J. Biosci., Vol. 3, Number 4, December 1981, pp. 431-438. © Printed in India.

Genetic and molecular events in transformation of *Haemophilus influenzae* with plasmid RSF 0885 carrying cloned segments of chromosomal DNA

N. K. NOTANI

Biology and Agriculture Division, Bhabha Atomic Research Centre, Bombay 400 085

MS received 6 June 1981; revised 21 August 1981

Abstract. In *Haemophilus influenzae* genetic transformation for a plasmid marker is significantly increased when recombinant plasmid RSF 0885 DNA carrying chromosomal DNA segments is used instead of the plasmid DNA alone. Chromosomal DNA by itself, added even a few minutes after the addition of plasmid DNA to competent cells, stopped further uptake of the plasmid DNA. These observations are consistent with the idea that plasmid RSF 0885 contains a 'degenerate' version of the required eleven base-pair 'uptake sequence' in *Haemophilus*. The transformation activity of the recombinant plasmid DNA is recoverable after its entry into cells, although the specific biological activity of the re-isolated plasmid DNA is less than that of the parental recombinant plasmid DNA. The *rec 1* gene function of the host is necessary for obtaining higher transformation frequencies with recombinant DNA from five different clones. The reduced transformation frequencies seen in *rec* 1^- strain is not all due to a permanent damage to the donor DNA since the recovered recombinant plasmid DNA from such cells can increase the transformation efficiency on *rec* 1^+ strain.

Keywords. *Haemophilus influenzae*; Plasmid transformation; recombinant DNA; uptake sequences; *rec* gene requirement.

Introduction

Competent cells of *Haemophilus influenzae* take up efficiently only the DNA that contains a specific eleven base-pair sequence, about 600 copies of which are distributed through the *Haemophilus* genome (Sisco and Smith, 1979). Plasmid RSF 0885 originally isolated from *H. influenzae b* carrying ampicillin resistance (amp^r) marker, transforms competent cells of *H. influenzae* Rd with a frequency lower than 0.01% (Notani *et al*., unpublished observations). These results suggest that either the required "uptake sequence" may be absent or that a 'degenerate' version of the sequence with a much lower affinity for the receptor protein(s) may be present. It is also possible that low transformation with RSF 0885 may be due to the events occurring subsequent to uptake. However, transformation frequencies are significantly increased by the plasmid DNA carrying a library of sequences of chromosomal DNA. The present paper describes the genetic and molecular events in transformation with the plasmid DNA carrying chromosomal DNA inserts.

Materials and methods

Bacterial strains

Haemophilus influenzae Rd is a wild-type strain from Dr. J. Setlow's collection, *rec* 1 strain was also from her collection and is a recombination-deficient strain. Plasmid RSF 0885 contained originally in a *Haemophilus* strain obtained from Stanley Falkow was transferred to *H. influenzae* Rd strain in J. Setlow's laboratory (BNL). Plasmid-bearing strains pD2, pD4, pD5, pD7 and pD9 were generated by selecting amp^r colonies by transformation with RSF 0885 DNA to which segments of chromosomal DNA had been spliced.

Preparation of recombinant DNA

The detailed method will be described elsewhere. Briefly, purified plasmid DNA and chromosomal DNA (Marmur, 1961) were digested with *Pvu II* and joined with T4 ligase (New England Biolabs). A sample of the primary recombinant DNA was generously provided by Dr D. McCarthy.

Preparation and isolation of unlabelled and labelled plasmid DNA

Cleared lysates were prepared by minor modifications in the methods described by Hirt (1967) as described below. Bacterial cultures were grown to stationary phase, washed and resuspended in standard saline citrate (0.15 M NaCl and 0.015 M sodium citrate) and lysed with 1% sodium dodecyl sulphate (SDS) (final concentration) at 37°C for a minimum period of 30 min. NaCl (1 M final concentration) was gently mixed with the lysate. Lysates were stored at 4°C for at least 8 h. The lysates were then centrifuged at 20,000 *g* (rotor SS 34) in a Sorvall RC 2B centrifuge for 30 min. The supernatant (cleared lysate) contained bulk of the plasmid DNA and less than 5% of the chromosomal DNA. Lysates were treated with RNase and deproteinized with chloroform: amyl alcohol (Marmur, 1961). The plasmid DNA was purified from this lysate by ethidium bromide-cesium chloride equilibrium density-gradient centrifugation (Radioff *et al*., 1967).

