Identification and Characterization of a New Replication Region in the *Neisseria gonorrhoeae* β-Lactamase Plasmid pFA3

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The 7.1-kilobase-pair (kbp) plasmid pFA3 specifies TEM β-lactamase production in *Neisseria gonorrhoeae*. We studied the minimal region required for replication of this plasmid in *Escherichia coli* by constructing a set of nested deletions of the 3.4-kbp *PstI-HindIII* fragment. The smallest fragment capable of maintenance in *E. coli* when ligated to a streptomycin-spectinomycin resistance cassette was 2.0 kbp in size and was different from another autonomously replicating fragment of pFA3 reported by K. H. Yeung and J. Dillon (Plasmid 20:232–240, 1988). The fragment contained single *BamHI* and *XbaI* sites and specified a 39-K protein. Fragments subcloned from the minimal region or constructed by deletion from the 3' or 5' ends were not capable of autonomous replication. Mutants constructed by end filling and religating DNA cleaved at the *BamHI* or *XbaI* sites were not capable of autonomous replication and no longer produced the 39K protein. These results suggest that replication is dependent on the 39K protein. DNA sequence analysis of the region showed an A-T-rich region followed by four 22-bp direct repeats followed by an open reading frame encoding a 39K basic protein.

The TEM β-lactamase-specifying plasmid pFA3 was originally found in Far Eastern isolates of *Neisseria gonorrhoeae* (29). It is highly related to a series of small β-lactamase-specifying plasmids found in *N. gonorrhoeae*, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, and *Haemophilus ducreyi* (3, 4, 9, 11). A similar plasmid has been reported in a clinical isolate of *Neisseria meningitidis* (10). The related plasmid RSF0885 has been used to construct the shuttle vector pHVT1, which is maintained in *Escherichia coli* and *H. influenzae* (8). All the plasmids are thought to have evolved from a group of cryptic plasmids which are commonly found in isolates of *H. parainfluenzae* (5). Figure 1 depicts the relationships of two representative plasmids of this group. pJB1 carries a full and functional Tn*A* sequence, while pFA3 contains an insertion of about 1.8 kilobase pairs (kbp) which carries a *HindIII* site (4, 9, 11, 20, 34). Relative to pJB1, 60% of the Tn*A* sequence and 270 bp of the core plasmid sequences adjacent to the left-hand inverted repeat of Tn*A* are deleted in pFA3 (3–5, 7, 20). The exact position of the insertion in pFA3 between the *BamHI* and *AvaI* sites is still the subject of a controversy which will be resolved only by nucleotide sequencing (9, 11, 20, 33, 34).

Yeung and Dillon recently defined a replication region designated *a* on plasmid pFA3 which lies within the 1.8-kbp insertion sequence (Fig. 1). They also postulated that a second replication region designated *b* exists in pFA3 but did not clearly define it (33). In this paper, we have identified and characterized a replication region on pFA3 which is distinct from the *a* region characterized by Yeung and Dillon. Moreover, since it encompasses a region common to all of the plasmids of this group, it may be the main replication region.

MATERIALS AND METHODS

E. coli strains were grown in L broth or agar or brain heart infusion (Difco Laboratories) supplemented when appropriate with carbenicillin (50 μg/ml) or kanamycin (35 μg/ml). Strains containing the Ω cassette were grown in medium supplemented with streptomycin (20 μg/ml) and spectinomycin (100 μg/ml).

The plasmids used are listed in Table 1. Plasmid pUC18ΩP was constructed by inserting the Ω spectinomycin-streptomycin resistance cassette of pHP45Ω (24) into the *HindIII* site of pUC18 to produce pUC18Ω. This plasmid was purified and partially digested with *HindIII*. The cohesive termini were filled with deoxyribonucleoside triphosphates by using Klenow fragment, and the plasmid was religated and transformed. One plasmid which contained the Ω cassette flanked on one side by the *HindIII* site of the polylinker and on the other side by the rest of the polylinker (including the *EcoRI* site and the obliterated *HindIII* site) was identified by the characteristic restriction endonuclease cleavage pattern and was designated pUC18ΩP. The Ω fragment could be cleaved from this plasmid by combined digestion with *EcoRI* and *HindIII* and was used to test the in vivo replication capacity of *EcoRI-HindIII*-flanked fragments generated as described below.

Plasmid DNA was prepared by a modification of the method of Birnboim and Doly (1). However, after precipitation with isopropanol, plasmid DNA was suspended in 1 volume of TE buffer (10 mM Tris, 0.1 mM EDTA; pH 8.0). An equal volume of 10 M LiCl was added, and the mix was placed on ice for 30 min. Precipitate was removed by centrifugation, and plasmid DNA was precipitated from the supernatant with 4 volumes of ethanol at −20°C. The DNA pellet was suspended in 0.1 M sodium acetate–50 mM MOPS (morpholinepropanesulfonic acid; pH 8.0) and was precipitated with 2 volumes of ethanol. For in vitro protein synthesis experiments, plasmid DNA was prepared by ultracentrifugation of cleared lysates in cesium chloride-ethidium bromide density gradients (19).

