Direct transfection of viral and plasmid DNA into the liver or spleen of mice

(DNA-mediated gene transfer/papovavirus/transient replication)

THOMAS W. DUBENSKY, BRUCE A. CAMPBELL, AND LUIS P. VILLARREAL

Department of Microbiology and Immunology, Health Sciences Center, School of Medicine, University of Colorado, Denver, CO 80262

Communicated by David W. Talmage, August 9, 1984

ABSTRACT A method for the direct transfection of polyoma viral DNA and polyoma–plasmid recombinant DNA into the liver or spleen of newborn or adult mice was developed. Calcium phosphate-precipitated DNA was injected directly into mouse organs in combination with hyaluronidase and collagenase. Transfected DNA was shown to replicate at moderate efficiency, relative to direct injection of organs with virus. Transfection with viral DNA rapidly led to an acute infection. A polyoma–bacterial plasmid recombinant DNA also was shown to replicate when transfected into mice. With this plasmid, however, genomic-length polyoma DNA rapidly recombined away from the bacterial component and replicated as viral DNA. This method should allow the direct determination of the biological activity of a cloned DNA within a mouse organ.

DNA-mediated gene transfer has been an invaluable tool for investigating such problems as gene expression and regulation (for review, see ref. 1), identification of oncogenes (2), and the genetics of somatic recombination (3). DNA can be introduced into cells in vitro by a variety of techniques. Calcium phosphate precipitation of DNA onto cells (4), and facilitated uptake of DNA in the presence of DEAE-dextran (5), are commonly used methods. In addition, chloroquine may be used to increase the efficiency of transfection (6).

To determine the biological activity of a cloned DNA in the fully differentiated cells of an organ in situ, we developed a method for the direct transfection of DNA into mouse organs. Because the differentiated phenotype of cells in culture is, in general, rather limited, questions relating to tissue-specific expression or replication cannot always adequately be answered in vitro. DNA has been introduced into mice by DNA microinjection into pronuclei of fertilized mouse ova and then implanted into the reproductive tracts of pseudopregnant females to yield transgenic mice (7–9). This technique has resulted in the expression of rabbit β-globin, herpesvirus thymidine kinase, and rat growth hormone in mice. In these mice, the heterologous DNA has been observed to integrate into chromosomes at an early stage of embryonal development, allowing the exogenous DNA to be transmitted through the germ line (10).

Acquiring such transgenic mice is, however, technically difficult and time consuming. In addition, integration of foreign DNA into the germ line may be lethal with some genes (11). Therefore, it would be useful to directly transfect foreign DNA into the organs of mice, as this would allow a rapid and/or transient analysis of introduced DNA. Previously, in vivo transfection of hepatitis B virus (HBV) DNA into chimpanzees and polyoma virus (Py) DNA into mice has been reported (12, 13). Although chimpanzees were injected with HBV DNA in solution, it was not clear if the source of infection was the HBV DNA or the in vitro HBV- transfected liver cells also present in the inoculum. In addition, HBV transfections have an extremely long incubation period, making experimental analysis difficult. Infectious Py could be detected after DNA injection into mice, but only after amplifying virus titers by passage through weanling mice. Therefore, it is difficult to assess the efficiency of this procedure.

We have developed an efficient procedure to transfect calcium phosphate-precipitated Py DNA into mice. This method allows the analysis of transiently replicating DNA in organs. The Py–mouse system is well suited for optimizing the conditions for in vivo DNA transfections. We have directly transfected the liver and spleen of newborn and adult mice and observed replication of DNA at the initial site of transfection. The replication and recombination of a Py/PiAN7 recombinant plasmid DNA also was observed when transfected into the spleens of newborn mice.

MATERIALS AND METHODS

Cells and Virus. The protocol for the propagation of the A2 strain of Py in mouse 3T6 cells has been described (14). For infection of 3T6 monolayers with organ homogenates, organs were dissected under aseptic conditions, followed by extensive dounce homogenization in 2 ml of Dulbecco medium supplemented with 10% fetal calf serum. The cell suspension was then sonicated, and the cell debris was removed by low-speed centrifugation (2000 rpm for 20 min). This supernatant was used to infect subconfluent 3T6 cells.

