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DNA BINDING ACTIVITY OF VESICLES PRODUCED BY COMPETENCE DEFICIENT MUTANTS OF HAEMOPHILUS

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SUMMARY:

Membrane vesicles 40-70NM in diameter have been observed in the supernatant of cultures of a mutant strain of Haemophilus parainfluenzae (C-10) defective in transformation. Electron microscopy of thin sections of H. parainfluenzae (C-10) demonstrate that the vesicles are produced by budding off the outer membrane. Vesicles purified by differential centrifugation possess a DNase resistant DNA binding activity, and the membrane-DNA complex has been analyzed on CsCl gradients and shown to band at a density of 1.35g/cc. Mutants of H. influenzae having similiar properties have also been isolated. We report the method of isolation and some of the biochemical properties of vesicles from H. parainfluenzae and H. influenzae.

INTRODUCTION:

Transformation in Haemophilus is unique in that cells demonstrate specificity in the binding and uptake of DNA. Foreign DNAs are not taken up by these bacteria and can not compete with the ability of recipient cells to take up homologous DNA (1). The mechanism of this specificity is unknown. Models postulating the existance of DNA receptors on the bacterial membrane have been proposed by authors studying Pnuemococcus, Bacillus subtilus, as well as Haemophilus (2,3,4). Scocca (5) has shown that in H. influenzae, new envelope polypeptides are synthesized during the induction of competence, implying that one or all of these proteins may serve as specific DNA receptors. Recently Smith (6) and Goodgal and Chung (7) have stated that recognition of cloned DNA fragments by Haemophilus is sequence specific. Smith (6) also reports the isolation of a membrane

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protein from competent bacteria that can preferentially bind Haemophilus DNA. Our current finding of H. parainfluenzae and H. influenzae mutants that release membraneous vesicles into the media capable of specific DNA binding may enable us to further elucidate the nature of specific DNA uptake.

METHODS:

Strains- Three competence deficient mutants (com$^-$10, 44,56) of H. parainfluenzae were isolated by nitrosoguanidine treatment. Bacteria were grown for 18 hours in BHI media supplemented with NAD. The cells were diluted 1:4 in fresh BHI media and grown for another 18 hours at 37°C. These conditions induce competence in H. parainfluenzae. H. influenzae mutants were screened for properties similiar to those of H. parainfluenzae (C-10) by testing the supernatants of competent cultures for DNA binding activity. One mutant com$^-$51 was found to have similiar DNA binding properties. Com$^-$51 was also derived by nitrosoguanidine mutagenesis (8) and is unable to transform due to its inability to take up DNA.

Chemicals- Radioactive DNA was prepared by the procedure of Marmur (9). 1uCi/ml of ^{3}H thymidine (Amersham) was added to the growth media containing BHI and inosine (1.5mg/ml). Reagents for electron microscopy were purchased from Polyscience Inc., Fort Washington, Pa. Schleicher and Schuell BA-85 nitrocellulose filters were purchased from A. H. Thomas Co., Phila., Pa.

Electron Microscopy- Negative staining was carried out by adding a drop of 3% phosphotungstic acid pH 7.0 to a drop of vesicles on a 100 mesh grid coated with formavar and carbon. The vesicles had been dialyzed against .25M NH_4-acetate before examination. Grids were examined under a Hitachi HU-9 electron microscope at 50KV. For thin sections, a 100 ml culture of three competence mutants and control H. parainfluenzae were collected by centrifugation and fixed with 2% gluteraldehyde and 1% OsO_4. The pellets were stained with 1% Uranyl-acetate at 50°C for 18 hours and embedded in Epon. Ultra-thin sections were cut using a glass knife and were post-stained with lead citrate by the method of Reynolds (10). The sections were examined under either a Hitachi HU-11E or Zeiss M-10 at 75 or 80 KV respectively.

