Replication and expression in mammalian cells of transfected DNA; description of an improved erythrocyte ghost fusion technique

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ABSTRACT

After modification of an erythrocyte - ghost fusion technique, DNA can be transferred in large amounts to mammalian cells. The overall recovery permits analysis of transferred DNA immediately after fusion by e.g. Southern - blot hybridization and (electron) microscopy. In general more than 20% of the cells contain the transfected DNA in their nuclei within 4 hours post fusion. Expression of Polyoma T antigens is delayed, as compared to a normal virus infection, and is detected at about 60 hours after transfer of DNA. During the first days an increase in amount of transferred DNA could be detected which might be due to limited replication. To analyze the replication of hybrid DNA molecules consisting of Polyoma and prokaryotic plasmid DNA sequences, fusion was followed by a normal Polyoma virus infection. The resulting induction of cells to enter the S phase made possible replication analysis of transfected hybrid DNA. Replicating molecules of both the θ form and rolling - circle type were observed.

INTRODUCTION

Transfer of DNA to living mammalian cells is a powerful experimental approach. The technique has so far mainly been used in studies of gene expression, recombination and in isolation of eukaryotic genes. The technique should also be very useful for studying the replication of e.g. hybrid or mutated viral DNA molecules but because of the overall low efficiency of the transfection methods, only very few investigations have been performed so far.

Several methods have been developed such as DEAE dextran mediated DNA transfer (1), direct microinjection with micropipettes (2,3), the calcium phosphate technique (4) and red cell or liposome mediated microinjection (5-12). The most extensively applied method for introducing DNA into cells has been the calcium-phosphate-DNA precipitation technique (4) but because of its low effi-

ciency, enough DNA to permit replication analysis cannot be transferred.

In an alternative technique red blood cells are used as injection vehicles. By this method macromolecules (5,6,7,13) and tRNAs (13-16) have been transferred into large amounts of cells containing receptors for Sendai virus. A similar technique using polyethylene glycol (PEG) as a fusogen has been described (8,9). This technique has also been used to transfer DNA (17,18), and after modification of the loading procedure this technique was used in the present investigation.

In this paper we describe an alternative transfection technique with a transfer efficiency that permits an immediate analysis of injected DNA by e.g. hybridization and electron microscopy. Moreover, by stimulating cells containing injected DNA with normal virus infection at a short time after fusion, replicating hybrid DNA molecules were detected by electron microscopy.

MATERIALS AND METHODS

Cell lines

Mouse 3T6 cells and Fischer Rat-1 cells were a gift from Dr. G. Magnusson and have previously been described (19). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and 10% fetal calf serum. Cell lines used were regulatory tested for mycoplasma (20), and only mycoplasma free cells were used in the experiments.

Polyoma and hybrid DNA molecules

Polyoma virus of the large plaque A-2 strain (21) was used. Polyoma DNA was isolated by the Hirt procedure (22) from infected 3T6 cells.

The recombinant plasmid pAT-Py contains the entire polyoma DNA cloned into the bacterial plasmid pAT 153 (23) in the restriction enzyme Bam-HI cleavage site.

The recombinant plasmid pBR322-Py-Py contains two molecules of polyoma DNA cloned in tandem into the bacterial plasmid pBR322. This plasmid has been described by Fried et al. (24).

Loading of DNA into erythrocyte ghosts

Human erythrocytes collected in heparin or 2 mM EDTA was washed three times in Ca^{2+} , Mg^{2+} - free phosphate buffered saline

(PBS) pH = 7.2 and centrifuged at 1 200 rpm for 10 min. Hemoglobin - free ghosts were prepared by resuspending in 50 vol 10 mM Na-phosphate buffer and 2 mM ATP, pH = 7.2, followed by centrifugation at 10 000 rpm for 10 min in a Sorvall SS 34 rotor at 4°C and washed three to four times in the same buffer.

The white pellet was resuspended in the washing buffer and transferred to an Eppendorf tube. After centrifugation, 50 µl of the pellet (2×10^8 ghosts) were transferred to the DNA (5 µg) in 15 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.6), held on ice.

The ghost-DNA solution was then frozen (-70°C)/thawed (37°C) three times before resealing by adding 1/10 vol of 10 x PBS with reverse concentrations of NaCl and KCl (to restore isotonicity), the ghosts were kept on ice for another 5 min and then incubated at 37°C for 45 min to complete resealing.

