INFECTION AND IMMUNITY, Aug. 1985, p. 389–395
0019-9567/85/080389-07$02.00/0

Vol. 49, No. 2

Genetics of Spontaneous, High-Frequency Loss of b Capsule Expression in *Haemophilus influenzae*

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Received 27 February 1985/Accepted 6 May 1985

We determined that the frequency of spontaneous capsule loss in *Haemophilus influenzae* type b is 0.1 to 0.3%. All of 10 independent capsule-deficient variants (derived from four different type b strains) were found to be missing an identical 9-kilobase *Eco*RI restriction fragment when probed with a cloned piece of DNA containing sequences known to be necessary for type b capsule expression. These results suggest the existence of a specific mechanism for shutting off type b capsule synthesis at a high frequency. Intranasal infection of infant rats showed that capsule loss occurred in vivo at frequencies comparable to those observed in vitro.

The type b capsule is considered an important virulence determinant of *Haemophilus influenzae*; indeed, more than 90% of systemic infections caused by *H. influenzae* (including meningitis) are caused by organisms possessing the type b capsule (22). It is not clear, however, whether it is the type b capsule per se that makes type b strains more virulent than types a, c, d, e, and f, or whether type b organisms have evolved from an ancestor possessing one or more as yet unidentified virulence factors. Previous work from this laboratory has described the use of molecular cloning to isolate DNA sequences necessary for type b capsule expression (15). Several different capsule-deficient mutants were transformed to the b^+ phenotype and regained virulence after transformation with the cloned DNA. This λ clone, λ Charon 4 clone 48 (Ch.4:48), contains two *Eco*RI fragments of 4.4 and 9 kilobases (kb), and both fragments have been shown to be necessary for b capsule expression (15). By transformation analysis, Catlin et al. (6) have shown that the genes necessary for type b capsule synthesis are linked in one region of the *H. influenzae* chromosome and that the amount of DNA involved may be as large as 50 kb. Although Ch.4:48 does not contain all the genes necessary for b capsule expression (15), the 4.4- and 9-kb fragments have been useful as probes. We report here their use in the characterization of chromosomal DNA from *H. influenzae* clinical isolates and capsule-deficient variants.

The evolutionary relationship between type b organisms and nontypable strains is unknown, but it has been known for many years that encapsulated strains lose their capsules very readily (7, 10, 17). Much of this work was done in the 1930s, before the concept of the gene was understood, and little is known regarding the genetic basis of this instability. The more recent observations of Catlin (5) describe two classes of nonencapsulated variants. Variants of class 1 formed small, rough, noniridescent colonies, which when examined by immunoflourescent staining produced only small amounts of capsular antigen in the form of "embossments." The class 2 variants produced smoother (although still noniridescent) colonies and no longer made detectable capsular antigen. These variants arose at frequencies which were appreciable (precise frequencies not determined) but which differed for different strains. Since variants appear to arise at frequencies much higher than can be accounted for by classical base-change mutations and since the type b capsule is an important virulence determinant for *H. influenzae*, we felt it important to examine the genetic basis and biological significance of this instability.

In this study we systematically examined the frequency of capsule loss in many independently isolated type b strains and found that nonencapsulated variants arose at frequencies of 0.1 to 0.3%. Using our cloned DNA fragments as probes, we examined the DNA in these high-frequency variants and suggest that a highly specific mechanism may play a role in modulating the virulence of *H. influenzae*.

MATERIALS AND METHODS

Media and culture conditions. *H. influenzae* strains were grown in supplemented brain heart infusion (sBHI) broth containing 3 μg of NAD and 10 μg of hemin per ml. Plates consisted of BHI with 10% Levinthal base and 1.5% agar. *Escherichia coli* strains were grown in LB broth (14) with antibiotics added as necessary for maintenance of plasmids.

DNA preparation and analysis. High-molecular-weight chromosomal DNA was prepared as previously described (15). Plasmid screening was done by the rapid alkaline method of Birnboim and Doly (3). Larger preparations required the use of a scaled-up version of the rapid alkaline method, followed by banding in cesium chloride-ethidium bromide density gradients. Restriction enzymes were purchased from New England BioLabs, Inc., Beverly, Mass., and were used according to the recommendations of the supplier. Gel-purified restriction fragments (for subcloning or for use as probes or in transformation assays) were prepared by separation on 0.9% agarose-Tris acetate gels (14); the separated fragments were electroeluted, concentrated on NACS prepack columns (Bethesda Research Laboratories, Gaithersburg, Md.), and ethanol precipitated.

