

DNA-Binding Proteins in Cells and Membrane Blebs of *Neisseria gonorrhoeae*

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Naturally elaborated membrane bleb fractions BI and BII of *Neisseria gonorrhoeae* contain both linear and circular DNAs. Because little is known about the interactions between DNA and blebs, studies were initiated to identify specific proteins that bind DNA in elaborated membrane blebs. Western immunoblots of whole-cell and bleb proteins from transformation-competent and DNA-uptake-deficient (*dud*) mutants were probed with single- or double-stranded gonococcal DNA, pBR322, or synthetic DNA oligomers containing intact or altered gonococcal transformation uptake sequences. The specificity and sensitivity of a nonradioactive DNA-binding protein assay was evaluated, and the assay was used to visualize DNA-protein complexes on the blots. The complexes were then characterized by molecular mass, DNA-binding specificity, and expression in bleb fractions. The assay effectively detected blotted DNA-binding proteins. At least 17 gonococcal DNA-binding proteins were identified; unique subsets occurred in BI and BII. Certain DNA-binding proteins had varied affinities for single- and double-stranded DNA, and the intact transformation uptake sequence competitively displaced the altered sequence from a BI protein at 11 kilodaltons (kDa). A *dud* mutant, strain FA660, lacked DNA-binding activity at the 11-kDa protein in BI. The segregation of DNA-binding proteins within BI and BII correlates with their distinct protein profiles and suggests that these vesicles may play different roles. Although the DNA-binding proteins expressed in BII may influence the nuclease-resistant export of plasmids within BII vesicles, the BI 11-kDa protein may bind transforming DNA.

Neisseria gonorrhoeae is a gram-negative bacterial pathogen that causes the sexually transmitted mucosal and disseminated infections of gonorrhea. This organism readily develops antimicrobial resistance and expresses several highly variable immunogenic cell surface determinants (5). Numerous studies have addressed the genetic bases of these observations, and mechanisms including directed intracellular gene translocation, transformation, and conjugation are considered responsible (2, 3, 7, 14a, 15, 22, 24). Recently, an additional genetic exchange mechanism involving membrane-associated DNA was proposed (10).

Log-phase cultures of *N. gonorrhoeae* release membrane vesicles termed blebs (5, 10, 11, 26), and bleb formation by gonococci in vivo has been reported (5). On sucrose density gradients, the blebs sediment into two fractions, BI and BII. Fraction BI has a protein content characteristic of the inner membrane, and fraction BII contains outer membrane proteins and lipopolysaccharide (10). Each fraction also contains chromosomal and plasmid DNAs, but only plasmids associated with outer membrane-derived BII exhibit nuclease resistance (10). Genetic exchange experiments with blebs showed that recipient gonococci, incubated in cell-free broth culture filtrates that contained blebs from penicillinase-producing bacteria (*bla*⁺), naked DNA with streptomycin resistance (*Str*^r) markers, and exogenous DNase efficiently incorporate and express plasmid-encoded *bla* markers without expressing chromosomal *str* genes. Equivalent results of experiments with a *bla*⁺ *Str*^r bleb donor indicate that blebs can mediate intercellular transfer of nuclease-protected plasmids, but may be incapable of transferring chromosomal DNA in a DNase-resistant state (10). Such differences suggest that specific mechanisms may exist

for determining the association between DNA and blebs and the role of bleb DNA in genetic exchange.

The present study was undertaken to identify and characterize DNA-binding proteins in blebs that could contribute to interactions between DNA and these membrane vesicles. A qualitative approach, developed by Wager and Stephens to examine DNA-binding proteins in *Chlamydia* spp. (27), was used. Western immunoblots of gonococcal whole-cell (WC) and bleb proteins were probed with DNA, and the resulting DNA-protein complexes were visualized by a recently described nonradioactive assay (9). The assay system uses digoxigenin-derivatized dUTP to label DNA probes in a Klenow enzyme-catalyzed polymerization. The label is then detected by a solid-phase enzyme-linked immunosorbent assay.