Preparation of Vesicles- A 100ml culture of the mutant was placed through a competence ritual and then centrifuged at 8000RPM using a Sorvall SS34 rotor for 20 minutes to remove bacteria. The supernatant was spun at 40K RPM in a type 65 rotor for 90 minutes. The resulting pellet was resuspended in 2ml of buffer and subjected to another low speed centrifugation. The supernatant was now relatively free of bacterial contamination and could then be assayed for DNA binding activity. Vesicles were stored in a buffer consisting of 20mM Tris-HCl pH 7.4, 5mM Mercaptoethanol, 85mM NaCl, 5mM $MgCl_2$, plus 20% glycerol. We have found that vesicles could be stored indefinitely at -70°C in this buffer without a significant loss of DNA binding activity.

DNA binding assay- DNase resistant DNA binding was measured by adding ^3H DNA to vesicles in a 0.1ml system. The standard reaction mixture for H. parainfluenzae consisted of 5mM ATP, 5mM NAD, 50ul of storage buffer, and 40ul of vesicles. 0.3ug of H. parainfluenzae ^3H DNA (16700cpm/ug) was added to the mixture. For H. influenzae, the reaction mixture contained 20ul of vesicles, 80ul of BHI, 5mM $MgCl_2$, and 0.15 ug of H. influenzae ^3H DNA(13000cpm/ug). After a 15 minute incubation at 37°C, the mixture was treated with 50 to 200 ug/ml of DNase to remove unbound DNA. The material was either filtered through nitrocellulose filters or precipitated with trichloroacetic acid or perchloric acid after the addition of 20ug of Salmon testis DNA. The filters were dried and counted for radioactivity in "Aquasol-2". As a control, assays were run with no vesicles, or with vesicles inactivated by boiling for 10 minutes prior to incubation.

CsCl gradient centrifugation- A 50 ml culture of H. parainfluenzae C-10 was grown to induce competence. Vesicles were prepared by the procedure outlined above and resuspended in 1ml of buffer. 8ug of ^3H H. parainfluenzae DNA was added and the mixture incubated for 25 min. at 37°C, followed by treatment with DNase; 200ug/ml for 30 minutes. 0.5 ml of this material was layered on a preformed CsCl gradient (4ml) made of solutions of CsCl 1.25 and 1.55 g/cc in density. The gradients were centrifuged for 18 hours at 30K RPM in a SW 50.1 rotor. Fractions of 8 drops were collected from the bottom of the tube and 50ul of each fraction was used to count radioactivity. The refractive index of each fraction was measured immediately after collection.

RESULTS:

Electron Microscopy- Three competence deficient mutants and wild type were examined by thin sectioning. As compared to wild type, the surface characteristics of one mutant C-10 was peculiar in that vesicles could be observed budding off the outer membrane (Fig. 1). Many particles were also seen unattached to any bacterial surface. The diameter of the vesicles ranges from 40-70NM and the particle seems to be composed of a lipid bilayer. There seems to be no preferance as to where these vesicles will be formed along the cell surface, and bacteria have been seen with as many as 20 vesicles either attached to or in close proximity of the cell. The vesicles are usually elliptical in shape, but many spherical ones can also be seen.

DNA Binding- Vesicles were isolated by differential centrifugation and tested for their ability to bind DNA as described. The results of a typical binding experiment are presented in