Subcloning. The 4.4- and 9-kb *Eco*RI fragments from the original λ clone Ch.4:48 (15) were subcloned into the *Eco*RI site of the plasmid vector pBR328 (21) by standard cloning methods (14). The vector was phosphatased with calf intestinal phosphatase (Boehringer Mannheim, Indianapolis, Ind.), and the vector and insert DNAs were ligated with T4

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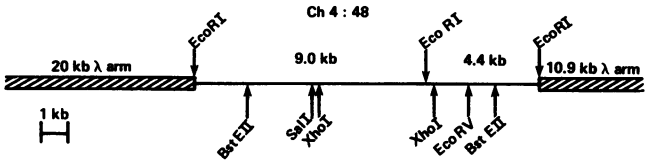

FIG. 1. Restriction map of λ Charon 4 clone 48 (Ch.4:48). The solid line represents the 13.4-kb fragment of *H. influenzae* DNA. The hatched boxes represent the λ arms (restriction sites in the arms not included). The map was constructed from single and double digests of Ch.4:48 (*Eco*RI, *Xho*I, and *Sal*I) and from single, double, and triple digests of the 4.4- and 9-kb subclones. The linkage of the 4.4- and 9-kb *Eco*RI fragments in the chromosome of *H. influenzae* has been confirmed by probing *Bst*EII or *Xho*I digests of type b, strain Eagan DNA with either the 4.4- or 9-kb subclones. Both subclones recognized the predicted 4.6-kb *Xho*I or 9.5-kb *Bst*EII junction fragments.

ligase (New England Biolabs) overnight at 14°C. The 4.4-kb ligation mixture was transformed into competent *E. coli* HB101 (14) with selection on LB-ampicillin plates (50 μg/ml), and the 9-kb ligation mixture was transformed into competent *E. coli* DHI (14) cells. Ampicillin-resistant colonies were tested on LB-chloramphenicol plates (30 μg/ml), and chloramphenicol-susceptible isolates were saved for plasmid analysis.

Southern analysis of genomic DNA. DNA (~2 μg) was digested to completion with the appropriate restriction enzyme and run overnight on 0.9% agarose-Tris acetate gels at 50 V. The DNA was transferred bidirectionally to nitrocellulose filters as described by Smith and Summers (20). Nick-translated hybridization probes were prepared by the method of Rigby et al. (18) with [α-^{32}P]dCTP (3,000 Ci/mmol) (Amersham Corp., Arlington Heights, Ill.) and *Micrococcus luteus* DNA polymerase (Miles Laboratories, Inc., Elkhart, Ind.). The filter-bound DNA was hybridized to the nick-translated probe at 37°C in 50% formamide–10% dextran sulfate for 16 h by the method of Scott et al. (19) as modified by Wahl et al. (23). The filters were washed at 45°C in 0.015 M NaCl, 0.0015 M sodium citrate, 0.1% sodium dodecyl sulfate for 2 h with three changes of buffer. These conditions yield an overall requirement for base pair homology of approximately 80% (14). Autoradiograms were obtained with Kodak XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) and a Cronex Quanta III intensifying screen (DuPont Co., Wilmington, Del.).

Animal studies. Sprague-Dawley rats (strain COBS-CD) were purchased from Charles River Laboratories, Wilmington, Mass. Atraumatic intranasal inoculations were performed by letting a 25-μl drop fall onto the nares from the tip of an Eppendorf pipette.

DNA transformation. Cloned *H. influenzae* DNA was tested for the ability to transform capsule-deficient mutants to the encapsulated phenotype. Mutant strains were made competent for DNA uptake by growth in supplemented heart infusion broth, followed by incubation in Herriott's MIV medium (11) as described previously (15). Encapsulated transformants were detected by their iridescent appearance when examined in obliquely transmitted light. When the transformation frequencies were too low to visualize, encapsulated transformants were isolated by injection of the entire transformation mixture into 40-day-old rats. Encapsulated transformants were recovered from blood cultures 48 h after intraperitoneal injection of the transformation mixture.

Quantitation of capsular antigen. Type b capsule elaboration was quantitated by using a modification of the enzyme-linked immunosorbent assay described previously (16). The coating antiserum was the burro anti-polyribose ribitol phosphate antiserum used previously, but the rabbit antiserum to polyribose ribitol phosphate (Health Research Inc., New York, N.Y.) was used directly as a peroxidase conjugate instead of indirectly, as before.

Slide agglutinations were performed with type-specific antisera (Centers for Disease Control, Atlanta, Ga.).

RESULTS

Restriction maps of cloned DNA. The restriction map of the original λ clone Ch.4:48 is shown in Fig. 1. The 4.4- and 9.0-kb *Eco*RI fragments of Ch.4:48 were subcloned onto pBR328, and the resulting plasmids were designated pSKH1 and pSKH2, respectively. The restriction map of pSKH1 is shown in Fig. 2.

