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Haemophilus influenzae Immunoglobulin A1 Protease Genes: Cloning by Plasmid Integration-Excision, Comparative Analyses, and Localization of Secretion Determinants

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Many bacteria which establish infections after invasion at human mucosal surfaces produce enzymes which cleave immunoglobulin A (IgA), the primary immunoglobulin involved with protection at these sites. Bacterial species such as *Haemophilus influenzae* which produce IgA1 proteases secrete this enzyme into their environment. However, when the gene encoding this protein was isolated from *H. influenzae* serotype d and introduced into *Escherichia coli*, the activity was not secreted into the medium but was localized in the periplasmic space. In this study, the IgA1 protease gene (*iga*) from an *H. influenzae* serotype c strain was isolated and the gene from the serotype d strain was reisolated. The IgA1 proteases produced in *E. coli* from these genes were secreted into the growth medium. A sequence linked to the carboxyl terminus of the *iga* gene but not present in the original clone was shown to be necessary to achieve normal secretion. Tn*5* mutagenesis of the additional carboxyl-terminal region was used to define a 75- to 100-kilodalton coding region required for complete secretion of IgA1 protease but nonessential for protease activity. The *iga* genes were isolated by a plasmid integration-excision procedure. In this method a derivative of plasmid pBR322 containing a portion of the protease gene and the kanamycin resistance determinant of Tn*5* was introduced into *H. influenzae* by transformation. The kanamycin resistance gene was expressed in *H. influenzae*, but since pBR322 derivatives are unable to replicate in this organism, kanamycin-resistant transformants arose by integration of the plasmid into the *Haemophilus* chromosome by homologous recombination. The plasmid, together with the adjoining DNA encoding IgA1 protease, was then excised from the chromosome with DNA restriction enzymes, religated, and reintroduced into *E. coli*. Comparisons between the *H. influenzae* protease genes were initiated which are useful in localizing functional domains of these enzymes.

For a pathogen to establish an infection, host defense mechanisms must be overcome. There are a number of ways in which microorganisms can subvert these host responses, one of which is to interfere with the effectiveness of protective antibodies. Secretory immunoglobulin A (IgA) is the primary form of antibody found at human mucosal surfaces (50). A number of bacteria which infect humans at mucosal surfaces produce and secrete an enzyme that cleaves IgA1. These IgA1 proteases are produced by bacterial species belonging to the genera *Neisseria*, *Streptococcus*, *Bacteroides*, *Clostridium*, and *Haemophilus* (15, 26, 27, 29, 35, 40). It seems likely that the IgA proteases contribute to pathogenesis, since pathogenic strains make the protease, whereas nonpathogenic species within the same genera do not (36).

The IgA1 proteases produced by different strains of bacteria are similar to one another in that they all cleave within the 16-amino-acid hinge region which separates the antigen-binding region (Fab) from the carboxyl (Fc) end of the IgA1 molecule (Fig. 1). However, the IgA1 proteases differ in the exact site of cleavage within the hinge region (27, 34, 35, 40). For example, some strains of *Haemophilus influenzae* produce a type 1 IgA1 protease that cleaves at a site 4 amino acids away from the cleavage site of the *H. influenzae* type 2 protease (Fig. 1). The type of enzyme produced by a particular *Haemophilus* strain is correlated with the serotype, or capsular structure, of the strain (22, 34). The IgA1 proteases of other bacteria cut the IgA1 molecule either at the same sites cleaved by the *Haemophilus* enzymes or at other sites within the hinge region.

Several laboratories are studying the properties of IgA1 proteases at the molecular level. The genes encoding the IgA1 protease of *Neisseria gonorrhoeae* (type 2 [19, 28] and type 1 [14]) and *H. influenzae* (type 1 [8]) have been isolated and introduced into *Escherichia coli*. The gonococcal proteases produced in *E. coli* are secreted into the growth medium, as they are in *N. gonorrhoeae* (14, 19, 28, 42). The type 2 gonococcal enzyme is synthesized as a 180-kilodalton (kDa) precursor protein; this is processed to the form found in the growth medium, a protein of about 100 kDa. A role for the carboxyl terminus of the protein in the secretion process has been previously suggested (41, 42).

In contrast to the gonococcal proteases, the protease produced in *E. coli* from a clone containing the *H. influenzae* type 1 gene (*iga*) (8) is not secreted and has low enzymatic activity relative to that produced by the native *H. influenzae* strain. Furthermore, the polypeptide product produced in *E. coli* is larger than the mature protein found in *H. influenzae* growth media (137 versus 100 kDa). It has been suggested that the low level of activity and the periplasmic location of the protease produced in *E. coli* from the *H. influenzae* clone might be due to defects in processing or secretion (8). It seemed likely that the carboxyl terminus of the *H. influenzae* *iga* product might have a role in secretion, since the corresponding region of the gene is not necessary for complete activity (8) and the carboxyl-terminal coding region of the *N. gonorrhoeae* gene is needed for complete secretion (42). To address this possibility we have isolated type 1 and 2 *H.*

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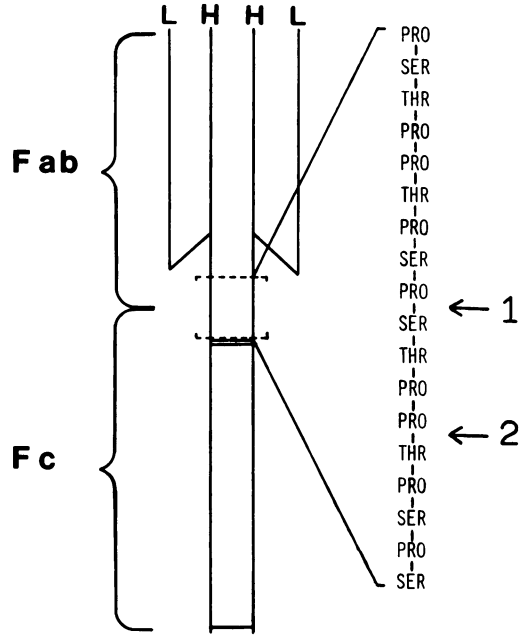