Polyethylene - glycol (EPG) fusion of DNA loaded ghosts with cells

The cells Rat-1 or 3T6 were trypsinized and resuspended in DMEM without fetal calf serum (approximately 5×10^5 cells were used).

Then the DNA-loaded ghosts (5×10^6 - 1.5×10^8) were added and the cell/ghosts solution were spun down at 3 000 rpm for 6 min.

The pellet was treated with 1 ml PBS:polyethylene glycol 6 000 1:1 (v/w) (PEG) (Koch-Light) for 1 min at 37°C and 9 ml medium were slowly added and mixed very well.

The cells were spun down at 1 200 rpm for 5 min, resuspended and seeded out in complete medium either in flasks or petridishes (some with coverslips). The cells were incubated in the CO₂-incubator at 37°C.

The fusion could also be performed by using cells growing in monolayer on coverslips. Then the DNA-loaded ghosts were spun down on the coverslip with the cells and 1 ml PEG was added. After 1 min the PEG was carefully washed away and the cells on the coverslips were incubated in complete medium.

Detection of T antigen by immunofluorescence

The expression of polyoma T antigen was analyzed by indirect immunofluorescence. Infected or transfected cells growing on coverslips were fixed with acetone for 10 min at -20°C, followed by incubation with rat anti-Py-tumor serum for 30-45 min at 37°C.

After careful rinsing with PBS three times they were treated with fluorescein-conjugated rabbit anti-rat immunoglobulin serum (DAKO, Denmark) for 30 min at 37°C. After washing the coverslips in PBS they were mounted in a drop of glycerol/PBS (1:1 vol/vol) on a glass slide and examined by fluorescence microscopy.

Slide of transformed cells

Rat 1 transformants were selected in soft agar after transfection with Py DNA. DNA-treated cells were transferred to semi-solid DMEM containing 0.3% agar (Sea Kem ME) over a preformed layer of medium containing 0.45% agar prepared as described by MacPherson and Montagnier (25). After solidification at room temperature for 30 min the petridishes were incubated in a moist incubator at 37°C for up to 1 month.

Colonies of transformed cells (at least 20 cells) were removed from the agar, expanded and transferred to Coster trays and flasks for further growth before being tested for the presence of Py DNA.

Chromatography on DND cellulose

Purified DNA samples were chromatographed on benzoylated-naphtoylated DEAE (BND)-cellulose to enrich for replicating molecules (26).

Agarose gel electrophoresis of DNA

DNA was extracted from cells as described by Hirt (22) and purified by treatment with proteinase K (Boehringer) and RNase A (Worthington Biochemicals) followed by phenolextraction. After ethanol precipitation DNA was analyzed by electrophoresis on 1% agarose (Sea Kem ME) gels. DNA was visualized either by staining with ethidium bromide or after transfer to nitrocellulose filter papers by hybridization to nick translated probes (27,28).

Electron microscopy

Portions of the Hirt supernatants were spread with an aqueous droplet technique. The DNA was diluted in 50 µl of 0.05 mg/ml cytochrome c (Grade VI, Sigma), 0.5 M ammonium acetate, pH = 8.0, and was allowed to stand as a droplet on Parafilm plastic for 10 min. A Parlodion (Balzers Union) covered 300 mesh copper grid (Polaron Equipment Ltd) was brought in brief contact with the surface of the droplet, then with a droplet of 0.25 M ammonium acetate. The grid was stained in 25 µM uranyl acetate (Fluka AG) in

90% ethanol for 40 sec and destained in 90% ethanol for 40 sec.

Grids were shadowed with platinum/palladium (Polaron Equipment Ltd). The DNA was visualized in a JEOL JEM-100CX electron microscope at 10 000 X magnification. Circular or replicating molecules were photographed and redrawn at 10 X further magnification. Contour lengths were measured with a Hewlett-Packard 9874A digitizer connected to a Hewlett-Packard 9845B computer loaded with a program derived from the length measuring program of Hewlett-Packard. Lengths were calculated relative to an internal length standard, Py circular DNA (5294 base pairs (29)).