[^{3}H]-Labelled plasmic DNA was prepared by growing 8 ml cells in brain-heart infusion broth containing [^{3}H]-thymidine (33 µCi/ml) (Sp. activity 52 Ci/mmol NEN). Cleared lysate (0.4 ml) was prepared as described above. An aliquot (0.3 ml) was layered on 5 to 20% sucrose (in SSC) gradients and centrifuged at ~75,000 *g* for 2 to 2.5 h in a Beckman ultracentrifuge. On these gradients chromosomal DNA sediments faster than the peak plasmid DNA. The amount of chromosomal DNA which was contaminating peak plasmid DNA fractions was estimated to be less than a ng/ml. A part of the peak fractions was used for the assay of radioactivity and biological activity and was also used in transformation experiments.

Size determination of recombinant plasmid (pD7) DNA fragments

The recombinant 15 µl (pD7) plasmid DNA (~1µg) was cut with Pvu II (2.5 units) or Eco RI (10 units) for 1 h at 37°C and the digests were electrophoresed with Hpal-digested T7 DNA standards (kindly provided by Alan Rosenberg), through 1% agarose gels as described by McDonnel *et al*. (1977).

Transformation

Transformation was done according to the MIV method (Steinhart and Herriott, 1968). For the 'fate' studies of input plasmid DNA, a 12 ml competent cell culture was exposed to DNA and 4 ml fractions were taken for each time point (in general, 0, 30 and 60 min). Cleared lysates were prepared from these cells as described above and the DNA from these lysates was used in a second transformation to assay for biological activity and radioactivity. In some cases, these assays were carried out on samples fractionated through sucrose density gradients.

Results

Inhibition of RSF 0885 DNA uptake by chromosomal DNA

At 1, 5, 10 or 20 min after mixing competent cells, with RSF 0885 DNA, excess of chromosomal DNA containing novobioin resistance marker was added. It was observed that even the subsequent addition of chromosomal DNA stopped further uptake/transformation of the plasmid marker (figure 1). This is consistent with our other observation (to be published elsewhere) that the kinetics of uptake of plasmid DNA are slower than that of chromosomal DNA and that chromosomal DNA effectively competes with plasmid DNA.

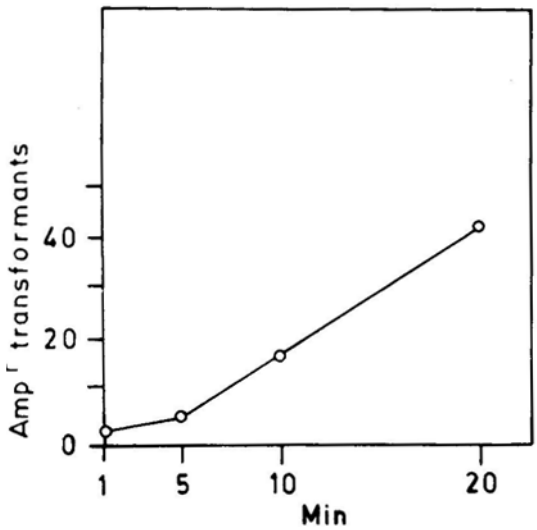

Figure 1. Effect of addition of excess of chromosomal DNA on transformation of *H. influenzae* by RSF 0885 DNA.

Transformation with plasmids carrying chromosomal DNA fragments

Transformation frequency with plasmid RSF 0885 is less than 0.01%. However, using recombinant plasmid DNA containing chromosomal DNA inserts yielded a burst of transformation. Ampicillin-resistant colonies picked from the cells that

had been transformed with the primary recombinant DNA molecules were characterized further. Five of these were grown and cleared-lysates were prepared of the plasmid DNA. DNAs of the five isolates designated pD2, pD4, pD5, pD7 and pD9 were elctrophoresed though 1% agarose gels. All the isolates showed plasmids of varying sizes, with pD7 moving the slowest and therefore presumably had the largest insert(s) (data not shown). Transformation frequencies for *amp*^{r} marker with the five plasmids are given in table 1. These frequencies are higher by two to three orders of magnitude than that obtained with RSF0885 alone.

Table 1. Transformation of wild type *H. influenzae* with recombinant plasmids carrying chromosomal DNA segments.

Plasmid DNA	*amp*^{r} transformants ($\times 10^6$)	Frequency of transformation (%)
pD2	3.1	0.6
pD4	7.5	1.5
pD5	4.3	0.9
pD7	5.2	1.0
pD9	4.2	0.8

Effect of DNA concentration on the number of transformations

Figure 2 shows the dose-response curve for pD7 DNA. Transformation increases linearly and then begins to saturate at less than 0.12 µg of plasmid DNA.