FIG. 1. Cleavage sites of IgA1 proteases. The amino acid sequence of the hinge region of human IgA1 is shown at the right with the following three-letter code: pro, proline; ser, serine; thr, threonine. The location of the hinge region within the heavy chains (H) of IgA1 is bracketed by dashed lines. Disulfide bonds between cysteine residues join the heavy chains together and the light chains (L) to the heavy chains. Cleavage by IgA1 proteases results in three fragments, two Fab and one Fc. Each Fab, or antigen-binding region, consists of one light chain and the amino-terminal half (Fd) of one heavy chain. The Fc region consists of the carboxyl-terminal ends of two heavy chains. The arrows show the cleavage sites of type 1 and 2 *H. influenzae* IgA1 proteases.

influenzae iga clones containing additional downstream sequences. These clones were isolated by a plasmid integration-excision system developed for *H. influenzae*; similar methods have been used successfully with other genera, including *Bacillus* and *Streptococcus* (31, 38). In contrast to the original type 1 clone, the new isolates encoded proteases which were secreted from *E. coli*. These results, together with the results of Tn*5* mutagenesis of the plasmids and complementation analysis, confirm that carboxyl-terminal sequences of *H. influenzae* IgA1 proteases are important for secretion.

(A preliminary report of this work was presented at the International Congress on Mucosal Immunity, Niagara Falls, N.Y., 29 June to 3 July 1986.)

MATERIALS AND METHODS

Media. L broth (32) was used for culturing *E. coli*. When necessary, kanamycin sulfate, tetracycline hydrochloride, or ampicillin (sodium salt, all from Sigma Chemical Co., St. Louis, Mo.) was included at a final concentration of 10, 30, or 100 μg/ml, respectively. *H. influenzae* was routinely grown in brain heart infusion (BHI) medium (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 10 μg of β-NAD–hemin (both from Sigma) per ml. Heart infusion containing similar supplements (Difco Laboratories, Detroit, Mich.) was used for *H. influenzae* cultures that would subsequently be made competent in the M-IV medium of Herriott et al. (20; see below). BHI supplemented agar (BBL) was used as a solid growth medium for *H. influenzae*. For appropriate *H. influenzae* strains, ampicillin (10 μg/ml), kanamycin (20 μg/ml), or streptomycin (Sigma; 50 μg/ml) was included in the growth media.

Bacterial strains. *E. coli* strains used were MM294 (*hrs hrm*$^+$ *thi endA supE*) (3) and a *recA* derivative of MC1000 [*leu araD139* Δ(*ara-leu*)*7697* Δ*lacX74 galE galK rpsL*] (10). *H. influenzae* strain Rd (2) is an uncapsulated derivative of a serotype d, type 1 IgA1 protease producer (*iga*$^+$). Rd2-8DK is an *iga* derivative of Rd with a large deletion in the IgA1 protease gene; the kanamycin resistance gene from the transposon Tn*5* was substituted for the deleted region. This strain will be described in detail elsewhere (F. J. Grundy, A. Plaut, and A. Wright, manuscript in preparation). HF2.8c$^+$(c3) is a type 2 IgA1 protease producer derived from strain Rd (7) in the following way. An *iga* frameshift mutation constructed at the *Xba*I site of the *E. coli* plasmid pJB2 (see below) was introduced into the chromosome of strain Rd by homologous recombination after transformation with linearized plasmid DNA. This *iga* mutant was then transformed with chromosomal DNA from a serotype c type 2 IgA1 protease-producing *H. influenzae* strain. HF2.8c$^+$(c3) is a type 2 IgA1 protease producer found among the transformants. The *Eco*RI restriction enzyme map of the region encompassing the *iga* gene in this strain is identical to that of the *iga* gene from the type 2-producing serotype c strain from which the *iga* gene in HF2.8c$^+$(c3) originated. However, it cannot be ruled out that these restriction fragments are actually hybrid DNA containing type 1 and 2 sequences, since this strain was constructed by recombining DNA from a type 2 strain with DNA from a type 1 strain.

In vitro DNA manipulations. All DNA restriction enzymes and T4 DNA ligase were purchased from New England BioLabs, Inc., Beverly, Mass. Restriction enzymes were used with the buffer conditions described by Maniatis et al. (30). DNA fragments were ligated with 40 to 80 U of T4 DNA ligase in 20 μl at 15°C for 15 h under conditions recommended by the manufacturer. DNA fragments were analyzed by electrophoresis through 1% agarose gels (FMC Corp., Marine Colloids Div., Rockland, Maine).

Transformation. Transformation of *E. coli* strains was carried out by the calcium chloride shock method essentially according to the protocol of Cohen et al. (11). *H. influenzae* strains to be made competent were grown as stationary overnight cultures in supplemented DHI at 37°C. These cultures were diluted 40-fold in supplemented DHI and grown to a density of 5×10^8 CFU/ml) as monitored by Klett-Summerson readings. The competence protocol of Herriott et al. (20) involving the defined nongrowth medium M-IV was then followed. DNA was added to competent cells at a concentration of 4 to 40 μg/ml for plasmids or 4 μg/ml for chromosomal DNA from a streptomycin-resistant *H. influenzae* strain (used as a control for competence). After 40 min at 37°C, the mixture was diluted in BHI, and 0.1 ml of the appropriate dilution was embedded in 20 ml of 0.75% supplemented BHI agar containing 0.15 μg of tetracycline per ml (48) to stimulate plasmid establishment. After 3 h at 37°C, the agar was overlaid with 10 ml of 0.75% supplemented BHI agar containing antibiotics at three-fold-higher concentrations than the desired final concentration. The plates were then incubated at 37°C under reduced oxygen tension for 36 to 48 h.