Restriction endonucleases were purchased from Boehringer Mannheim Canada or Bethesda Research Laboratories, and digestions were performed according to the instructions of the suppliers. Ligations were performed as previously described (15). Agarose gel electrophoresis was performed with Tris borate-EDTA buffer (19) and bacteriophage λ DNA cleaved with *HindIII* and *EcoRI* or pBR322

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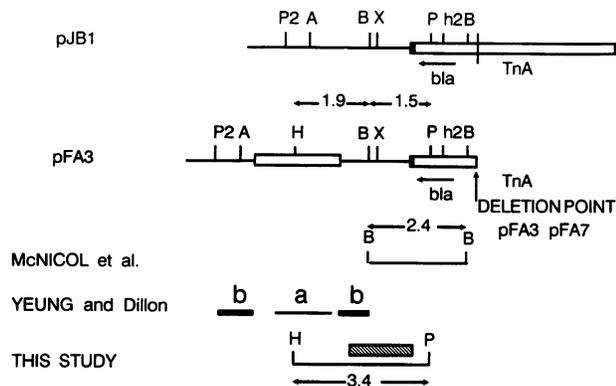
FIG. 1. Restriction maps of plasmids pJB1 and pFA3 showing the insertion (open box spanning HindIII site) in pFA3 and the deletion of TnA sequences. The replication regions reported by McNicol et al. (21) and regions a and b reported by Yeung and Dillon (33) are indicated. The 3.4-kbp HindIII-PstI fragment which is the subject of this report is indicated at the bottom, and the minimal replication region defined in this paper is indicated by the hatched box. The sizes of the fragments most commonly used in this study are in kilobase pairs. A, AvaI; B, BamHI; H, HindIII; h2, HincII; P, PstI; P2, PvuII; X, XbaI.

cleaved with HinfI as molecular weight standards. DNA fragments greater than 600 bp were purified from 0.7% low-melting-temperature agarose (Bio-Rad Laboratories) gels with a Gene Clean kit purchased from Bio Can Scientific. Smaller fragments were electroeluted from 5% acrylamide gels as recommended by Maniatis et al. (19).

Nested deletions were produced by the method of Henikoff (14) with a kit purchased from Promega Biotec. The 3.4-kbp HindIII-PstI fragment of pFA3 was cloned in the

polylinker of pUC18, and the recombinant was designated pKG3834. Nested, unidirectional deletions from the PstI site toward the HindIII site were generated by exonuclease III-S1 nuclease digestion of DNA which had been previously cleaved with SalI and KpnI within the pUC18 polylinker site. The deleted plasmids were incubated with Klenow enzyme and deoxyribonucleoside triphosphates before being recircularized with T4 DNA ligase and transformed into E. coli. To produce deletions from the HindIII end of the fragment, a PstI site was introduced next to (and outside) the HindIII site and the fragment was recloned in the PstI site of the pUC18 polylinker by using the PstI ends. A plasmid carrying the fragment so that its HindIII site was oriented towards the EcoRI side of the polylinker was identified by its endonuclease cleavage pattern and was designated pKG38343. Unidirectional deletions were made with SalI-KpnI-cleaved plasmid DNA as described above.

Plasmid-specified polypeptides were produced by using a procaryotic in vitro DNA transcription-translation system (no. 3802; Amersham Canada Ltd.). The procedure employed was that recommended by the supplier and has been previously outlined (15). Polypeptides were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 15% resolving gel and a 5% stacking gel (15, 18). ^{14}C-labeled molecular weight standards were from Bio-Rad. The high-molecular-weight series consisted of myosin (200K), phosphorylase B (97K), bovine serum albumin (66K), ovalbumin (42K), carbonic anhydrase (30K), and lysozyme (14K). The low-molecular-weight series consisted of carbonic anhydrase, trypsin inhibitor (21K), cytochrome c (12.5K), and aprotinin (6.5K).

Nucleotide sequencing was performed by the Sanger chain termination method (27) with a Sequenase Version 2 kit purchased from U.S. Biochemical Corp. Appropriate fragments were subcloned in M13mp18 and M13mp19. Single-