DNA Preparation. Py viral DNA was extracted by the method of Hirt (15) from infected 3T6 cells. Py/pBR322 recombinants and pBR328 plasmid were grown in the HB101 strain of Escherichia coli (16). Py/PiAN7 recombinants were grown in the E. coli strain MC1061 bearing a p3-derived plasmid as described (17). DNA was extracted from E. coli and purified by centrifugation in CsCl/ethidium bromide gradients.

Enzymes. The restriction endonucleases BamHI and Mbo I were from New England Biolabs. Bcl I, Pst I, and Bgl I were obtained from Bethesda Research Laboratories. T4 DNA ligase and DNA polymerase I were from Boehringer Mannheim. Hyaluronidase (type VI-S) and collagenase (type VII), chromatographically purified, were purchased from Sigma.

Organ Transfection of Mice. Calcium phosphate precipitation of DNA was performed as described by Graham and Van der Eb (4), as modified by Parker and Stark (18). DNA (10 µg) was diluted into 1 ml of Hepes-buffered saline containing (per liter) 8 g of NaCl, 0.36 g of KCl, 0.16 g of Na2PO4·7H2O, 1 g of dextrose, and 5 g of Hepes (pH 7.05) and was adjusted to 125 mM CaCl2. The solution was incubated for 30 min at room temperature. As the calcium in solution was expected to be toxic to mice, the precipitated DNA was

The abbreviations used are: Py, polyoma virus; HBV, hepatitis B virus; dpt, days post-transfection; hpt, hours post-transfection; dpi, days post-infection.

7529
removed from the calcium solution. The fine precipitate that formed had a coarse, milky appearance and was collected by centrifugation for 10 min at 10,000g at 4°C. The supernatant was virtually free of DNA, as determined by UV light absorption, and was discarded. The pellet was then allowed to dry in air.

The inoculation volume used for transfection was 100 μl for adults and 50 μl for neonates. The calcium phosphate-precipitated DNA was resuspended in TNE buffer (10 mM Tris, pH 7.5/0.15 M NaCl/2 mM EDTA). When hyaluronidase or collagenase were used, they were added from 1 mg/ml stocks (in 50% glycerol). Generally, collagenase at 0.1 mg/ml and hyaluronidase at 0.2 mg/ml were injected. Because these enzymes are often contaminated with DNase, transfection samples were resuspended in cold solutions just prior to transfection and used immediately.

The abdominal fur of BALB/c adult mice was sheered to allow visual localization of the organs. The skin was wet with ethanol and then was pulled tightly over the liver or spleen. Under a light ether anesthesia, the mouse was injected with the DNA suspension directly into the organ with a 28-gauge needle. As specified in the Fig. 1 legend, some mice were also injected i.p. with 0.15 mg of chloroquine in 100 μl at 30 min after transfection.

**Analysis of Transfected Organs.** Organs were removed by dissection at the indicated time after inoculation. Nuclei were then isolated by low-speed centrifugation (1500 rpm for 30 min) after extensive homogenization (Dounce homogenizer) of the tissue in reticuloocyte standard buffer (0.25 M sucrose/25 mM NaCl/5 mM MgCl2/10 mM Tris, pH 7.5) with 0.2% Nonidet P-40. Total cellular DNA was extracted from nuclei as described (14). For the dot-hybridization analysis, 10 μg of tissue DNA was loaded onto each dot. Reconstruction experiments were performed to determine the copy number of viral genomes that were present in the dot-hybridization blot analysis. DNA from 2 × 106 3T6 cells was extracted and quantitated to be 10 μg; 11.6 pg of Py viral DNA was calculated to be equivalent to one copy of Py DNA per 2 × 106 cells. Based on this calculation, purified viral DNA was added to calf thymus carrier DNA to obtain the copy levels of viral genomes shown in the legend to Fig. 1. DNA blots were prepared as described (19). Nick-translation of cloned Py DNA (in pBR322) was to a specific activity of 4 × 106 cpm/μg of DNA (20). Washing conditions were as described (14). Dried filters were exposed to Kodak XAR-5 film at −70°C in the presence of a Lightning-Plus intensifying screen (DuPont) for times indicated in the figure legends.