Plasmids. pJB2 is a pBR322 derivative which has a 6.9-kilobase (kb) insert of *H. influenzae* DNA at the *Eco*RI site (8). This plasmid encodes a type 1 IgA1 protease. The shuttle vector pHVT1 (12) can be maintained in either *E. coli* or *H.*

influenzae and encodes resistance to ampicillin and tetracycline. The 4.8-kb *Cla*I-*Eco*RI fragment of pJB2 containing the IgA1 protease gene (*iga*) was inserted within the tetracycline resistance determinant of pHVT1 to form pFG24. pJB2::Tn*5*-201 contains a Tn*5* element located 1 kb downstream of the *iga* promoter. This plasmid was digested with *Cla*I, for which there are no sites in Tn*5*, and *Sal*I, for which there is one site in Tn*5* (25). The resulting 4.5-kb *Cla*I-*Sal*I fragment containing the amino-terminal end of *iga* and the kanamycin resistance determinant of Tn*5* was inserted into pBR322 between the *Cla*I and *Sal*I sites, with selection for ampicillin and kanamycin resistance, to form pFG16. pFG25 and pFG26 are pBR322 derivatives described in this work that contain *H. influenzae* DNA sequences encoding type 2 and 1 IgA1 protease sequences, respectively. pFG31 is similar to pFG26 but contains Tn*5* located within the pBR322 DNA just upstream of the ampicillin resistance determinant. The *Cla*I-*Nde*I fragment containing all inserted *H. influenzae*-specific DNA of pFG25 was substituted for the similar fragment of pFG31 to form pFG41 (type 2 IgA1 protease producer, kanamycin resistant). The kanamycin resistance determinant in pFG31 was replaced by the chloramphenicol resistance determinant of pBR325 (6) to form pFG43 (type 1 IgA1 protease producer, chloramphenicol resistant).

Transposition of Tn*5*. Tn*5* insertions in pJB2 and pFG26 were selected by a previously described method (13).

IgA1 protease assays. For qualitative assays to distinguish protease-negative from -positive colonies on agar plates, an overlay assay with ^{125}I-labeled IgA was used (16). To determine quantity and type of IgA1 protease, the following method was used. *E. coli* cultures to be assayed for activity were grown at 37°C with aeration in L broth containing relevant antibiotics. A sample (1 ml) of each culture was chilled, and the cells were pelleted by centrifugation for 2 min in an Eppendorf microfuge. The supernatant was used to assay for secreted protease. The pellet was suspended in 1 ml of chilled growth medium and sonicated at 4°C for 1 min in a Sonifier cell disruptor (Branson Sonic Power Co., Danbury, Conn.) at a pulsed power setting of 3. This extract was assayed to determine the amount of cell-associated protease activity. A portion (12.5 μl) of an extract to be tested (or growth medium as a negative control or, when needed, partially purified protease preparations from standard *H. influenzae* type 1 or 2 protease producers) was mixed with an equal volume of 0.05 M Tris (pH 7.5)–0.5% bovine serum albumin containing 0.8 μCi of ^{125}I-labeled IgA. After digestion at 37°C for 3 to 16 h, the products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described elsewhere (34). Amount and type of IgA1 protease activity were determined by the intensity and distance of migration of the Fd fragment of IgA1 in comparison with similar bands found by using standard types of proteases in control digests.

DNA isolation. Small-scale plasmid isolation from *E. coli* cells (1.5 ml) was carried out by the method of Ish-Horowicz and Burke (23). Large-scale plasmid isolation was carried out by a modification of the method of Birnboim and Doly (5) followed by ethidium bromide-cesium chloride density gradient separation. Small-scale DNA isolation from *H. influenzae* was carried out by an alkaline-sodium dodecyl sulfate procedure (33). Large-scale total DNA preparations were made as previously described (8).

Heteroduplex analysis. Samples (1 μg) of pFG25 and pFG26 plasmid DNA, purified by ethidium bromide-cesium chloride density gradient fractionation, were digested in 50 μl of buffer with the DNA restriction enzyme *Nde*I to linearize each plasmid. The digested plasmid DNAs at a concentration of 0.25 μg/ml were mixed, denatured by heating to 90°C for 2 min, and allowed to reanneal in the presence of 55% formamide at 22°C for 2 h. The resulting molecules were spread on cytochrome *c*, rotary shadowed with platinum, and viewed with a Jeol 100S electron microscope at a magnification of ×15,000. The micrographs were measured with a Numonics electronic graphics calculator. The ends of heteroduplex molecules were oriented by the known minimal size (2.1 kb) of homology resulting from common pBR322 sequences.

RESULTS

Localization of *iga* sequences necessary for high activity. Previous work with the type 1 *iga* gene of *H. influenzae* showed that in *E. coli* the DNA cloned in plasmid pJB2 coded for a low-activity IgA1 protease that was not secreted. Moreover, Tn*5* insertions near the end of the cloned DNA, but apparently within the *iga* gene, did not destroy IgA1 protease activity. We considered the possibility that these results could be explained if the cloned DNA lacked a segment of the *iga* gene necessary for high-level activity and secretion. This seemed possible since the product produced from the cloned gene approaches the coding capacity of the isolated DNA fragment. To test this idea, we first isolated and mapped additional Tn*5* insertions in pJB2 and assayed the resulting plasmid-containing strains for IgA1 protease activity to localize the region necessary for high-level activity (Fig. 2). It was found that protease activity was eliminated by insertions starting with pJB2::Tn*5*-225, which is known from DNA sequence information to be very close to the amino terminus of the gene, and extending to pJB2::Tn*5*-208. This region is 2.5 kb in length, representing an amino-terminal portion of the protease of approximately 85 kDa. Tn*5* insertions 231 (located 300 base pairs downstream of 208), 224, and 227 reduced but did not eliminate protease activity; promoter distal insertions affected activity less than did promoter proximal insertions.

