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Delivery of bacterial artificial chromosomes into mammalian cells with psoralen-inactivated adenovirus carrier

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ABSTRACT

Molecular biology has many applications where the introduction of large (>100 kb) DNA molecules is required. The current methods of large DNA transfection are very inefficient. We reasoned that two limits to improving transfection methods with these large DNA molecules were the difficulty of preparing workable quantities of clean DNA and the lack of rapid assays to determine transfection success. We have used bacterial artificial chromosomes (BACs) based on the *Escherichia coli* F factor plasmid system, which are simple to manipulate and purify in microgram quantities. Because BAC plasmids are kept at one to two copies per cell, the problems of rearrangement observed with YACs are eliminated. We have generated two series of BAC vectors bearing marker genes for luciferase and green fluorescent protein (GFP). Using these reagents, we have developed methods of delivering BACs of up to 170 kb into mammalian cells with transfection efficiency comparable to 5–10 kb DNA. Psoralen-inactivated adenovirus is used as the carrier, thus eliminating the problems associated with viral gene expression. The delivered DNA is linked to the carrier virus with a condensing polycation. Further improvements in gene delivery were obtained by replacing polylysine with low molecular weight polyethylenimine (PEI) as the DNA condensing agent.

INTRODUCTION

Most transfection techniques have been developed and optimized for the delivery of DNA molecules of <15 kb in size (1,2). However, molecular biology has certain applications where the introduction of much larger DNA molecules is required. These include gene mapping studies as well the characterization of promoter elements which can be distributed over tens of kilobases of DNA. The current methods of large DNA transfection, cationic lipid-mediated methods (3–6), spheroplast fusion with YAC- containing yeast (3,7–10) and microinjection (11–13), are inefficient with regard to time and material. We have sought to develop an improved system for introducing large DNA molecules into mammalian cells.

Two impediments to improving transfection methods with these large DNA molecules are the difficulty of preparing reproducible and workable quantities of pure DNA (CsCl or column purified) and the lack of rapid assay methods to determine transfection success. Yeast artificial chromosome (YAC) systems are useful for many applications, however, they are difficult to manipulate and it is extremely laborious to prepare microgram quantities of intact YAC DNA free of yeast genomic DNA. We have used instead bacterial artificial chromosomes (BACs) based on the well-characterized *Escherichia coli* F factor plasmid system (reviewed in 14,15). BACs are simple to manipulate and purify in microgram quantities (16–18; see also Web site http://www.tree.caltech.edu). In addition, because replication of the F factor in *E.coli* is strictly controlled and the plasmids are kept at one to two copies per cell, the problems of recombination and rearrangement observed with YACs are reduced (19).

To address the second limit, the lack of a rapid method of quantitating successful large DNA delivery, two series of BAC vectors bearing the easily assayed marker genes luciferase and green fluorescent protein (GFP) were developed. These marked BAC vectors facilitate the titration and optimization of transfection conditions with large DNA molecules.

A method of using adenovirus particles as DNA transfection carriers has been developed (20) and modified to include streptavidin/biotin coupling of the delivered DNA to the virus particle (21) and to use transcriptionally and replicationally silent, psoralen-inactivated adenovirus particles (22–24). This adenovirus-augmented transfection method has been previously shown to deliver DNA molecules of up to 48 kb in size to mammalian cells (22). One advantage of this system over many of the other viral gene delivery systems is that the delivered DNA is condensed and linked to the outside of the carrier virus. This method uses the efficient entry mechanism of adenovirus without being subject to the 8–10 kb size constraint of the existing recombinant adenovirus vectors. Psoralen inactivation eliminates the problems associated with viral gene expression (25).

Here we show that this adenovirus-augmented transfection system is a useful method of delivering BAC DNA molecules of up to 170 kb into mammalian cells. Larger molecules have not yet been tested. The transfection efficiency is comparable with delivery of the same marker genes on small plasmids (5–10 kb).

Initial experiments demonstrated utility of the adenovirus/polylysine system. However, the interactions between polylysine and the large BAC DNA molecules produce complexes with limited solubility. We sought a better polycation to perform the DNA condensation. The cationic polymer polyethylenimine (PEI) can be used to condense and deliver plasmid DNA (26,27). We demonstrate here that a BAC/PEI/adenovirus method is an improvement over both the polylysine/adenovirus method and

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two different cationic lipid methods. We find that the BAC/PEI/adenovirus methods gives ~10-fold greater levels of transient gene delivery than the polylysine/adenovirus system and 2–3 log higher levels than cationic lipid systems (Lipofectamine or Transfectam). The large DNA is delivered intact, as shown by pulsed-field analysis of BAC DNA recovered from transfected cells. Furthermore, in expression studies we find that the large DNA molecules produce exceedingly stable gene expression, relative to small DNA molecules in non-dividing cultures.

MATERIALS AND METHODS

Construction of BAC reporter vectors

The original cloning vector pBeloBac11 was a generous gift of Dr Ung-Jin Kim (California Institute of Technology; 16,18; http://www.tree.caltech.edu). The reporter BAC vectors pLUCBAC and pGreenBAC were constructed by inserting a 2.9 kb CMV-driven luciferase cassette or a 3 kb CMV-driven GFP cassette into the *Xho*I site of pBeloBac11.

