Construction of DNA recognition sites active in *Haemophilus* transformation

(synthetic deoxynucleotides/plasmid pBR322 derivatives/DNA sequencing/A+T-rich flanking DNA/protein–DNA interactions)

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ABSTRACT   Competent *Haemophilus* cells recognize and preferentially take up *Haemophilus* DNA during genetic transformation. This preferential uptake is correlated with the presence on incoming DNA of an 11-base-pair (bp) sequence, 5'-A-A-G-T-G-C-G-G-T-C-A-3'. To prove that this sequence is the recognition site that identifies *Haemophilus* DNA to the competent cell, we have now constructed a series of plasmids, each of which contains the 11-bp sequence. Using two different assay systems we have tested the ability of fragments from these plasmids to compete with cloned *Haemophilus* DNA fragments that naturally contain the 11-bp sequence. We find that the addition of the 11-bp sequence to a DNA fragment is necessary and sufficient for preferential uptake of that fragment. However, plasmid DNAs containing this sequence may vary as much as 48-fold in uptake activity, and this variation correlates with the A+T-richness of the DNA flanking the 11-mer.

During genetic transformation (ref. 1; see Fig. 1) *Haemophilus* cells preferentially take up *Haemophilus* DNA (2). Fragments of *Haemophilus* DNA cloned in *Escherichia coli* retain the ability to direct selective uptake, indicating that they bear identifying sequences or "uptake sites" rather than a particular pattern of modified bases (3). Four small cloned fragments exhibiting preferential uptake were found to contain a common 11-base-pair (bp) sequence, 5'-A-A-G-T-G-C-G-G-T-C-A-3' (4). Ethylation of phosphate groups on one of these DNA fragments in and near the region of the 11-bp sequence was found to significantly diminish uptake (4). From these experiments, we concluded that the 11-bp sequence is probably involved in *Haemophilus* DNA recognition. However, it was possible to obtain a definitive proof of this idea by cloning synthetic 11-bp sequences into the plasmid pBR322 and examining their ability to direct DNA uptake.

In this paper we describe the construction of a series of pBR322 derivatives, each of which contains the synthetic 11-bp sequence in a different DNA context. Two different assay systems were used to compare the activity of pBR322 fragments containing these synthetic uptake sites to the activity of cloned *Haemophilus* DNA fragments bearing natural uptake sites. One assay examines the ability of the synthetic site to direct the uptake of the DNA fragment on which it resides into a DNase- and salt-resistant form. The other assay tests whether the synthetic site can interfere with the transformation of *Haemophilus* cells to streptomycin resistance by competing with the streptomycin-resistant (Str^R) DNA for cell uptake. From the results of these experiments, we conclude that the presence of the 11-bp sequence on a DNA fragment is necessary and sufficient for preferential uptake of that fragment. However, we have also ob-
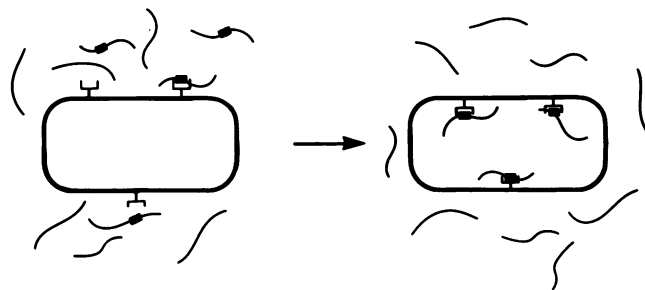
FIG. 1. A model for the genetic transformation of *Haemophilus* cells. Each cell has about six receptors that bind to the uptake sites on exogenous DNA. The DNA subsequently becomes protected from the effects of external DNase and cannot be removed from cells by washing them in 0.5 M NaCl.

served that the more A+T-rich the DNA flanking this sequence, the better the uptake, and that the level of uptake specified by two different regions containing the 11-bp sequence may vary as much as 48-fold.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media. *Haemophilus influenzae* Rd strain *com*56 [a high-uptake mutant (5)], KW35 (a Str^R strain), and KW21 [a streptomycin-sensitive (Str^S) strain] were grown in Difco heart infusion broth supplemented with hemin at 10 μg/ml (Eastman) and NAD at 2 μg/ml (Sigma). *E. coli* strains HB101 (*hsdR hsdM recA*), C600 (*hsdR*), and MM294 (*endA hsdR*) were grown in L broth (6). Strain MM294 was used for all fusaric acid selections. Recombinant plasmid pKS17 has been described (3); it contains an 8.1-kilobase (kb) insert of *H. parainfluenzae* DNA bearing two naturally occurring uptake sites, cloned into the BamHI site of pBR322.