RESULTS

The efficiency of transferring DNA to cells

The transfer of DNA to cells was analyzed by three independent methods, (i) by following the incorporation of nick translated DNA into ghosts and cells, (ii) by analysis of polyoma T antigen expression and (iii) by analysis of transformation efficiency.

(i) Incorporation of labelled DNA into recipient cells. The amount of DNA integrated into loaded ghosts was assessed by determining the percentage of radioactive pAT plasmid DNA remaining associated with ghosts after treatment by detergent NP-40 (1%). When using Tris or TE buffer alone no significant amount of material remained associated with ghosts; TE buffer in combination with freeze-thawing resulted in a significant fraction remaining associated with ghosts after NP-40 treatment (Table 1). As can be seen, we routinely obtain between 6-14% of added DNA to be stably associated with ghosts. The results also demonstrate the importance of including the freeze-thawing in the loading procedure. Ghosts loaded with DNA remained stable for several days and no decrease in the amount of DNA associated with ghosts was noticed for seven

Table 1. Loading efficiency of radioactive pAT 153 DNA into human erythrocyte ghosts.

Method	DNA bound	DNA resistant to 1% NP-40
TE-ghosts + DNA	5 - 7%	0.5%
TE-ghosts + DNA, freeze-thaw	10 - 20%	6 - 14%

Figure 1. Autoradiograph of 3T6 cells transfected with ^3H -labelled pAT 153 DNA. The cells were fixed at 2.5 h post fusion. Exposure: 14 days.

ral days under storage of loaded ghosts at 4°C .

The loading of plasmid pAT 153 DNA (MW 2.6×10^6 , 3740 bp) and polyoma form I DNA gave equivalent results. Even loading of plasmid pBR322-Py-Py (15.000 bp) was as efficient (data not shown). The amount of polyoma form I DNA found in ghosts as NP-40 resistant material was linearly related to the amount of DNA (0.1-10 μg) used for loading, using the conditions outlined in Methods. This procedure resulted in incorporation of 8-800 genome equivalents of ^3H -labelled polyoma DNA, respectively, into ghosts. After loading, ghosts containing ^{32}P -labelled pAT 153 DNA, were fused with 3T6 cells on coverslips as described. The cells were incubated after fusion in complete medium and at 2 and 6 hours after fusion DNA was extracted. At those timepoints 2-5% of the radioactive DNA contained in ghosts were found as TCA precipitable material in the cells. This implies that 0.1-0.7% of added DNA can be extracted from recipient cells.

Autoradiography was also used in an attempt to localize the transferred DNA in the cells. Again ghosts loaded with ^3H -labelled pAT 153 DNA were fused with 3T6 cells on coverslips and

Table 2. Polyoma gene expression after transfection with polyoma DNA or cloned polyoma DNA into mouse 3T6 cells.

DNA	Fraction of T-antigen positive cells after		Hemagglutination test positive after
	72 h post fusion	96 h post fusion	
Py	40%	60%	9 days
pAT-Py	x	x	-
pBR 322-Py-Py	x	10%	6 days

x None or very weak

at 2.5 and 20 hours after fusion cells were fixed and mounted for analysis by autoradiography. At 2.5 hours post fusion roughly 30% of the cells contained grains mainly localized over the nuclei. Such cells are shown in Fig. 1. Also at 20 hours post fusion grains could still be found over the nuclei.

(ii) Analysis of polyoma T antigen expression. The polyoma system was used in order to monitor the genetic activity of transferred DNA. Polyoma DNA was transferred to mouse 3T6 cells and T antigen expression and virus production was analyzed at various time-points after fusion. Hybrid DNA molecules, pAT-Py and pBR322-Py-Py, were also included in the experiment. In pilot experiments it was found that no T antigen positive cells were observed until 60-70 hours post fusion. Thus, as shown in Table 2, T antigen expression and virus production was analyzed at 72 and 96 hours post fusion. After transfer of Polyoma DNA 40% of the cells were T antigen positive at 72 hours post fusion. The percentage of positive cells increases with time and by 96 hours 60% of the cells are T antigen positive. In this experiment polyoma virus was produced and could be detected by haemagglutination analysis at 9 days post fusion. We thus attribute the high number of positive cells to the fact that 3T6 cells are permissive for polyoma and that some of the cells are positive because of a secondary virus infection. To test this hypothesis experiments were carried out in the presence of Anti-polyoma serum. This serum was earlier tested and found to inhibit a normal virus infection. In the presence of antiserum, approximately 20% of the cells were T antigen positive at 72 hours post fusion. No increase in the amount