Preparation of high molecular weight DNA and partial digestion of DNA

High molecular weight human DNA for cloning was prepared from HeLa cells, embedded in agarose blocks and partially digested with *Bam*HI following the protocol of Shizuya (16). The digested DNA, ranging from 150 to 300 kb, was isolated from 1% low melting point preparative agarose gels (BioRad) run in 0.5× TBE (0.045 M Tris–borate, 0.001 M EDTA, pH 8.0). Electrophoresis was at 14°C using a BioRad CHEF-DR III pulsed-field electrophoresis system. The switch time was 3–11 s at a voltage of 6 V/cm, an angle of 120° and a 12 h running time.

Cloning human DNA into pGreenBAC and pLUCBAC

Gel slices of 100 µl containing the *Bam*HI-digested DNA were treated with β-agarase (New England Biolabs) according to the manufacturer's instructions. Following digestion, the DNA was dialyzed against 10 mM Tris, 0.1 mM EDTA, 750 µM spermidine, 300 µM spermine, pH 8.0, in dialysis cassettes (10 K cut-off Slide-A-Lyzer; Pierce) for 1 h.

Vector DNA pGreenBAC or pLUCBAC was digested with *Bam*HI (Boehringer Mannheim) for 3 h, dephosphorylated and then gel purified by the QIAquick method (Qiagen). Ligations were carried out as described by Dr Ung-Jin Kim (16,18). Before electroporation, samples were dialyzed against 10 mM Tris, 0.1 mM EDTA, 750 µM spermidine, 300 µM spermine, pH 8.0, by spotting on Millipore 0.025 µm VS filters (catalog no. VSWP02500).

Transformation of *E.coli* DH 10B cells (BRL) was carried out by electroporation using a Gibco-BRL cell porator using settings of 330 µF and 4 kV. After electroporation, cells were incubated at 37°C with gentle shaking for 60 min before being spread on LB plates containing 12.5 µg/ml chloramphenicol, 50 µg/ml X-Gal (Bachem) and 25 µg/ml IPTG. White colonies were selected for further analysis.

DNA miniprep method

A 5 ml overnight culture provided sufficient plasmid for restriction digest analysis. The culture was centrifuged and resuspended in 200 µl cold (4°C) P1 (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A), lysed in 250 µl P2 (200 mM NaOH, 1% SDS) for 3 min at room temperature and then incubated on ice for 5 min after adding 150 µl P3 (3.0 M potassium acetate, pH 5.5). After centrifugation (20 000 *g*), the supernatant was collected and the centrifugation repeated. The DNA in the supernatant was then precipitated by adding 0.6 vol isopropanol. After pelleting the DNA by centrifugation at 20 000 *g* for 30 min at 4°C the DNA was washed once in cold 70% ethanol, centrifuged, dried and resuspended in 50 µl TE.

Large scale DNA preparation

Large scale BAC DNA preparation was carried out as described by Shizuya (16,18) with the following modifications. An overnight culture (2 l) was collected by centrifugation, the bacteria were lysed with P1 (10 ml/250 ml starting culture) and centrifuged as described in the manufacturer's instructions (Qiagen) for a low copy number plasmid. Two centrifugations were performed and then the equivalent of 500 ml bacterial culture were added to one Qiagen 500 tip. After the final wash, the DNA was eluted from the column by the addition of 5 × 3 ml prewarmed (65°C) elution buffer (1.25 M NaCl, 50 mM Tris–HCl, pH 8.5, 15% ethanol). The eluted DNA was precipitated by the addition of 0.7 vol isopropanol and incubation overnight at –20°C and the precipitated DNA was collected by centrifugation, washed, dried and resuspended in TE. The DNA was further purified by a CsCl centrifugation (28.5 g CsCl/28 ml TE) at 64 000 r.p.m. for 20 h. The BAC DNA band (the lower band as visualized by UV light) was removed and dialyzed extensively against TE in a dialysis cassette. A 2 l culture of bacteria typically yielded 80–100 µg 130–160 kb BAC DNA.

Transfections

Polylysine/adenovirus transfections. These were carried out as previously described (21,22,24) with the single modification that DNA solutions contained 1 M NaCl in an attempt to limit precipitation of the large DNA. Briefly, adenovirus (Ad5 dl1014) (32), grown in W162 cells (30), was biotinylated and inactivated with psoralen as described (23). Virus samples were quantitated by protein concentration (BioRad Bradford assay with BSA as standard) using the relationship 1 mg/ml protein = 3.4×10^{12} adenovirus particles/ml (37). Virus (1.2×10^{10} particles) was diluted in 150 µl HBS (150 mM NaCl, 20 mM HEPES, pH 7.4) and mixed with 1 µg streptavidin–polylysine (21) in 150 µl HBS. After a 30 min incubation at room temperature, this was mixed with 100 µl 20 mM HEPES, pH 7.4, 1 M NaCl and 6 µg DNA. After a further 30 min incubation, 4 µg transferrin–polylysine (20) in 100 µl HBS were added. After a final 30 min incubation, aliquots of the mixture were added to the cell cultures as indicated in the figure legends.

Cationic lipid transfections were performed as described by the suppliers for Transfectam (36; Promega) and Lipofectamine (Life Technologies).