Chemical Synthesis of 11-mers and 15-mers. 5'-A-A-G-T-G-C-G-G-T-C-A, 5'-T-G-A-C-C-G-C-A-C-T-T, 5'-A-A-T-T-A-A-G-T-G-C-G-G-T-C-A, and 5'-A-A-T-T-T-G-A-C-C-G-C-A-C-T-T were chemically synthesized by the modified triester method in solution (7). The sequence of each oligomer was confirmed by the mobility-shift method.

Assay for Cell Uptake of DNA Fragments. *H. influenzae* cells were made competent by using the MIV-medium procedure of Herriott *et al.* (8). In a typical uptake experiment, radioactively labeled DNA fragments ($\approx 10^6$ cpm; 2.5 pmol) in 100 μl of 10 mM Tris·HCl/1 mM EDTA, pH 8 (TE buffer) were added to 4×10^8 cells in 1 ml of MIV medium, and the mixture

was incubated for 10 min at 37°C. The cells were then chilled to 0°C and DNase I (Worthington) was added to 100 μg/ml. After 20 min at 0°C, the cells were centrifuged for 1 min in an Eppendorf 5412 centrifuge and then were washed twice with 1 ml of 0.5 M NaCl in MIV medium and once with TE buffer. They were then resuspended in 360 μl of TE buffer, 40 μl of 10% NaDodSO₄ was added, and the mixture was incubated at 65°C for 10 min to lyse the cells (3). The lysate was extracted twice with equal volumes of phenol and once with phenol/chloroform (1:1). Residual phenol was removed by ether extraction and the DNA was recovered by ethanol precipitation (9). Lysate DNA was subjected to electrophoresis and autoradiograms of dried gels were obtained as described (4).

Assay for Competition Between Plasmid DNA Bearing Synthetic Sites and Genomic DNA Bearing a Streptomycin-Resistance Gene. Str^S *Haemophilus* cells (KW21) were made competent and 100-μl aliquots at 5×10^8 cells per ml were added to sterile 1.5-ml Eppendorf tubes on ice. Ten microliters of TE buffer containing a mixture of DNAs was added to each tube. This mixture contained a fixed amount (0.1 μg) of genomic fragments from *Haemophilus* Str^R strain KW35 and various amounts of each plasmid DNA. All plasmid DNAs had been linearized with *Sal* I, an enzyme which cuts once in each. Each tube was then incubated at 37°C for 10 min; the cells were then embedded in 10 ml of supplemented brain/heart infusion broth plus 0.75% agar and grown at 37°C for 1.5 hr to express the Str^R phenotype. The plates were then overlaid with 10 ml of supplemented brain/heart infusion broth plus 0.75% agar containing streptomycin sulfate (Sigma) at 400 μg/ml and were grown overnight at 37°C.

Construction of Plasmids Carrying Synthetic Uptake Sites.
pPUP3. To obtain an A+T-rich DNA context, poly(dT) extensions were added to the synthetic 11-bp sequence, poly(dA) extensions to *Pst* I-cut pBR322 DNA, and the complementary ends thus formed annealed by the procedure of Nelson and Brutlag (10). The annealed DNA was used to transfect HB101 cells by the calcium treatment procedure of Wensink *et al.* (11) and colonies were replica-plated to isolate ampicillin^S tetracycline^R (Amp^S Tet^R) clones. Several of these pPUP derivatives were purified in small amounts by the method of Birnboim and Doly (12) and insert size was examined by restriction digests. One plasmid, pPUP3, was purified in larger amounts and sequenced in the region of the 11-bp sequence (Table 1) by the method of Maxam and Gilbert (9).

