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THE SPECIFIC UPTAKE OF CLONED HAEMOPHILUS DNA

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SUMMARY. During the process of transformation Haemophilus influenzae cells bind its own DNA but little or no foreign DNA. This specificity for recognition of DNA was studied by cloning Haemophilus DNA in E.coli. Haemophilus DNA fragments were cloned using plasmid pBR322 as a vector. The fragment cH7 cloned in pBR322 was found to be homologous to Haemophilus DNA and shown to bind irreversibly to competent Haemophilus cells. The fact that cH7 isolated from E.coli lacks Haemophilus modification leads to the conclusion that modification does not play a role in the uptake mechanism. Uptake specificity is a function of recognition sequences that reside in DNA itself.

One of the first steps in the interaction of cells and DNA during the process of bacterial transformation involves an irreversible binding to competent cells. Although all carefully analyzed systems appear to exhibit tight binding, the specificity of different systems for homologous or heterologous DNAs is not the same. In the case of Pneumococcus (1) or Bacillus subtilis (2) there is a relatively low degree of specificity and many DNAs can be bound in a DNase resistant form; on the other hand, only Haemophilus DNA is taken up efficiently or can compete with Haemophilus DNA for sites on the competent cell (3,4). What is the biochemical basis for the specificity of uptake of DNA by Haemophilus? With the advent of restriction enzyme technology and the ability to prepare purified fragments of DNA it is now possible to study the phenomenon in some detail. The study reported here is designed to determine whether specificity is due to modification or alteration of Haemophilus DNA, or whether specific recognition is associated with the sequence of bases or the structure of DNA. The experimental design is direct. A fragment of Haemophilus DNA is cloned in a vector replicating in E. coli, and then isolated and purified. One then asks if the Haemophilus DNA cloned in E.coli is capable of being specifically taken up by Haemophilus. If modification in Haemophilus is responsible for specific uptake one would expect the fragment cloned in E.coli to be poorly

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absorbed and a poor competitor for transformation by Haemophilus DNA. On the other hand, if the specificity is encoded in the DNA sequence one would expect the cloned fragment to behave like the general population of Haemophilus DNA molecules. Our results clearly demonstrate that prior modification in Haemophilus is not required for specific uptake but that uptake is a function of recognition coded in Haemophilus DNA.

MATERIALS AND METHODS

Bacterial and Plasmid Strains: Haemophilus influenzae strain 255, derived from H. influenzae Rd, was used as a recipient for studying DNA uptake. E. coli C r^-m^- was used as a host for plasmid DNA transformation. The cloning vector pBR322 (originally from H.W. Boyer) is a 2.6×10^6 dalton plasmid bearing both ampicillin and tetracycline resistance markers.

Enzymes: The restriction endonuclease EcoR1 was purchased from Boehringer-Mannheim. T4 ligase was purchased from New England Biolabs.

Cloning Procedure: 10 µg of pBR322 DNA and 5 µg of Haemophilus DNA were digested separately by EcoR1. The two DNAs were then mixed and chilled in ice for at least 30 min. ATP and T4 ligase were added and the mixture incubated at 14°C for 24 hours. This ligated material was dialyzed overnight against 10 mM Tris, 0.1 mM EDTA, pH 7.8. The dialyzed mixture was used to transform E. coli according to the procedure of Mandel & Higa (5). Transformants were selected in McConkey agar plate supplemented with 50 µg/ml of ampicillin. Ninety clones were picked and cultured. Plasmid DNA was prepared from about 30 of these clones and the sizes of the plasmids were examined by agarose gel electrophoresis.

Purification of DNA: Plasmid DNA was purified following a cleared lysate procedure (6). Bacterial DNA was prepared by a modified Marmur's procedure (7).

Gel Electrophoresis and Blotting: Electrophoresis was performed in horizontal slab gels of agarose (6 mm thick) in an apparatus designed by McDonnell et al. (8). DNA in the gel was denatured and blotted onto a piece of nitrocellulose filter paper (Millipore Co., HAWP304 FI) by a modified Southern technique (9).