PEI/adenovirus transfections. A 10 mM, pH 7, solution of polyethylenimine (PEI, mol. wt 2000; Fluka) was prepared as described by Boussif *et al.* (26). A 10 mM solution of PEI was prepared by diluting 9 mg 50% PEI in 10 ml water, adjusting the pH to 7 with HCl and passing the solution through a 0.2 µm filter. To prepare transfection complexes, PEI (24 µl 10 mM PEI, pH 7) and 6 µg BAC DNA were diluted with 250 µl HBS in separate tubes. The two solutions were then carefully mixed by adding the PEI solution dropwise to the DNA and incubating at room

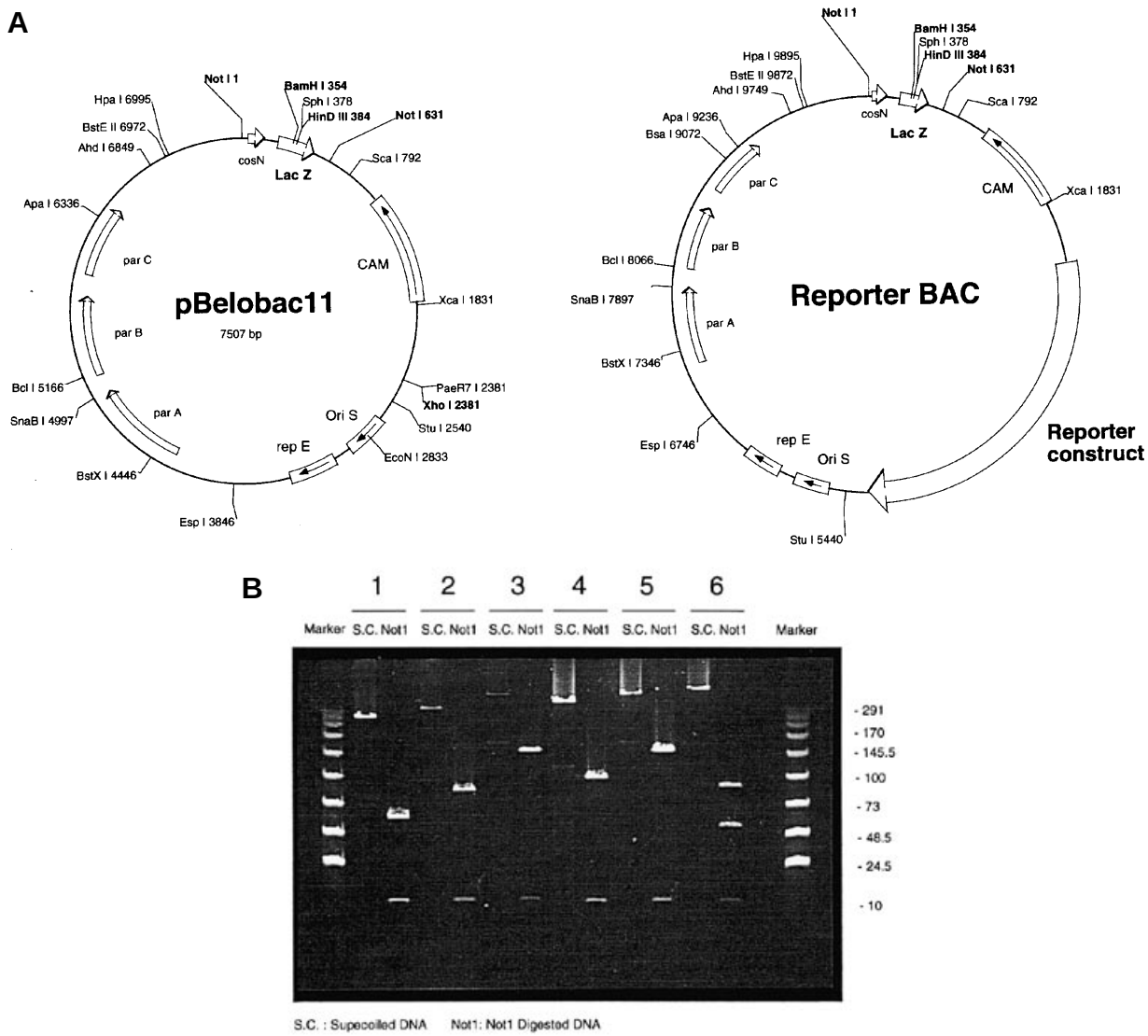

Figure 1. (**A**) Construction of BAC reporter vectors. The BAC reporter vectors were derived from pBeloBac11 (16,18) and include pLUCBAC containing a CMV-driven luciferase cassette and pGreenBAC containing a CMV-driven GFP construct (see Materials and Methods for additional details). (**B**) Analysis of various BAC clones by pulsed-field electrophoresis. BAC DNA from six clones was analyzed by pulsed-field gel electrophoresis in either uncut, supercoiled form (S.C.) or digested with the restriction enzyme *Not*I (Not1). Marker sizes are indicated in kilobases.

temperature for 10 min. Adenovirus (3 µl 2.5×10^9 particles/µl in HBS containing 40% glycerol, psoralen-inactivated as described above) was then added and the material was allowed to incubate for a further 10 min before the complex was supplied to cells. For transfection, 50 µl transfection complex were used for a 1.5 cm dish (20 000 primary cells or 30 000 HeLa cells) in 250 µl medium (no serum). Cells were kept in medium lacking serum during the transfection period of 4 h, followed by a change to medium with normal serum levels. When transfecting primary fibroblasts, polymyxin B (10 µg/ml) was included during the transfection period to inhibit cellular responses to lipopolysaccharide (33).