**RESULTS**

**Conditions for in Vivo Transfection.** The conditions for transfection were varied to maximize the replication of Py DNA in the transfected organ. Our previous experience had established that the livers of adults and the spleens of neonates differed with respect to the levels to which viral DNA would replicate after infection (unpublished data). Neonatal spleens were more permissive for virus replication. To determine if this difference were also true after DNA transfection into organs, both adult livers and neonatal spleens were injected with calcium phosphate-precipitated DNA. It seemed likely that the efficiency of DNA entering cells in the transfected organ would be more uniform and that the DNA might have access to a greater number of cells if the cellular integrity of the organ were somewhat disrupted by hydrolytic enzymes. Therefore, various concentrations of hyaluronidase and collagenase were included in the transfections. Also, chloroquine was injected into transfected mice in an attempt to increase transfection efficiency.

The results of these optimization experiments are shown in Fig. 1. Fig. 1A shows the amount of Py DNA present in livers at 4 days post-transfection (dpt) and at 3.5 hr post-transfection (hpt). DNA that varied between 1 and 10 μg was injected. The levels of Py DNA detected in these mice showed substantial variability at both time points. Even in duplicate samples (i.e., 3.5 hpt) DNA was not uniformly detected. This variability was seen in several other mice transfected with calcium phosphate-precipitated DNA (data not shown). In an effort to minimize this variability, organs were transfected by coinjecting the calcium phosphate-precipitated DNA along with various concentrations of hyaluronidase and collagenase. In addition, 5 μg of DNA was used in the transfections so that any increase in efficiency would be more readily apparent. Less variability was observed in the level of Py DNA detected after these transfections. Fig. 1B shows that Py DNA was present in all samples. Because the conditions for probe hybridization, the amounts of DNA loaded, and time of exposure were all exactly equivalent for all the panels in Fig. 1, the level of viral DNA replication can be determined by directly comparing the dot intensities to those in Fig. 1F. It was not clear whether the addition of chloroquine to the transfection inoculum enhanced the levels of DNA found in the transfected liver as the detected level of DNA appears to be only slightly greater with the chloroquine treatment. From these results it was decided that 10 μg of calcium phosphate-precipitated DNA would be used in combination with collagenase at 0.1 mg/ml and hyaluronidase at 0.2 mg/ml as the standard conditions for DNA transfection of adult liver.