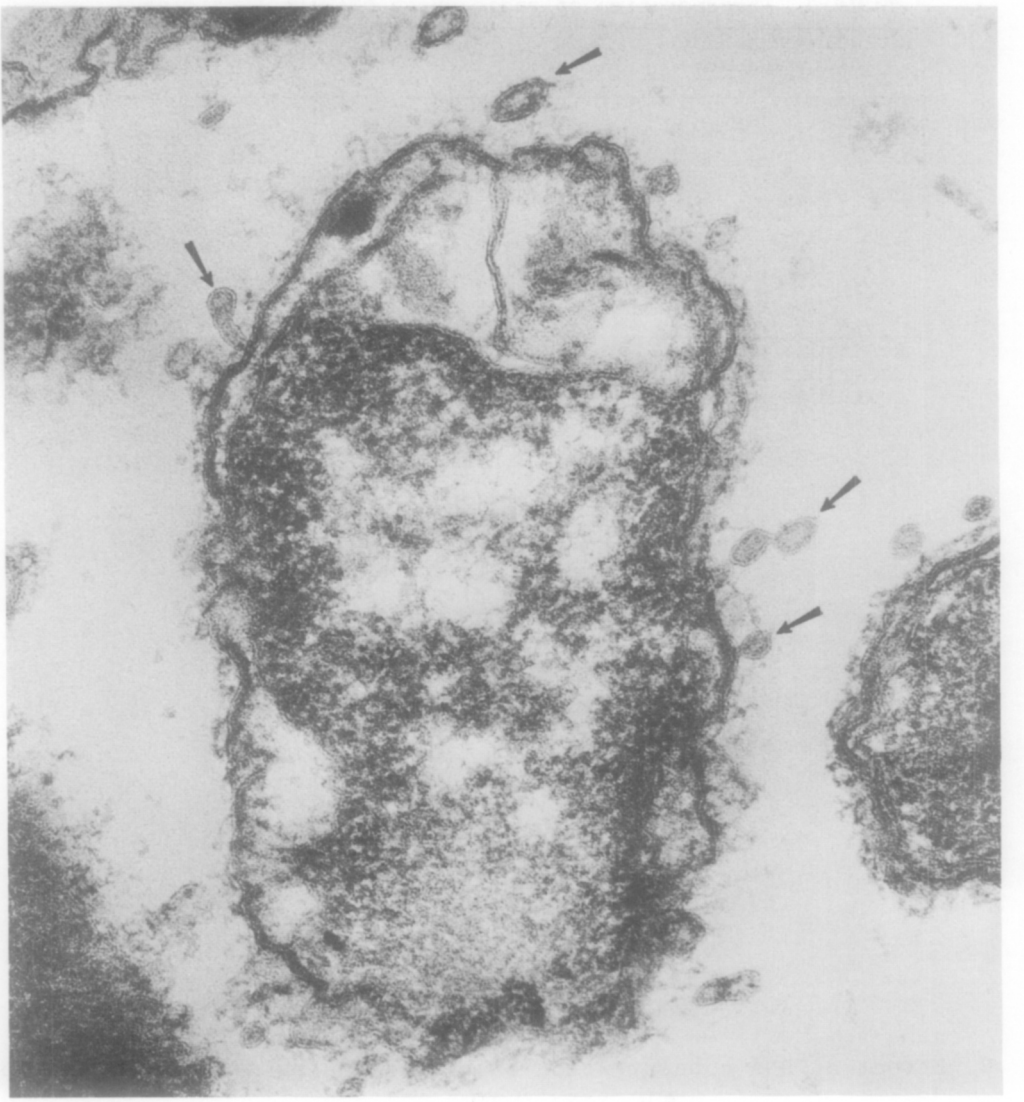

Figure 1. Thin section of H, parainfluenzae C-10 showing membraneous vesicles (arrows) along the cell surface and in the intercellular space.
0.1uM

Table 1. It can be seen that 40ul of vesicles are capable of binding at least 40ng of DNA. The concentration dependence of this reaction was examined by increasing the amount of H. parainfluenzae vesicles in the reaction mixture while keeping the DNA concentration constant. A 3.6 fold increase in binding

TABLE I Incorporation of radioactive DNA into vesicles

Standard reaction mixture plus	cpm ^{3}H DNA incorporated	
	H. para C-10	H. influenzae Com$^-$51
0	53	77
Vesicles	4090	3000
Vesicles + DNase	812	1250

Reactions were run for 15 min. using standard conditions described in Methods. Material was filtered on nitrocellulose filters.

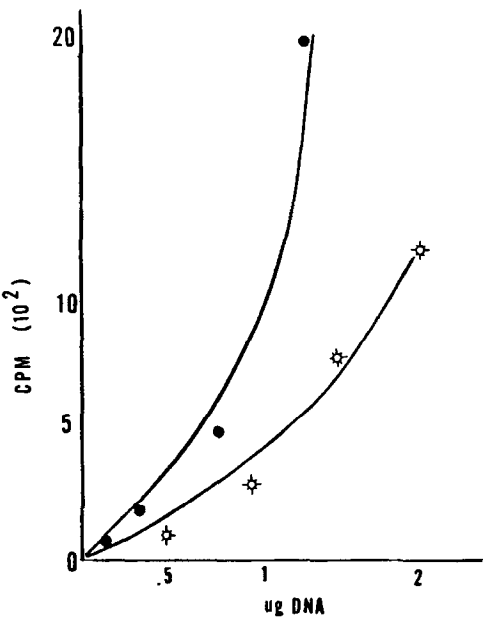

Fig. 2. Effect of DNA concentration on binding using standard conditions
● H. para C-10
☼ H influenzae Com$^-$51