Southern analysis

W162 cells were transfected using the PEI/adenovirus method described above. The cells were harvested at the indicated times and genomic DNA extracted (34). Genomic DNA (10 µg) was loaded into each lane of a 1% agarose (pulsed-field grade; BioRad) gel. Pulsed-field electrophoresis was at 14°C in 0.5× TBE using a switch time of 3–11 s at a voltage of 6 V/cm, an angle of 120° and a 12 h running time. After depurination in 0.25 M HCl for 30 min and denaturation in 0.5 M NaOH, 1.5 M NaCl the DNA in the gel was transferred to a nylon filter (Hybond). The membrane was probed with a radiolabeled 600 bp BAC probe and then exposed to film (Kodak XAR) overnight at –70°C.

RESULTS

Construction of reporter BAC plasmids

To facilitate the optimization of transfection parameters we developed a series of reporter BAC constructs. Using pBeloBac11 as the starting vector (18), either a CMV-driven GFP cassette or luciferase cassette was subcloned into the vector (Fig. 1A). These

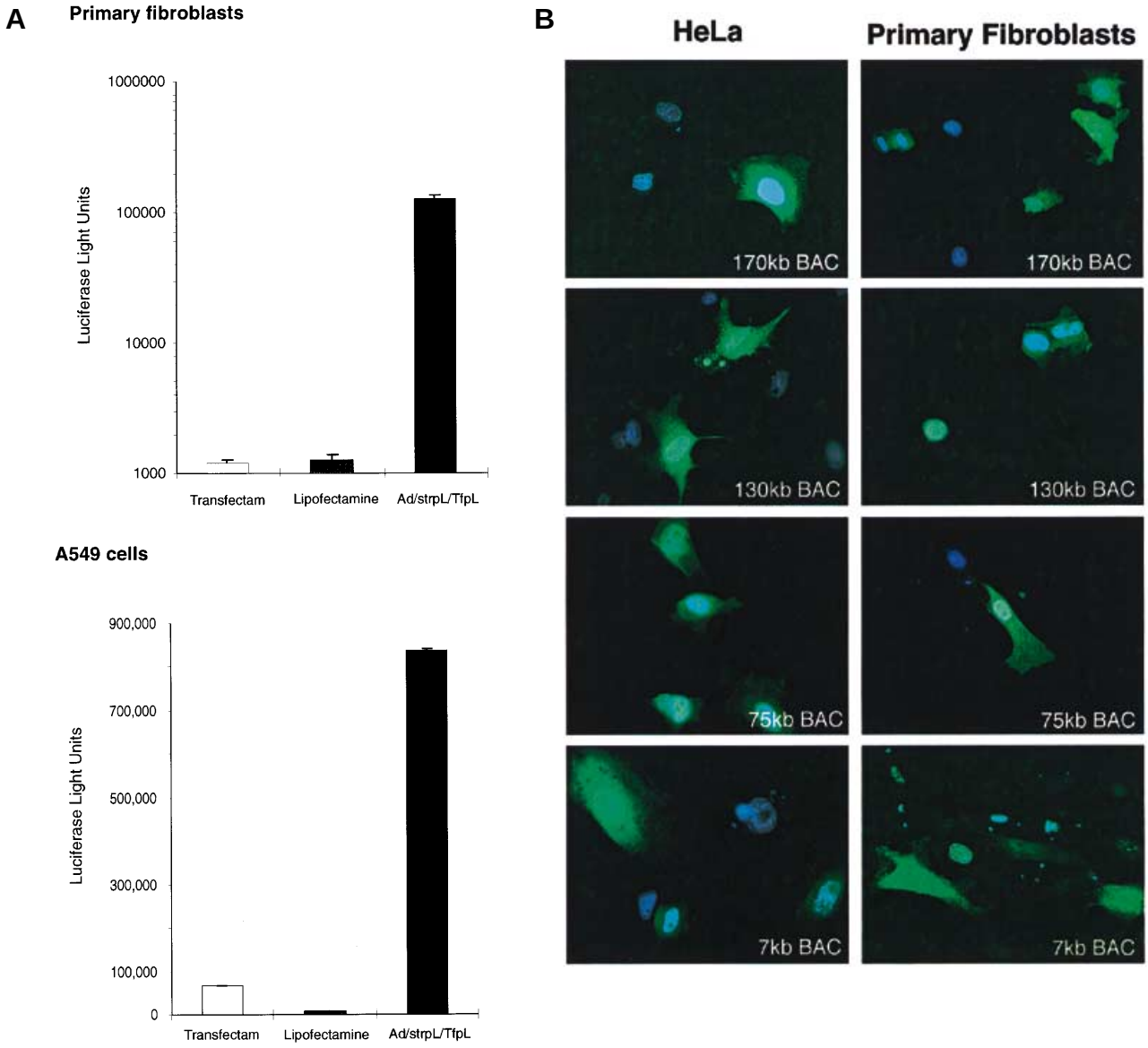

Figure 2. (**A**) Transfection of luciferase BACs. Using the indicated methods, human primary fibroblasts (left panel) were transfected with a 100 kb luciferase BAC and A549 cells (right panel) were transfected with a 130 kb luciferase BAC. Luciferase levels in extracts of the transfected cells were measured at 6 days post-transfection and the average of three experiments ± 1 SD are indicated. Details of transfection complex formation are in Materials and Methods. (**B**) Transfection of GFP BACs. HeLa cells and primary fibroblasts were transfected with pGreenBAC of the indicated sizes using the adenovirus/polylysine method. At 3 days post-transfection, cells were fixed, stained with 4′,6-diamidino-2-phenylindole to visualize DNA and examined by fluorescence microscopy. Images were captured by CCD and assembled using Adobe Photoshop. GFP expression generates a green signal, both cellular and transfected DNA generate a blue signal.