pEUP1. The objective in this case was to place a single copy of the 11-bp sequence between two filled-in *Eco*RI termini, so that it would have four A·T pairs on each side. Two 15-mer

strands were synthesized so that the duplex would have single-stranded ends that could base pair with *Eco*RI ends: 5'-A-A-T-T-A-A-G-T-G-C-G-G-T-C-A-3' and 5'-A-A-T-T-T-G-A-C-C-G-C-A-C-T-T-3'. When these duplex molecules are ligated to *Eco*RI ends, the *Eco*RI sites are not regenerated. Therefore, the strategy was to ligate them to *Eco*RI-cut pBR322 DNA in the presence of *Eco*RI enzyme so that recircularized pBR322 molecules would be recleaved and the reaction would be driven toward the formation of the recombinant plasmid. The reaction mixture (100 μl) contained *Eco*RI-cut pBR322 DNA (1.3 pmol) and the synthetic 15-mers (1.3 pmol of each strand), 20 units of *Eco*RI, and 400 units of T4 DNA ligase (New England BioLabs) in a solution of bovine serum albumin (Bethesda Research Laboratories) at 0.1 mg/ml, 5 mM ATP (Sigma), 100 mM Tris·HCl (pH 8.0), 10 mM MgCl₂, and 5 mM dithiothreitol. Reactions were carried out at 14°C for 16 hr. Six Amp^R Tet^R colonies obtained by transfection of C600 cells were screened, and a pBR322-sized derivative that had lost its *Eco*RI site, pEUP1, was purified and sequenced in the region of the 11-bp sequence (Table 1).

pRSUP1. Fusaric acid selection for loss of tetracycline resistance was used to select for the insertion of one copy of the synthetic 11-mer into one of the three *Rsa* I sites of pBR322 (the one at base 164, because it interrupts the Tet^R gene). To prepare the vector DNA, pBR322 DNA was partially digested with *Rsa* I, and the resulting fragments were first separated by agarose gel electrophoresis and were then transferred by electrophoresis to DE-81 paper (Whatman) in a Trans-Blot apparatus (Bio-Rad). The band of single-cut linear DNA was cut out of the paper and the DNA was recovered by elution with 1 M NaCl. This DNA was ligated to a 20-fold molar excess of each of the synthetic 11-bp strands (which will not ligate to each other because they lack 5' phosphate groups). The reaction mixture (100 μl) contained: 1 μg of linearized pBR322 DNA, 11-mer DNA as described, 5 mM ATP, 50 mM Tris·HCl (pH 8.0), 10 mM MgCl₂, 5 mM dithiothreitol, and 800 units of T4 DNA ligase. Incubation was at 14°C for 16 hr. Strain MM294 cells were transfected with the ligated DNA and Tet^S colonies were selected on fusaric acid plates by the procedure of Bochner *et al.* (13). Colonies were double-checked for ampicillin resistance and tetracycline sensitivity by replica plating and three plasmids were screened for loss of the *Rsa* I site at base 164. Of these, one promising plasmid, pRSUP1, was isolated and sequenced in the region of the 11-bp sequence (Table 1).

pBUP2. To create a G+C-rich environment for the 11-mer, a mixture of the synthetic 11-mer (not phosphorylated) and phosphorylated *Bam*HI linkers (Collaborative Research, Wal-

Table 1.   Uptake activities of synthetic and natural recognition sites

Plasmid	Sequence of 11-bp region	Activity, %
pPUP3	AAAAAAAAAAAAAAAA AAGTGCGGTCA TTTTTTTTTTTTTTT	191
pKS17	AAAATTAAAATTTAA AAGTGCGGTCA TTTTGACCGAGATTT	117
pKS17	GGTTGTGCATTGTTG AAGTGCGGTCA AAAAATCGGAAATTT	100
pEUP1	TCGTCTTCAAGAATT AAGTGCGGTCA AATTCTCATGTTTGA	40
pRSUP1	CTTGGTTATGCCGGT AAGTGCGGTCA ACTGCCGGGCCTCTT	27
pBUP2	GCGTAGAGGATCCGG AAGTGCGGTCA CCGGATCCACAGGAC	4
pBR322	No 11-bp sequence	<1