TABLE 1. Plasmids used in this study

Plasmid	Derivation or description	Resistance profile	Reference
pFA3	Far Eastern strain of N. gonorrhoeae	Ap^r	Sox et al. (29)
pFA3K	Kan^r cassette of pUC4K inserted at PstI site of bla gene of pFA3	Kan^r	This study
RSF1010::TnI Ap101	TnA inserted in Su gene of RSF1010	Ap^r	Rubens et al. (25)
pUC18/pUC19	pBR322 origin of replication	Ap^r	Norrander et al. (23)
pUC4K	pUC4 with Kan^r gene of pML21	Ap^r Kan^r	Vieira and Messing (32)
pHP45Ω	pHP45 with Sm^r-Sp^r gene from R100.1	Sm^r Sp^r	Prentki and Krisch (24)
pUC18ΩP	2.0-kbp Ω fragment from pHP45Ω; cloned in HindIII site of pUC18 (see Materials and Methods)	Ap^r Sm^r Sp^r	This study
pKG3815	1.5-kbp PstI-BamHI fragment of pFA3 cloned in pUC18	Ap^r	This study
pKG3819	1.9-kbp BamHI-HindIII fragment of pFA3 cloned in pUC19	Ap^r	This study
pKG3820	2.0-kbp XbaI-HindIII fragment of pFA3 cloned in pUC18	Ap^r	This study
pKG3830	2.8-kbp BamHI-HindIII fragment of pFA3 cloned in pUC18	Ap^r	This study
pKG3834	3.4-kbp PstI-HindIII fragment of pFA3 cloned in pUC18	Ap^r	This study
pKG38343	Reverse orientation of PstI-HindIII fragment compared with pKG3834 (see Materials and Methods)	Ap^r	This study
pKG8162	Kan^r cassette of pUC4K recombined with 3.4-kbp PstI-HindIII fragment of pFA3 (Fig. 2)	Kan^r	This study
Δ231Ω	EcoRI-HindIII fragment of Δ231 ligated to Ω cassette prepared as EcoRI-HindIII fragment from pUC18ΩP	Sm^r Sp^r	This study
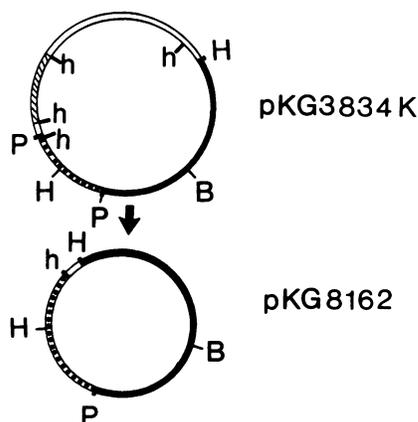
FIG. 2. Use of the kanamycin resistance cassette of pUC4K to determine replication properties of various restriction fragments. The cassette was inserted into the *Pst*I site of pKG3834 to produce plasmid pKG3834K. The cassette combined with the 3.4-kbp *Pst*I-*Hin*dIII fragment of pFA3 was then cut out and gel purified as a *Hae*II fragment before recircularization and transformation. Symbols: ▨, the *Hae*II fragment which carries the ColE1 replication origin (32); ▢, kanamycin resistance cassette; ▬, 3.4-kbp *Hin*dIII-*Pst*I fragment of pFA3. B, *Bam*HI; H, *Hin*dIII; h, *Hae*II; P, *Pst*I.

FIG. 3. Summary of replication properties of subclones and deletion derivatives of the 3.4-kbp *Hin*dIII-*Pst*I fragment of pFA3. The smallest deletion capable of autonomous maintenance was Δ231, which was 2.0 kbp. The minimal region required for replication as defined by the deletions from either end is 1.6 kbp and is indicated at the bottom. REP, Capacity for replication of each fragment; +, able to replicate; −, not able to replicate. The masses of the proteins specified by the fragments determined in the in vitro system are indicated in kilodaltons under PROTEIN. The deletions have an *Eco*RI site at one end because of the method by which they were constructed. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; X, *Xba*I; Kb, kilobase pairs.

stranded DNA was prepared as described by Messing and Vieira (22). Nucleotide sequences were analyzed by using the University of Wisconsin Genetics Computer Group sequence analysis programs.

RESULTS

We used the kanamycin resistance cassette from pUC4K to assess the capacity for replication of various restriction fragments of pFA3. The cassette was purified from a *Bam*HI digest of pUC4K and was ligated to a *Bam*HI digest of pFA3. The kanamycin-resistant colonies obtained always carried the large 4.7-kbp *Bam*HI fragment of pFA3. In no case was the 2.4-kbp fragment alone found ligated to the resistance cassette (data not shown).

The capacity for replication of several other restriction fragments was tested in a different fashion (Fig. 2). The kanamycin resistance cassette was purified from a *Pst*I digest of pUC4K and was inserted into the *Pst*I site of the pUC18 polylinker adjacent to the 3.4-kbp *Pst*I-*Hin*dIII fragment of pFA3 in pKG3834 (to produce plasmid pKG3834K) and adjacent to the 1.5-kbp *Bam*HI-*Pst*I fragment of pFA3 in pKG3815. The resulting plasmids were then cleaved with *Hae*II, yielding in each case a large fragment which carried the kanamycin cassette and the 3.4-kbp *Pst*I-*Hin*dIII or 1.5-kbp *Bam*HI-*Pst*I fragment of pFA3. The origin of replication of pUC was present on a small *Hae*II fragment (32). In each case, ligation of a purified large fragment yielded kanamycin-resistant colonies carrying a relatively low copy number plasmid made up of the cassette and the *Pst*I-*Hin*dIII fragment. This plasmid was verified by checking the restriction endonuclease cleavage pattern (data not shown) and was designated pKG8162. In the case of the *Pst*I-*Bam*HI fragment, no such colonies were obtained. In the cases of the 1.9-kbp *Bam*HI-*Hin*dIII fragment, 2.0-kbp *Xba*I-*Hin*dIII fragment, and 2.8-kbp *Bam*HI-*Hin*dIII fragment, the kanamycin cassette was prepared as an *Eco*RI fragment and was inserted into the *Eco*RI site of plasmids pKG3819, pKG3820, and pKG3830, respectively. Again the resulting plasmids were cleaved with *Hae*II, and the largest fragment was