Expression in *H. influenzae* of the cloned *iga* gene. To rule out the possibility that differences between the *iga* gene analyzed for *E. coli* and the native *H. influenzae* gene were due to inherent differences of expression or secretion between these two species, we tested expression of the cloned DNA in *H. influenzae*. Since pBR322 replicons cannot be maintained in *H. influenzae* (12), the insert DNA from pJB2 was transferred to a shuttle vector, pHVT1, which could replicate in both *E. coli* and *H. influenzae*. The resulting plasmid, pFG24, was used to transform an *H. influenzae* recipient, HF2.8c$^+$(c3), which produces type 2 IgA1 protease (7). In the pFG24 transformant of HF2.8c$^+$(c3), type 2 protease was found to be secreted, whereas only a small amount of type 1 protease was found, and this activity was cell associated (data not shown). This result indicates that the cloned *iga* gene does not contain all of the information for proper secretion of its product and that this information cannot be supplied *in trans* in *H. influenzae*.

Transformation of strain Rd with plasmid pJB2::Tn*5*-209 (Iga$^-$) gave rise to equal numbers of Iga$^+$ and Iga$^-$ transformants. These kanamycin- and ampicillin-resistant derivatives were the result of plasmid integration into the chromosome via *iga* homology, since pBR322 replicons cannot be maintained as autonomous plasmids in *H. influenzae* (12). If all the sequences necessary for IgA protease production and secretion were present on pJB2, then integration of pJB2::Tn*5*-209 into the chromosome would result in Iga$^+$

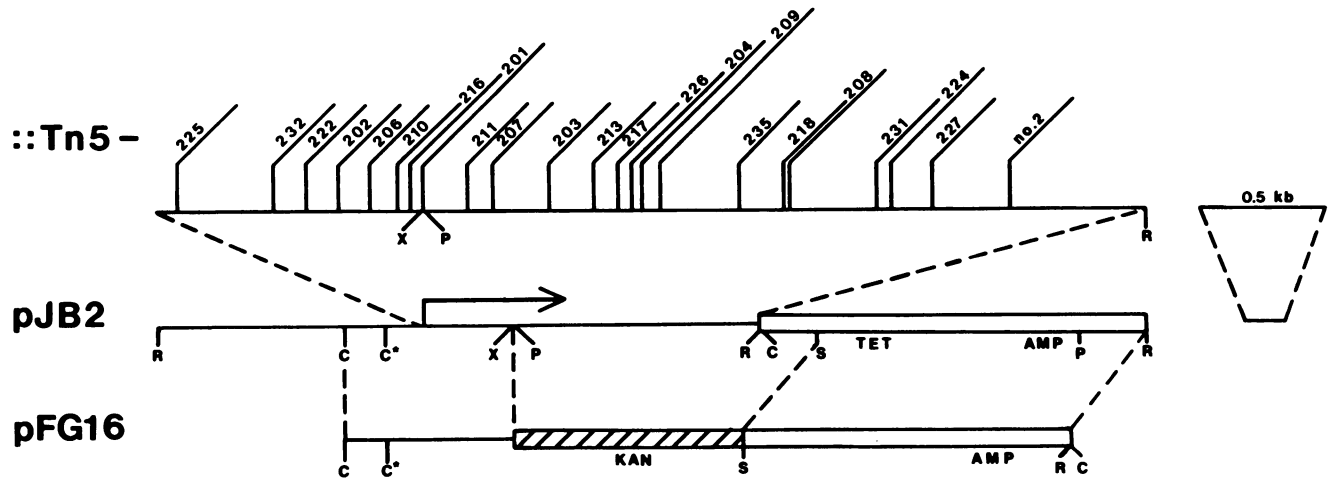


FIG. 2. Tn*5* insertions affecting *iga* activity. The restriction map of pJB2 shows (→) the location of the *iga* promoter and the direction of transcription (7). Vector (▭) and cloned (——) DNAs are indicated. The top line is an enlargement of the portion of the cloned DNA which is located downstream of the promoter. Numbered lines locate the sites of Tn*5* insertions. Tn*5* no. 2 is from Bricker et al. (8). Tn*5*-201 was used to construct the plasmid (pFG16) shown at the bottom. The portion (▨) of Tn*5* containing the kanamycin resistance determinant remains in pFG16, as indicated. Restriction sites: C, *Cla*I; C*, *Cla*I in unmethylated DNA; P, *Pst*I; R, *Eco*RI; S, *Sal*I; X, *Xba*I.

transformants only; one functional end of the gene or operon would be supplied by the incoming plasmid, and the other would be provided by the chromosomal gene. The finding that half of the transformants were Iga$^-$ is evidence that the plasmid lacks one intact end of the gene-operon.

