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Homospecific and heterospecific transformation with chimeric plasmid DNA in *Haemophilus influenzae* and *Haemophilus parainfluenzae*

S M KHANOLKAR and N K NOTANI

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

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Abstract. A gene for resistance to novobiocin has been self-cloned in *H. parainfluenzae*, using a high-efficiency plasmid vector pJ1-8. Homospecific and heterospecific transformation using chimeric plasmids containing an insert of the *nov*ʳ gene of *H. influenzae* or *H. parainfluenzae* has revealed some novel features. There is some evidence from our observations that *H. parainfluenzae* also recognises specific uptake sites in the incoming DNA. Transformation with chimeric and non-chimeric plasmids suggests that the homology of the insert to the resident DNA has a major role in the extrachromosomal fixation of chimeric plasmids. It is also inferred that apart from the (passive) internalisation of input DNA from transformasomes, there is also recombination-assisted internalisation.

Keywords. Gene cloning; homospecific/heterospecific transformation; DNA homology; chimeric plasmid fixation.

1. Introduction

Heterospecific transformation between *H. influenzae* and *H. parainfluenzae* occurs with a low efficiency and is marker-dependent. Uptake of donor DNA seemed relatively normal and could not account for the two- to five-order of magnitude reduction in heterospecific as compared to homospecific transformation (Notani and Setlow 1972). From isopycnic sedimentation analysis, two other observations were also noteworthy: i) even in *H. parainfluenzae* homo- or heterospecific transformation, intracellular donor DNA was recoverable in a biologically-active (double-stranded) form and ii) while substantial association of donor DNA could be detected with resident DNA, recombination was probably not completed since this association seemed to break-down upon further incubation. Recently, the physical fate of chimeric DNA in *H. parainfluenzae* has been examined by Barany and Kahn (1985). Their main observations are that DNA in *H. parainfluenzae* also first enters into transformasome (s) from where it translocates into the cell (by the mechanism of internalisation) integrating the homologous DNA into the resident chromosome.

We have self-cloned in *H. parainfluenzae* a gene (*nov*ʳ) which conditions resistance to novobiocin. Fixation of this chimeric plasmid was also followed in *H. parainfluenzae*. Our observations are generally consistent with the physical picture of Barany and Kahn (1985) but, in addition, we propose a role for *rec* genes or recombination in translocation and/or fixation of donor DNA in the recipient cells. Our experiments throw some light on the features of extrachromosomal fixation of plasmid DNA as well.

2. Materials and methods

Bacterial strains, plasmids and media

H. parainfluenzae resistant to 2·5 μg/ml of novobiocin, *H. parainfluenzae* resistant to 2000 μg/ml of streptomycin, *H. influenzae* Rd (wild type) and *H. influenzae* Rd resistant to 2·5 μg/ml of novobiocin were obtained from Drs S H Goodgal and J K Setlow. H. *influenzae* Rd strains carrying the following plasmids were also used: p RSF 0885 (De Graaff *et al* 1975), pD7 (Notani 1981), pJ1-8 (Joshi and Notani 1983), pJ1-8N19 (Joshi and Notani 1984) and pJ1-8S38 (Samiwala and Notani, unpublished)

Cells were grown at 37°C in 3 % brain heart infusion (BHI) broth supplemented with 2 μg/ml NAD in case of *H. parainfluenzae* and 10 μg/ml hemin in addition to 2 μg/ml NAD in case of *H. influenzae*.

Isolation of DNA

Chromosomal DNA was extracted according to the procedure of Marmur (1961). Plasmid DNA was isolated as described by Notani (1981).

Competence development

H. parainfluenzae and *H. influenzae* cells were made competent by the methods of Nickel and Goodgal (1964) and Goodgal and Herriott (1961), respectively.

Genetic transformation

The transformation mixture consisted of 0·8–0·9 ml BHI brotn + 0·1 ml of DNA to which 0·1 ml competent cells (either *H. influenzae* or *H. parainfluenzae*) were added. Uptake was allowed at 37°C for 10–15 min after which the cells were diluted, plated in BHI agar and incubated at 37°C for 1·5–2 hr. Plated bacteria were then challenged with appropriate antibiotic(s). Transformants were counted after 24 hours of incubation at 37°C.

Restriction digestion and ligation

Restriction enzymes *Eco* RI, *Bam* HI, *Pst* I, *Pvu* II and T_4 DNA ligase were purchased from Bethesda Research Laboratories and used according to Maniatis *et al* (1982). For partial *Eco* RI digestion of chromosomal DNA, incubation at 37°C was terminated after 15 min and the reaction mixture was kept at 65°C for 10 min to inactivate the restriction enzyme. Restriction digestion was monitored by agarose gel electrophoresis.