activity was observed over the range of 10ul to 50ul of vesicles. The reaction was linear over this range and plateaued above 50ul. When the time course of ^{3}H DNA binding was studied it was found that the reaction was virtually complete after 5 minutes of incubation, using the standard reaction conditions described in Materials and Methods for both Haemophilus mutants. The effect of DNA concentration on binding is presented in figure 2.

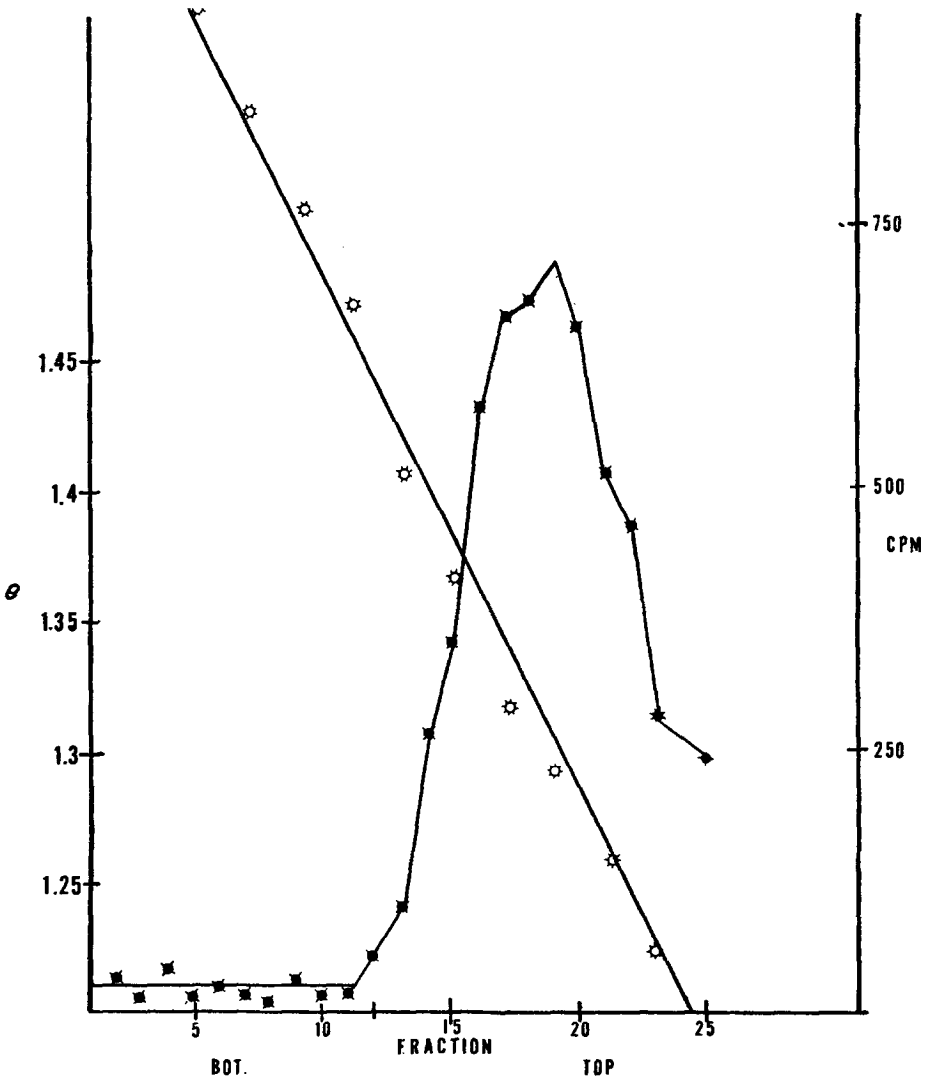

Figure 3 . CsCl gradient of DNA vesicle complex in H. parainfluenzae C-10
☼Density
◆CPM ^{3}H DNA