isolated, circularized, and transformed. In these cases, no kanamycin-resistant colonies were obtained. These results are summarized in Fig. 3 and confirm the failure of others (16, 33) to demonstrate replication directed by the 2.4-kbp *Bam*HI fragment. Although the 3.4-kbp *Pst*I-*Hin*dIII fragment directed replication, the 1.5-kbp *Bam*HI-*Pst*I and 1.9-kbp *Bam*HI-*Hin*dIII fragments did not. We concluded that there was a strong possibility that the replication region spans the *Bam*HI site.

To better define the replication region, two sets of nested deletions of the 3.4-kbp *Pst*I-*Hin*dIII fragment were constructed, one set starting from the *Pst*I end with pKG3834 and the other set starting from the *Hin*dIII end of the fragment with pKG38343. Since the deletions were unidirectional, the deleted fragment could always be recovered as an *Eco*RI-*Hin*dIII fragment from the polylinker of pUC18, and deletions were recovered regardless of the replication properties of the pFA3-derived fragment. The gel-purified fragments were then tested for replication by ligation to gel-purified Ω cassette DNA recovered from plasmid pUC18ΩP as an *Eco*RI-*Hin*dIII fragment. Figure 3 shows the deleted fragments tested for replication by transformation of the ligation mix into *E. coli* TB1. The smallest fragment still capable of replication, Δ231, included the whole 1.5-kbp *Pst*I-*Bam*HI fragment and 460 bp of the *Bam*HI-*Hin*dIII fragment. The recombinant plasmid was designated Δ231Ω. Only deletions Δ69 and Δ65 from the *Pst*I end were capable of replication, whereas Δ78, which had an additional 500 bp deleted, was not capable of replication.

The restriction maps of the plasmids Δ231 and Δ231Ω are shown in Fig. 4b. To confirm the nature of the recombinant plasmid Δ231Ω, we purified DNA and cut it with various restriction endonucleases. It can be seen that *Eco*RI-*Hin*dIII cleavage of Δ231Ω resulted in two fragments: the larger comigrated with the Ω cassette, while the other comigrated with the Δ231 fragment (Fig. 4a, lanes B through D). The

FIG. 5. Agarose gel electrophoresis of plasmid DNA extracted from a strain containing plasmids RSF1010::Tn*I* Ap101 (1) and Δ231Ω (2). DNA was cleaved with *Eco*RI, and samples were run on an agarose gel. Lane A, λ DNA cleaved with *Eco*RI and *Hin*dIII; lane B, *Eco*RI-cleaved plasmid DNA. One-half the amount of the same DNA was loaded in lane C.

FIG. 4. (a) Agarose gel electrophoresis analysis of plasmid Δ231Ω compared with fragments of plasmids used in its construction. Lanes: A and L, Bacteriophage λ DNA digested with *Hin*dIII and *Eco*RI; B, C, and D, Δ231, Δ231Ω, and pUC18ΩP, respectively, digested with *Eco*RI and *Hin*dIII; E, Δ231 digested with *Bam*HI and *Hin*dIII; F, Δ231 digested with *Xba*I and *Hin*dIII; G, Δ231 digested with *Eco*RI, *Bam*HI, and *Hin*dIII; H, Δ231Ω digested with *Bam*HI and *Hin*dIII; I, Δ231 digested with *Eco*RI, *Xba*I, and *Hin*dIII; J, Δ231Ω digested with *Xba*I and *Hin*dIII; K, pUC18ΩP digested with *Eco*RI and *Hin*dIII. Arrowheads: 1, 1.5-kbp *Bam*HI (*Pst*I)-*Hin*dIII fragment; 2, 1.35-kbp *Xba*I (*Pst*I)-*Hin*dIII fragment; 3, 0.63-kbp *Eco*RI-*Xba*I fragment; 4, 0.46-kbp *Eco*RI-*Bam*HI fragment. (b) Maps of plasmids Δ231 and Δ231Ω. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; X, *Xba*I. The *Xba*I site located between the *Bam*HI and *Pst*I sites of the polylinker has been omitted from the map of Δ231Ω.

cleavage patterns for the enzymes *Hin*dIII-*Bam*HI and *Hin*dIII-*Xba*I were the same in the recombinant plasmid Δ231Ω as those found for Δ231 cut with *Eco*RI, *Bam*HI, and *Hin*dIII and *Eco*RI, *Xba*I, and *Hin*dIII, respectively (Fig. 4a, lanes G through J). This is to be expected, since *Bam*HI and *Xba*I cut both within the pFA3 fragment and close to the *Eco*RI site in the polylinker adjacent to the Ω fragment (Fig. 4b). Δ231Ω was transformed into *E. coli* carrying RSF1010::Tn*I* Ap101. Plasmid DNA was isolated and cleaved with *Eco*RI which cut both plasmids once. The DNA was electrophoresed in a 1% agarose gel. Figure 5 shows the two DNA species. Scanning densitometry suggested that the two species were present in approximately equimolar amounts (data not shown). This suggests that the plasmid copy numbers were similar.