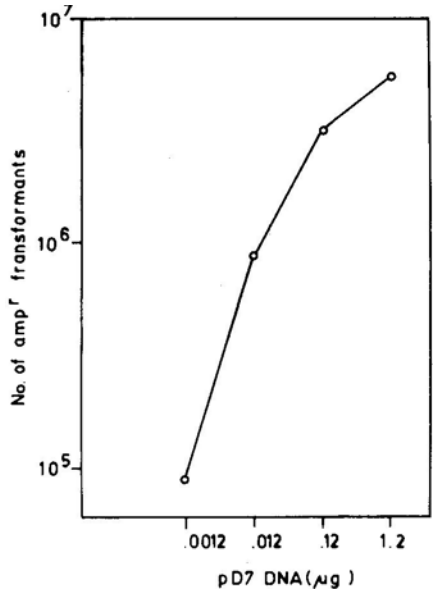

Figure 2. Effect of pD7 DNA concentra tion on plasmid transformation.

Determination of the size of pD7 DNA

When pD7 was cut with Eco RI, four fragments were obtained. Since, RSF 0885 is not cleaved by Eco RI, all the four cuts should have occurred in the chromosomal DNA. Molecular weight of these fragments was determined by gel electrophoresis of Eco RI digests of pD7 along with Hpal-cut T7 DNA standards. Molecular weight of pD7 was estimated to be about 12×10^6 (data not shown). Pvu II generated about 10 fragments which was surprising because the original splicing was done with Pvu II. It is possible that the original chromosomal DNA was either partially digested or that a number of independent segments were spliced or was rearranged.

Requirement for rec 1 *gene expression for transformation with plasmids carrying chromosomal inserts*

Following an observation of D. McCarthy (unpublished data) and of Setlow *et al* (to be published elsewhere), it was observed that all the five plasmids under consideration here also require *rec* 1 gene expression for peak transformation frequencies (table 2). The reduction in the frequencies is almost three to four orders of magnitude. Reduction in transformation with chromosomal DNA or pD5 DNA in *rec* 1 is comparable (table 3).

Table 2. Transformation of *Rec*⁻ 1 strain with recombinant plasmids carrying chromosomal DNA segments.

Recipient cells	plasmid DNA	*amp*^{r} transformants ($\times10^2$)	Frequency (%)
nov rec 1	pD2	2	
nov rec 1	pD4	33	
nov rec 1	pD5	35	Less than 0.001
rec 1	pD7	6	
rec 1	pD9	15	

Table 3. Transformation with pD5 (*amp*^{r}) or chromosomal (*str*^{r}) DNA of wild type or rec_1^- strain of *H. influenzae.*

Recipient cells	Donor DNA cells	No. of transformants
Wild type;	*str*^{r}	291×10^4
rec_1	*str*^{r}	0×10^1, 0×10^2
Wild type	*amp*^{r}	240×10^4
rec_1	*amp*^{r}	0×10^1, 0×10^1

Fractionation of [^3H]-labelled pD7 DNA and eficiency of transformation

A cleared-lysate of [^{3}H]-labelled pD7 was fractionated through 5-20% sucrose gradients. Since the resident c hromosomal DNA had *nov*^{r} marker, the extent of separation of chromosomal DNA could be examined. Residual chromosomal DNA peaks two fractions faster than the plasmid DNA peak (figure 3). Our best

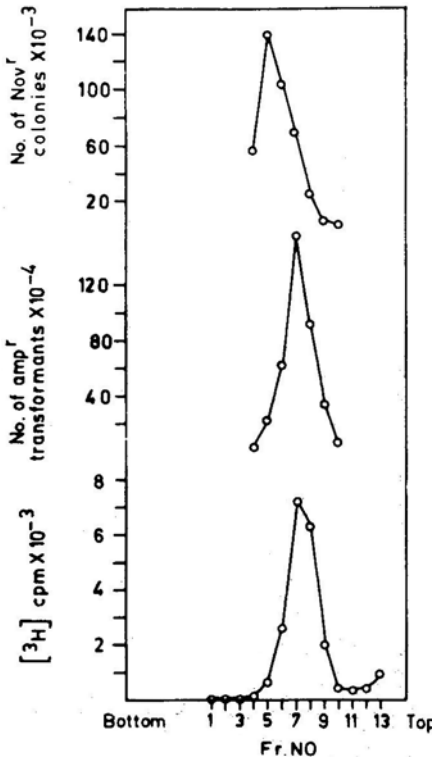

Figure3. Radioactivity and biological activity profiles of [^{3}H] -labelled pD7 DNA and residual chromosomal DNA.

estimate from biological data is that there is half a ng or less of chromosomal DNA contaminating the plasmid DNA in the peak fraction. By comparing with the intensity of the bands of serial dilutions of pBR 322 DNA of known concentrations, the pD7 concentration in fr. 7 was estimated as 1.4 µg/ml (and chromosomal DNA concentration from biological data was 0.25 ng/ml). Specific activity of the pD7 DNA is 170,000 cpm/µg of DNA; and 1µg of DNA yields 2.7×10^7 *amp*^{r} transformants. Assuming a molecular weight of 12×10^6 for pD7 DNA, efficiency of transformation works out to between 5×10^{-4} and 1×10^{-3}.