Use of the 4.4-kb probe to analyze clinical strains. Previous work (15) showed that all of eight type b strains examined possessed chromosomal 4.4-kb *Eco*RI fragments homologous to the cloned 4.4-kb fragment of Ch.4:48. These observations have been extended to 30 additional type b strains, and all were found to have a chromosomal *Eco*RI fragment of 4.4 kb when probed with the 4.4-kb subclone (data not shown). It had also been noted (15) that the 4.4-kb probe shared homology with other capsular serotypes of *H. influenzae*, hybridizing to an *Eco*RI fragment of a different, but characteristic size for each serotype. When we cut pSKH1 into two *Eco*RV fragments (see map, Fig. 2) and used the gel-purified fragments as probes, we found that the fragment containing the left 1.7-kb *Eco*RI-to-*Eco*RV portion of the insert DNA hybridized to all serotypes (Fig. 3A). The pattern of hybridization observed was indistinguishable from that of the intact 4.4-kb (15). In contrast, the right 2.7-kb *Eco*RV-to-*Eco*RI portion of the insert DNA recognized only serotypes a and b (Fig. 3B, lanes 1 through 5 and 10 and 12), suggesting that this region contains DNA sequences unique to type a and b strains.

Use of cloned DNA to map the mutations in laboratory-derived mutants Sec-1 and Rb$^-$. The capsule-deficient mutants Sec-1 and Rb$^-$ (Table 1) could be transformed with the

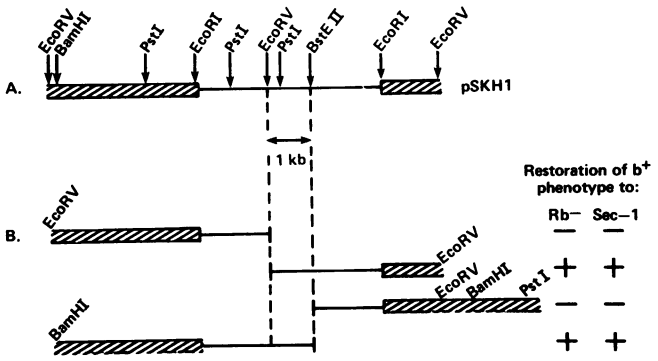

FIG. 2. (A) Restriction map of the 4.4-kb subclone pSKH1. The 4.4-kb *H. influenzae* fragment is denoted by the solid line, and the pBR328 vector DNA is denoted by the hatched boxes. (B) Gel-purified restriction fragments were tested for their ability to transform the capsule-deficient mutants Sec-1 and Rb$^-$ to the b$^+$ phenotype. The transforming activity was localized to the 1-kb *Eco*RV-to-*Bst*EII fragment shown between the dotted lines.

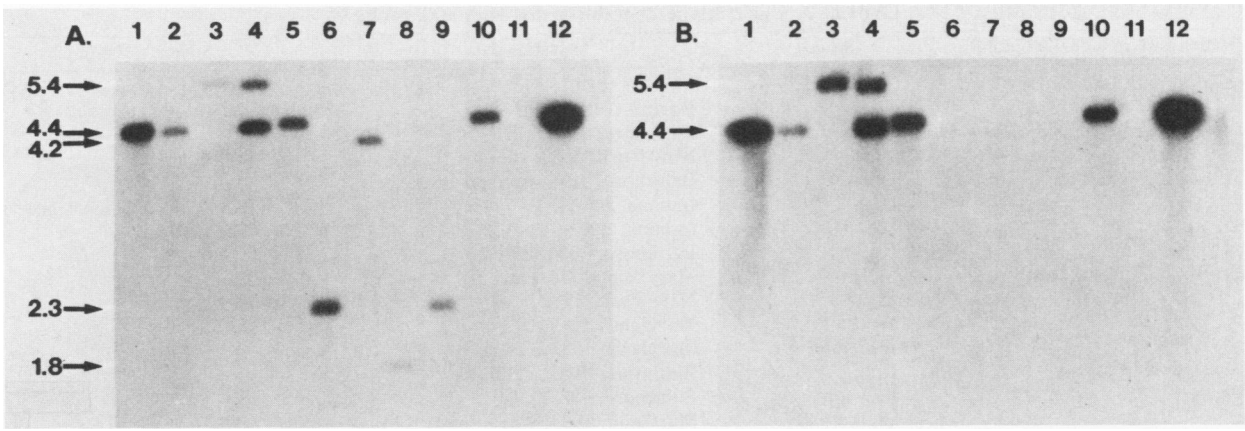

FIG. 3. Identification of a type a, b-specific DNA probe. The 4.4-kb subclone (pSKH1) was cut into two *Eco*RV fragments (Fig. 2B), and the two gel-purified fragments were used as probes. Chromosomal DNA from each of the encapsulated serotypes was cut with *Eco*RI, run on a 0.9% agarose-Tris acetate gel, and bidirectionally transferred to nitrocellulose. One filter was probed with the*Eco*RV fragment containing the left 1.7-kb portion of the insert DNA (A), and the other filter was probed with the *Eco*RV fragment containing the right 2.7kb (B). Lanes: 1, the 4.4-kb fragment of Ch.4:48; 2, b^+ Eagan; 3, a clinical type a strain; 4, a type ab transformant (which makes both type a and b capsules [derived by Grace Leidy]); 5 to 10, clinical isolates of type b, c, d, e, f, and b, respectively; 11, blank; 12, the 4.4-kb fragment of Ch.4:48. The arrows indicate the size of the DNA fragments in kilobases (measured by comparison to a λ *Hin*dIII standard run on the same gel).

