Investigating the utility of the P1 Artificial Chromosome shuttle vector pJCPAC-Mam2 for gene therapy studies

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Abstract

The goal of gene therapy is to deliver as many copies of a functional gene to a patient that lacks this protein. There have been some recent successes in rescuing some types of blindness and red blood cell disorders. Current lentivirus-based vectors can only accommodate around 10 kilobases of foreign DNA. Also, integration of the vector is random, so transcription of a given gene can vary several-fold depending on the chromosomal architecture where insertion occurs. We constructed a P1 Artificial Chromosome (PAC) shuttle vector that has a greater than 150 kilobase insert size limit and remains as an episome in human cells. We previously demonstrated that a PAC clone containing the p53 gene was transcribed and translated when transiently introduced into p53 homozygous null Saos-2 human osteosarcoma cells. Furthermore, the apoptotic pathway was triggered in some of the cells. A Saos-2 cell line that overexpresses the bcl-2 cDNA was generated using G418 selection so that the effects of different lipofection reagents on copy number and transcription and translation levels of p53 could be studied. EndoFectin Max resulted in a several-fold higher transfection efficiency than Lipofectamine 3000. Seven stable cell lines were generated from each transfection procedure using puromycin and G418 selection. Unfortunately, only 7 of 13 cell lines contained measurable levels of p53 cDNA when total RNA was subjected to reverse transcriptase polymerase chain reaction (RT-PCR). A PAC clone containing the green fluorescent gene from *Pontellina plumata* that is expressed from the strong cytomegalovirus promoter is being constructed to investigate whether Lipofectamine 3000 or EndoFectin Max maximizes transfection efficiency, plasmid copy number, and transcription and translation levels in the continuous cell lines HEK293, KG1 and Saos-2.

Key Words: apoptosis, gene therapy, lipofection, transcription, translation

1. Introduction

In gene therapy, a functional version of a gene is introduced to as many of the cells of an individual that has inherited two faulty copies in an attempt to ameliorate the symptoms of a genetic disorder. The ultimate goal of this technology is to cure the person of their genetic defect. Recently there have been some very exciting successes for treating various types of eye blindness such as Chorioderaemia and Leber congenital amaurosis (LCA1 and LCA) since the eye is amenable to direct injection of vector systems carrying a functional gene and is protected from the immune system [1-4]. Gene therapy trials have shown success in rescuing a variety of red blood cell disorders such as hemophilia B [5-6], beta-thalassemia [7-8] and sickle cell anemia [7,9]. Gene therapy trials also have been successful in treating SCID [10-11].

Gene therapy has a lot of potential, but the ideal vector system has not been constructed as of yet. Adenovirus-based vectors were initially used for gene therapy trials because the virus remained as an episome and could accommodate transgenes up to 30 kb in size. Unfortunately, the viral life cycle limited the expression of the transgene over time and the capsid of the virus turned out to be highly immunogenic causing the death of Jesse Gelsinger during a gene therapy trial at the University of Pennsylvania [reviewed in 12]. Lentivirus-based vectors can accommodate transgenes up to 10 kb in size and can infect both dividing and non-dividing cells. These vectors randomly integrate into the genome, which can affect expression depending on the chromatin architecture where integration occurs [13]. Adeno-associated vectors (AAV) do not integrate into the host genome, but they can only accommodate transgenes up to 4 kb in size [reviewed in 14].

Yeast Artificial Chromosome (YAC) vectors and Bacterial Artificial Chromosome (BAC) vectors have been used for gene therapy trials in knockout mice. These vector systems accommodate insert sizes >300 kb and remain as episomes. Investigators demonstrated that a YAC vector containing the *CFTR* gene kept mice lacking the cystic fibrosis transmembrane regulator receptor protein alive [15]. Also, a BAC vector containing the *frda* gene could rescue mice lacking the frataxin protein from death when it was delivered to a mouse embryo [16]. We constructed the PAC vector pJCPAC-Mam2 that could be stably maintained in both bacterial and mammalian cells for functional studies in human cell lines; it also accommodates insert sizes >150 kb and remain as episome [17]. The p53 gene was cloned into this vector, and then this construct was transfected into the homozygous p53 null Saos-2 osteosarcoma cell line to demonstrate that p53 was transcribed and translated. Furthermore, transfection of a p53-GFP fusion gene contained in the PAC shuttle vector caused a small minority of the cells to undergo apoptosis [18].