Neonatal mice are more permissive to Py infection than are adults (14, 21), and it has been our observation that the levels of Py DNA found in the spleen after intranasal Py infection are higher than the DNA levels found in the liver (unpublished data). The spleens of neonates, however, are more difficult to inoculate and, therefore, were not used in the preliminary experiments. However, as it would be useful to be able to transfect the spleens of neonates, an attempt was made to inject Py DNA into baby mouse spleens. The results of this experiment are shown in Fig. 1C. It appears that the kinetics of Py DNA replication in spleens may be different than in adult livers. The level of Py DNA was low in baby spleens at 4 dpt compared to adult livers. At 10 dpt, though, the Py DNA levels in the neonatal spleen were similar to DNA levels in the adult liver at 4 dpt. The replication of the organ DNA transfection to that of virus infection is shown in Fig. 1D. In neonatal spleens virus replication following infection was most efficient, reaching a level of about 100 copies of viral DNA per cell in the infected organ. This level then fell rapidly to about 1 copy per cell at 10 days post-infection (dpi). In adult livers, however, virus replication following infection appeared to be much more restricted, reaching a level of only 1 copy per cell at 4 dpi. It appears, therefore, that neonatal spleens are, in fact, much more permissive for Py infection than are adult livers. When compared to the various DNA transfections, several observations were made. DNA transfections of neonatal spleens gave a high level of 20 copies per cell but more typically a level of 5 copies per cell. These peak levels, however, occurred at 10 dpi instead of the four-day peak seen after infection. Thus, it appears that the level of Py DNA in the neonatal spleen after transfection is usually 5%, but as high as 20% of the Py DNA level following virus infection of spleens. In comparing the efficiencies of adult liver infection versus transfection, a curious observation was made. Transfected livers gave a high level of 10 copies per cell and a usual level of 5 copies of Py DNA per cell. This is on the order of a 5- to 10-fold higher level than that seen following infection of this organ and is roughly equal to the level of DNA replication observed in the transfected neonatal spleens. These peak levels in adults were at 4 dpi. It seems that transfection of adult liver is more efficient than infection of the same organ.
Fig. 1. Dot-hybridization analysis of transfected mice. (A–E) Values of 10, 1, and 0.1 above the columns refer to micrograms of tissue DNA. (A) Various microgram amounts of Py DNA were injected into the livers of adult mice. Livers were removed at 48 hpt except for those in the bottom two rows, which were removed at 3.5 hpt. (B) Various doses of enzymes were coinjected along with 5 μg of Py DNA into adult livers. A 1× enzyme dose was collagenase at 0.1 mg/ml and hyaluronidase at 0.2 mg/ml. Rows designated as +Ch included an injection of 0.15 mg of chloroquine at 30 min after transfection. (C) Neonatal spleens were transfected with 10 μg of religated plasmid Py DNA without enzymes and assayed at 4 dpt (arrowhead a), with religated plasmid Py DNA/0.5× enzymes and assayed at 4 dpt (arrowheads b and c), with religated plasmid Py DNA/1× enzymes and assayed at 10 dpt (arrowhead d), with viral Py DNA/1× enzymes and assayed at 4 dpt (arrowhead e), and with PCW10 DNA/1× enzymes and assayed at 10 dpt (arrowhead f). (D) Py (10^9 plaque-forming units) was injected into neonatal spleen and assayed at 4 dpt (arrowhead a), into neonatal spleen and assayed at 10 dpt (arrowhead b), and into adult liver and assayed at 4 dpt (arrowhead c). (E) Transfection of adult livers with control DNAs. CT DNA, calf thymus DNA; aq DNA, Py DNA in aqueous solution. (F) Reconstruction experiments for the determination of copies per cell of Py DNA were based on 10 μg of total DNA. All hybridizations were performed under identical conditions. The specific activity of the hybridization probes, (nick-translated Py/pBR328) were all equal. Exposure time was for 72 hr.

The transient transfection efficiency of DNA in solution and the post-transfection stability of nonreplicating (plasmid) calcium phosphate-precipitated DNA are shown in Fig. 1E. DNA in solution appears to be replicated inefficiently, as no Py DNA was detected at 4 dpt (liver injection). This result was reproduced with several mice (data not shown). DNA also was not detected at 4 dpt in mice transfected with calcium phosphate-precipitated pBR328 DNA, indicating an inability of nonreplicating plasmid DNA to persist after transfection into organs. In addition, Py DNA also was not found in untransfected mice. The limit of detection of these studies was about 0.1 copies of Py DNA per cell as can be seen in Fig. 1F.