Cloning of additional downstream DNA by plasmid integration-excision. To determine if the Iga phenotype of pJB2 was unique to this type 1 clone, a second *H. influenzae* protease clone was isolated. This was done by taking advantage of plasmid integration properties by using the strategy shown in Fig. 3. In this method pFG16, a pBR322 derivative containing 1 kb of DNA from pJB2 corresponding to the amino-terminal coding region of *iga* (type 1), was integrated into the chromosome of the type 1 IgA1 protease-producing *H. influenzae* strain Rd by selection for kanamycin resistance. The integration event resulted in the juxtaposition of the plasmid vector sequences with an intact type 1 protease gene. This gene could be excised, in conjunction with the plasmid, by digestion of the chromosomal DNA with the restriction enzyme *Nde*I, which does not cut in the gene or in the regions of the plasmid necessary for independent maintenance. After ligation, the DNA was used to transform *E. coli* MM294, a strain defective in restriction of foreign DNA. The plasmids obtained (e.g., pFG26) all exhibited identical restriction digestion patterns. The restriction map of pFG26 was identical to those of analogous regions of pJB2 except that pFG26 contained an additional 1.65 kb of DNA beyond the carboxyl-terminal *Eco*RI fragment of pJB2.

E. coli transformants containing plasmid pFG26 were tested for protease activity and were found to be type 1 producers (Fig. 4). Furthermore, the activity was secreted into the growth medium. The levels of activity were comparable to those produced by wild-type *H. influenzae* cells, much higher than the levels produced from pJB2. However, pFG26 is present in many copies per cell whereas the *H. influenzae* chromosomal *iga* gene is presumably present in one copy per cell. These differences in gene dosage could affect the total amount of protease produced in a cell and would have to be taken into account before exact comparisons of activity could be made between these strains.

The change in secretion resulting from the additional DNA contained in pFG26 confirmed that carboxyl-terminal sequences are important in IgA protease secretion. Further evidence for the role of downstream sequences in protease production came from plasmid insertions into the *Haemophilus* chromosome with the use of carboxyl-terminal clones. As with amino-terminal clones (Fig. 3), such insertions should result in Iga$^+$ transformants if all the necessary downstream information of this transcriptional unit is contained on the plasmid. If the plasmid does not contain the intact carboxyl terminus of the gene, then plasmid integration results in an Iga$^-$ phenotype. Also, plasmid integration is likely to disrupt transcription, resulting in a polar effect on the expression of cotranscribed downstream genes. If the plasmid does not contain these genes, then integration will once again result in lack of protease production. Integration of plasmids containing the downstream region of the insert DNA in pJB2 resulted in loss of protease activity. However, integration of plasmids with the additional sequences of pFG26 had no effect on protease production, indicating that the carboxyl terminus of the gene was intact.

Localization of *iga* sequences necessary for secretion. Tn*5* mutagenesis of pFG26 was carried out to define the region necessary for high-level, secretable protease activity (Fig. 5). All insertions beyond the *Eco*RI site (which defined the endpoint of insert DNA in pJB2) interfered with the secretion of the protease but did not eliminate its activity. Together with the results of the similar analysis of pJB2, these data delimit a 2.2 to 3.1-kb region between pJB2::Tn*5*-208 and the end of insert DNA in pFG26 that is necessary for complete protease activity. This region has a total coding capacity of 75 to 100 kDa.

Cloning of type 2 *iga* gene of *H. influenzae*. As a step toward understanding the basis of the differences (such as cleavage site specificity) between the various *H. influenzae iga* genes, we isolated a type 2 *iga* clone by the plasmid integration-excision technique described above (Fig. 3). In this case, integration of plasmid pFG16 into the chromosome of strain HF2.8c$^+$(c3) (type 2 *iga*$^+$) resulted in type 2$^+$ transformants. Although the type 1 and 2 *iga* genes show a high degree of homology in hybridization analyses (7), the frequency of integration was about 10^{-4} lower than that for integration of

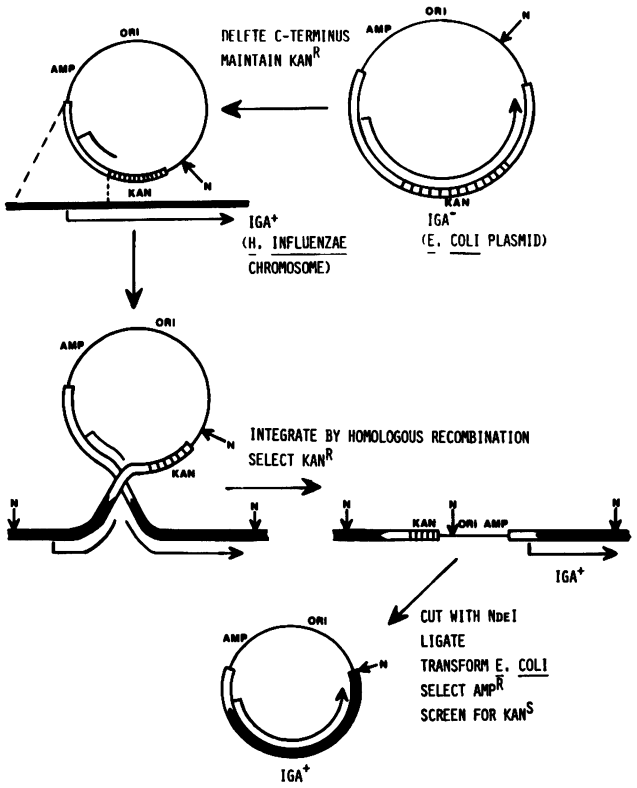


FIG. 3. Cloning strategy. The plasmid at the beginning of the flow chart is pJB2::Tn*5*-201. This plasmid has the ampicillin resistance gene (AMP) and origin of replication (ORI) of pBR322. The arrow in this depiction of the plasmid originates at the *iga* promoter and proceeds in the direction of *iga* transcription. The carboxyl terminus of *iga* was deleted from pJB2::Tn*5*-201 by using a restriction site in Tn*5*. The resulting plasmid, pFG16, retained the kanamycin resistance gene (▭) and the amino terminus of *iga*. Integration of pFG16 into the *H. influenzae* chromosome (thick bar) by homologous recombination resulted in kanamycin-resistant transformants. The vector DNA, together with chromosomal DNA, was retrieved from the transformants after cleavage with the restriction enzyme *Nde*I (*Nde*I sites are shown by arrows labeled N.)

plasmid pFG16 into strain Rd or for a chromosomal marker. Excision of the plasmid and *iga* sequences with the restriction enzyme *Nde*I resulted in the isolation of plasmid pFG25. As with plasmid pFG26, plasmid pFG25 elicited the production in *E. coli* of high levels of protease activity that was secreted into the growth medium (Fig. 4).