4.4-kb subclone, and these mutations were further mapped by transformation with gel-purified fragments containing portions of the 4.4-kb subclone (Fig. 2). The right *Eco*RV fragment transformed both Sec-1 and Rb^-, while the left *Eco*RV fragment did not; the right *Bst*EII-to-*Pst*I fragment did not elicit encapsulated transformants, but the left *Bst*EII-to-*Bam*HI fragment did. These transformation experiments thus localize the mutations in Sec-1 and Rb^- to the central 1-kb *Eco*RV-to-*Bst*EII fragment. This 1-kb fragment lies within the 2.7-kb *Eco*RV fragment found to be specific for type a and b strains (see above). As Sec-1 and Rb^- could also be transformed to the b^+ phenotype with type a chromosomal DNA, these results suggest that this region contains genetic information common to both type a and b strains.

TABLE 1. Laboratory-derived *H. influenzae* mutants and transformants

Mutant or transformant	Description	Source or reference
RM5009	Primary b^- mutant of Eagan	This work
RM5013	Secondary b^- mutant from RM5009	This work
RM5011	Primary b^- mutant of Gast	This work
RM5014	Secondary b^- mutant from RM5011	This work
S-2	Spontaneous b^- variant from Eagan; makes 1/1,000 wild-type levels of polyribose ribitol phosphate	26
Sec-1	b^- from an unstable b^+ transformant of S-2; reverts to b^+ at $\sim 10^{-7}$ frequency and is probably a point mutant; hybridization pattern with 4.4- and 9-kb probes unaltered from that of b^+ parent	15
Rd	Spontaneous capsule-deficient mutant of a type d strain	Grace Leidy, 1
Rb^+	Type b transformant of strain Rd	Andre Zwahlen
Rb^-	Spontaneous capsule-deficient mutant of strain Rb^+; makes no detectable polyribose ribitol phosphate	Andre Zwahlen

Use of the 9-kb fragment as a probe. The 9-kb fragment has been shown previously to be necessary for type b capsule expression (15). When we used the 9-kb fragment as a probe, we found that our prototypic type b strain, Eagan, had three chromosomal *Eco*RI fragments (~ 20, 10.2, and 9.0 kb) (Fig. 4, lane 1) homologous to the 9-kb probe. These results suggest that there are multiple chromosomal copies of at least some of the DNA sequences contained within the 9-kb fragment. Increasing the stringency of the wash (60°C instead of the usual 45°C) did not change this hybridization pattern. An additional 32 type b strains (isolated from diverse geographic locations [see Table 2]) showed multiple bands of homology with the 9-kb probe. These strains exhibited three basic hybridization patterns: (i) those with *Eco*RI fragments of ~20, 10.2, and 9 kb; (ii) those with fragments of 10.2, 9, and 5.6 kb; and (iii) those with bands at ~20, 10.2, 9, and 5.6 kb (Table 2). A hybridization pattern representative of each class is shown in Fig. 4.

The other capsular serotypes of *H. influenzae* also share homology with the 9-kb probe (data not shown). Strain Rd, a capsule-deficient mutant, showed only one faint band with homology to the 9-kb probe (Fig. 5, lane 1); transformation of strain Rd to the capsular phenotype with chromosomal DNA from each of the capsular types a, b, c, d, and f (type e transformant was not obtained) resulted in acquisition of multiple bands showing homology with the 9-kb probe (Fig. 5). These results suggest that DNA sequences in the 9-kb region may be necessary for expression of all capsular serotypes of *H. influenzae*.

Analysis of spontaneous, capsule-deficient mutants. The capsule-deficient mutants Sec-1 and Rb^- were descendants of laboratory-derived transformants and may not be representative of naturally occurring capsule-deficient mutants. We therefore decided to isolate spontaneous, capsule-deficient mutants as well as systematically examine the frequency of capsule loss. We found that all of four independently isolated type b strains (strains Eagan, Sterm, Gast, and Valee) produced spontaneous, capsule-deficient variants at frequencies of 0.1 to 0.3%. These frequencies