Replication of Py DNA Post-Transfection. To establish that the Py DNA being detected post-transfection was due to DNA replication and not from the inoculated DNA, the following experiment was done. Py DNA was purified from a recombinant plasmid DNA (Py/pBR322). The Py sequences were excised by cleavage with BamHI and religated prior to transfection. Mbo I endonuclease can differentiate between DNA replicated in mammalian or bacterial cells because it will only cut the unmethylated DNA following replication in mammalian cells. The results of this experiment are shown in Fig. 2, in which lanes 1 and 2 contain supercoiled Py DNA and Mbo I-digested Py DNA, respectively, from Hirt extracts of Py virus-infected 3T6 monolayers. Digestion with Mbo I yielded six bands, whose lengths are noted in the figure. Bands 7 and 8 of 126 and 5 nucleotides in length, respectively, were not visible on this blot. Lane 3 contains an undigested, excised, religated Py/pBR322 clone, and lane 4 is Mbo I-digested. Lane 4 shows that DNA replicated in bacteria is resistant to Mbo I digestion. Lanes 5 and 6 contain spleen DNA, extracted from a neonatal mouse at 10 dpt into the spleen with 10 μg of calcium phosphate-precipitated, religated Py DNA along with enzymes. The supercoiled and linear Py DNA bands visible in lane 5 disappeared after Mbo I digestion, indicating that these bands represent DNA that had replicated after transfection. The bands in lane 6 comigrated with the bands from Mbo I-digested Py DNA propagated in 3T6 cells. This indicates that substantial rearrangement of the Py DNA genome did not occur. The low molecular weight bands from the Mbo I-digested Py DNA were visible in lanes 2 and 6 after longer exposure (not shown). Thus, it is apparent that the majority of the Py DNA detected at 10 dpt in spleens is replicated DNA. Partially degraded DNA often was observed during extraction of DNA from transfected but not infected organs. This degradation might be accounted for by induction of endogenous endonuclease activity because of elevated levels of calcium in the transfected cell (22). Additionally, damage to the organ either due to the precipitated DNA or the hydrolytic enzymes may predispose the organ to degrade DNA. It may be necessary to address this lability of DNA following transfections by using methods for DNA extraction that are more denaturing for proteins, such as those used for RNA extraction.

To establish that transfected organs were producing infectious virus, the cleared lysates from transfected organ homogenates were inoculated onto 3T6 cells. After 12 days, substantial cytopathic effect was evident. DNA was extracted from these cells. Electrophoretic gel analysis and hybrid-
Fig. 2. DNA blot-hybridization with extracted neonatal spleen DNA. The Py DNA used for these transfections was from a Py/pBR322 plasmid. The Py DNA had been excised and then recircularized by ligation. Lanes: Mbo I, digested with Mbo I; Py, Py viral DNA; religated Py, plasmid DNA. Exposure time was for 48 hr.

Transfection with a Py/PIAN7 Plasmid Recombinant. A principal purpose for developing the in vivo transfection procedure was to acquire a method for testing the biological activity of a recombinant DNA in vivo. Therefore, we constructed a plasmid (PCW10) and tested the ability of this plasmid DNA to replicate after transfection into the liver of adult mice. PCW10 is a Py/PIAN7 recombinant plasmid that contains dual Py origin/promoter regions with a complete Py genome (Fig. 3A). Due to the duplicated ori/promoter region, the structure of this plasmid is such that it would facilitate the recombinational excision of an intact Py DNA and possibly lead to virus propagation. The bacterial component used for the plasmid construct was an 885-base-pair plasmid, PIAN7. (17) and was supplied to us by B. Seed. PCW10 was made by ligating the Bcl I–Pst I fragment of Py (Py map units 0.645 to 0.797) to the BamH I–Pst I fragment of PIAN7. The resultant recombinant was cleaved at the unique Bgl I site in the Bcl I–Pst I fragment of Py and ligated to Py DNA linearized by Bgl I cleavage. The structure of PCW10 was confirmed by extensive restriction enzyme analysis (not shown). The DNA of PCW10 includes complete Py early and late transcriptional units, each with dual promoters and separated by the entire PIAN7 sequence. The replication of PCW10 and expression of both early and late transcripts of PCW10 proceeds efficiently when transfected onto 3T6 cells (unpublished data).