The type 2 recipient which was used in the isolation of plasmid pFG25 was derived from an *iga* mutant of Rd by transformation with DNA from a serotype c, type 2-producing strain (8). The resulting type 2 Iga^+ strain, HF2.8c^+(c3), may therefore contain an *iga* gene that is a hybrid between the type 1 gene of strain Rd and the type 2 gene; however, this hybrid exhibits the type 2 cleavage specificity and *Eco*RI restriction map (7; see Materials and Methods).

The total length of insert DNA in plasmid pFG26 (type 1) was 0.7 kb larger than that of pFG25 (type 2). These two plasmids had similar patterns of restriction sites upstream of the *iga* gene, as well as near the carboxyl-terminal ends of the DNA inserts. These similarities may indicate sequence homology between the two types of *iga* genes or may be due to recombination events between the type 1 and 2 genes which might have occurred during construction of strain HF2.8c^+(c3) or plasmid pFG25.

Heteroduplex analyses. Plasmids pFG25 and pFG26 were directly compared for sequence homology by DNA heteroduplex analyses (Fig. 6). The heteroduplexes showed extensive homology with the exception of two regions. One is a 0.8-kb region starting about 0.2 kb from the start of *iga* and extending downstream. It will be reported elsewhere that this region of nonhomology corresponds to the region which specifies the cleavage site preference of the *H. influenzae* IgA proteases (Grundy et al., in preparation). The other region resembles a deletion-substitution loop where a 0.2-kb region in one plasmid is replaced by a 0.9-kb region in the other. This region is about 1.2 kb from the carboxyl-terminal end of the insert DNA.

Complementation analyses. We addressed the question of the number of genes involved in protease production in two ways. In the experiment 1, plasmid pFG41, a derivative of pFG25 (type 2 protease) carrying Tn*5* in the vector portion of the plasmid, and plasmid pJB2 (type 1 protease) were maintained together in a *recA E. coli* strain. This strain secreted type 2 activity while producing a low level of type 1 activity which accumulated within the cell (Table 1). This result rules out the possibility that a *trans*-acting function encoded within pFG41 can complement the defect of pJB2. In experiment 2, plasmid pJB2 was maintained with plasmid pFG26::Tn*5*-11 in a *recA E. coli* strain; insertion Tn*5*-11 eliminates protease activity but lies upstream of the region defined as necessary for secretion. Only a small amount of cell-associated type 1 activity was found to be produced

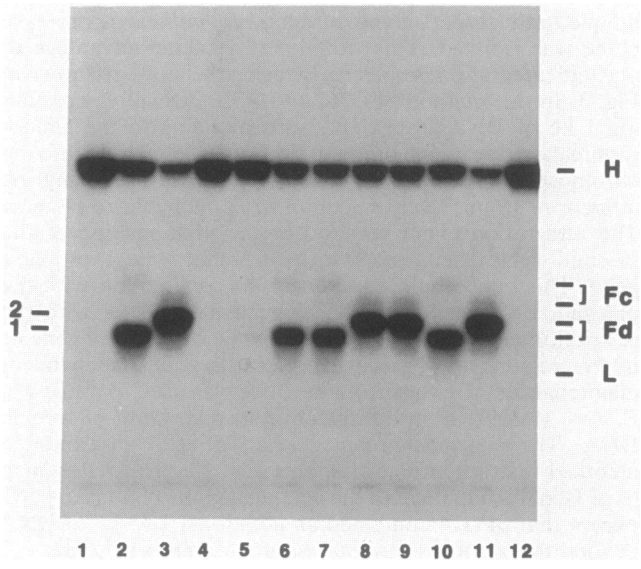


FIG. 4. IgA1 protease activity produced from *iga* clones. ^{125}I-labeled IgA1 was incubated with culture supernatants (lanes 4, 6, 8, and 10) or sonicated whole cultures (lanes 5, 7, 9, and 11). Strains contained the plasmid pJB2 (lanes 4 and 5), pFG26 (lanes 6 and 7), or pFG25 (lanes 8 and 9). The digestion products were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The positions of the intact IgA1 heavy chain (H), intact light chain (L), carboxyl terminus of the heavy chain (Fc), and the amino terminus of the heavy chain (Fd) are shown. The positions of the Fd fragments which resulted from digestion with type 1 or 2 enzymes are shown by numbers at the left. Control digestions are indicated by the following lanes: 1 and 12, growth medium; 2 and 10, native *H. influenzae* type 1 protease; 3 and 11, native *H. influenzae* type 2 protease.