Hybridization and Autoradiography: Probe DNA (^{32}P labeled #7 plasmid DNA, see below) was sonicated for three 30 second periods in a sonifier-cell disruptor (Ultrasonics, Inc.) prior to heat denaturation. The filter was rolled around a Wassermann tube and dropped into a 16 x 150 tube filled with 2 ml of preincubation buffer (0.02% each of Ficoll 400,000, Pharmacia; bovine albumin, fraction V, Armour; and polyvinylpyrrolidone 360,000, Sigma in 3 x SSC). After 6 hours of preincubation, the buffer was replaced by the probe DNA solution. After 24 hours of hybridization at 68°C, the filter was washed and then air dried and baked in a vacuum oven at 80°C for 30 min. An X-ray film (Kodak XRP-5) was placed on top of the filter and stored at -78°C. The X-ray film was developed after 1-10 days of exposure depending on the number of counts in the probe.

Assay for DNA Uptake: The reaction mixture contained 1.25 ml of Brain Heart Infusion broth (Difco), 0.10 µg of DNA, 0.25 ml of competent Haemophilus cells (3×10^9 cells/ml). After incubation at 37°C for 30 min, the mixture was centrifuged and resuspended in 1.5 ml of saline. Pancreatic DNase (dissolved in Mg^{++}) was added to give 20 µg/ml and incubated at

37°C for 10 min. After a second wash, cells were centrifuged, the pellet resuspended in saline, and the radioactivity counted. The counts associated with the cell pellet measured the amount of DNA taken up by the cells.

RESULTS AND DISCUSSION

Cloning of Haemophilus DNA in E.coli

The cloning vector used was the 2.6 megadalton plasmid pBR322 carrying both ampicillin and tetracycline resistance genes. It has a single recognition site for restriction endonuclease EcoRI and becomes a single linear molecule after EcoRI digestion (Fig. 1A, lane 5). Haemophilus DNA was also digested by EcoRI to yield a mixture of fragments differing in length from 10^5 to 10^7 daltons (Fig. 1A, lane 1). These two DNAs were mixed and reannealed via sticky ends created by EcoRI and finally ligated by T4 ligase, E.coli was transformed by this ligated material