In this study we first considered the specificity and sensitivity of the nonradioactive DNA-binding protein assay by comparing the detection of known DNA-binding proteins with that of proteins lacking DNA-binding activity and then examined DNA-binding proteins in gonococcal WC and blebs. Probes for DNA binding included single- and double-stranded gonococcal WC and plasmid DNA (ssDNA and dsDNA), pBR322, and synthetic oligomers containing either an intact (I) or an altered (A) gonococcal transformation uptake sequence (14). Both transformation-competent and DNA-uptake-deficient (*dud*) (4) strains were examined.

MATERIALS AND METHODS

Bacteria. *N. gonorrhoeae* 31426 (transformation competent) (American Type Culture Collection, Rockville, Md.) and FA660 (*dud*) (3) were maintained by daily single-colony transfers on gonococcal clear typing medium as previously described (11). For experiments, gonococcal cells and membrane blebs from 16-h cultures were harvested and separated

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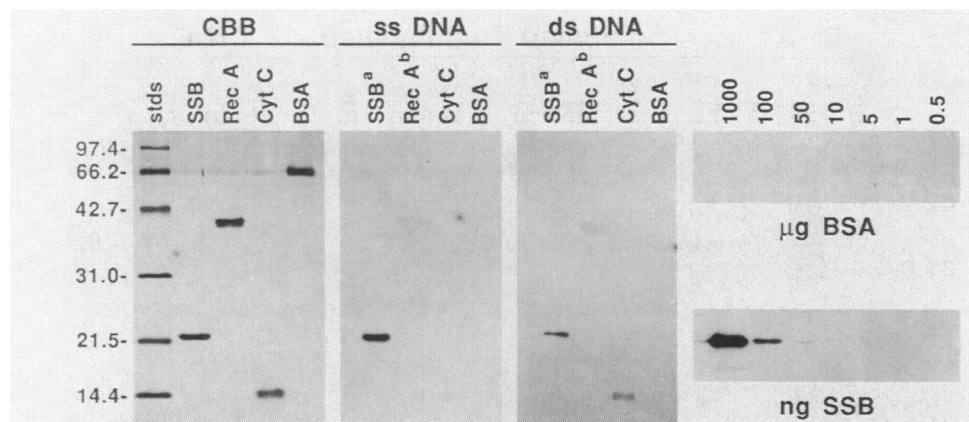


FIG. 1. Evaluation of the DNA-binding protein assay. A Coomassie brilliant blue-stained (CBB) SDS-12.5% polyacrylamide gel, containing molecular mass standards (lane stds) and 1 μ g each of SSB, RecA, cyt *c*, or BSA was compared with bands exhibiting DNA-binding activity on Western blots made from similar gels. Blots were probed with digoxigenin-labeled single-stranded (ss DNA) or double-stranded (ds DNA) pBR322, and DNA-protein complexes were detected as described in Materials and Methods. The Coomassie brilliant blue stain showed effective resolution of the proteins, with dimers evident in the cyt *c* lane. Single-stranded probes showed strong reactivity with SSB and relatively weak reactivity with RecA and Cyt *c*. Detection of RecA and Cyt *c* was enhanced, and labeling of SSB was reduced with dsDNA probes. Titrations of SSB and BSA showed that 10-ng loadings of SSB were detectable, although 1 mg of BSA was not detected. In lanes labeled a and b, 0.1 and 10 μ g was loaded, respectively. Molecular mass standards are given in kilodaltons.

by differential and sucrose density gradient centrifugation, as previously described (10).

DNA preparation. WC DNA was purified from *N. gonorrhoeae* by the alkaline lysis and phenol-chloroform-ether extraction methods of Maniatis et al. (20). Ethanol-precipitated DNA was washed once with 70% ethanol and then dissolved at approximately 1 mg/ml in buffer consisting of 10 mM Tris (pH 8.0) and 1 mM EDTA (TE).

For gonococcal plasmid purifications, WC DNA extracts were separated by electrophoresis in agarose tube gels (Bio-Rad Laboratories, Richmond, Calif.) and supercoiled forms of the 4.2-kilobase-pair cryptic plasmid were eluted from excised gel segments. Eluted plasmids were extracted once with 3 volumes of TE-saturated *n*-butanol, precipitated with ethanol, and suspended in TE. The plasmids were linearized by single-site restriction with *Hind*III (18) (New England BioLabs Inc., Beverly, Mass.), as specified by the manufacturer.