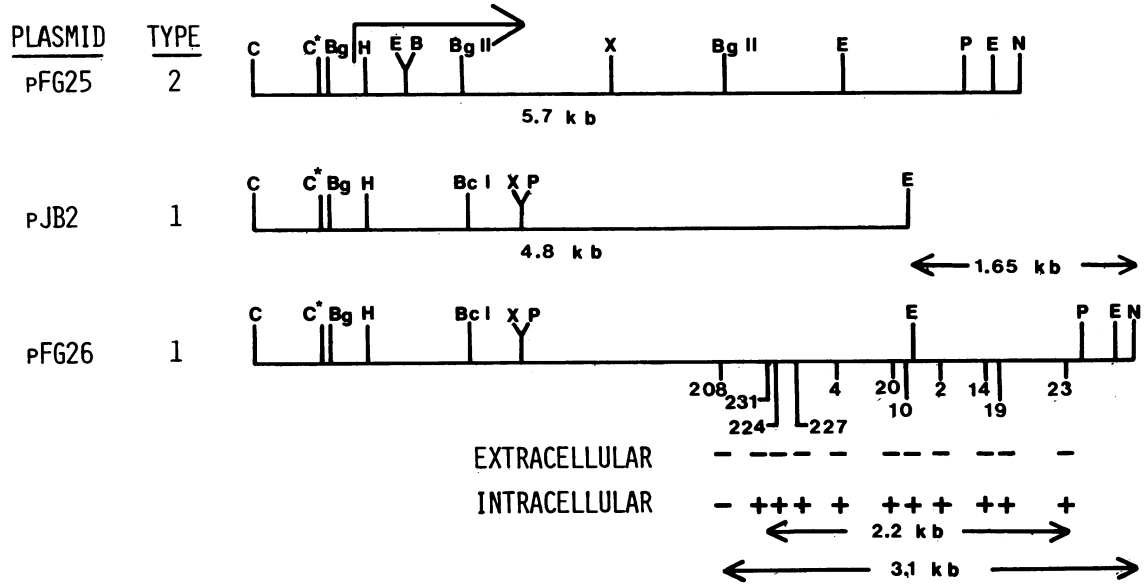
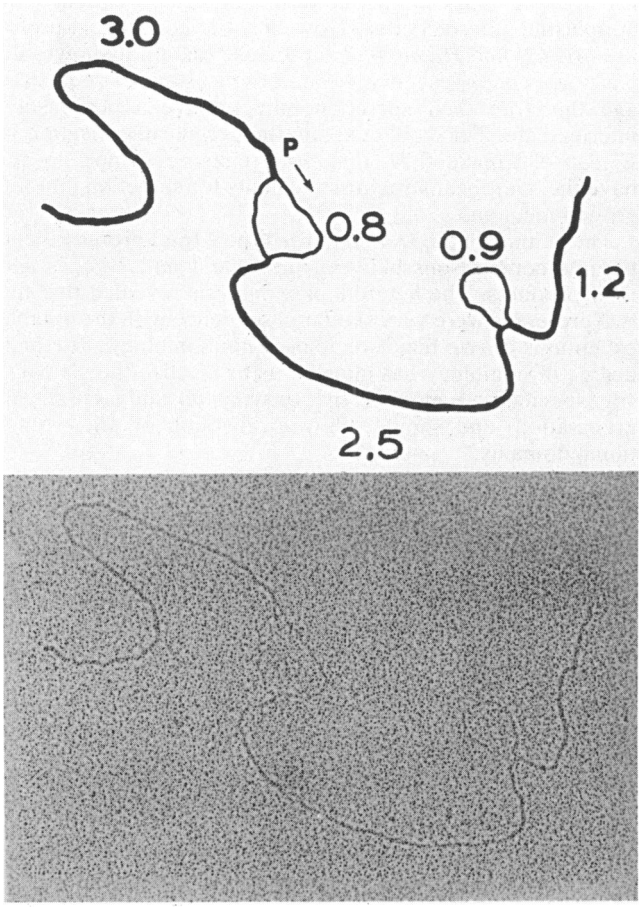


FIG. 5. DNA restriction maps of *iga* clones and localization of secretion determinant. The insert DNA of plasmids pFG25 (type 2 IgA1 protease) and pFG26 (type 1 IgA1 protease) and the analogous portion of pJB2 are aligned by restriction sites upstream of *iga*. The large arrow at the top starts at the promoter of *iga* as determined for pJB2 (7) and extends in the direction of transcription. pFG26 contains 1.65 kb of carboxyl-terminal DNA that is not found in pJB2. The positions of Tn*5* insertions are shown by numbered bars below the map of pFG26. Numbers in the 200-series were Tn*5* insertions in pJB2 (Fig. 2); the remaining insertions were in pFG26. The bottom of the figure shows the effect of the Tn*5* insertions on protease activity (+, retains activity; −, lacks activity) in culture supernatants (EXTRACELLULAR) or sonicated whole cultures (INTRACELLULAR). B, *Bam*HI; Bg, *Bgl*I; Bg II, *Bgl*II; C, *Cla*I; C*, *Cla*I site in unmethylated DNA; E, *Eco*RI; H, *Hin*fI; N, *Nde*I; P, *Pst*I; X, *Xba*I; Bc I, *Bcl*I.



from a strain containing both of these plasmids (Table 1). Again, this argues against a *trans*-acting function encoded by a second gene in pFG26 which can restore secretion to pJB2-encoded protease, although polarity due to the insertion of Tn*5* cannot be ruled out in this case. These results are in accordance with previous experiments (14) which failed to show complementation of the secretion defects of pJB2 by the cloned gonococcal IgA protease gene.

DISCUSSION

Plasmid integration has proved to be a valuable tool for both procaryotic and eucaryotic genetic analyses, including isolation of gene variants and excision of neighboring sequences of previously cloned genes (31, 38, 39, 46). In *H. influenzae*, plasmid DNA has been found to be capable of association with chromosomal sequences in that genetic markers can be exchanged between these replicons (4, 44, 49). Also, conjugal drug resistance plasmids apparently can integrate into the *Haemophilus* chromosome (37, 47). In the

FIG. 6. Heteroduplex analysis of pFG25 and pFG26. Plasmid DNAs were linearized by digestion with the restriction enzyme *Nde*I, denatured, reannealed, and examined in the electron microscope. The drawing at the top summarizes the results of measurements of electron micrographs, an example of which is shown at the bottom. Double- (thick lines) and single-stranded (thin lines) DNA is represented. The lengths of these segments are shown in kilobases. The 3.0-kb double-stranded region includes the 2.1-kb vector portion of the plasmids which is common to both plasmids. This determined the orientation of the insert DNA such that the promoter (P) is near the 0.8-kb single-stranded region, with transcription (arrow) proceeding toward this region.