constructs were then used to produce a series of mini BACs with varying insert sizes. Human genomic DNA was partially digested with *Bam*HI, size fractionated twice by pulsed-field electrophoresis to enrich for DNA molecules larger that 70 kb and then ligated into the BAC reporter vectors. It has been previously demonstrated that BACs can be made with inserts of up to 300 kb (16). We found that after two pulsed-field size selections of the starting DNA, the average insert size in the BAC vectors was between 70 and 170 kb. GFP and luciferase BACs containing a range of insert sizes (70–170 kb) were selected and used for analysis of transfection conditions. A pulsed-field gene analysis of six different GFP BAC clones is shown in Figure 1B. The clones were run uncut and digested with *Not*I. Supercoiled DNA migrates very slowly on pulsed-field gels (28,29) but the actual size of the BACs can be determined after *Not*I digestion, which excises the insert from the BAC vector.

Comparison of Lipofectamine, Transfectam and adenovirus

We compared several different methods of delivering BAC DNA into mammalian cells. We introduced a 100 kb luciferase reporter BAC into either A549 cells or primary human fibroblasts using two commercially available cationic lipid DNA delivery compounds (Lipofectamine and Transfectam) and adenovirus/polylysine complexes. Successful gene delivery was assessed by measuring luciferase (Fig. 2A). In both cell types, the lipid-based methods gave weak but detectable delivery of the 100 kb plasmid into the cells. In sharp contrast, delivery of the BAC with the

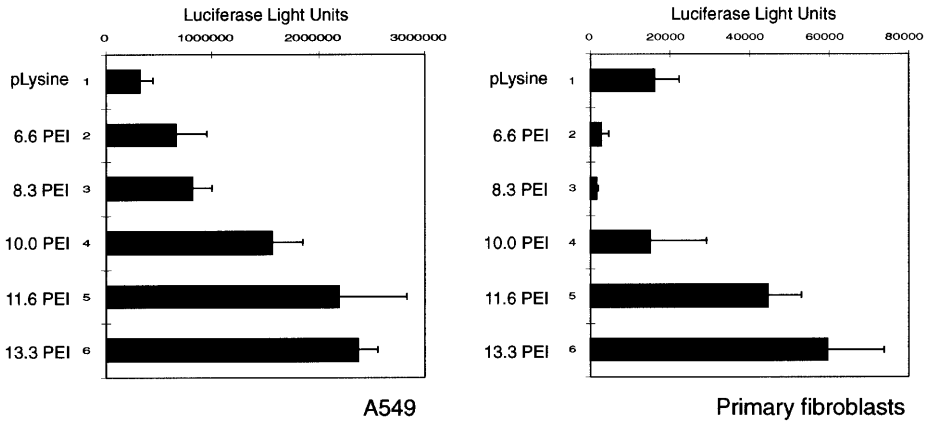

Figure 3. Comparison of polylysine and PEI as condensing reagents. A549 cells (left panel) or human primary fibroblasts (right panel) were transfected with a 100 kb luciferase reporter BAC using the adenovirus/polylysine system or the adenovirus/PEI system. Luciferase activity was measured at 72 h post-transfection with the average of three experiments ± 1 SD being shown. pLysine, polylysine; the PEI nitrogen:DNA phosphate ratio is indicated for the PEI samples.

adenovirus/polylysine complexes resulted in 10- to 100-fold higher levels of luciferase activity in these cells (Fig. 2A).

The fraction of the cell population successfully transfected was determined using the GFP BAC series. GFP BACs bearing a 75, 130 or 170 kb insert as well as the parental GFP BAC (7 kb) were delivered into either HeLa cells or primary human fibroblasts (Fig. 2B). Examples of GFP-positive cells generated by the transfection are presented; quantitation of the percentage positive HeLa cells gave the following values: 170 kb, 7.4%; 130 kb, 11%; 75 kb, 7.7%; 7 kb, 39%. The percentage of GFP-positive primary human fibroblasts was lower (~2-fold) than for HeLa cells, but had the same ratio when comparing BAC with plasmid transfection rates.

Replacing polylysine with PEI

Our initial efforts to optimize delivery of BACs indicated that these large DNA molecules had a propensity to precipitate when exposed to polylysine, even under the relatively dilute DNA conditions employed (6 µg/ml). Under the best conditions of transfection, the BAC/adenovirus/polylysine complexes were only partially soluble, as evidenced by the turbidity of the mixtures and our ability to sediment the material with a low speed centrifugation (results not shown). This precipitation was partially alleviated by the inclusion of higher ionic strengths during complex assembly. Inclusion of 0.5–1 M NaCl during complex assembly resulted in a modest improvement in gene transfer (results not shown). However, as the presence of high ionic strength in the transfection complex is cumbersome and can be toxic to cells, a better polycation for the condensation role was sought.

The polycation PEI is a simple DNA condensation and delivery agent (26,27). DNA was condensed using a titration of 6–12 PEI nitrogens/DNA phosphate [9 is the optimum identified by Boussif *et al.* (26) for small plasmid DNA]. This material was mixed with psoralen-inactivated adenovirus and the resulting complexes were supplied to cells. Presumably, regions of positive charge on the PEI/DNA complex allow binding to exposed negatively charged residues on the hexon of the adenovirus capsid. Delivery with PEI-condensed DNA was compared with the optimum transfection obtained with the adenovirus/polylysine complexes. In both A549 cells and primary human fibroblasts, PEI titration gave 5- to 10-fold higher levels of gene delivery than with the polylysine/adenovirus complexes (Fig. 3).

