments

References

1. Robbins, J. B. 1978. Vaccines for the prevention of encapsulated bacterial diseases: current status, problems and prospects for the future. *Immunochemistry.* 15:839–854.

2. Zamenhof, S. G., G. L. Leidy, P. L. Fitzgerald, H. E. Alexander, and E. Chargaff. 1953. Polyribophosphate, the type specific substance of *Haemophilus influenzae*, type b. *J. Biol. Chem.* 203:696–704.

3. Crisel, R. M., R. S. Baker, and D. E. Dorman. 1975. Capsular polymer of *Haemophilus influenzae* type b. I. Structural characterization of the capsular polymer of strain Eagan. *J. Biol. Chem.* 250:4926–4930.

4. Pittman, M. 1931. Variation and type specificity in the bacterial species *Haemophilus influenzae*. *J. Exp. Med.* 53:471–492.

5. Turk, D. C. 1982. Clinical importance of *Haemophilus influenzae*. *In Haemophilus influenzae*. Epidemiology, Immunology and Prevention of Disease. S. H. Sell and P. W. Wright, editors. Elsevier North-Holland, Inc. New York. 3–9.

6. Alexander, H. E. 1953. Holt's Pediatrics. L. E. Holt and R. McIntosh, editors. Appleton-Century-Crofts. 12th ed. New York. 1200.

7. Alexander, H. E., and G. Leidy. 1951. Induction of heritable new type in type-specific strains of *H. influenzae*. *Proc. Soc. Exp. Biol. Med.* 78:625–626.

8. Sisco, K. L., and H. O. Smith. 1979. Sequence-specific DNA uptake in Haemophilus transformation. *Proc. Natl. Acad. Sci. USA.* 76:972–976.

9. Catlin, B. W., J. W. Bendler III, and S. H. Goodgal. 1972. The type b capsulation locus of *Haemophilus influenzae:* map location and size. *J. Gen. Microbiol.* 70:411–422.

10. Moxon, E. R., A. L. Smith, D. R. Averill, and D. H. Smith. 1974. *Haemophilus influenzae* meningitis in infant rats after intranasal inoculation. *J. Infect. Dis.* 129:154–162.

11. Moxon, E. R., and K. A. Vaughn. 1981. The type b capsular polysaccharide as a virulence determinant of *Haemophilus influenzae*. Studies using clinical isolates and laboratory transformants. *J. Infect. Dis.* 143:517–524.

12. Zwahlen, A., J. A. Winkelstein, and E. R. Moxon. 1983. Surface determinants of *Haemophilus influenzae* pathogenicity: comparative virulence of capsular transformants in normal and complement-deficient host. *J. Infect. Dis.* 148:385–394.

13. Anderson, P., R. B. Johnston, Jr., and D. H. Smith. 1972. Human serum activities against *Haemophilus influenzae*, Type b. *J. Clin. Invest.* 51:31–38.

14. Weller, P. F., A. L. Smith, P. Anderson, and D. H. Smith. 1977. The role of encapsulation and host age in the clearance of *Haemophilus influenzae* bacteremia. *J. Infect. Dis.* 135:24–41.

15. Leidy, G., E. Hahn, and H. E. Alexander. 1953. *In vitro* production of new types of *Haemophilus influenzae*. *J. Exp. Med.* 97:467–472.

16. Pepple, J. M., E. R. Moxon, and R. H. Yolken. 1980. Indirect enzyme-linked immunosorbent assay for the quantitation of the type-specific antigen of *Haemophilus influenzae* b: a preliminary report. *Pediatrics.* 97:233–237.

17. Goldman, M. 1968. Fluorescent Antibody Methods. Academic Press Inc., New York. 48–51.

---

2. Losonsky, G. A., M. Santosham, A. Zwahlen, and E. R. Moxon. Submitted for publication.

18. Yomamoto, K. R., B. M. Alberts, R. Benzinger, L. Lawhorne, and G. Treibe. 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. *Virology.* 40:734–738.

19. Danner, D. B. 1982. Recovery of DNA fragments from gels by transfer to DEAE-paper in an electrophoresis chamber. *Anal. Biochem.* 125:139–142.

20. Herriott, R. M., E. M. Meyer, and M. J. Vogt. 1970. Defined non-growth media for stage II development of competence in *Haemophilus influenzae. J. Bacteriol.* 101:517–524.

21. Blattner, F., B. Williams, A. Blechl, K. Denniston-Thompson, H. Farer, L. Furlong, D. Grunwald, D. Keifer, D. Moore, J. Shumm, E. Sheldon, and O. Smithies. 1977. Charon phages: safer derivitives of bacteriophage lambda for DNA cloning. *Science (Wash. DC).* 196:161–169.

22. Maniatis, T., R. C. Hardison, E. Lacy, J. Lauer, C. O'Connell, and D. Quon. 1978. The isolation of structural genes from libraries of eucaryotic DNA. *Cell.* 15:687–701.

23. Smith, G. E., and M. D. Summers. 1980. The biochemical transfer of DNA and RNA to nitrocellulose of diazobenzyloxymethyl paper. *Anal. Biochem.* 109:123–129.

24. Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labelling deoxyribonucleic acid to high specific activity *in vitro* by Nick Translation with DNA polymerase I. *J. Mol. Biol.* 113:237–251.

25. Scott, A. F., J. A. Phillips, and B. R. Migeon. 1979. DNA restriction endonuclease analysis for localization of human and globin genes on chromosome 11. *Proc. Natl. Acad. Sci. USA.* 76:4563–4565.

26. Wahl, G. M., M. Stern, and G. R. Stark. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyl-oxymethyl-paper and rapid hybridization by using dextran sulfate. *Proc. Natl. Acad. Sci. USA.* 76:3683–3687.

27. MacLeod, C. M., and M. R. Krauss. 1950. Relation of virulence of pneumococcal strains for mice to the quantity of capsular polysaccharides formed in vitro. *J. Exp. Med.* 92:1–9.

28. Makaela, P. H., and B. A. D. Stocker. 1969. Genetics of polysaccharide biosynthesis. *Annu. Rev. Genet.* 3:291.

29. Bricker, J., M. H. Mulks, A. G. Plaut, E. R. Moxon, and A. Wright. 1983. IgA I proteases of *Haemophilus influenzae.* Cloning and characterization in *Escherichia coli* K.12. *Proc. Natl. Acad. Sci. USA.* 80:2681–2685.

30. Thomson, J., L. M. Bruce, M. G. Green. 1967. *H. influenzae* meningitis in relation to treatment: a clinical study of four cases. *Brit. Med. J.* 2:414–418.

31. Greene, G. R. 1978. Meningitis due to *Haemophilus influenzae* other than type b: case report and review. *Pediatrics.* 62:1021–1025.

32. Turk, D. C., and J. R. May. 1967. *Haemophilus influenzae:* Its Clinical Importance. The English Universities Press, London E.C. 4, United Kingdom. 35.