Agarose gel electrophoresis

0·7 % agarose gels were used to electrophorese whole plasmid DNA and 1 % gels were used to electrophorese DNA digested with restriction endonucleases. Electrophoresis

Table 1. Transformants obtained from *H. parainfluenzae* exposed to *nov*^{r} chimeric plasmid DNA.

Chimeric plasmid DNA	Transformants/5 × 10^8 cells		
	Nov^R	Amp^R	Amp^{R}Nov^R
pJ1–8HpN1	$2{\cdot}0 \times 10^7$	$4{\cdot}1 \times 10^6$	$1{\cdot}9 \times 10^6$
pJ1–8N19	$6{\cdot}1 \times 10^4$	$6{\cdot}3 \times 10^4$	$6{\cdot}1 \times 10^4$

was carried out in tris acetate buffer (pH 8) for 4–5 hr. Gels were visualised by a UV (350 nm) lamp.

3. Experimental results

H. parainfluenzae nov^r chromosomal DNA was partially digested with *Eco* RI and ligated to *Eco* RI-cut pJ1-8 DNA. Exposure of competent *H. parainfluenzae* Nov^s cells to the spliced DNA yielded 8 Amp^R Nov^R transformants. These were screened further. Only one clone, named pJ1-8HpN1, turned out to have *nov*^{r} marker linked to *amp*^{r} marker of the plasmid. This was inferred by a) checking Amp^R: Amp^R Nov^R transformant ratios, b) the ability of the electroeluted plasmid DNA band to yield double transformants (Amp^R Nov^R) and c) the lower reduction (8-fold versus 108-fold) in Nov^R transformants from heat denatured pJ1-8HpN1 DNA than in those from the control i.e. chromosomal DNA (data not shown).

Chimeric plasmids pJ1-8HpN1 ($\simeq$ 20 kb) and pJ1-8N19 (21 kb) containing inserts carrying *nov*^{r} markers of *H. parainfluenzae* and *H. influenzae*, respectively, were used in studying events in homo- and hetero-specific transformation. Transformants obtained from *H. parainfluenzae* or *H. influenzae* with pJ1-8HpN1 or pJ1-8N19 or chromosomal DNA are given in tables 1, 2 and 3.

pJ1-8HpN1 gives a high level of Nov^R and Amp^R transformants in *H. parainfluenzae* but transformation with pJ1-8N19 is lower by about two orders of magnitude (table 1). Also, whereas Nov^R transformants are about five-fold higher than Amp^R in *H. parainfluenzae*, those with pJ1-8N19 are equal for the two markers. Conversely, in *H. influenzae*, homospecific transformation is high but heterospecific transformation with pJ1-8HpN1 is high for Nov^R but low for Amp^R (table 2). This is somewhat surprising because heterospecific transformation is low in either direction with chromosomal DNA (table 3).

Table 2. Transformants obtained from *H. influenzae* exposed to *nov*^{r} chimeric plasmid DNA.

Chimeric plasmid DNA	Transformants/1 × 10^9 cells		
	Nov^R	Amp^R	Amp^{R}Nov^R
pJ1–8N19	$7{\cdot}2 \times 10^7$	$2{\cdot}8 \times 10^6$	$1{\cdot}1 \times 10^6$
pJ1–8HpN1	$5{\cdot}6 \times 10^6$	$2{\cdot}8 \times 10^3$	$2{\cdot}0 \times 10^3$

Table 3. Transformants obtained from *H. influenzae* and *H. parainfluenzae* exposed to homospecific and heterospecific nov^r chromosomal DNA.

Recipient species	Source of nov^r chromosomal DNA	Nov^R transformants/ml
H. parainfluenzae	*H. parainfluenzae*	$9{\cdot}0 \times 10^6$
	H. influenzae	$1{\cdot}0 \times 10^4$
H. influenzae	*H. influenzae*	$1{\cdot}0 \times 10^7$
	H. parainfluenzae	$1{\cdot}0 \times 10^4$

Thus, with chimeric DNA, efficient integration of the nov^r marker of *H. parainfluenzae* into resident *H. influenzae* is observed. In order to confirm that *H. parainfluenzae* nov^r marker is being integrated in *H. influenzae* chromosome without any change, the DNA of pJ1-8HpN1–transformed cells was isolated and digested with *Bam* HI. *Bam* HI inactivates the *H. parainfluenzae* nov^r marker but not the *H. influenzae* nov^r (tables 4 and 5). It could thus be inferred that the *H. parainfluenzae* nov^r marker is integrated in *H. influenzae* DNA unchanged enough to be inactivated by *Bam* HI.