TABLE 2. Clinical type b strains tested with 9-kb probe

Hybridization pattern with 9-kb probe[a]	Strain	Isolation data	Source and reference
1. 20-, 10.2-, and 9-kb bands	ATCC b 9795	Illinois, 1944, CSF[b]	ATCC[c] (M. Pittman NIH 641)
	Eagan	Boston, 1968, CSF	P. Anderson (2)
	Murphy	Baltimore, 1983, CSF	
	Gast	New York, 1982, blood	T. R. Leggiadro
	Dorsey	Baltimore, 1983, epiglotitis	
	IU-8	Indiana, blood	M. Kleiman (Indiana University)
	IU-15	Indiana, eye	M. Kleiman
	Nunley	Baltimore, 1984, blood	
2. 10.2-, 9-, and 5.6-kb bands	Sterm	Maryland, 1982, trachea	
	Rutzler	New York, 1982, CSF	T. R. Leggiadro
	Watkins	Maryland, 1983, CSF	
	Johnson	Maryland, 1983, CSF	
	Bragg	Baltimore, 1983, cellulitis	
	Lawson	Baltimore, 1983, CSF	
	Walker	Maryland, 1983, CSF	
	RM1038	Arizona, 1983	M. Santosham
	RM1039	Arizona, 1983	M. Santosham
3. 20-, 10.2-, 9-, and 5.6-kb bands	93	Arizona, 1983	M. Santosham
	2.7	Arizona, 1983	M. Santosham
	89	Arizona, 1981–1982, blood	M. Santosham
	Mitchell	Baltimore, 1983, CSF	
	IU-7	Indiana, blood	M. Kleiman
	Valee	Chicago, CSF	R. Yogev (27)
	Chisholm	Baltimore, 1983, blood (pneumonia)	
	Blome	Baltimore, 1983, CSF	
	Buckhart	Maryland, 1982–1983, CSF	
	RM1032	Arizona, 1983	M. Santosham
	RM1033	Arizona, 1983	M. Santosham
	RM1034	Arizona, 1983	M. Santosham
	RM1035	Arizona, 1983	M. Santosham
	RM1036	Arizona, 1983	M. Santosham
	RM1040	Arizona, 1983	M. Santosham
	RM1041	Arizona, 1983	M. Santosham

[a] Pattern numbers refer to lanes in Fig. 4.
[b] CSF, Cerebrospinal fluid.
[c] ATCC, American Type Culture Collection.

were determined by inoculating a single, iridescent colony into sBHI broth, incubating (without shaking) at 37°C for 17 to 18 h, and plating onto sBHI agar to obtain 1,000 to 2,000 colonies per plate. The mutants were detected as tiny, gray, noniridescent colonies with very rough edges (Fig. 6). The doubling time of these mutants in broth was twice that of the encapsulated parent strains (cf. 60 and 30 min). We noticed that platings from broth cultures of these mutants produced a mixture of colony types. These were not encapsulated revertants, but simply larger, healthier colonies. We will refer to the original small colonies as primary mutants, and the healthier colonies derived from them as secondary mutants.

From two different clinical isolates, strains Gast and Eagan, we obtained both primary and secondary mutants (see Table 1). The larger colonies were subcultured through two generations; each time, the largest colony on the plate was picked. Since the secondary mutants grow faster than the primary mutants, they presumably overgrow the primary mutants, and it is therefore difficult to establish an accurate estimate of the frequency at which they arise. The frequency does appear to be high (estimated at 0.1 to 1%), and if one is not careful to pick single colonies, it is nearly impossible to maintain primary mutants as such.

Quantitation of capsular antigen made by primary and secondary mutants of strains Eagan and Gast. By enzyme-linked immunosorbent assay, both the primary and secondary mutants of strains Eagan and Gast made detectable amounts of capsular antigen. The primary mutants (strains RM5009 and RM5011) made about 1/50 the amount made by the encapsulated parent strains, and the secondary mutants (RM5013 and RM5014) made 10-fold less than did the

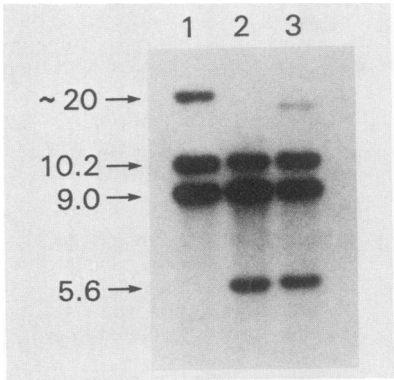

FIG. 4. Hybridization patterns of clinical type b strains. Chromosomal DNA from 33 type b strains was digested with *Eco*RI, run on a 0.9% agarose-Tris acetate gel, and blotted to nitrocellulose. Use of the 9-kb subclone (pSKH2) as a probe revealed three different hybridization patterns. Lanes: 1, the strains with ~20-, 10.2-, and 9-kb fragments homologous to the probe; 2, those with 10.2-, 9-, and 5.6-kb fragments; and 3, those with ~20-, 10.2-, 9-, and 5.6-kb fragments.