TABLE 1. Lack of complementation of pJB2 secretion defects[a]

Plasmid(s)	Activity by protease type in[b]:			
	Supernatant		Sonic extract	
	1	2	1	2
pFG41 (type 2)	−	+	−	+
pFG41 and pJB2 (type 1)	−	+	±	+
pJB2	−	−	±	−
pFG41 and pFG43 (type 1)	+	+	+	+
pFG26 (type 1)	+	−	+	−
pJB2 and pFG26::Tn*5*-11	−	−	±	−
pFG26::Tn*5*-11	−	−	−	−
None	−	−	−	−

[a] *E. coli recA* strains containing the plasmid(s) listed were tested for their ability to produce and secrete type 1 or 2 IgA1 proteases. Maintenance of two plasmids in the same cell was assured by unique antibiotic resistances encoded within each plasmid.

[b] Culture supernatants and whole-culture sonic extracts were tested for the presence (+) or absence (−) of activity; ± indicates a small amount of activity.

work presented here it is shown that plasmids exhibiting homology with chromosomal sequences but incapable of replicating in *H. influenzae* can integrate into the chromosome of this organism. Plasmid integration should be a useful device for analysis of many *Haemophilus* systems and for retrieval of the genes involved.

In addition to plasmid integration, it was found that restriction fragments could recombine with the *H. influenzae* chromosome to give duplications of *iga* sequences. Transformation of *H. influenzae* Rd with *Eco*RI-treated pJB2::Tn*5*-209 DNA gave rise to kanamycin-resistant transformants, some of which were Iga^+. All of these transformants were ampicillin sensitive. Replacement of the chromosomal *iga* gene with the defective plasmid-encoded *iga* gene yielded Iga^- transformants, indicating that the Iga^+ derivatives must have arisen in a different manner. Hybridization analysis of one of these Iga^+ transformants showed that the restriction fragment, without pBR322 sequences, was integrated into the chromosome at the *iga* genomic region and that the fragment was amplified 5- to 10-fold relative to the findings for other chromosomal fragments (data not shown). These results are consistent with the formation of a circular form of the restriction fragment which integrated into the chromosome by a single crossover (9). Alternatively, duplication of the resident *iga* gene before recombination between the linear fragment and the chromosome could have given rise to the observed structures. In either case, the resulting duplication could lead, by further recombination, to amplification of the Tn*5*-containing DNA. Amplification of plasmid-encoded sequences has previously been observed in *H. influenzae* (45). The amplification of chromosomally inserted sequences has been found in other systems (1, 18). It is not known at this time whether the amplification observed here is inherent to plasmid integration processes in *H. influenzae*, is a result of selection pressures to attain high levels of drug resistance, or is due to the high concentration of input DNA used during transformations. This amplification phenomenon may be useful in the study of gene dosage effects in *H. influenzae*.

The plasmid integration system was used as a means to ascertain the cause of the defects in activity and secretion in *E. coli* of the cloned IgA1 protease gene (*iga*) from *H. influenzae*. It had been proposed (8) that the low levels of activity could be the result of defects in secretion or processing. In fact, it was shown here that these deficiencies could be overcome by the addition of chromosomal sequences located downstream from the original cloned DNA. The results of Tn*5* mutagenesis were used to localize a segment of DNA spanning the endpoint of the original clone which is necessary for full activity and secretion. This region, having a coding capacity of 75 to 100 kDa, may be a portion of the *iga* structural gene or may represent additional accessory genes. However, complementation analyses rule out the possibility of independently transcribed genes, the products of which could act in *trans*. It is more likely that this region is part of the structural gene, because strains containing plasmids with Tn*5* insertions in this region, as well as the original clone, produce proteins that are larger (125 to 150 kDa) (8; data not shown) than the mature form of IgA1 proteases (about 100 kDa). This suggests that maturation of *H. influenzae* IgA1 protease involves protein cleavage, as has been shown to be true for gonococcal IgA protease, where a precursor of 180 kDa is processed to a final form of 105 kDa. The additional protein or helper region of the *N. gonorrhoeae* enzyme is encoded within the carboxyl-terminal region of the gene and is essential for secretion (42). Deletions which remove the carboxyl terminus of the gonococcal IgA protease result in loss of secretion without loss of activity (41).

Some bacterial products which are known to be secreted have carboxyl-terminal sequences which are removed in the mature form of the protein (21, 24, 43). Certain deletions which remove a portion of the carboxyl terminus of *E. coli* hemolysin result in loss of secretion while some activity is maintained (17). In this case, secretion is not thought to involve the classical amino-terminal signal sequence or periplasmic intermediates. However, the gonococcal protease (41, 42) and *Haemophilus* protease (data not shown) do have sequences resembling the consensus signal sequence, and therefore their secretion may involve a periplasmic intermediate. This could explain the periplasmic location in *E. coli* of truncated *H. influenzae* enzymes which do not have the additional structural elements for secretion into the growth medium.

The cloning of the *H. influenzae* type 2 IgA1 protease gene permits comparisons between the type 1 and 2 genes and their products. The heteroduplex analysis revealed that the two proteases were very similar in sequence with the notable exceptions of two major regions of nonhomology. The high degree of homology has allowed us to localize the cleavage site specificity region of the enzyme (Grundy et al., in preparation) and should allow localization of other functional domains.

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