The goal of this study was to evaluate whether transfection with Lipofectamine 3000 or EndoFectin Max would be the better reagent for potential gene therapy studies. A Saos-2 cell line that overexpresses the anti-apoptotic protein bcl2 was constructed so that permanent cell lines containing the p53-containing PAC clone could be established [19]. Unfortunately, the expression of the p53 mRNA was very low; therefore, this line of inquiry was suspended. We are in the process of constructing a GFP-containing PAC clone and plan to evaluate both lipofection reagents in a variety of continuous cell lines; this strategy will enable us to use immunofluorescence to quantitate both transfection efficiency and protein expression.

2. Materials and Methods

2.1 Generating the Saos-2 bcl-2 Stable Cell Lines

Saos-2 cells [20] were seeded into 6-well dishes at a concentration of 0.25-1 x 10^6 cells/well. The next day 4 μg of pcDNA336 Bcl-2 DNA (a gift of Stanley Korsmeyer, Addgene plasmid #8768) was complexed with 4 μl of Lipofectamine 2000 (Invitrogen) and transfected according to the manufacturer's specifications. The following day the media was removed and the cells were trypsinized with 500 μl of 0.05% of trypsin-EDTA (Gibco) and centrifuged for 5 min at 1500 rpm at 4°C in a 5810R table-top centrifuge (Eppendorf). Cell pellets were resuspended in 3 ml of D-MEM-F12 (ATCC) supplemented with 10% fetal bovine serum (Hyclone), 0.03% L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (complete media). Next, one ml aliquots were added to 60 mm tissue culture dishes containing one ml of complete media. The following day the media was replaced with 2 ml of complete media containing 50 μg/ml G418 (Sigma). The media was replaced every 3 days until colonies containing >100 cells were generated. Colonies were recovered using sterile small tip cotton applicators and placed into individual wells of a 12-well dish containing complete media + G418. When wells were >90% confluent, the cells were transferred to 6-well dishes, then to T25 flasks and finally to T75 flasks. Cells from confluent T75 flasks were trypsinized and the cell pellets were resuspended in 1.5 ml freezing media (D-MEM-F12 supplemented with 20% FBS and 10% w/v DMSO (Sigma-Aldrich) and stored in liquid nitrogen.

2.2 Identifying cell lines containing the integrated bcl-2 cDNA

Genomic DNA was recovered from confluent T75 flasks of each cell line using the Puregene Core Kit B (Qiagen). Then the samples were subjected to the polymerase chain reaction (PCR). The forward primer was designed so that it spanned the end of exon 1 and the beginning of exon 2 (5'GCTGGGATGCCTTTGTGG AAC3'), while the reverse primer started around 200 bp from the 5' end of exon 2 (5'GCTGGGATGCCTTTGTGGAAC3'). Each sample contained 0.6 μM of each primer, 1X BioMix Red (Bioline) and 2 μl of genomic DNA. The PCR tubes were placed in a Perkin Elmer 2400 thermocycler and the following program was used: 1 min at 94°C, 1 min at 62°C, 1 min at 72°C for 30 cycles and then 7 min at 72°C. DNA from the PCR reactions was resolved on composite 2% NuSieve/0.5% agarose gels.

2.3 Constructing Saos-2 bcl-2 cell lines with the p53-containing PAC clone

Transfections with Lipofectamine 3000 (Invitrogen) at a 1:3 ratio (μg: μl) and with EndoFectin Max (GeneCopoeia) at a 1:2 ratio were performed. Then the same procedure described in section 2.1 was performed; in addition, 20 μg/ml of puromycin (Sigma) was added. Colonies were transferred to 12-well dishes and then amplified until T75 flasks were confluent. Then cells were resuspended in 1.5 ml freezing media and stored in liquid nitrogen.

2.4 Analyzing the cell lines with the p53-containing PAC clone for trans-cription of p53

Each cell line was thawed and transferred to T75 flasks containing complete media + G418 + puromycin. Confluent flasks were then trypsinized and cells were placed into T150 flasks. When these flasks were confluent, cell pellets were generated. Then total RNA was prepared using the RNeasy mini kit (Qiagen). One-Step Reverse Transcriptase-PCR (RT-PCR, Qiagen) was then performed on each RNA sample using an annealing temperature of

56°C and products were resolved on composite 2% NuSieve/0.5% agarose gels [18].