Transformation of *H. parainfluenzae* was examined with several plasmids (table 6). These could be broadly grouped into 3 classes – low, medium or high – depending on their efficiency to transform for amp^r marker. Thus, pJ1-8 gives little or no transformation (because it probably lacks the uptake sites), while pJ1-8S38, pD7 and pJ1-8N19 give medium-efficiency transformation. All these plasmids contain an *H. influenzae* DNA splice. Finally, pJ1-8HpN1 containing *H. parainfluenzae* DNA gives the highest transformation frequency in *H. parainfluenzae*.

4. Discussion

Vector pJ1-8 is usable for self-cloning *H. parainfluenzae* DNA and we have successfully cloned the nov^r gene on a (15–16) kb insert. This DNA clone was used in both homospecific and heterospecific transformation. From our observations, we construct the following picture of transformation in *H. parainfluenzae*: 1) the highest transform-

Table 4. Effect on transformation of restriction endonuclease digestion of *H. parainfluenzae* nov^r chromosomal DNA.

H. parainfluenzae nov^r chromosomal DNA	Nov^R transformants/ml in *H. parainfluenzae*	Relative survival of nov^r marker (%)
Uncut	$1{\cdot}2 \times 10^5$	100·0
Eco RI digested	$1{\cdot}0 \times 10^2$	0.08
Bam HI digested	$5{\cdot}0 \times 10^3$	4.1
Pst I digested	$6{\cdot}0 \times 10^2$	0.5
Pvu II digested	$1{\cdot}0 \times 10^5$	83.0

Table 5. Effect on transformation of *Bam* HI digestion of *H. influenzae nov*^{r} chromosomal DNA.

H. influenzae nov^r chromosomal DNA	Nov^R transformants/ml in *H. influenzae*	Relative survival of *nov*^{r} marker
Uncut	$9{\cdot}2 \times 10^5$	100
Bam HI digested	$1{\cdot}0 \times 10^6$	108

ation is obtained when there is a homologous splice in the chimeric DNA. This seems to make a difference of about 2 orders of magnitude since transformation is markedly lower with plasmids containing *H. influenzae* inserts. At least two interpretations of these observations can be thought of: a) because of partial homology of *H. influenzae* DNA with *H. parainfluenzae* DNA, translocation is less efficient. However, physical homology is about 50% (Boling 1972) but the reduction here is about 99%. Thus, quantitatively, this would not be the most tenable explanation; b) besides translocation by internalisation, there might also be another mechanism which is mediated by recombination and which increases transformation about 100-fold. This argument is supported by the 1:1:1 ratio of Nov^R: Amp^R: Nov^R Amp^R transformants obtained with pJ1-8N19. 2) pJ1-8 gives little or no transformation and we assume that this is so because it lacks uptake sites. 3) Nov^R: Amp^R transformant ratios are higher in *H. influenzae* than in *H. parainfluenzae* homospecific transformations. Apparently, there is a higher relative rescue of the *nov*^{r} marker in *H. influenzae* which could be attributed to the different mechanisms of translocation observed in the two species by Barany and Kahn (1985). 4) High Nov^R transformation with pJ1-8HpN1 DNA in *H. influenzae* was unexpected but perhaps is explained on the basis of pJ1-8 containing short sequences of *H. influenzae* which facilitate recombination and rescue. This observation also suggests that chromosomal and plasmid transformations do not always go hand in hand.

The physical picture for *H. parainfluenzae* transformation as inferred by Barany and Kahn (1985) is as follows: DNA is first taken up into transformasomes of competent cells. A requirement is that incoming DNA contain uptake sites. DNA is then internalized along with the transformasomes. We have given evidence that apart from such (passive) internalisation of incoming DNA, recombination-assisted internalization may also occur. The role of recombination in fixation of Amp^R transformants is not quite clear,

Table 6. Transformation of *H. parainfluenzae* with different plasmid DNA of *H. influenzae*.

Plasmid DNA	Chromosomal DNA marker	Amp^R transformant/ml in *H. parainfluenzae*
pJ1–8	Unmarked	$0{\cdot}0 \times 10^2$
pRSF0885	Unmarked	$5{\cdot}0 \times 10^2$
pD7	Unmarked	$5{\cdot}8 \times 10^4$
pJ1–8N19	*nov*^{r}	$6{\cdot}0 \times 10^4$
pJ1–8S38	*str*^{r}	$3{\cdot}0 \times 10^4$

i.e., whether once internalised by recombination their fixation is inevitable. It is likely that additional events are required but these are not clear at present.

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