Linearized pBR322 was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind., and synthetic DNA oligomers were produced on a DNA synthesizer (model 380 B; Applied Biosystems Inc., Foster City, Calif.) as specified by the manufacturer.

Purified protein preparations. Single-stranded-DNA-binding protein (SSB) and RecA protein were purchased from United States Biochemical Corp., Cleveland, Ohio. Cytochrome *c* (cyt *c*), purified from *Saccharomyces cerevisiae*, and bovine serum albumin fraction V (BSA) were purchased from Sigma Chemical Co., St. Louis, Mo., and ICN Bio-medicals Inc., Costa Mesa, Calif., respectively.

Protein electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed, with the discontinuous buffer system of Laemmli (19), by methods previously described (16). Gels were typically run for 2.5 h at 7.5 W (constant power) and then either stained with Coomassie brilliant blue or electroblotted onto nitrocellulose membranes as previously described for DNA-binding protein assays (27).

DNA-binding protein assays. Assays for DNA binding to electroblotted proteins were performed by using modifications to previously published procedures (9, 27). All steps

were performed at room temperature. Nitrocellulose membranes with blotted proteins were blocked for 1 h with 0.1% (wt/vol) nonfat dry milk, dissolved in Dulbecco phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20 (Sigma) (dPBS-Tween). The blots were then washed twice for 10 min in dPBS-Tween. Washed blots were probed for 2 h with 0.1 μ g of DNA per ml in dPBS-Tween or incubated in dPBS-Tween alone. Probes were either labeled with digoxigenin-conjugated dUTP (Boehringer Mannheim), as specified by the manufacturer, or left unlabeled. Probed blots were blocked again for 30 min with 4% nonfat dry milk in buffer consisting of 0.1 M Tris (pH 7.5) and 0.15 M NaCl (TN) and washed twice for 10 min each in TN. Protein-DNA complexes were located with an anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) and detected as specified by the manufacturer.

RESULTS

To optimize the specificity and sensitivity of this nonradioactive DNA-binding protein assay, we performed a series of procedural experiments. These involved titrating blocking reagent concentrations while maintaining appropriate conditions for DNA-protein interactions. As reported previously for radioactive assays (27), dPBS-Tween proved an effective buffer system for DNA-protein binding. Membrane blocking with dPBS-Tween alone, with dPBS supplemented with BSA, or with nonfat dry milk in dPBS, was evaluated (data not shown). Optimal results were obtained when the procedure described above was used.

The specificity of this system was evaluated by comparing the reactivities of SSB, RecA, cyt *c*, and BSA (Fig. 1). Both SSB and RecA bind DNA *in vivo*. The highly basic cyt *c* can bind DNA ionically (13), and BSA has no known DNA interaction. An SDS-12.5% polyacrylamide gel stained with Coomassie brilliant blue showed the migration of each protein and revealed a small proportion of dimers in the cyt *c* preparation. When Western blots of such gels were probed with single-stranded pBR322, labeled and detected as described above, strong labeling of SSB and relatively weak