We were also interested in the importance of potential internal open reading frames to the replication of deletions Δ231 and Δ69. Accordingly, the plasmids were cleaved with *Bam*HI or *Xba*I, and the ends were filled with deoxyribonucleoside triphosphates by using Klenow enzyme. The plasmids were circularized with T4 DNA ligase and transformed into *E. coli*. The constructions were confirmed by failure to

cut with the relevant enzyme, and the fragments were purified and tested for replication after ligation to the Ω cassette as described above. Removal of either the *Xba*I (Δ231X and Δ69X) or *Bam*HI site (Δ231B and Δ69B), as described, yielded a fragment which failed to replicate. The results are summarized in Fig. 6.

The various plasmid constructions were tested in the in vitro transcription-translation system, and the results are shown in Fig. 7. It can be seen that pFA3 specified peptides of 29K and 30K, corresponding to the processed and unprocessed β-lactamase species (13). These species were also produced by pUC18 (Fig. 7, lane A). In addition, peptides estimated to be 39K, 11K, and 7K were produced by pFA3 (Fig. 7, lane E). The *Pst*I-*Hin*dIII fragment (pKG3834) also specified peptides of 39K and 11K (Fig. 7, lane C). In contrast, the 1.9-kbp *Bam*HI-*Hin*dIII fragment (pKG3819) specified only the 11K protein (Fig. 7, lane D), while no additional protein species appeared to be produced by the

FIG. 6. Summary of replication properties (Rep) of and proteins specified by end-filled derivatives of Δ231 and Δ69. Solid boxes indicate which site (*Bam*HI [B] or *Xba*I [X]) was obliterated by end-filling and religation. H, *Hin*dIII; P, *Pst*I; +, able to replicate; −, not able to replicate; PEP, protein sizes in kilodaltons.
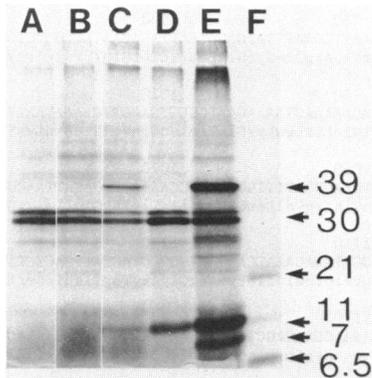
FIG. 7. Autoradiogram of sodium dodecyl sulfate-polyacryl-amide gel electrophoresis of [³⁵S]methionine-labeled polypeptides specified by recombinant plasmids. Lane A, pUC18; lane B, pKG3815; lane C, pKG3834; lane D, pKG3819; lane E, pFA3; lane F, low-molecular-weight standards. 39K protein is seen in lanes C and E. 11K protein is seen in lanes C, D, and E, and a 7K protein is seen in lane E. Two polypeptides at 30K and 29K are the β-lactamase expressed from the pUC vector (lanes A to E). Molecular weight standards in lane F are 21.5K, 12.5K, and 6.5K.

1.5-kbp PstI-BamHI fragment (pKG3815; Fig. 7, lane B). Figure 8 shows that deletion Δ231 produced the 39K protein (lane C), while Δ69 produced both the 39K species and a very faint 11K species (lane F). It appeared that plasmids which did not produce 39K protein, such as pKG3819 and Δ69B (Fig. 7, lane D, and Fig. 8, lane G), produced more 11K protein than the corresponding plasmids pKG3834 and Δ69, which did produce 39K protein (Fig. 7, lane C, and Fig. 8, lane F). In both Δ231 and Δ69, obliteration of the BamHI site resulted in plasmids which no longer produced the 39K species (Fig. 8, lanes D and G). End filling at the XbaI site also eliminated production of the 39K species and resulted in a new low-molecular-weight species estimated at 6K (Fig. 8, lane E). pKG3820, which carried the HindIII-XbaI fragment of pFA3, also produced the 11K and 6K proteins (data not
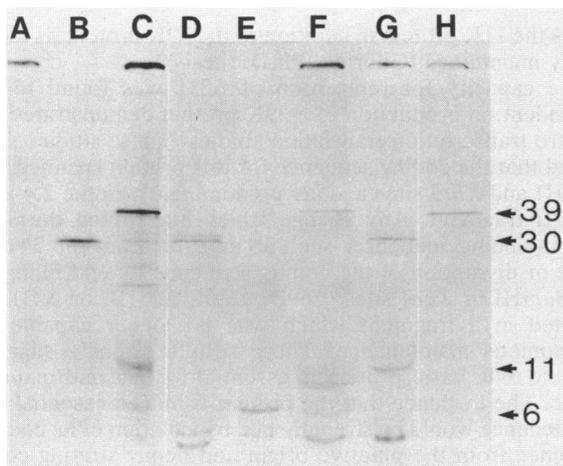
FIG. 8. Autoradiogram of sodium dodecyl sulfate-polyacryl-amide gel electrophoresis of [³⁵S]methionine-labeled polypeptides. Lane A, no DNA; lane B, pUC18; lane C, Δ231; lane D, Δ231B; lane E, Δ231X; lane F, Δ69; lane G, Δ69B; lane H, Δ231Ω. The 39K protein is present in lanes C, F, and H but is absent in lanes D, E, and G. A novel protein species estimated at 6K is seen in lane E.