Table 1. Sizes of DNA/PEI/adenovirus complexes[a]

	Size ± SD (nm)	
	Sample prepared in 20 mM HEPES, pH 7.4, 150 mM NaCl	Sample prepared in 20 mM HEPES, pH 7.4
Adenovirus type 5	126 ± 3	121 ± 7
130 kb BAC/PEI		
20 min	398 ± 24	465 ± 66
45 min	486 ± 51	591 ± 39
130 kb BAC/PEI plus adenovirus		
20 min	525 ± 28	496 ± 95
45 min	662 ± 51	448 ± 20
60 min	562 ± 27	538 ± 73
180 min	635 ± 32	716 ± 54

[a]Sizes were determined by dynamic light scattering using a Brookhaven BI-90 particle sizer fitted with a BI-9000 AT correlator (Brookhaven Instruments Corp., Holtsville, NY). Samples were illuminated with a Lexel 95E argon laser operating at 0.5 W. The listed values are the average of four determinations with the standard deviation indicated.

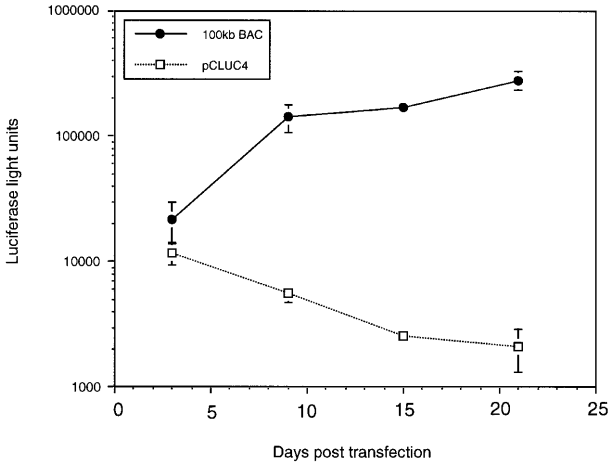

Figure 4. Large DNA gives stable reporter gene expression. Human primary fibroblasts were transfected with adenovirus/PEI complexes prepared with either a 100 kb luciferase reporter BAC or a 7 kb CMV–luciferase reporter plasmid (pCLuc). At the indicated times post-transfection luciferase activities in extracts (standardized for protein content) were measured. After transfection, the cells reached confluence at approximately day 7 and were not further subdivided. The values are the average of three experiments ± 1 SD.

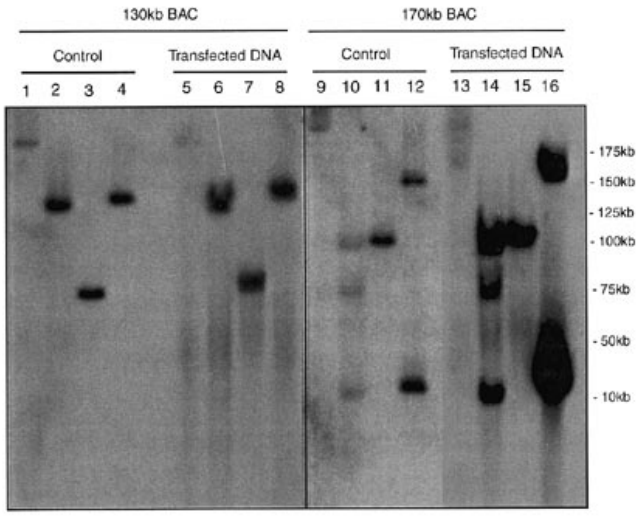

Figure 5. The transfected BAC DNA is intact and in an extrachromosomal supercoiled form. W162 cells were transfected with a 130 kb *LUC* BAC or a 170 kb GFP BAC using PEI/adenovirus. At 3 days post-transfection, the DNA was extracted from the cells and analyzed by pulsed-field gel electrophoresis. Southern analysis was performed using a radiolabeled BAC probe. DNA was run uncut or digested with rare cutting enzymes. Lanes 1, 5, 9 and 13 are undigested DNA; in lanes 2, 6, 10 and 14 the samples were digested with *Not*I; in lanes 3, 7, 11 and 15 the samples were digested with *Pme*I; in lanes 4, 8, 12 and 16 the samples were digested with *Sfi*I.

The size of PEI/BAC/adenovirus transfection complexes

The size of the virus/PEI/DNA complexes might play a role in successful delivery of this material. Light scattering was used to measure the sizes of both PEI/DNA and PEI/DNA/adenovirus complexes. Adenovirus particles alone, when measured in either HBS (150 mM NaCl, 20 mM HEPES, pH 7.4) or in 20 mM HEPES, pH 7.4, alone, display a diameter of 115–125 nm (Table 1), as previously demonstrated with this technique (31). The 130 kb BAC DNA molecule is condensed to a 400–600 nm particle, with slightly smaller sizes obtained at lower ionic strength and a slight tendency to grow to larger dimensions (Table 1). Addition of the 120 nm adenovirus particle to condensed PEI/DNA generates 500–700 nm species that are relatively constant in size over a 3 h measurement period. These sizes are consistent with BAC/PEI/adenovirus transfection complexes containing condensed DNA complexed through ionic interactions with one to two adenovirus particles. This is also consistent with the stoichiometry of the system: 6 µg 130 kb BAC is 4×10^{10} molecules, the complex was prepared with 12×10^{10} virus particles.