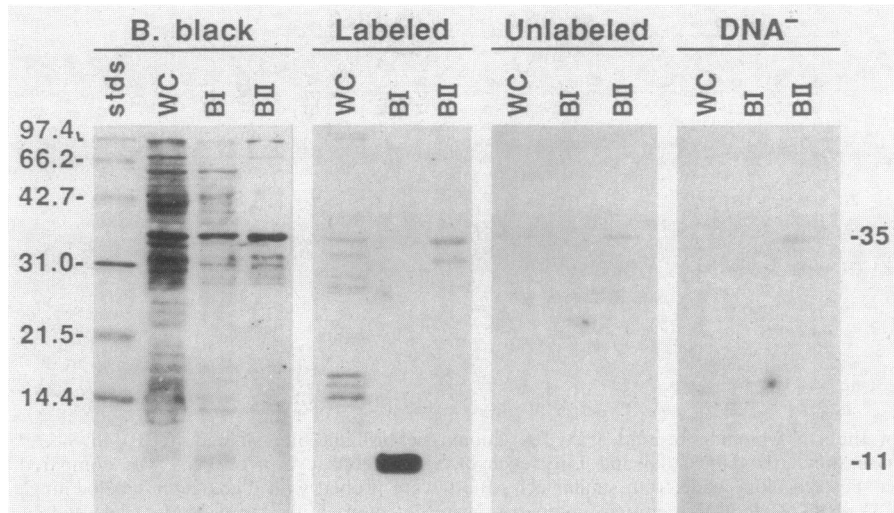


FIG. 2. Gonococcal DNA-binding proteins. Western blots containing proteins from WC and bleb fractions BI and BII of *N. gonorrhoeae* 31426 were stained for total protein with buffalo black (B. black), probed with digoxigenin-labeled (Labeled) or unlabeled (Unlabeled) pBR322, or incubated in buffer lacking DNA (DNA⁻). A single protein, corresponding to PI at 35 kDa, reacted nonspecifically in the WC and BII lanes of the negative controls. Seventeen bands that bound labeled DNA in the WC lane were observed. The apparent masses for these and bleb-associated DNA-binding proteins are listed in Table 1. Molecular mass standards (lane stds) are given in kilodaltons.

labeling of RecA and cyt *c* were observed. Conversely, equivalent blots probed with double-stranded pBR322 showed increased labeling of RecA and cyt *c*, with a corresponding decrease in labeling of SSB. No label was observed in BSA lanes in either experiment. Samples containing 10 ng of SSB loaded on gels were detectable, whereas a 1-mg loading of BSA failed to bind the DNA probes.

The assay system was then used to identify proteins capable of binding DNA in *N. gonorrhoeae* WC and blebs

(Fig. 2). Western blots containing lysates of WC and fractions BI and BII from *N. gonorrhoeae* 31426 were stained with buffalo black, and probed with either control-labeled pBR322 (Boehringer Mannheim) or unlabeled pBR322 or incubated in dPBS-Tween alone. A single protein, corresponding to outer membrane protein I (PI) at 35 kilodaltons (kDa) in WC and BII lanes, showed a nonspecific reaction in the unlabeled and dPBS-Tween control experiments. At least 17 gonococcal proteins, ranging from 11 to 97 kDa,

TABLE 1. DNA-binding proteins in *N. gonorrhoeae*

Fraction	Molecular mass (kDa)	Protein present ^a in:					
		pBR322	WC dsDNA	WC ssDNA	Plasmid	(I) probe	(A) probe
WC	97	+	+	+	+	+	-
	84	+	+	+	+	+	-
	57	-	-	+	-	+/-	-
	39	+	+	+	+	+	+
	34	-	-	-	-	+	+/-
	32.5	+	+	+	+	+	+
	32	+	+	-	+	+/-	-
	29	+	+	+	+	+	+
	27	+	+	+	+	+	+
	26.5	+	+	+	+	+	+
	21	+	+	+/-	-	+/-	+
	17	+	+	+	+	+	+
	16	+	+	+	+	+	+
	15	+	+	+	+	+	+
	14	+	+	+	+	+	+
	13	+	+	+	+	-	-
	11	+	+	+	+	-	-
BI	22	-	+	-	+/-	-	+
	11	++	++	++	++	++	++
BII	57	-	-	+/-	-	+	-
	34	-	-	+/-	-	+	-
	32	+	+	+	+	+	-
	27	+	+	+	+/-	+	+/-
	17	+	+/-	+	+	+	+/-

^a +/-, Light reaction product, not always reproducible; +, reproducible detection; ++, strong reproducible reaction.

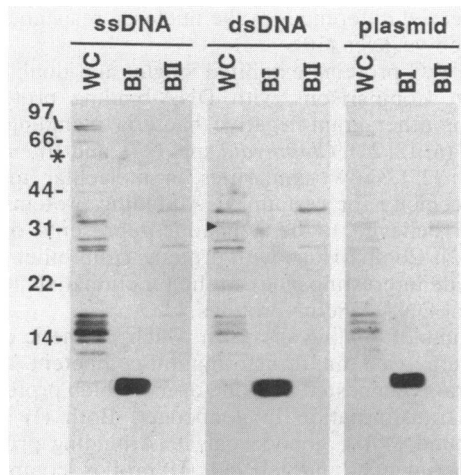


FIG. 3. Molecular specificity of gonococcal DNA-binding proteins. Western blots containing proteins from WC and bleb fractions BI and BII or *N. gonorrhoeae* were probed with digoxigenin-labeled WC ssDNA, WC dsDNA, or linearized 4.2-kilobase-pair gonococcal cryptic plasmids (plasmid), and the resulting DNA-protein complexes were visualized. Several proteins showed enhanced binding with ssDNA. A 57-kDa WC band was detected only with ssDNA probes (asterisk). A protein at 22 kDa in the WC lane was detected only with WC dsDNA. A highly reactive BI band at 11 kDa bound all three probes. Molecular mass standards are given on the left in kilodaltons.