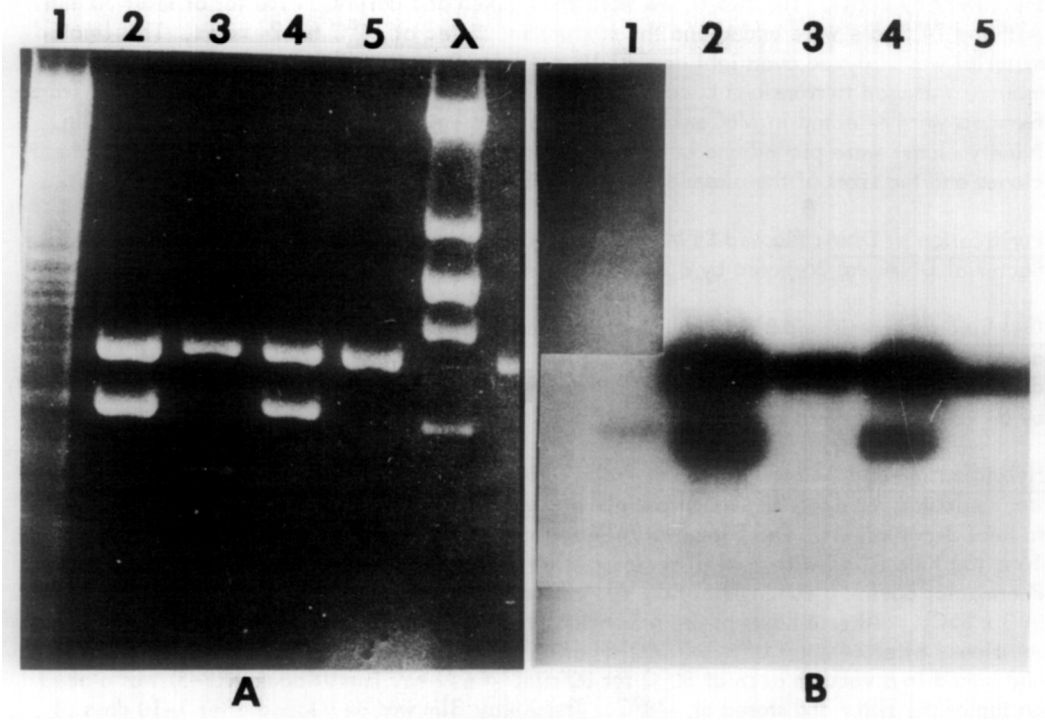

Fig. 1. Gel electrophoresis and hybridization of recombinant DNA. Fig. 1A is the gel pattern stained by ethidium bromide. Fig. 1B is the autoradiogram after hybridization with [^{32}P] #7 DNA. lane 1 = Haemophilus DNA, lane 2 = #7 DNA, lane 3 = #26 DNA, lane 4 = #2 DNA, lane 5 = pBR322 DNA; 1 μg of DNA was digested with EcoRI at 37°C for 1 hour. DNA was applied to the gel after heating at 65°C for 5 min and electrophoresed at 30V for 16 hours.

in the presence of calcium, and ampicillin resistant clones were selected. The procedure yielded about 2000 ampicillin resistant clones. Of these, 90 clones were picked and cultured. About 20% had plasmids larger in size than pBR322. Three of them, #2, #7, and #26 were further characterized and studied.

Characterization of Recombinant DNA

DNA from clone 7 was cleaved into two pieces by EcoRI. In addition to the pBR322 segment, it had another fragment with a molecular weight of 2.1×10^6 daltons, which was designated as cH7 (Fig. 1A, lane 2). It was assumed that cH7 was a fragment of Haemophilus inserted into pBR322. To prove this point, two tests were performed: density gradient equilibrium centrifugation and hybridization. The density of pBR322 was similar to E. coli DNA: 1.705 g/ml; Haemophilus DNA, on the other hand, sedimented at a density of 1.688 g/ml (Fig. 2B). DNA from #7 after EcoRI digestion and sedimentation in CsCl exhibited two peaks at densities corresponding to both pBR322 and Haemophilus DNAs. Additional evidence that the fragment cH7 was Haemophilus DNA was obtained by extracting linear pBR322 and cH7 from agarose gel (Fig. 1A, lane 2) and subjecting them to CsCl gradient separately (Fig. 2A). The fragment of cH7 banded at 1.688 g/ml, the same as the control Haemophilus DNA (Fig. 2C), while linear pBR322 banded at a position similar to that of control pBR322. That cH7 and Haemophilus DNA have the same density suggested that they were homologous to each other, but the unambiguous demonstration of homology came from hybridization analysis.

Haemophilus and recombinant DNAs were digested by EcoRI and electrophoreses in agarose gels to give the pattern shown in Fig. 1A. DNAs were denatured in the gel and transferred to nitrocellulose filter paper using a modified southern blotting technique. DNAs in the filter paper were hybridized with [^{32}P] #7 probe DNA. The autoradiogram of hybridized bands is shown in Fig. 2B. The smear of Haemophilus DNA in lane 1 hybridized with the probe to produce a band only at a single position, which was the same as that of cH7 in the agarose gel. The fact that cH7 hybridized with Haemophilus and that it had the same size as the hybridized band clearly