3. Results

3.1 Identifying the Saos-2 bcl-2 Stable Cell Lines

Since the p53-containing PAC clone was transiently expressed in the Saos-2 cell line, we attempted to modify this cell line by inserting the bcl-2 cDNA into the genome, which allowed the cells to divide indefinitely. pcDNA336 Bcl-2 DNA was transfected into Saos-2 cells and then G418 selection was applied. Nine cell lines were generated and genomic DNA was recovered from each one. PCR with a forward primer that spanned the end of exon 1 and the beginning of exon 2 for the *bcl-2* gene was used so that only the cDNA could be amplified. The desired 243 bp PCR fragment was present in all of the newly created cell lines but not in the parental Saos-2 cell line (Figure 1).

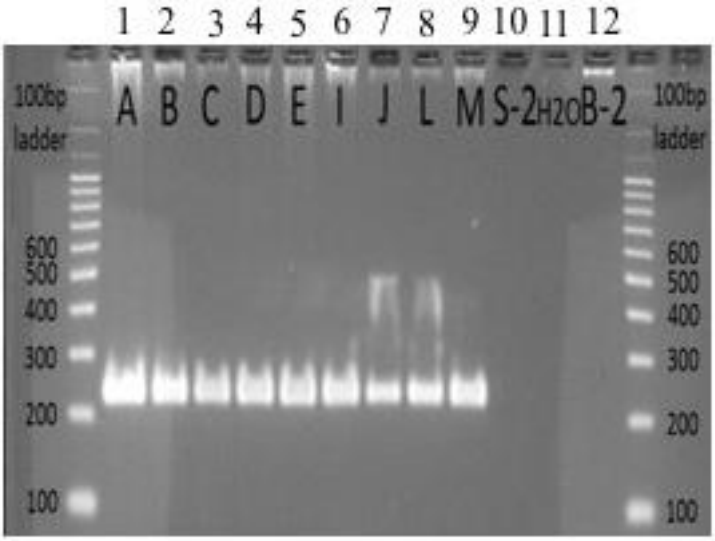

Figure 1. PCR of Cell Lines with Bcl-2 primers. Genomic DNA was recovered from each cell line and subjected to PCR with a forward primer that spanned exons 1 and 2 and a reverse primer in exon 2. Lanes 1-9 contain the different cell lines; lane 10, parental cell line Saos-2; lane 11, H_20; lane 12, Bcl2 cDNA plasmid DNA.

3.2 Lipofection of the p53-containing PAC clone into the Saos-2 bcl-2 Cell Line

One of the newly generated cell lines was seeded into 6-well dishes and transfections were carried out the next day by mixing the p53-containing PAC clone with either the proprietary Lipofectamine 3000 or EndoFectin Max lipid mixtures. Then the cells were transferred to 60 mm dishes and subjected to G418 + puromycin selection. After several weeks of selection, small colonies began to appear on all of the plates. The EndoFectin Max plates had an approximately five-fold higher number of colonies than the plate that had undergone transfection with Lipofectamine 3000 (data not shown). Fourteen cell lines were established, seven from each transfection procedure.

Total RNA was recovered from each cell line and then One-Step RT-PCR was performed. The forward primer spanned the end of exon 6 and the beginning of exon 7

of the p53 gene; therefore, only spliced RNA was amplified (Figure 2). The HEK 293 positive control produced a bright 127 bp band and the Saos-2 bcl-2 RNA was not amplified. Five of the cell lines had the same 127 bp band that varied in intensity; four of the cell lines were generated using lipofection with EndoFectin Max (Figure 3A). Additional RNA was prepared from four of the cell lines (L1 and L2 were generated from the Lipofectamine 3000 transfections and E8 and E9 were generated from the EndoFectin Max transfections). When the RT-PCR was repeated, RNA from all four of these cell lines produced small amounts of the desired 127 bp cDNA (Figure 3B). Therefore, seven of the thirteen cell lines analyzed did produce some p53 mRNA. Unfortunately, the transcription levels were too low to perform any quantitative analysis. Also, no plasmid DNA was able to be extracted from any of the cell lines. Therefore, this line of inquiry was abandoned.