Figure 2. Rat-1 cells growing in soft agar 14 days after transfection with polyoma DNA. To the left: 2 μ g polyoma DNA transferred to $2 \cdot 10^4$ cells. To the right: mock fusion with same amounts of DNA and cells but without ghosts.

of positive cells was detected with time. Thus, we conclude that at least 20% of the cells receive Polyoma DNA through the fusion with DNA-loaded ghosts.

When using pAT-Py hybrid DNA no clearly T antigen positive cells were obtained, despite the fact that polyoma DNA here is cloned in such a way that the coding potential and regulatory unit for production of the proteins are unbroken. In contrast the hybrid DNA molecules pBR322-Py-Py which contains 2 polyoma molecules in head to tail tandem at 96 hours post fusion gives rise to T antigen positive cells and virus production.

(iii) Transformation frequency of cells after fusion with Polyoma or cloned Polyoma DNA. In the experiments the DNA loaded ghosts were fused with the established cell line Rat 1. When this cell line is infected at a high multiplicity with polyoma virus approximately 1-5% of the cells become transformed.

In a first series of experiments 1 μ g of polyoma DNA was fused into Rat-1 cells. As a control unloaded ghosts were fused with cells in the presence of 0.1 μ g polyoma DNA. Cells were allowed to grow on the coverslips for 24 hours post fusion and at that timepoint they were transferred to soft agar. Colonies were counted at 2-4 weeks after fusion. Fig. 2 shows the results from one experiment and in Table 3 is listed the number of positive colonies in two separate experiments. As can be seen approximately

Table 3. Transformation frequency of Rat-1 cells after transfection with polyoma DNA.

Expt	Ghosts	No. of cells transfected	No. of colonies in agarose / μg DNA
1	DNA loaded	$4 \cdot 10^3$	40
1	Control	$4 \cdot 10^3$	0
2	DNA loaded	$2 \cdot 10^4$	160
2	Control	$2 \cdot 10^4$	0

1% of the fused cells become transformed.

The transformation frequency using hybrid DNA (pAT-Py and pBR322-Py-Py) were also tested. In this case frequencies of transformation were also found to be about 1%.

Separate colonies were isolated from soft agar and grown in mass cultures. DNA isolated from such cells was analyzed by the Southern-transfer procedure (27) and found to contain polyoma DNA, both free and integrated.

The so established cell lines were all found to be tumorigenic in Fischer rats (data not shown).

Analysis of transferred DNA extracted from mouse 3T6 cells after fusion

From the results described above we conclude that the amount of transferred DNA remaining in cells is enough for analysis by e.g. the Southern procedure and by electron microscopy. To increase the number of cells containing transferred DNA, all fusions in the experiments mentioned below were done with trypsinized cells and ghosts in suspension. In one such experiment $10 \mu\text{g}$ pAT-Py DNA was loaded into ghosts and fused with $4 \cdot 10^7$ mouse 3T6 cells. The cells were seeded on Petri dishes and allowed to settle. At time points from 4 to 96 h post fusion the cells were washed with PBS and Hirt supernatants were prepared as described in Methods. Analysis of the extracted DNA with the Southern transfer procedure (27) (Fig. 3) shows an increase of relaxed circular (form II) and linear (form III) pAT DNA up to at least 48 h, indicating a limited replication of the transfected DNA. The very faint bands of supercoiled (form I) pAT-Py DNA suggest that the transfected DNA is not readily super-coiled in the cells. An al-

Figure 3. Southern blot analysis of transferred pAT-Py DNA extracted from 3T6 cells at various time points after fusion.