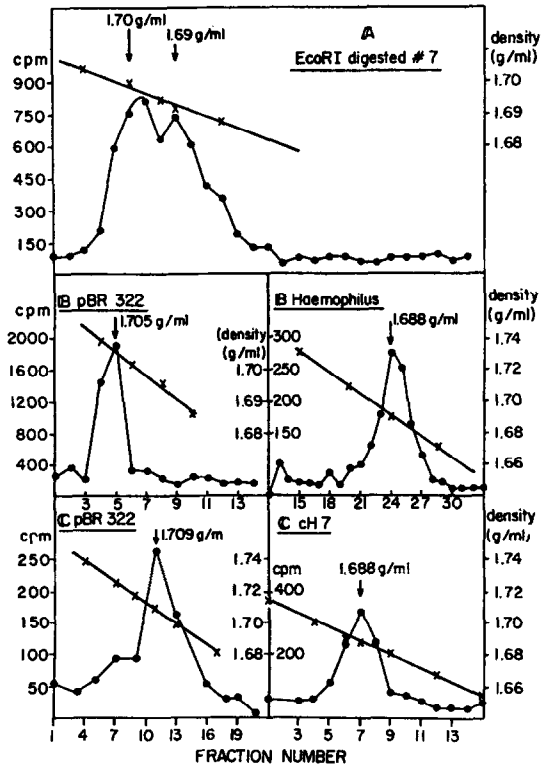

Fig.2. CsCl density gradients of cloned DNA fragments. All the gradients were centrifuged in a type 65 rotor at 55,000 rpm for 20 hours. Fig.2A. EcoRI digested #7 DNA. Fig.2B. Control pBR322 and Haemophilus DNA, in separate gradients. Fig.2C. cH7 and linear pBR322 extracted from agarose gel, in separate gradients.

demonstrated that it was homologous with it and was derived from a similar EcoRI fragment of Haemophilus DNA.

Uptake Property of #7 DNA

Table 1 shows that #7 DNA was taken up almost as efficiently by competent Haemophilus cells as homologous Haemophilus DNA. DNA from #7 also competed with Haemophilus DNA for both uptake and transformation of streptomycin and erythromycin resistance markers (data not shown The uptake of #7 DNA could also be competed out by either Haemophilus or #7 DNA. On the other hand, pBR322 DNA had no effect on the uptake of #7 even at a 10 fold excess (Table 2). This indicated that #7 DNA was taken up by Haemophilus cells and this uptake was specific only for homologous DNA. Although #7 DNA lacked Haemophilus modification

Table 1. Uptake of Cloned DNA Fragment by Haemophilus influenzae

DNA	Input cpm	cpm[a] recovered	% recovery	DNA input (ng)	Uptake cpm	Uptake ng	% Uptake
Haemophilus	23,000	21,500	93	300	701	39	13.0
EcoRI digested #7	22,000	20,070	91	500	366	33	6.6
pBR322	8,400	8,400	100	750	20	0.7	0

[a] Total cpm recovered was calculated by summing the counts in the supernatant and pellet

Table 2. Competition for Uptake of [H^3] #7 DNA

Competing DNA[a]	Uptake (cpm)
----	1109
Haemophilus	72
#7	504
pBR322	1066

[a] 0.5 μg of [H^3] #7 transforming DNA and 5 μg of non-radioactive competing DNA was used in this experiment.

because it was purified from E. coli, it was still recognized as homologous by Haemophilus. In other words, modification did not contribute to the uptake mechanism, and the real recognition resided in the properties carried by DNA itself, probably in its base sequences.

There are at least two models consistent with the available data that explain the specific uptake mechanism. One is that a specific recognition site, or a recognition sequence (a sequence 6 to 20 bases long) is required for uptake. This sequence should be present only in Haemophilus, absent in all non-haemophilus strains, and must occur all over the Haemophilus chromosome due to the high frequency of uptake. The second hypothesis states that recognition requires a longer homology between donor and recipient DNA. No particular "site" is involved in the recognition. On this model, specificity would depend upon the pairing of donor with recipient DNA at the cell surface.

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