exhibited DNA-binding activity in this assay. The apparent molecular masses of WC and bleb proteins that bound one or more of six different DNA probes are listed in Table 1. Unique subsets of the DNA-binding proteins were observed in membrane bleb fractions. The 11-kDa protein had strong reactivity and was the sole DNA-binding protein consistently resolved in BI lysates from strain 31426. Three proteins between 17 and 32 kDa in BII lysates specifically bound pBR322. Control blots probed with labeled pBR322 but incubated without the antibody-alkaline phosphatase conjugate showed no reaction (data not shown).

Equivalent blots were probed with gonococcal WC dsDNA, WC ssDNA (denatured by boiling dsDNA), or 4.2-kilobase-pair cryptic plasmids extracted from strain 31426. The results are shown in Fig. 3. Reactivity at 22 kDa appeared to be specific for dsDNA, and a band at 32 kDa in WC lysates (arrow) was detected only with the dsDNA and the plasmid probes. Most proteins were labeled more intensely with ssDNA probes. One WC band at 57 kDa (asterisk) was observed only when ssDNA was used. The banding pattern of blots probed with gonococcal cryptic plasmids was similar to the pBR322 profile shown in Fig. 2.

To identify proteins possibly involved in binding and uptake of transforming DNA by *N. gonorrhoeae*, we probed

blots with synthetic DNA oligomers containing either an (I) or an (A) transformation uptake sequence. The oligomer sequences are given in Fig. 4. Figure 5 shows the results of the blotting experiments. The (I) probe contains the 10-base inverted repeat (underline) recently correlated with optimal competitive inhibition of gonococcal transformation (Fig. 4) (14). The (A) probe has an identical base composition, except that every second base within the inverted repeat was substituted with the complementary base, to alter the sequence while maintaining the physical structure.

Both oligomers bound to proteins on the filters (Fig. 5) and reacted strongly in the assay. When blots were preincubated with unlabeled (A) probes and then challenged with labeled (I) probes, few changes in banding occurred. Conversely, blots preincubated with unlabeled (I) probes and challenged with (A) labels lacked a reaction product at 11 kDa in the BI lane. Nonspecific reactions at 35 kDa were disregarded (Fig. 2).

In a final experiment to identify proteins associated with transformation, we compared (I) probe binding with WC and bleb lysates from transformation-competent strain 31426 and *dud* mutant FA660 (Fig. 6). No significant differences in banding between these strains were detected in WC and BII lanes. However, the FA660 BI preparation appeared to have enhanced labeling at 22 kDa, and it lacked the strong DNA-binding activity at 11 kDa.

DISCUSSION

In this study we identified several DNA-binding proteins in *N. gonorrhoeae*, and showed that specific DNA-binding proteins are located in membrane bleb fractions BI and BII. The data supported the effectiveness of nonradioactive DNA-binding protein assays on Western blots of SDS-polyacrylamide gel electrophoresis protein separations and suggested that a BI protein may actively bind transforming DNA.