The replication of PCW10 after transfection into the liver of an adult mouse is shown in Fig. 3B, where lanes 4 and 5 contained undigested and Mbo I-digested liver DNA at 10 dpt with PCW10. The bands seen in lanes 4 and 5 all comigrated with the undigested and Mbo I-digested Py DNA bands from virus-infected 3T6 cells (lanes 1 and 2). In the PCW10-digested lane, there were no bands that comigrated with the PCW10 DNA grown as plasmid in bacteria (lane 3). The undigested and Mbo I-digested patterns in lanes 4 and 5 suggest that genome-length Py recombinant away from the bacterial component of PCW10 plasmid soon after transfection and then propagated as a virus. No parental PCW10 plasmid DNA was observed.

**DISCUSSION**

Injection of calcium phosphate-precipitated Py DNA along with hyaluronidase and collagenase leads to a reasonably uniform transfection of livers and spleens. However, a substantial variability in DNA replication was observed when these enzymes were absent, especially when low amounts of DNA were used for the transfection. The reasons for this variability were not determined but may be related to the microvariability of the specific site of transfection.

Several differences were noted when the direct virus infection of organs was compared to DNA transfection of organs. In neonatal spleens, the level of Py DNA replication after transfection was usually 5% and up to 20% of that of virus infection of the same organ. The peak of replication, however, was slower in transfected spleens compared with infected spleens, occurring at 10 dpt versus 4 dpi. This difference may reflect some general aspect of the lowered efficiency for transfection. In adult livers, however, an unexpected observation was made. Transfected livers replicated Py DNA to higher levels than infected livers, about a 5- to 10-fold increase. From our previous studies, we have observed that in systemically infected mice, adult livers are not highly permissive sites of Py infection, replicating Py DNA to about 1 copy per cell. This same level of replication was seen here when livers were infected directly with virus. The ability of transfected livers to replicate Py DNA more efficiently than infected livers could be related to the specific nature of why liver cells are not very permissive. It will require further investigation to clarify this question.

DNA was not detected in organs that were transiently transfected with aqueous DNA. Israel et al. (13) inoculated aqueous Py DNA into mice and reported that 0.1 ng of Py DNA was an ID₃₀ dose for Py infection, as determined by an indirect second passage and seroconversion test. Our experiments indicate that aqueous DNA is much less efficient at transient transfection than a calcium phosphate precipitate of DNA. It is our opinion, however, that Py DNA in aqueous solution inoculated parenterally would eventually lead to seroconversion due to a low level of virus replication and amplification that would be below the sensitivity of our analysis.

It appears that nonreplicating (plasmid) DNA is not stable after transfection and is degraded in the organ. At 4 dpt with pBR328 plasmid, DNA was not detected in the adult liver. Nonreplicating Py DNA also appeared to be degraded, since only DNA that had replicated and not the initial injected Py DNA was present. Thus, inactive DNA does not appear to persist in transfected organs and should allow a simpler interpretation of the dot-hybridization analysis.

Transfection with PCW10 gave a genome-length Py DNA that must have recombined away from the bacterial component, PIAN7. No PIAN7 sequences were detected in the
transfected organ. Excision of Py unit-length DNA from PCW10 was not unexpected, but the rapid rate at which this occurred was surprising. Perhaps this construct is unusually able to recombine.

A direct organ transfection procedure should be a generally useful technique allowing the rapid assessment of transient expression and replication of a recombinant DNA in vivo. Our interest has been to define the genetic control of the pattern of Py virus tissue tropism in mice. With this technique, it should be possible to alter the Py DNA sequence in vitro and then to determine the functional roles of both coding and noncoding regions in organ tropism, persistent infection, and tumorigenesis. It also may be possible to exploit Py-derived recombinant DNA to analyze the in vivo activity of many different foreign genes.

We thank N. Jorgensen Soo for her comments on this manuscript. L.P.V. is a Rita Allen Scholar for cancer research and is also supported by National Institutes of Health Career Development Award 2532413. B.A.C. is supported by Public Health Service Training Grant 5T32AI07209 from the National Institutes of Health.