CsCl Gradient Centrifugation - The vesicle-DNA complex was analyzed in CsCl gradients as described in methods (Fig. 3). Purified untreated vesicles have a density of around 1.1g/cc. However, the addition of DNA to these particles causes a shift in density to approximately 1.35g/cc indicating that a membrane-DNA complex has been formed. Experiments using Com-51 gave

similiar results. Each fraction of a CsCl gradient was examined by negative staining under the electron microscope in order to determine the nature of the material banding in association with the ^{3}H DNA. All of the radioactive DNA was found to be contained in a band of membraneous vesicles characteristic of those produced by the H. parainfluenzae C-10 mutant. Less dense fractions contained cellular debris and unreacted vesicles.

DISCUSSION:

In this paper we have reported the isolation of membraneous vesicles from mutants of H. parainfluenzae and H. influenzae which possess a DNase resistant DNA binding activity. This DNA-membrane complex bands at a density of 1.35 in CsCl indicating that a physical association between the DNA and the vesicle has been formed. Experiments soon to be published indicate that this reaction is specific in that homologous DNA is preferentially taken up by these particles, and foreign DNA can not effectively compete for the binding of homologous DNA. The fact that isolated vesicles or the supernatants of C-10 or Com-51 cultures are capable of inhibiting transformation when added to competent H. parainfluenzae or H. influenzae cells respectively (unpublished data) suggests that the vesicles are capable of competing for transfroming DNA and may contain the same DNA receptor found on the surface of competent cells.

The mechanism by which Haemophilus take up DNA during transformation is not known. Although a specific DNA receptor protein is now believed to be present, it is unclear how this protein is capable of interacting with the cell membrane, and how the DNA molecule taken up by the cell is able to traverse both outer and inner membrane. One explanation is that during competence a specific receptor protein resides on a membraneous extension

of the outer bacterial membrane. When transforming DNA having the proper sequence comes in contact with the receptor, a physical association is formed between the DNA, receptor, and membraneous extension so that the complex is able to penetrate the inner bacterial membrane by fusing and budding. Membraneous extensions have been observed on the surface of competent H. parainfluenzae and H. influenzae wild type cells by electron microscopy. Such structures are rarely found on the surface of non-competent cells (M.K. in preparation). A correlation between the degree of competence and the presence of these membrane structures can be made. Studies have also shown that competent H. parainfluenzae lose these structures on their surface after being treated with homologous DNA. We speculate that the defect in transforming activity of our mutants may be due to the abnormal release of these vesicles since we have been unable to demonstrate the release of vesicles by wild type cells.

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REFERENCES

1. Scocca, J. J., Poland, R.L., and Zoon, K.C. (1974) J. of Bacteriology 118, 369-373.
2. Lacks, S.A. (1977) in "Microbial Interactions" (Receptors and Recognition, series B, Vol. 3, Chapt. 5). J.L. Reissing, Ed. Chapman and Hall, London.
3. Seto, H., Lopez, R., and Tomasz, A. (1975) J. Bacteriology 122, 1339-1350.
4. Stuy, J. H. (1962) J. Gen. Micro. 29, 537-549.
5. Zoon, K.C., Habersat, M.,and Scocca, J.J. (1976) J. of Bacteriology 127, 545-554.

6. Deich, R.A., Sisco, K., and Smith, H.O. (1978) p. 46 Abstracts of the 4th European Meeting on Bacterial Transformation and Transfection, University of York, York, England.
7. Goodgal, S., and Chung, Bon-Chu (1978) p. P2-9. Abstracts of the 4th European meeting on Bacterial Transformation and Transfection, University of York, York, England.
8. Caster, J. H., Postel, E.H., and Goodgal, S.H. (1970) Nature 227, 515-517.
9. Marmur, J. (1961) J. Molec. Bio. 3, 208-218.
10. Reynolds, E.S. (1963) J. Cell Bio. 17, 208-212.