Shown are the nucleotide sequence of the uptake site region and the uptake activity for four synthetic uptake sites (see *Materials and Methods*) and two natural sites (4). One strand is shown for each, running in the 5' to 3' direction. On the right is shown the uptake activity of each site relative to the second pKS17 site, which is arbitrarily assigned a value of 100%. Uptake activity is calculated from the ratios of the band intensities in the autoradiograms shown in Fig. 2. These bands were quantitated by weighing the paper peaks obtained from tracings with a Joyce–Loebl Mark IIIC densitometer. The following formula was used: uptake activity of site in a given band B (of the six bands shown in Fig. 2) = (intensity of B in uptake lane/intensity of band 4 in uptake lane) × (intensity of band 4 in control lane/intensity of band B in control lane) × 100%.

tham, MA) was ligated and recleaved with *Bam*HI to generate *Bam*HI-ended 11-mers. These were treated with bacterial alkaline phosphatase (Worthington) to prevent multiple insertions and ligated in 10-fold molar excess into the *Bam*HI site in the Tet^R gene of pBR322. The ligation mixture was made up as for the pRSUP1 construction. The fusaric acid technique was used to select cells carrying recombinant plasmids. Plasmid DNAs from six clones were linearized with *Hind*III, subjected to agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with one of the 11-mer strands labeled at its 5′ end (14). Of six plasmids screened by this method, four seemed to have a single copy of the 11-mer. One of these, pBUP2, was sequenced in the region of the 11-bp sequence (Table 1).

RESULTS

Comparison of the Activity of Synthetic and Natural Sites in Directing Cell Uptake of Radioactive DNA Fragments. *Eco*RI digestion of pKS17 DNA [a pBR322 derivative that contains an 8.1-kb piece of *H. parainfluenzae* DNA cloned into the *Bam*HI site (3)] gives four fragments, of which the 5.3-kb and 1.75-kb fragments each contain a copy of the 11-bp sequence and the 4.6-kb and 0.8-kb fragments do not (3). The sequences

of these naturally occurring sites (4) are shown in Table 1. If circular pBR322 DNA is cut with both *Ava* I and *Hind*III, two fragments of 2.9-kb and 1.4-kb are generated, which can easily be identified when mixed with *Eco*RI-digested pKS17. If a mixture of these six fragments is presented to competent *Haemophilus* cells, the two fragments bearing natural sites should be preferentially taken up by competent cells and the other fragments excluded. If a functional synthetic site has been created in one of the pBR322 fragments, it should also be taken up. In this assay system, DNA uptake is quantitated by determining the amount of exogenous DNA that becomes protected from external DNase treatment and high-salt washing. When fragments bearing synthetic or natural sites are recovered from lysed cells, a comparison of their relative abundance before and after uptake of fragments gives a direct and reasonably accurate measurement of the relative efficacy of the synthetic site. Because the input ratio of fragments is taken into account, perturbations due to unequal amounts of input DNA are removed. Saturating amounts of DNA fragments (1 μg/ml) are used to ensure maximal competition. Because each synthetic site is compared to the same natural sites, synthetic sites can be quantitatively compared with each other.