shown; Fig. 3). Δ231Ω produced the 39K protein (Fig. 8, lane H). These results are consistent with an open reading frame starting just to the left of the BamHI site proceeding towards the PstI site. Protein species encoded by the other deletions were also determined (data not shown). The results are summarized together with the replication phenotype in Fig. 3 and 6. It is apparent that replication correlates with production of the 39K protein.

We next determined the nucleotide sequence of the region by using additional deletion derivatives. The sequence (Fig. 9) reads from 480 bp upstream of the BamHI site towards the PstI site, ending just inside the right-hand inverted repeat of TnA. It is apparent that the sequence can be divided into several regions. A 112-bp A-T-rich region which contains 75% A-T residues is followed by four 22-bp directly repeated sequences. An open reading frame for a 39K basic protein is apparent starting 105 bp upstream of the BamHI site and continuing to a point 160 bp upstream of the TnA inverted repeat. The sequence predicts a protein rich in lysine and arginine with a net positive charge of 13.

DISCUSSION

McNicol et al. originally reported that the replication region of pFA3 was located on the small (2.4-kbp) BamHI fragment (Fig. 1) because this fragment conferred DNA polymerase I independence when cloned in the ColE1-derived vector pAT2 (21). It now appears that the results were most likely due to reversion of the polA mutation in the host strain which was used (16; P. McNicol and W. Albritton, personal communication). Yeung and Dillon recently confirmed that the small BamHI fragment does not replicate and localized a replication region designated a to the 1.8-kbp insert in pFA3 (33). In this paper, we have confirmed the findings of Johnson (16) and Yeung and Dillon that the small BamHI fragment does not replicate by itself. Our data show that a 2.0-kbp deletion derivative of the 3.4-kbp PstI-HindIII fragment of pFA3 is capable of independent maintenance when ligated to the resistance cassette. This region starts 460 bp proximal to the BamHI site and includes almost all of the non-TnA sequence of the 2.4-kbp BamHI fragment. It appears that our minimal replication region is completely distinct from region a of Yeung and Dillon, since the latter spans the HindIII site and extends to a point 500 bp proximal to the BamHI site (Fig. 1). Yeung and Dillon stated that region a lies wholly within the insertion sequence on pFA3 and is absent from plasmids such as pFA7 (33). They also proposed a second replication region designated b, which was presumed to drive replication of plasmids such as pJB1, pFA7, and pHPA300, which do not contain the 1.8-kbp insertion found in pFA3 (3–5, 29, 33; Fig. 1). The proposed location of the b region was based on the premise that the 2.4-kbp BamHI fragment carries no replication functions at all. This contention is not supported by the results these authors presented, since they were not able to produce plasmids which could be maintained in E. coli after deletion of the 2.4-kbp BamHI fragment (33). We therefore believe that our results are not inconsistent with those of Yeung and Dillon. It is quite possible that region b may in fact be identical to the region characterized in our study. Tomb et al. have recently shown that the region surrounding the BamHI and XbaI sites derived from RSF0885 is essential for maintenance of the shuttle vector pHVT1 in H. influenzae Rd (31; J. F. Tomb, G. Barcak, and H. O. Smith, personal communication). This region is known to be highly homologous in plasmids RSF0885, pFA3, pFA7, and pJB1 (3, 4, 9,

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        150              170              190
GGCTTCGGTTGCTGAACCCTTGCAGAACAAGCAAACAAGTTTACTTGCTCTGTAAGGGTT
                                                           ▄▄

        210              230              250
TTGTAATTTTTAATCCTGAAAAAAGAAAAAATTAGCTCAAAGCCCAAAAAAGACGAAAGT

        270              290              310
CCCTATATTTTTATATTTTTGTTTTTTACTTGTTTTCGGCGTTTTTAAAACCAGTAAAAT

        330              350              370
CAATAAGTTACAAACTTATACGACAACAAATTTCCTGTTATACGACAACAAATTTCCTGT
                         ──────────────────▶

        390              410              430
TATACGACAACAAATTTCCTGTTATAAGACAACAAATTTCCTGTTATAGACAACTTTTAA
▄▶──────────▶            ──────────────────────────▶