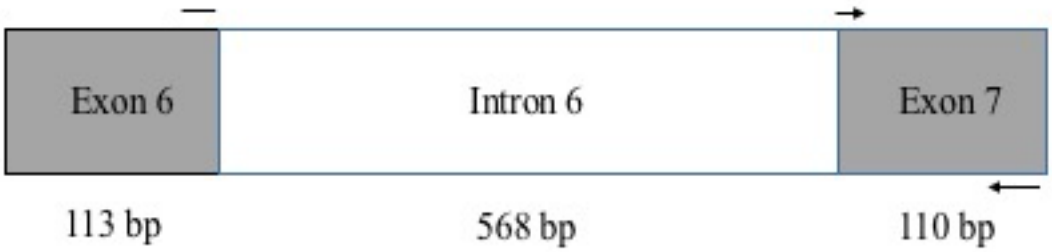

Figure 2. RT-PCR Strategy. The forward primer was designed to start at the end of exon 6 and end in the beginning of exon 7. The reverse primer started near the end of exon 7.

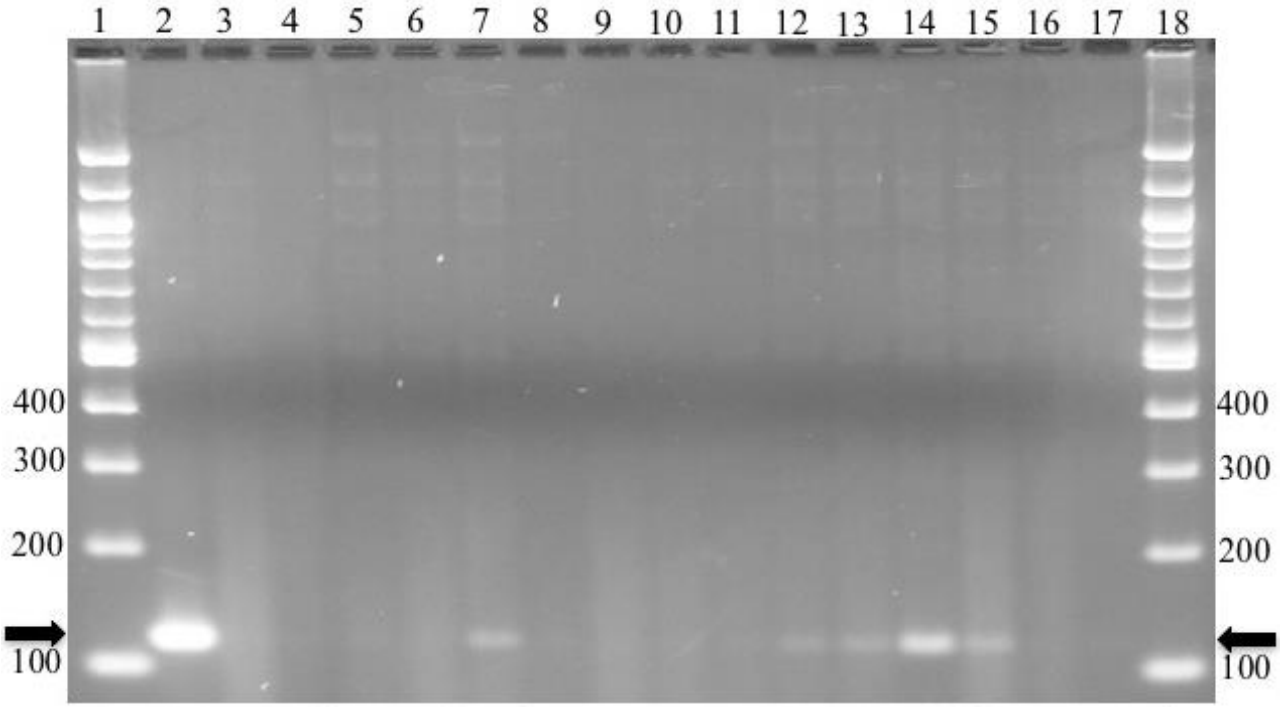

Figure 3A. RT-PCR of Saos-2 Bcl2 Cell Lines Transfected with a p53-containing PAC Clone. Lanes 1 and 18; 100 bp ladder, lane 2: HEK 293 RNA, lane 3; Saos-2 Bcl2 RNA, lane 4; H_20; lanes 5-11; RNA from cell lines 1-7 derived from Lipofectamine 3000 transfection; lanes 12-17; RNA from cell lines 8-13 derived from EndoFectin Max transfection. The arrowhead represents the 127 bp cDNA.