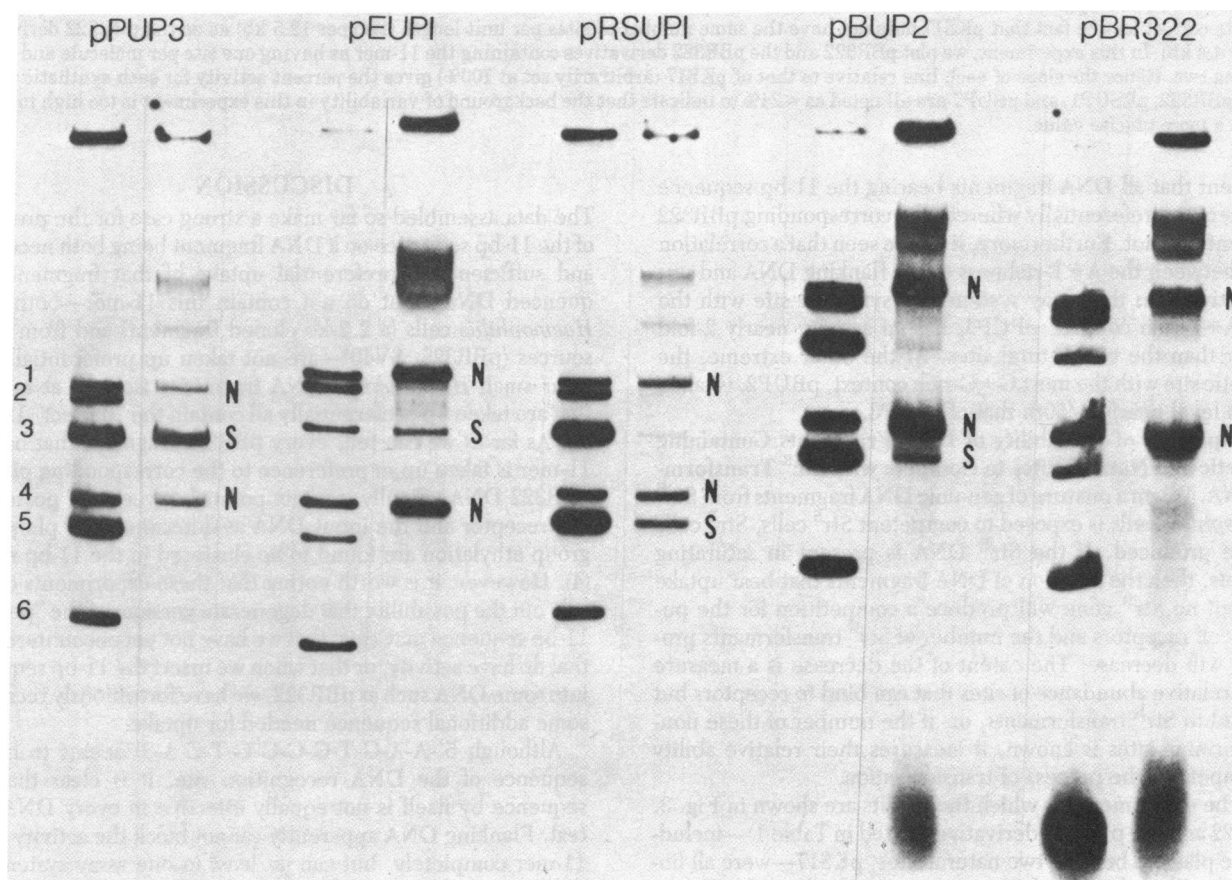Fig. 2 and Table 1 show the results of these experiments. It