        450              470              490
GAAAATACTTGTTGTTTTATATATAAACAACAGGTATATTTTGCTTGTTGTTTTAAACAG
                                                      ◀────

        510              530              550
GAAATTTGTTGTCTTATGCCAAATGATTTAGTTGTTGTAAAGGCTAATAGCCTTATTGAA
▄▄▄▄▄▄▄▄                 MetProAsnAspLeuValValValLysAlaAsnSerLeuIleGlu

        570              590              610
GCCAATTACCGATTGAGTATTGATGAAATCAGAATTTTAGCTTTAACTATTGGGACTATG
AlaAsnTyrArgLeuSerIleAspGluIleArgIleLeuAlaLeuThrIleGlyThrMet

BamHI
        ▼        630              650              670
GATCCGAAATCTAATCAAAAGATTTTTGATTTTACGGTGGCTGATTTTGTCCGTGAGTTT
AspProLysSerAsnGlnLysIlePheAspPheThrValAlaAspPheValArgGluPhe

        690              710              730
CCAGAAATAAGCCAAGATAACGCCTATAAGCAAATTCAGGCGGCTATAAAACGGATTTAT
ProGluIleSerGlnAspAsnAlaTyrLysGlnIleGlnAlaAlaIleLysArgIleTyr

                                              XbaI
        750              770              ▼
GACCGAAGTGTTAAAACTGAAGATAAAGACCGTGTAACTGAATTTCGTTGGGTTTCCTCT
AspArgSerValLysThrGluAspLysAspArgValThrGluPheArgTrpValSerSer

        810              830              850
AGAACATATTTCAAGAAAGAGGGGCGTTTTAGAATTGCAATGACTGATGAAGTTATGCCA
ArgThrTyrPheLysLysGluGlyArgPheArgIleAlaMetThrAspGluValMetPro

        870              890              910
TACCTAACGCAACTTAAAGGGCAATTCACGCAATATCAACTCAAACATATCGCTTATTTC
TyrLeuThrGlnLeuLysGlyGlnPheThrGlnTyrGlnLeuLysHisIleAlaTyrPhe
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        930              950              970
AACAGTGTACATTCAATTCGAATTTATGAGCTAATTACACAATATCGCTCTGTTGGTAGT
AsnSerValHisSerIleArgIleTyrGluLeuIleThrGlnTyrArgSerValGlySer

        990              1010             1030
CGTGAAATTACAGTAGAAAAACTAAAAGAATGGCTTCAGGTTGAAAATAAATACCCTAGA
ArgGluIleThrValGluLysLeuLysGluTrpLeuGlnValGluAsnLysTyrProArg

        1050             1070             1090
TTTAATTCATTAAATCAACGTGTTTTGGAACCTGCAATAACTGAGATTAACGAGAAATCT
PheAsnSerLeuAsnGlnArgValLeuGluProAlaIleThrGluIleAsnGluLysSer

        1110             1130             1150
GATTTAGTCGTTGAGGTTGAACAAATCAAGCGTGGGCGTACTATTCACTCTTTAAATTTT
AspLeuValValGluValGluGlnIleLysArgGlyArgThrIleHisSerLeuAsnPhe

                                              HaeIII
        1170             1190             ▼
GTGATTGGTAGTAAAAAACGAACAGCTCAAAAAATTGAAGAAGTTGCAAAACGGCCAGTA
ValIleGlySerLysLysArgThrAlaGlnLysIleGluGluValAlaLysArgProVal

        1230             1250             1270
TTTCCACATAAAAACAAGTATGGGAAGTTCGTCAAACTCGATAAGCAAAACCCTAAGATG
PheProHisLysAsnLysTyrGlyLysPheValLysLeuAspLysGlnAsnProLysMet

        1290             1310             1330
AGCAATCACGAGTACGGTTTATGGGCTAGAGATTGCTTAAAAATACTCGAAGATCATTAC
SerAsnHisGluTyrGlyLeuTrpAlaArgAspCysLeuLysIleLeuGluAspHisTyr

        1350             1370             1390
ACCGATATTACGAAAGTTACCAATGAAGATTTGCGTAATTACTGGGTATTTCTAGCGGGT
ThrAspIleThrLysValThrAsnGluAspLeuArgAsnTyrTrpValPheLeuAlaGly

        1410             1430             1450
AACGATAGCAATAGATCAAAATTAGGCTCAAAATCTGATTTTTTAAATGAATTGAAAAAA
AsnAspSerAsnArgSerLysLeuGlySerLysSerAspPheLeuAsnGluLeuLysLys

        1470             1490             1510
CGAGGTTATAAGCTCGTAGATTGCGAACTAGTAAAAATTTAGGTAGGTATTTTACTTAAC
ArgGlyTyrLysLeuValAspCysGluLeuValLysIle

        1530             1550             1570
TTTTTTTTGAAGAAGAACAAGATGAATAGCAATTTTATAACAGAGACAGAAAAATTAAGC

        1590             1610             1630
CGAGAAAAAGCGGTTAGAGCGGCTATTGATAATAATCGTTTAGAGGGATTAGAACCAAGC

        1650             1670             1690
CAAGAGTTTATAGATAGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTT