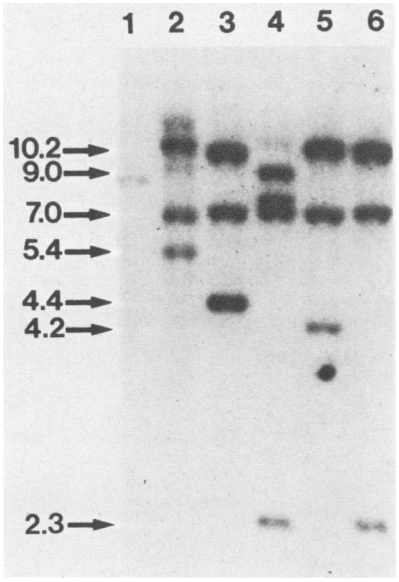

FIG. 5. Acquisition of DNA sequences homologous to the 9-kb probe after transformation of strain Rd$^-$ to each of the encapsulated serotypes. *Eco*RI-cut chromosomal DNA was probed with Ch.4:48. Lanes: 1, Rd$^-$; 2, Rd$^-$/a$^+$; 3, Rd$^-$/b$^+$; 4, Rd$^-$/c$^+$; 5, Rd$^-$/d$^+$; 6, Rd$^-$/f$^+$. (The smallest band in lanes 2 through 6 is due to homology with the 4.4-kb fragment of Ch.4:48, as verified by independent experiments with only the 4.4-kb probe).

primary mutants. The primary mutants were slide agglutinable with type b antisera, whereas the secondary mutants were not.

Analysis of spontaneous capsule-deficient mutants with the 9-kb probe. To determine what alteration, if any, had occurred in the DNA of the primary, high-frequency variants, we probed *Eco*RI digests of their chromosomal DNA with the 9-kb subclone. In all of 10 independent variants examined (7 from strain Eagan, and 1 each from Sterm, Gast, and Valee), we found that an identical 9-kb band was missing (Fig. 7 and 8). The encapsulated strains produced bands of ~20, 10.2, and 9 kb (strains Eagan and Gast); 10.2, 9, and 5.6 kb (Sterm); or ~20, 10.2, 9, and 5.6 kb (Valee). The primary variants were missing the 9-kb band when probed with 9-kb subclone (pSKH2) but showed an intact 4.4-kb fragment when probed with the 4.4-kb subclone (pSKH1).

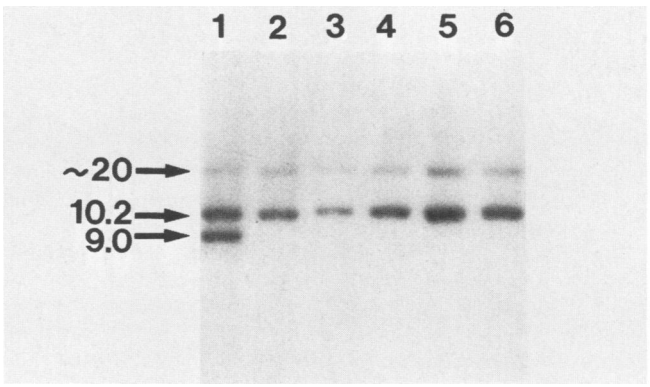

FIG. 7. Southern blot of five independent b$^-$ variants (lanes 2 through 6) derived from strain Eagan (lane 1). Chromosomal DNA was digested with *Eco*RI and probed with the 9-kb subclone (pSKH2). Fragment sizes (in kilobases) were determined by comparison with a *Hin*dIII digest of λ DNA.

The secondary mutants of strains Eagan and Gast (strains RM5013 and RM5014) showed no further alteration in the hybridization pattern over that seen in the primary variants. However, on another occasion we observed what appears to be sequential loss of DNA. In the process of collecting representative type b strains from various regions of the United States, we received several strains on chocolate-agar slants. Some of these cultures had been at room temperature for over a week, and we noticed upon initial plating that some contained more than 90% b$^-$ colonies. Since the precise history of the inoculum for these cultures was not available, we decided to try and reproduce this phenomenon. We inoculated a chocolate-agar slant with a single, iridescent colony of strain Eagan and deliberately let the slant sit at room temperature for many weeks. We subcultured periodically to sBHI plates and found that throughout the first 3 weeks, primary b$^-$ variants arose at approximately the same frequency as that we had observed from overnight broth cultures (0.1 to 0.3%). As the viability of the culture decreased (after more than 4 weeks), we detected an increase in the proportion of capsule-deficient variants (to greater than 50% on day 54). These were no longer the primary-type variants but were the healthier, secondary type. Single-colony isolates were inoculated into broth and grown overnight, and DNA was extracted. Sec-

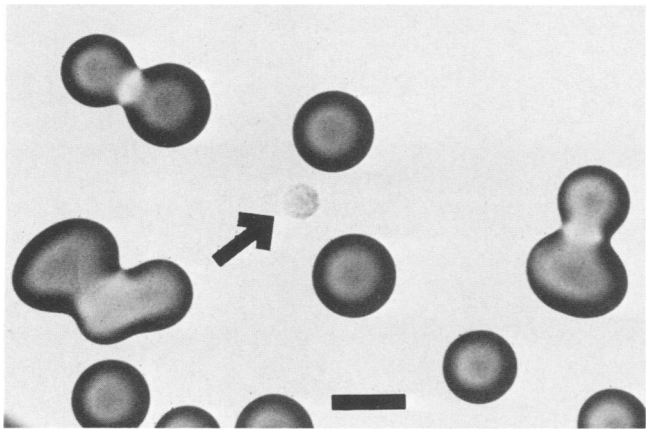

FIG. 6. Colonial morphology of primary b$^-$ variant (arrow). Bar, 1 mm.