Delivering a gene on a large BAC provides more stable expression than delivery with a small plasmid

A comparison of gene expression after delivery of small versus large DNA molecules was performed in primary cells. Gene expression from a transfected small plasmid (e.g. 7 kb pCLuc) declines over a 15 day period to background (Fig. 4). The basis of this decline is currently not known, but it could be due to entry of the adenovirus/DNA complex triggering a stress response in the transfected cell or to transcriptional silencing of the transfected gene. Gene expression from a 100 kb BAC follows strikingly different kinetics (Fig. 4). Expression increased over the first 10 days and was maintained at a high level throughout the course of the 21 day experiment (Fig. 4). The primary fibroblasts were dividing over the first 7 days and the culture then slowly became quiescent. Such stable gene expression was also seen with all the other luciferase BACs tested (data not shown), suggesting that the stability is not due to the specific human sequences carried by the 100 kb BAC, but rather a feature of large DNA molecules. The mechanism responsible for this phenomenon is not clear. Recent studies have shown that plasmid DNA that has no human origin of replication or nuclear retention signal is quickly lost from dividing cells (35), with plasmids that possess a human origin of replication or nuclear retention signal showing much more stable gene expression whose kinetics were similar to those seen with the BAC plasmids. The large human DNA insert found in the BACs may act as a nuclear retention signal by allowing the BAC to associate with the chromatin or even provide an origin of replication. This phenomenon may be of use for gene therapy applications and will be investigated further.

Integrity of delivered DNA

We were interested in determining the integrity of the transfected BAC DNA. There remains the possibility that fragmentation of the large DNA molecules occurs during the manipulation and the transfection process, with the observed gene expression signal a function of small DNA molecules. The African green monkey cell line W162 (30) was used for this experiment to facilitate hybridization analysis of the human sequences carried in the BAC. We transfected these cells with either a 130 or a 170 kb BAC using optimum PEI/BAC/adenovirus conditions. Total DNA was isolated from W162 cells 3 days after transfection and pulsed-field Southern analysis was performed to determine both the integrity and the chromosomal state of the BAC DNA (Fig. 5). The left panel shows the control 130 kb BAC (lanes 1–4) and DNA from cells transfected with the 130 kb BAC (lanes 5–8); the right panel shows the same variations with the 170 kb BAC. With

both BACs, undigested BAC DNA runs in the supercoiled form and when digested with *Not*I (lane 2), *Pme*I (lane 3) or *Sfi*I (lane 4) gives a distinctive restriction pattern. The same hybridization pattern is observed with DNA isolated from transfected cells. Uncut DNA runs like the supercoiled, uncut BAC before transfection; samples cut with the restriction enzymes generate similar patterns. An important feature of the hybridization pattern is the predominance of a DNA pattern that would be produced by non-degraded or intact BAC molecules. Although there is a small amount of smaller hybridizing species in DNA isolated from the transfected cells, >95% of the hybridization signal is accounted for by intact species. Similar levels of intact DNA were observed in samples harvested at day 10 (results not shown).

DISCUSSION

The DNA transfer system described here is a novel method of introducing BACs into eukaryotic cells using BAC DNA condensed with PEI and linked to adenovirus particles by ionic interactions. The reagents are either commercially available (PEI) or simple to prepare (adenovirus) and no special equipment is required to establish the method. Part of the simplicity of this system derives from the ability of PEI to both condense DNA as well as to serve a bridging function to link the DNA to the adenovirion. Furthermore, under appropriate conditions, higher molecular weight PEI can function alone to facilitate DNA delivery (26,27) due to the ability of PEI to absorb large quantities of protons, either generating an osmotic shock in the endosome or slowing the degradative action of the pH-dependent lysosome compartment. From the work presented here, this buffering capacity of PEI does not impair entry of adenovirus and may account for part of the improvement over polylysine systems. An additional advantage of PEI over polylysine is in increasing the solubility of the large DNA/polycation complex. The low molecular weight PEI/DNA interactions are much weaker than polylysine/DNA interactions and this may aid in assembly of the delivered DNA into active chromatin. Furthermore, once internalized, the displaced polycation may influence the cell and perhaps the low molecular weight PEI used here has fewer ill effects on the cell than polylysine.

This method will be useful in screening BAC molecules for gene functions in eukaryotic cells. In contrast to microinjection methods, the PEI/BAC/adenovirus can be introduced into large numbers of cells. Other possible applications include the study of higher order chromatin structure and its role in gene regulation, as well as the study of regulatory elements which, in their natural state, may be spread over hundreds of kilobases of the genome. One unexpected application may derive from our observation that large DNA molecules can provide stable gene expression in non-dividing cultures. This may have important applications in the generation of tissue implants supplying medically important proteins, such as clotting factors, cytokines or enzymes.