Recent studies showed that [³²P]DNA specifically labeled certain developmentally regulated *Chlamydia* DNA-binding proteins after SDS-polyacrylamide gel electrophoresis separation and electrophoretic transfer to nitrocellulose (27) and that a nonradioactive assay could effectively detect similar DNA-protein complexes (9). The present study confirmed those findings and evaluated the specificity and sensitivity of the assay system. DNA-binding experiments with purified proteins showed that nanogram quantities of SSB, RecA, and *cyt c* were detectable by the assay, whereas no reaction occurred with up to 1 mg of BSA. A 10-ng sample of SSB, corresponding to 0.45 pmol, was visualized although it is unlikely that this amount represents the minimum detectable quantity of DNA-binding protein in this assay. Electrotransfer efficiencies are variable (9), and the extent of protein renaturation after SDS-polyacrylamide gel electrophoresis is unknown (27). Furthermore, the DNA-binding properties of

Intact probe:

5'AATTCAACAACAACAACAGCCGCTCTGAACCAAATTCAGACGGCAACAACAACAACA3'

Altered probe:

5'AATTCAACAACAACAACAGGCCTGTCTATCCAAAATGACAGGCCAACAACAACAACA3'

FIG. 4. Sequences of synthetic oligomer probes. DNA-binding protein probes containing an (I) or an (A) gonococcal transformation uptake sequence were synthesized. The underlined inverted repeats denote the uptake sequences.

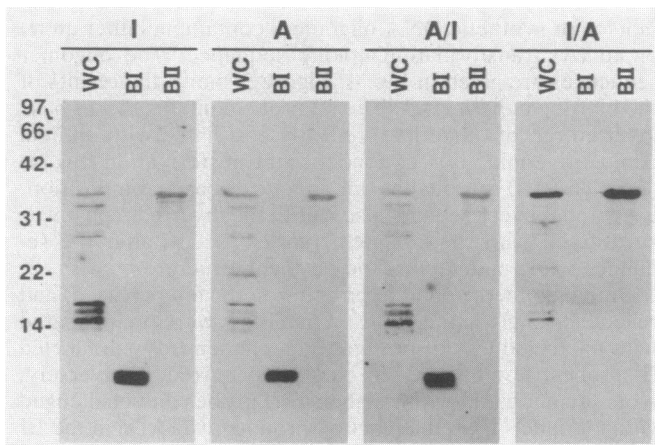


FIG. 5. Gonococcal proteins with affinity for transformation uptake sequences. Western blots containing proteins from WC and bleb fractions BI and BII from *N. gonorrhoeae* were probed with synthetic DNA oligomers containing either an (I) or an (A) gonococcal transformation uptake sequence. Both probes bound to most gonococcal DNA-binding proteins (Table 1). Similar results were obtained when the blots were preincubated with unlabeled (A) probe and then labeled with the (I) probe (A/I). No reaction product was observed at 11 kDa in the BI lane with the reciprocal experiment (I/A), indicating that the 11-kDa protein has greater affinity for the (I) probe. Molecular mass standards are given on the left in kilodaltons.

these known proteins appeared variable, with SSB exhibiting the strongest reaction. The present study did not exclude the possibility that blotted proteins with greater affinity for DNA exist or that proteins with altered DNA-binding activity may be detected if additional procedural modifications are tested.

In this study we found that membrane blocking prior to incubation with both DNA and the antibody conjugate helped eliminate background reactions (data not shown). Titrations involving concentrations of nonfat dry milk or BSA ranging from 0.05 to 5% (wt/vol) showed optimum sensitivity and specificity with the procedures described above.

When gonococcal WC were assayed, at least 17 proteins with DNA-binding activity were identified (Table 1). Detection of these proteins was often dependent upon the DNA probe used. Several highly reactive DNA-binding proteins were marginally detectable with total protein stains, further supporting the selectivity and sensitivity of this assay. Outer membrane PI at 35 kDa was visualized nonspecifically in control experiments with unlabeled probes or lacking probes. Additional control experiments lacking the antibody conjugate revealed no bands (data not shown), indicating that blotted PI has no detectable alkaline phosphatase activity. Thus, nonspecific labeling of PI was probably due to antibody cross-reactivity. Further experimentation with this assay provided information on the expression, specificity, and possible functions of several gonococcal DNA-binding proteins.

A limited number of DNA-binding proteins were observed in bleb lysates. In BI vesicles from strain 31426, which originate from the cytoplasmic membrane (10), a highly reactive band was detected at 11 kDa. This protein bound all probes tested. Outer membrane-derived BII preparations (10) typically had three to five reactive bands, ranging from 17 to 57 kDa. This segregation of different DNA-binding proteins in the two bleb fractions is consistent with previ-

ously observed differences in the nuclease resistance exhibited by bleb vesicles (10).