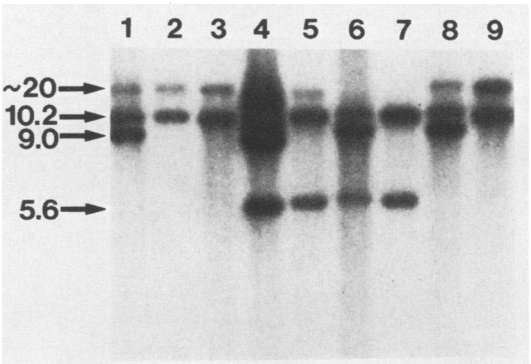

FIG. 8. Southern blot of additional b$^-$ variants (and their b$^+$ parental strains) probed with the 9-kb subclone (pSKH2). Lanes: 1, b$^+$ Eagan; 2 and 3, two independent b$^-$ variants of Eagan; 4, Valee b$^+$; 5, Valee b$^-$; 6, Sterm b$^+$; 7, Sterm b$^-$; 8, Gast b$^+$; 9, Gast b$^-$.

ondary variants isolated on days 28 and 41 showed loss of the 9-kb fragment, but had otherwise normal hybridization patterns with the 4.4- and 9-kb probes. Three of four capsule-deficient variants isolated when the viability of the culture was approaching zero (day 54) showed no homology with the 4.4-kb probe and only one very faint band (>20-kb) with the 9-kb probe. These results suggest that these isolates have undergone further loss or rearrangement of DNA sequences involved in type b capsule expression.

Reversion tests. Encapsulated revertants of these high-frequency variants were not observed by visual screening of more than 100,000 colonies. The primary mutants of strains Eagan and Gast (RM5009 and RM5011) were therefore tested for reversion by intraperitoneal injection into rats. Use of the rat as a biological cell sorter has been shown previously (28) to be an effective method for detecting small numbers of b^+ organisms from a predominantly b^- culture. Five 30-day-old rats received 10^6 organisms of strain RM5011, five received 5×10^6 organisms of strain RM5009, and five control rats received 30 b^+ organisms of strain Eagan. All 10 rats receiving the b^- variants had sterile blood cultures at 48 h, whereas all 5 control rats were heavily bacteremic ($>10^4$ b^+ organisms per ml of blood) at 48 h. These results indicate that small numbers of b^+ organisms (given intraperitoneally) effectively initiated bacteremia. Since all rats receiving RM5009 and RM5011 gave negative blood cultures, we conclude that strains RM5009 and RM5011 either do not revert or revert only at very low frequencies.

b^- mutants from rats. To determine whether high-frequency capsule loss occurs in vivo, and to explore its potential biologic significance, we infected 5-day-old rats intranasally with 10^5 organisms of one of three different type b strains (strains Sterm, Gast, and Chisholm). The strains were made streptomycin resistant so that colonization could be monitored on streptomycin plates without overgrowth of normal flora. All 39 rats were still colonized on day 23, but by day 29, 13 rats gave negative nasal cultures. Primary b^- mutants were detected throughout the first 28 days at frequencies similar to those seen with broth-grown cultures (0.1 to 0.3%). By day 28, however, one rat gave approximately 5% b^- variants; by day 35 the same rat had greater than 50% b^- variants, and by day 48 it gave more than 90% b^- variants. The variants isolated beginning on day 35 were no longer the sick, primary-type mutants, but rather, healthy, secondary mutants. All of five variants examined by Southern hybridization showed loss of the 9-kb *Eco*RI fragment when probed with the 9-kb probe.

Examination of clinical nontypable strains. Previous work from this laboratory suggested that many clinical nontypable strains have no homology with the 4.4-kb probe (15). We have examined 25 additional nontypable strains from a variety of geographic locations and body sites; several of these strains have a 4.4-kb *Eco*RI fragment when probed with the 4.4-kb probe and have a pattern with the 9-kb probe identical to that found in our spontaneous, capsule-deficient derivatives (i.e., show the typical type b pattern but are missing the 9-kb fragment). One such isolate, W13, was isolated from an adult with pneumonia (sent to us by R. Wallace), four were neonatal isolates from Indiana (sent to us by M. Kleiman), and one was isolated from the nasopharynx of an Apache Indian child who also had a b^+ strain isolated from the blood. The latter b^- strain (I.C.) has the same outer membrane protein and lipopolysaccharide profiles and the same biotype as the b^+ strain from the blood (13), suggesting that the b^- strain from the nose may be a capsule-deficient derivative of the strain isolated from the blood.