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REFERENCES

1 Behr,J.P. (1994) *Bioconjugate Chem.*, **5**, 382–389.
2 Cotten,M. and Wagner,E. (1993) *Curr. Opin. Biotechnol.*, **4**, 705–710.
3 Gnirke,A., Barnes,T.S., Patterson,D., Schild,D., Featherstone,T. and Olson,M.V. (1991). *EMBO J.*, **10**, 1629–1634.
4 Strauss,W.M. and Jaenisch,R. (1992) *EMBO J.*, **11**, 417–422.
5 Strauss,W.M., Dausman,J., Beard,C., Johnson,C., Lawrence,J.B. and Jaenisch,R. (1993). *Science*, **259**, 1904–1907.
6 Pearson,B.E. and Choi,T.K. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 10578–10582.
7 Pachnis,V., Pevny,L., Rothstein,R. and Costantini,F. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 5109–5113.
8 Silverman,G.A., Yang,E., Proffitt,J.H., Zutter,M. and Korsmeyer,S.J. (1993) *Mol. Cell. Biol.*, **13**, 5469–5478.
9 Featherstone,T. and Huxley,C. (1993) *Genomics*, **17**, 267–278.
10 Gobin,S.J., Alcaide-Loridan,C., Bono,M.R., Ottone,C., Chumakov,I., Rothstein,R. and Fellous,M. (1995) *Gene*, **163**, 27–33.
11 Gnirke,A. and Huxley,C. (1991) *Somat. Cell Mol. Genet.*, **17**, 573–580.
12 Gnirke,A., Huxley,C., Peterson,K. and Olson,M.V. (1993) *Genomics*, **15**, 659–667.
13 Schedl,A., Larin,Z., Montoliu,L., Thies,E., Kelsey,G., Lehrach,H. and Schütz,G. (1993) *Nucleic Acids Res.*, **21**, 4783–4787.
14 Willetts,N. and Skurray,R. (1987) In Neidhardt,F.C. (ed.), *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology.* American Society for Microbiology, Washington, DC, Vol. 2, pp. 1110–1133.
15 Low,K.B. (1987) In Neidhardt,F.C. (ed.), *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology.* American Society for Microbiology, Washington, DC, Vol. 2, pp. 1134–1137.
16 Shizuya,H., Birren,B., Kim,U.J., Mancino,V., Slepak,T., Tachiiri,Y. and Simon,M.I. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 8794–8797.
17 Kim,U.J., Shizuya,H., Birren,B., Slepak,T., de Jong,P. and Simon,M.I. (1994) *Genomics*, **22**, 336–339.
18 Kim,U.J., Birren,B.W., Slepak,T., Mancino,V., Boysen,C., Kang,H.L., Simon,M.I. and Shizuya,H. (1996) *Genomics*, **34**, 213–218.
19 Kim,U.J., Shizuya,H., de Jong,P.J., Birren,B. and Simon,M.I. (1992) *Nucleic Acids Res.*, **20**, 1083–1085.
20 Curiel,D.T., Agarwal,S., Wagner,E. and Cotten,M. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 8850–8854.
21 Wagner,E., Zatloukal,K., Cotten,M., Kirlappos,H., Mechtler,K., Curiel,D. and Birnstiel,M.L. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 6099–6103.
22 Cotten,M., Wagner,E., Zatloukal,K., Phillips,S., Curiel,D. and Birnstiel,M.L. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 6094–6098.
23 Cotten,M., Saltik,M., Kursa,M., Wagner,E., Maass,G. and Birnstiel,M. (1994) *Virology*, **205**, 254–261.
24 Cotten,M., Baker,A., Birnstiel,M.L., Zatloukal,K. and Wagner,E. (1996) In Dracopoli,N.C., Haines,J.L., Korf,B.R., Moir,D.T., Morton,C.C., Seidman,C.E., Seidman,J.G. and Smith,D.R. (eds) *Current Protocols in Human Genetics.* John Wiley and Sons, Inc., New York. pp. 12.3.1–12.3.33.
25 Cotten,M. (1995) In Doerfler,W. and Böhm,P. (eds) *Current Topics in Microbiology and Immunology.* Springer, Berlin, Vol. 199/III, pp. 283–295.
26 Boussif,O., Lezoualc'h,F., Zanta,M.A., Mergny,M.D., Scherman,D., Demeneix,B. and Behr,J.P. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 7297–7301.
27 Abdallah,B., Hassan,A., Benoist,C., Goula,D., Behr,J.P. and Demeneix,B.A. (1996) *Hum. Gene Ther.*, **7**, 1947–1954.
28 Barton,B., Harding,G.P. and Zuccarelli,A.J. (1995) *Anal. Biochem.*, **226**, 235–240.
29 Wang,M. and Lai,E. (1995) *Electrophoresis*, **16**, 1–7.
30 Weinberg,D.H. and Ketner,G. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 5383–5386.
31 Cotten,M. and Weber,J.M.(1995) *Virology*, **213**, 494–502.
32 Bridge,E. and Ketner,G. (1989) *J. Virol.*, **63**, 631–638.
33 Cotten,M. and Saltik,M. (1997) *Hum. Gene Ther.*, **8**, 555–562.
34 Maniatis,T., Fritsch,E.F. and Sambrook,J. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Habor Laboratory Press, Cold Spring Habor, NY.
35 Wohlgemuth,J.C., Kang,S.H., Bulboaca,G.H., Nawotka,K.A. and Calos,M.P. (1996) *Gene Ther.*, **3**, 503–512.
36 Behr,J.P., Demeneix,B., Loeffler,J.P. and Perez-Mutul,J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 6982–6986.
37 Lemay,P., Boudin,M., Milleville,M., Boulanger,P. (1980) *Virology*, **101**, 131–143.