                                    TnA
        1710
TGGTCATGAGATTATCAAAAAGGATCTTCACCTAGTC
```

FIG. 9. Nucleotide sequence starting 480 bp upstream of the BamHI site and proceeding to the right-hand inverted repeat of TnA. The A-T-rich region is indicated by the solid bar. A putative Shine-Dalgarno sequence (28) upstream of the open reading frame for the 39K protein is underlined. The TnA sequence is indicated by the arrow beneath the sequence. The direct repeats (and one in inverted orientation) are indicated by the arrows above the sequence.

11, 29, 34). This supports the notion that the replication region found in Δ231 is important for other homologous plasmids. A potential conflict between our data and those of Yeung and Dillon relates to the exact location of the insertion which differentiates pFA3 from pFA7. Yeung et al. have proposed that it is located 200 bp from the BamHI site on the basis of electron microscope heteroduplex analysis and restriction mapping (33, 34). While it is quite possible that the location is in error by 200 to 300 bp and that it lies outside the region which we have characterized in this paper, it is also possible that the error is larger and that the insertion point is upstream of the coding sequence and regulatory region of the 11K protein which is produced by both pFA3 and pJB1 (K. Gilbride, Ph.D. thesis, University of Toronto, Toronto, Ontario, Canada, 1988). Only nucleotide sequencing, which is in progress, will resolve this issue.

While the Δ231 deletion is the smallest fragment which is capable of replication, we believe that the actual replicon may function more efficiently with additional sequences 5' to the sequence presented in Fig. 9. Δ231Ω is very unstable in the absence of antibiotic selective pressure (S. Huibner and K. Gilbride, unpublished data). In contrast, pKG3834, which carries the entire 3.4-kbp PstI-HindIII fragment and pro-

duces the 11K protein in addition to the 39K protein, is more stably maintained (Gilbride, Ph.D. thesis).

The capacity for replication of Δ231 was found to be dependent on production of a 39K protein demonstrated by in vitro transcription-translation studies. These studies suggested that the coding sequence for this protein spanned the BamHI and XbaI sites and lay predominantly in the 2.4-kbp BamHI fragment. Any change which interrupted the 39K protein-coding sequence, such as deletion from the 3' end (Δ78) or disruption of the coding sequence by end filling at the BamHI or XbaI sites (Δ69B, Δ69X, Δ231B, or Δ231X), resulted in a fragment which was no longer capable of autonomous maintenance. These results strongly suggest that the 39K basic protein is essential for plasmid maintenance. The evidence that the protein is indeed essential for maintenance would be strengthened by isolation of its coding sequence from the putative origin and demonstrating complementation of replication in trans. It would also be useful to construct conditional mutants which are maintained in a suppressor host but not in the wild type.

Stein et al. reported the production of a 41K protein from the same region which encodes the 39K protein reported here but reported that the direction of transcription is the

opposite of that reported here (30). This is likely due to the fact that they used RNA polymerase binding to determine the locations of putative promoters. The proteins reported by Stein et al. as having masses of 14 and 11 kilodaltons correspond to those reported here as having masses of 11 and 7 kilodaltons, respectively (30; Gilbride, Ph.D. thesis).

Sanchez-Pescador et al. (26) recently published the nucleotide sequence of the 2.4-kbp BamHI fragment of pFA7. Analysis of this sequence shows that there is no large open reading frame as would be expected for a sequence encoding the 39K protein. Comparison of this sequence with our data shows that in some regions, single bases are missing in the pFA7 sequence when compared with that of the pFA3. Our data confirm the presence of these bases on both strands and are supported by the in vitro protein synthesis data. While it is possible that there is no open reading frame in pFA7, we feel that this is unlikely because the protein profile of pJB1 also shows that a 39K protein is encoded in the same region (K. Gilbride, unpublished data). Moreover, the strong conservation of this group of plasmids in this region, previously demonstrated by restriction mapping and electron microscope heteroduplex analysis and the report of Tomb et al. (31), would argue against significant differences (3, 4, 20, 29).

Sequencing of the Δ231 fragment showed that it carries a 112-bp region with 75% A-T residues followed by four 22-bp directly repeated sequences which are in turn followed by an open reading frame for a 39K basic protein. The right-hand inverted repeat of TnA is found 160 bp downstream from the end of the open reading frame. Δ231 is organized in a fashion typical of many procaryotic replication regions, which usually are composed of an A-T-rich region closely associated with directly repeated sequences and a sequence encoding a replication initiator protein. Initiator protein binding at the direct repeats is associated with strand separation in the A-T-rich region and assembly of the apparatus for DNA replication (for a review, see references 2 and 12). Initiator protein expression may also be autoregulated by the binding of the protein to its own promoter which lies within the direct repeats (6, 17). Further studies are in progress to determine the exact roles of the regions of Δ231 defined by nucleotide sequencing in replication and maintenance of pFA3 and related plasmids.

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