Hirt supernatants were prepared as described in the text and 10% of each sample, uncut or cleaved with Cla I, was applied to a 1% agarose gel. Probe was ^{32}P -labelled polyoma DNA. A, uncut Hirt supernatants. Lane (1) uncut marker polyoma, (2) uncut marker pAT-Py, (3) Hirt supernatant extracted at 4 h post fusion, (4) 8 h, (5) 24 h, (6) 48 h, (7) 74 h, (8) 96 h, (9) marker Cla I cleaved pAT-Py. B, same as A but Hirt supernatants cleaved with Cla I.

ternative explanation is that although the gels were acid-treated prior to alkali treatment and blotting the transfer of supercoiled DNA may still be poor leading to an underestimation of the amount of form I. Cla I cleavage of the extracted DNA (Fig. 3B), which linearizes pAT-Py, yields the expected form III band. The nature of the faint band migrating between form II and form III in Fig. 3B is unknown.

The main aim with this study was to develop a system with which the replication of hybrid DNA molecules in eucaryotic cells could be analyzed. In a first attempt hybrid DNA (pAY-Py) was isolated from cells at 72 hours post fusion. After purification of the DNA and enrichment of replicating molecules by chromatography on BND-cellulose, the DNA was analyzed by electron microscopy. Only mitochondrial DNA and small circular DNA molecules in a ratio of approximately 1:1 was observed. Very few replicating molecules

Figure 4. Electron micrographs of replicating hybrid DNA molecules extracted from transfected and polyoma superinfected 3T6 cells. DNA was spread with the aqueous technique as described in the text. A, B: Bidirectionally replicating pAT-Py. C: Rolling-circle type pAT-Py. D: Rolling-circle type pBR 322-Py-Py. In A, B and D circular polyoma DNA is included for size comparison. Bar represents 0.5 μm .

of the size of mitochondrial DNA and less was observed.

We thus reasoned that a normal virus infection of cells to which hybrid DNA had been transferred would A) cause a synchronization of the cells in S phase (30) and b) give rise to a high level of large T antigen which is important for replication from the virus origin (30).

Hence hybrid DNA either pAT-Py (10 μg) or pBR322-Py-Py (20 μg)

were transferred to 12×10^6 3T6 cells. At 6 hours post fusion, when the cells had attached to the surface of the Petri dish, the cells were infected with wild type polyoma virus at a multiplicity of infection of 5 pfu/cell. Twentyfour hours later when approximately 50% of the cells are T antigen positive the cells were harvested with the Hirt procedure, further purified as described (31) and analyzed by electron microscopy. The experiment, in which pAT-Py DNA was used, was most carefully analyzed. In this case, 0.6% of the total pool of small circular DNA was found to be of hybrid DNA length. Moreover, 4.5% of the pool of polyoma DNA was found to replicating viral DNA molecules. This value is in the same order as those published previously and demonstrates that the hybrid DNA in those cells do not severely affect virus DNA replication. After enrichment of replicating molecules by chromatography on BND cellulose replicating molecules of hybrid DNA length was observed. Approximately 0.5% of all replicating molecules were of this length and some are depicted in Fig. 4. These molecules were found to be of both the θ form (Fig. 4a,b) and rolling circle type (Fig. 4c) (31). In the case of pBR322-Py-Py only rolling circle type molecules were detected and one such molecule is depicted in Fig. 4d. At the present time a more thorough investigation is difficult to perform since the "background" of replicating polyoma DNA molecules is very high. Moreover, we have observed that approximately 1.5% of the replicating molecules are of various lengths up to that corresponding to that of mitochondrial DNA.

DISCUSSION

In the present paper we describe a method for studying the replication of transferred DNA in mammalian cells. The study can be divided into two parts namely i) development of a system by which large amounts of DNA ($> 10 \mu\text{g}$) can easily be transferred to large amounts of cells ($> 10^7$ cells) and ii) development of a synchronized permissive cell system.

To start with the red blood cell mediated DNA transfection technique was chosen because the fusion vehicle (ghosts) is readily obtained in large quantities and that fusion can be done with cells in suspension. Moreover no restriction, with respect

to cell type, is present as is the case with e.g. the calcium phosphate precipitation method (4). Earlier it has been found that both protein and RNA could be transferred by use of the red blood cell system (5-7,13-16). Moreover this technique has been used for transfer of DNA (18), but the efficiency did not turn out to be better than that described for other methods. The inner side of the erythrocyte membrane is laminated by a two-dimensional network of membrane proteins, which include spectrin, actin, integral proteins and other compounds (32,33). The structural stability of the NP-40 insoluble membrane skeleton is dissolved under hypotonic conditions and absence of divalent cations during ghost incubation. Thus in order to maximize the loss of spectrin and and possibly other membrane proteins it was decided to include EDTA and repeated freeze-thawings during the loading of DNA. Both parameters were necessary to achieve efficient loading of DNA into ghosts (see Table 1). Several variations of the freeze-thawing process has been tested (data not shown) but higher values than a 14% recovery of added DNA has never been obtained. We thus interpret the moderate efficiency that has earlier been obtained with this technique (14,17,18) to be a problem mainly connected with the loading process.