Several WC proteins exhibited single- and double-strand specificity. Comparisons with DNA-binding proteins described for other gram-negative bacteria including *Escherichia coli* (6, 12, 21), *Chlamydia* spp. (27), and *Haemophilus influenzae* (25) show similarities in molecular mass and strand specificity for certain DNA-binding proteins. A 15-kDa band believed to be a histone represents one such protein (23). Comparisons with proteins from other bacteria may provide interesting clues to the functional identities for gonococcal DNA-binding proteins.

The results of binding assays in which synthetic oligomer probes were used on transformation-competent and *dud* mutant strains suggested that one or more bleb proteins may influence transformation in gonococci. Both (I) and (A) probes bound to the gonococcal DNA-binding proteins in these experiments, and labeled (I) probes competitively displaced the (A) probes from most DNA-binding proteins. In the reciprocal experiment, although labeling was enhanced at the 35-kDa protein that exhibits cross-reactivity with the anti-digoxigenin antibody conjugate, the (A) probe failed to displace the (I) probe from the 11-kDa BI protein. The *dud* mutant, FA660, also failed to bind the (I) probe at 11 kDa in BI preparations. Together, these data suggest that this protein may selectively bind transforming DNA. The expression of this protein in blebs that associate with cell surfaces supports a hypothetical role for BI vesicles in gonococcal transformation.

This specific interaction between the gonococcal transformation uptake sequence and a bleb-associated protein further suggests certain similarities between gonococcal blebs and *Haemophilus* transformasomes (17). Reportedly, transformasomes sequester and later internalize exogenous DNA during transformation (1, 17). Arising as membrane evaginations on cell surfaces, transformasomes are considered

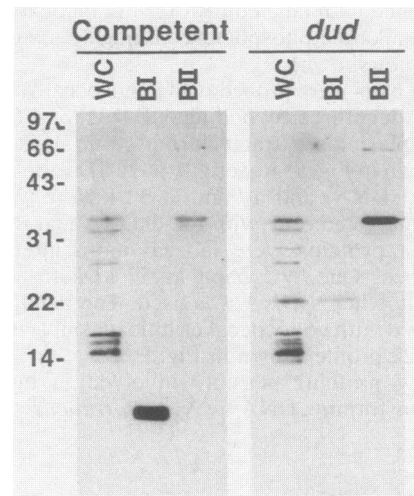


FIG. 6. Comparative binding of gonococcal transformation uptake probes by transformation-competent strain 31426 and DNA uptake-deficient FA660 (*dud*) mutants. Western blots of proteins from WC and bleb fractions BI and BII were probed with a synthetic DNA oligomer containing the gonococcal transformation uptake sequence. Resulting DNA-protein complexes were visualized and compared. A band at 22 kDa appeared more heavily labeled, and a band at 11 kDa was undetectable in the BI preparation from the *dud* mutant. The remaining DNA-binding proteins had similar reactivity. Molecular mass standards are given on the left in kilodaltons.

extensions of the outer membrane, although complete characterizations of transformosome protein and lipopolysaccharide contents are lacking (1, 8, 17). Gonococcal blebs originate as either cytoplasmic (BI) or outer (BII) membrane-derived vesicles (10). Vesicles in BII may contribute to DNase-resistant intercellular exchange or plasmids, but a genetic role for the DNA associated with extracellular BI membranes is unclear, since purified BI vesicles exhibit little, if any, nuclease resistance (10). Given evidence that transformosome release from *Haemophilus* cells correlates with loss of transformation competency (1, 8) and that the 11-kDa protein in BI vesicles from *N. gonorrhoeae* actively binds transforming DNA, experiments to examine whether cell surface-associated BI blebs function as transformosomes in *N. gonorrhoeae* may be warranted.

In the present study we have evaluated a sensitive, nonradioactive assay for detecting DNA-protein interactions on nitrocellulose membranes, provided a list of DNA-binding proteins expressed in cells and blebs of *N. gonorrhoeae*, and identified bleb-associated proteins that may regulate the interactions of plasmids with BII vesicles and linear DNA containing transformation uptake sequences with BI vesicles. Such information provides both a basis for further examination of genetic exchange regulation in gonococci and an effective, nonhazardous method for expanding these studies to a variety of organisms.

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