DISCUSSION

Sequences in both the 4.4- and 9-kb *Eco*RI fragments of Ch.4:48 have been shown previously to be necessary for type b capsule expression (15). We used these cloned fragments as probes to analyze the chromosomal DNA of clinical isolates of *H. influenzae* and found that all of the 33 type b strains examined had natural 4.4-kb *Eco*RI fragments in their chromosomes. This region thus appears to be highly conserved. When we used the 9-kb region as the probe, we found multiple bands of homology; strain to strain variation, however, was minimal and could be assigned to one of three basic hybridization patterns.

Both the 4.4- and 9-kb probes showed homology to all encapsulated serotypes of *H. influenzae*. However, we identified a portion of the 4.4-kb probe which was specific for types a and b. This 2.7-kb *Eco*RV-to-*Eco*RI fragment maps farthest from the 9 on the linear map of Ch.4:48 and contains the region needed to correct the defect (by transformation) in the capsule-deficient mutants Sec-1 and Rb^-. As this region appears to be necessary only for type a and b capsule synthesis, these mutants should help identify biosynthetic steps common to types a and b but lacking in types c, d, e, and f. The a and b capsules are structurally the most closely related of the *H. influenzae* capsules, the type b capsule being a linear polymer of ribose ribitol phosphate and the type a capsule consisting of glucose ribitol phosphate (4, 8). The type c, d, e, and f capsules are structurally very different (9) and are thus unlikely to share steps in the biosynthesis of basic sugar repeat units. Virtually nothing is known about the biosynthetic steps involved in *H. influenzae* capsule synthesis, and our observations should provide a starting point for the determination of the steps involved. Since the 9-kb region and the adjacent 1.7-kb *Eco*RI-to-*Eco*RV portion of the 4.4-kb fragment share homology with all encapsulated serotypes (and since DNA sequences homologous to the 9-kb probe are acquired in transformation of a capsule-deficient mutant to each of the capsular serotypes), these regions may code for functions necessary for the expression of all capsule types. Likely candidates for common functions would be transport and surface assembly of capsular polysaccharides.

It has been known for many years that the capsular phenotype in *H. influenzae* is an unstable characteristic (5, 17). Upon systematic examination of this phenomenon, we were surprised to find that loss of the capsule occurs at frequencies several orders of magnitude higher than can be accounted for by classical types of mutations. Starting with single, iridescent b^+ colonies, we found capsule loss occurring at frequencies of 0.1 to 0.3%. Examination of the DNA from 10 independent mutants by Southern hybridization revealed the loss of an identical 9-kb *Eco*RI restriction fragment. These results suggest the existence of a highly conserved mechanism for shutting off type b capsule synthesis at high frequency. The loss of capsule seems not to resemble other high-frequency events, such as *Salmonella* phase variation, in that we have never observed encapsulated revertants (even under strong selection in the rat). The loss of the 9-kb band does not appear to involve the loss of a plasmid, as the presence or absence of plasmid DNA does not correlate with type b capsule expression (E. R. Moxon, unpublished data). Southern hybridization analysis of chromosomal DNA cut with other restriction enzymes (*Xho*I and *Bst*EII; data not shown) suggests some

type of rearrangement. The presence of repeated DNA sequences is compatible with the idea that a high-frequency recombinational event is involved.

The high frequency and specificity with which these capsule-deficient variants arise led us to question the biologic significance of this event. Lampe et al. (12) have reported that nonencapsulated *H. influenzae* adhere better to epithelial cells than do encapsulated strains, and we therefore hypothesized that a mechanism for promoting high-frequency capsule loss might be a means for maintaining nasal (or other mucosal) colonization. Using the infant rat model for intranasal colonization, we followed the in vivo conversion from the b^+ to b^- form. Although only one rat truly converted, the shift was dramatic. The role that anticapsular antibody plays in this shift is something we still need to examine. That the shift occurred at approximately the same time that most rats were beginning to clear the organisms suggests that high-frequency capsule loss may be a device for evading the host immune response, as well as allowing the organism to adhere better to epithelial cells. Also of possible significance is the fact that most nontypable disease isolates come from neonates or adults (24, 25), two populations which are expected to have anticapsular antibody.

We found that although most clinical nontypable strains had no homology with either the 4.4- or 9-kb probes, there were a few strains that were missing the 9-kb fragment but which had otherwise typical type b patterns. These results, taken together with the high-frequency capsule loss observed both in vitro and in vivo (in rats), suggest a possible link between encapsulated organisms and at least some nontypable strains.

ACKNOWLEDGMENTS

We thank our colleagues in Hamilton O. Smith's laboratory (where much of this work was performed) for their advice and encouragement. We also thank Andre Zwahlen for constructing the type a, b, c, d, and f transformants of strain Rd.

This work was supported by the National Institutes of Health (Career Development Award NS12554 to R.M. and Postdoctoral Fellowship 1F32AI06978-01 to S.K.H.) and by the Eudowood Foundation.

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