FIG. 2. Uptake of DNA fragments containing synthetic or natural recognition sites. Shown is a composite photograph of six autoradiograms taken after agarose gel electrophoresis, representing six experiments in which competent *Haemophilus* cells were exposed to end-labeled DNA fragments. The plasmid pKS17 (containing two natural sites) was cut with *Eco*RI to yield fragments that migrate as bands 1, 2, 4, and 6. Bands 1 and 4 each have one natural site; bands 2 and 6 have no site. These four fragments were end-labeled as described and mixed with end-labeled *Ava* I/*Hind*III fragments of pBR322 or the pBR322 derivatives containing the synthetic 11-bp sequence, resulting in the addition of bands 3 and 5 on the autoradiogram. In each pair of lanes, the left lane shows this mixture of six fragments. Each pair of lanes is marked with a plasmid name (e.g., pPUP3) to show the source of bands 3 and 5. The right lane of each pair shows the radioactivity in DNA fragments recovered from cell lysates after uptake. To the right of each uptake lane, the observed bands are marked N if they contain a natural site and S if they contain a synthetic site. Other bands may also be seen in the uptake lanes. The two broad bands above the plasmid bands may be due to degradation of some input DNA with subsequent incorporation of released radioactivity into genomic DNA by repair enzymes. Very faint bands seen among the plasmid bands may be due to a ligation activity known to be present in these cells (15).
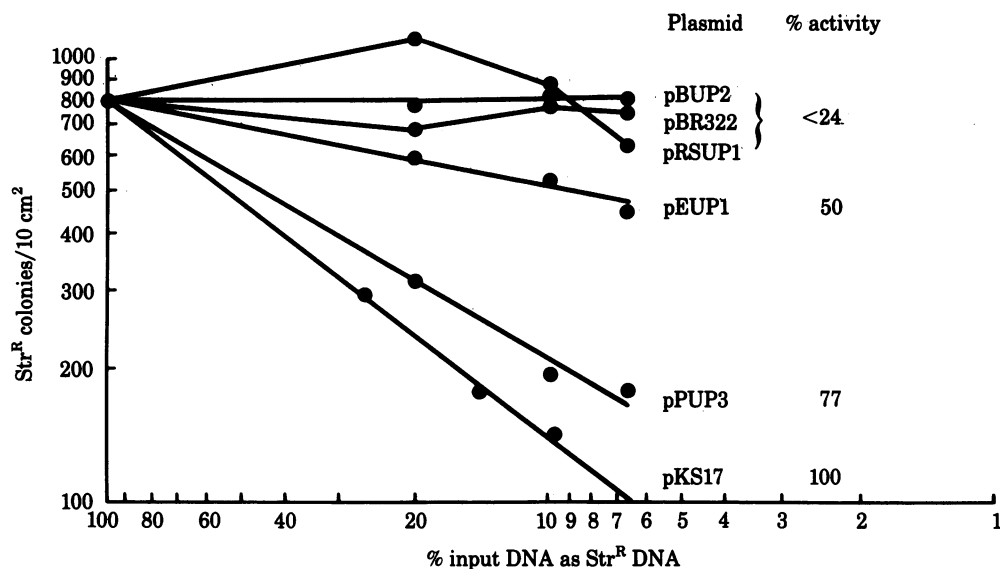
FIG. 3. A plot of the number of Str^R transformant colonies produced when a saturating amount of genomic DNA containing the Str^R marker is diluted with plasmid DNA that has uptake sites but no Str^R gene (and therefore can compete for uptake but cannot produce Str^R colonies). Each point represents data from one plate normalized to 10 cm^2. The x axis represents a mass ratio of the input DNAs. It has previously been shown that each site on a DNA fragment competes for uptake independently (16). Therefore, the x values for pKS17 have been shifted to the left by a factor of 1.43 to correct for the fact that pKS17 does not have the same number of sites per unit length (two per 12.5 kb) as do the pBR322 derivatives (one per 4.4 kb). In this experiment, we plot pBR322 and the pBR322 derivatives containing the 11-mer as having one site per molecule and pKS17 as having two. Hence the slope of each line relative to that of pKS17 (arbitrarily set at 100%) gives the percent activity for each synthetic site, as shown. pBR322, pRSUP1, and pBUP2 are all noted as <24% to indicate that the background of variability in this experiment is too high to assign to them a more precise value.

is evident that all DNA fragments bearing the 11-bp sequence are taken up preferentially whereas the corresponding pBR322 fragments are not. Furthermore, it can be seen that a correlation exists between the A+T-richness of the flanking DNA and uptake activity. In this assay system, the synthetic site with the most A+T-rich context, pPUP3, has an activity nearly 2-fold greater than the two natural sites. At the other extreme, the synthetic site with the most G+C-rich context, pBUP2, is taken up at a level nearly 1/50th that of pPUP3.

Comparison of the Ability of DNA Fragments Containing Synthetic and Natural Sites to Compete with Str^R Transforming DNA. When a mixture of genomic DNA fragments from Str^R *Haemophilus* cells is exposed to competent Str^S cells, Str^R cells will be produced. If the Str^R DNA is present in saturating amounts, then the addition of DNA fragments that bear uptake sites but no Str^R gene will produce a competition for the putative cell receptors and the number of Str^R transformants produced will decrease. The extent of the decrease is a measure of the relative abundance of sites that can bind to receptors but not lead to Str^R transformants, or, if the number of these nontransforming sites is known, it measures their relative ability to compete in the process of transformation.