We next analyzed the effect of the size of the DNA on the recovery after loading. Up to a size corresponding to 15.000 base pairs no decrease in recovery was obtained. Fragments with a size of approximated 50.000 base pairs (λ DNA) has been loaded and found to enter the ghosts. When using polyoma form 1 DNA (5.294 base pairs) a linear relationship was found between input DNA and recovery from ghosts when the amount of DNA was varied between 0.1 and 10 μ g of DNA. In this case 10^7 ghosts were used. Since 10^7 ghosts normally are used in a 10:1 ghosts to cell ratio and since the overall recovery was found to be roughly 0.5% we would expect to find 0.05 μ g of added DNA (10 μ g) in 10^6 cells. This value corresponds to the amount of mitochondrial DNA found in such a cell population.

Results obtained both by analysis of the autoradiographs and T antigen positive cells indicate that at least 20% of the cells contain transferred DNA. This value is supported by the high transformation frequency. In conclusion this means that after

transfer of DNA 20% of a cell population will under optimal conditions contain approximately 5.000 molecules per cell, which would be enough material to permit replication analysis of DNA injected into 10^7 cells.

When analyzing T antigen expression after transfer of polyoma DNA no positive cells were found in a period of 60 hours post fusion. After a normal virus infection expression is easily detected at 15 hours post infection at a multiplicity of infection of 5. This means that despite the fact that we get in more polyoma DNA by fusion expression is delayed. This phenomenon is now under investigation by doing experiments in which DNA complemented with various factors, chromatin or virus particles are being injected. The possibility of injecting complemented DNA or virus particles would be a very important property of the red cell system.

Expression of T antigen was hardly detected when hybrid DNA between polyoma and pBR (pAT-PT and pBR322-PY-PY) was used. On the other hand injection of both hybrid plasmids gave rise to transformed cells at a frequency comparable to that of polyoma DNA and in the case of pBR322-PY-PY, virus production was detected. An explanation to this is that the T antigens are expressed after transfer of the hybrid DNA molecules, but at a lower level than that after transfer of polyoma DNA.

When following the fate of injected DNA by the Southern transfer technique it became evident that the amount of pAT-PY DNA increased with time. It is also noteworthy that very little form I DNA molecules were present. This phenomenon could be explained by an inefficient transfer of form I DNA from the gel even though the gel was treated with acid. An alternative explanation is that the hybrid DNA is vulnerable to nuclease attack because of an abnormal chromatin structure, or of a deficient replication process.

DNA samples isolated at various time points of fusion and enriched for replicating molecules were analysed by electron microscopy. None or very few replicating molecules of a hybrid-DNA size were detected even though the amount of hybrid DNA increased with time as demonstrated in Fig. 3. We reasoned that this effect was due to both a low T antigen expression which is important for efficient replication and to few cells in S phase

at time of harvest. Hence an infection with normal polyoma virus was included in the experimental procedure in order to overcome the assumed problems. As shown in Fig. 4 replicating hybrid-DNA molecules were seen at a level which would be sufficient for a thorough analysis. The most important drawback with the technique is the high background of polyoma DNA. Moreover it was found that approximately 1% of the total pool of polyoma DNA corresponded to polyoma DNA dimers. Since our only way of discriminating hybrid DNA molecules and polyoma molecules is by length measurements only 25% of the analyzed replicating molecules larger than polyoma turned out to be of hybrid DNA length. However by introducing e.g. a sucrose gradient centrifugation step prior to BND chromatography probably 90% of the polyoma background could be omitted. Such experiments are presently being done.

In summary this transfection method is one of the few available methods that can be used to study the fate of transferred DNA a short time after transfer. By additional virus infection or by the use of synchronized cells and so called cos cells (34) a detailed replication analysis of transferred DNA will be possible.

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