Biological activity of input intracellular pD7 DNA

Wild type and *rec* 1 cells of *H. influenzae* were transformed with [^{3}H]-labelied pD7 DNA (fr. 8; figure 3). Samples were taken out, washed and cleared-lysates

prepared. From both the series, viz. wildtype or *rec 1* strain, the lysates were sedimented through sucrose gradients and DNA fractions were assayed for transformation on wild type cells. This provided information regarding the specific biological activity of intracellular input pD7 DNA. The data are recorded in table 4. The data indicate that: (i) substantial biological activity corresponding

Table 4. Relative specific biological activity of gradient fractions.

Fraction No.	Recipient strain			
	Wild type		Rec_1^-	
	0 min	60 min	0 min	60 min
	No. of amp^r transformants/ [^{3}H]-cpm			
4	75	160	133	—
5	560	310	419	208
6	711	414	1142	206
7	731	386	869	225
8	1237	316	900	221
9	597	118	970	—
10	344	213	—	—

The lysates from cells treated for 0 and 60 min with pD7 DNA were sedimented through sucrose density gradients and the fractions were assayed for biological activity.

to reisolated pD7 DNA is recoverable, (ii) specific biological activity of pD7 reisolated from wild-type, assayed on wild type of later samples is lower than those of earlier samples suggesting inactivation events and (iii) in *rec* 1 strain, although the first transformation was low, the second transformation of reisolated DNA assayed on wild-type is reasonably high, suggesting that in *rec* 1 strains, pD7 is not permanantly damaged or blocked to a significant extent.

Discussion

Uptake of plasmid RSF 0885 DNA is stopped even when the chromosomal DNA is added some minutes after the mixing of competent cells with the plasmid DNA. Increased transformation with the recombinant plasmid DNA containing chromosomal DNA segments also suggests that plasmid DNA contains presumably only a degenerate version of the uptake sites required in *Haemophilus* (Sisco and Smith, 1979) which has a lower affinity for the receptor(s) in competent cells. In spite of the increase in transformation with plasmids carrying inserts, the efficiency of transformation is still less than 10^{-3}. Considering that close to 100% of the cells generally become competent in cultures grown in MIV competence media, the low

transformation efficiency is presumably due to inactivation following uptake, at least a part of which was detected in the present experiments. Since the plasmid must be fixed intact in the cytoplasm, it would appear that any inactivation would interfere with its replication and fixation. On the other hand, fixation of the chromosomal DNA in *Haemophilus* should not be so sensitive to inactivation since a marker can be rescued by the resident chromosomal DNA even after inactivation occurs in the vicinity, but not in the marker. Inactivation in *rec* 1 strain is somewhat higher than in the wild type. Even so, it does not account for the much lower transofmation levels in the first transformation. Suprisingly, in *rec* 1, first transformation is low but the second one (lysate assay) is high. This suggests that up to 60 min, the block or damage introduced in *rec* 1 transformation is not permanent. This block could be due to lack of *rec* 1 gene expression. It is, difficult to deduce if recombination *per se* is required for this or that *rec* 1 product acts in some other manner. Some amount of inactivation is observed both during transformation and transfection for the respective input DNAs and fragmentation has been noted (Voll and Goodgal, 1961; Notani and Goodgal, 1966; Notani, 1971; Notani *et al*., 1973). Fragmentation for pD7 does not appear to be nearly as extensive asfor example for transfecting DNA.

Quite unlike for OPM2 (Lacks, 1979), bulk of the intracellular input pD7 DNA is recoverable in biologically active from. This seems to be the genus-characteristic, since in *H. parainfluenzae* also, the heterospecific transforming DNA remains mostly double-stranded (Notani and Setlow, 1972).

Acknowledgements

The author thanks Dr Jane K. Setlow in whose laboratory this work was done, for her cooperation and provocative discussions of the problem. Comments of several colleagues and their help is also acknowledged.

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