In the experiment for which the results are shown in Fig. 3, pBR322 and the pBR322 derivatives (listed in Table 1)—including the plasmid bearing two natural sites, pKS17—were all linearized with *Sal* I, which cuts once in each plasmid. Various amounts of each linear plasmid were mixed with saturating Str^R DNA and added to a fixed number of competent *Haemophilus* cells. In this competition assay, pPUP3, which has the most A+T-rich DNA context surrounding the 11-mer, competes 77% as well as the average of the two pKS17 sites and is the best of the synthetic sites. The next most A+T-rich DNA context is found on pEUP1, which competes at a 50% level. The remaining synthetic sites all compete less well. The order of activities of the synthetic sites relative to each other appears to be the same in the two assay systems.

DISCUSSION

The data assembled so far make a strong case for the presence of the 11-bp sequence on a DNA fragment being both necessary and sufficient for preferential uptake of that fragment. Sequenced DNAs that do not contain this 11-mer—both from *Haemophilus* cells (a 2.2-kb cloned fragment) and from other sources (pBR322, SV40)—are not taken up preferentially (4). Four small *Haemophilus* DNA fragments isolated at random that are taken up preferentially all contain the "perfect" 11-mer (4). As far as we can tell, every pBR322 fragment that has the 11-mer is taken up in preference to the corresponding piece of pBR322 DNA. Finally, contact points between the postulated cell receptor and the input DNA as determined by phosphate group ethylation are found to be clustered in the 11-bp region (4). However, it is worth noting that these experiments do not rule out the possibility that degenerate versions of the "perfect" 11-bp sequence may exist that we have not yet encountered but that do have activity, or that when we insert the 11-bp sequence into some DNA such as pBR322, we have fortuitously recreated some additional sequence needed for uptake.

Although 5'-A-A-G-T-G-C-G-G-T-C-A-3' seems to be the sequence of the DNA recognition site, it is clear that this sequence by itself is not equally effective in every DNA context. Flanking DNA apparently cannot block the activity of the 11-mer completely, but can (at least in one assay system) decrease it to 1/25th or increase it to almost 2-fold higher than a natural site. The evidence strongly favors the idea that the recognition feature that flanking DNA provides is A+T-richness. The correlation between local A+T-richness and uptake activity appears to hold for all six sites examined and in both assay systems, with the possible exception of the reversal of pPUP3 and pKS17 levels in the transformation assay. The ethylation data (4) suggest that the DNA contact points extend only several bases to each side of the 11-mer and this parallels the uptake results for pRSUP1 and pBUP2 in the radioactive fragment assay; the synthetic sites on these plasmids are both G+C-rich,

but the former has an A·T pair next to the site on each side, making it less G+C-rich in this local region, and these sites differ 6-fold in uptake in the expected direction. It is less likely but still possible that recognition is not due to A+T-richness in the region surrounding the 11-mer but instead relies on some other feature—such as base pair degeneracies (17) of the types recognized by restriction enzymes ("purine/pyrimidine," "G or T," "A or C"). However, the only degeneracies of these types that correlate with the uptake activities of all the sites are two A or C degeneracies at 7 and 8 bp to the left of the 11-mer and a G or T degeneracy 9 bp to the right of the 11-mer. No purine/pyrimidine degeneracies that fit the uptake data are observed.

How does a competent *Haemophilus* cell recognize the A+T-rich DNA flanking the 11-bp sequence? Any of several mechanisms may be involved. The postulated cell receptor may make contact with the minor groove of the incoming DNA, where A·T and T·A base pairs differ from G·C and C·G pairs by the absence of the centrally located 2-amino group of guanine (17). The receptor may induce a partial melting of DNA strands in the region of the 11-bp sequence, a process facilitated by the lower melting point of A+T-rich DNA. Such a mechanism has been proposed for the action of bacterial RNA polymerases (18). Finally, it has been shown recently that even short runs of A or T impart a significantly different twist to the DNA helix than do random DNA or alternating A and T (19, 20); perhaps this altered twist is an important recognition feature.

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