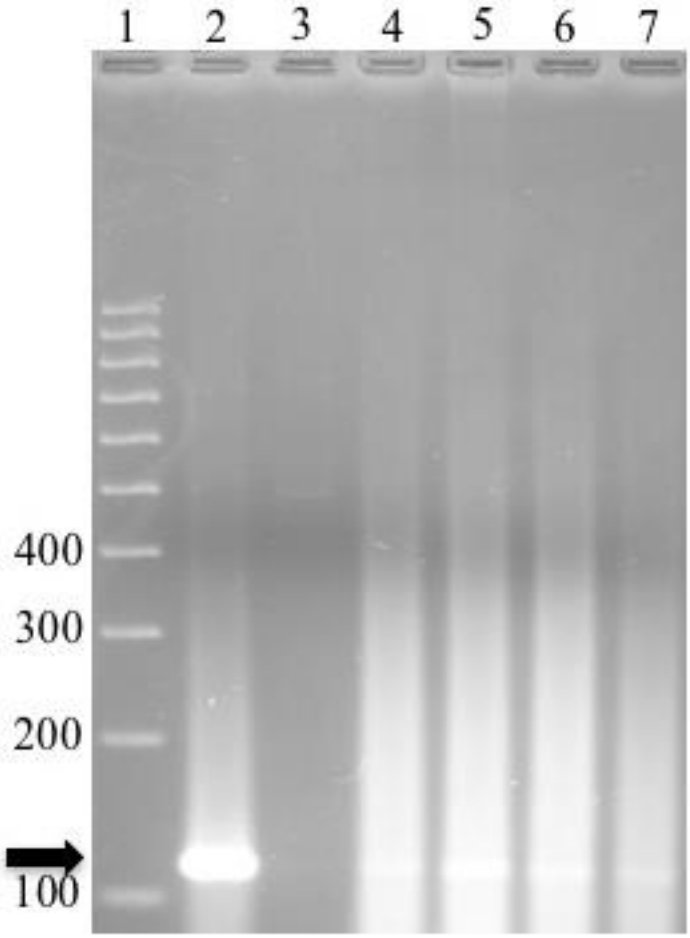

Figure 3B. RT-PCR of Saos-2 Bcl2 Cell Lines Transfected with a p53-containing PAC Clone. Lane 1; 100 bp ladder, lane 2: HEK 293 RNA, lane 3; Saos-2 Bcl2 RNA, lanes 4- 5; RNA from cell lines 1 and 2 (Lipofectamine 3000 transfection), lanes 6-7; RNA from cell lines 8-9 (EndoFectin Max transfection). The arrowhead represents the 127 bp cDNA.

4. Discussion

Many independent Saos-2 cell lines that had incorporated the bcl-2 cDNA were able to be generated. Unfortunately, when the p53-containing PAC DNA was transfected into one of these cell lines, all of the resulting stable cell lines only produced low levels of p53 mRNA. Perhaps the random integration of the bcl-2 cDNA in the cell line used for these studies was in a region of the genome that negatively influenced the expression of the bcl-2 mRNA [13]. It would have been more prudent to investigate the level of bcl-2 protein present in all nine cell lines; then the cell line producing the most bcl-2 protein could have been used to establish the stable cell lines that had taken up the p53-containing PAC DNA.

These preliminary studies suggested that transfection with EndoFectin Max resulted in better transfection efficiency since an approximately five-fold increase in colonies resulted with this reagent versus the Lipofectamine 3000. Since the PAC shuttle vector contains the multi-copy

Epstein Barr virus latent origin of replication *oriP*, anywhere from 10-100 copies of this vector can co-exist in the same cell; the number of copies present appears to be related to the initial amount of DNA taken up by the cell [21]. Furthermore, the cell lines generated from the EndoFectin Max transfections appeared to produce higher levels of p53 cDNA suggesting that more of the p53-containing PAC DNA was initially taken up when this reagent was used.

Repeating these studies with the pJCPAC-Mam GFP plasmid that is currently being constructed offers many advantages (Figure 4). Expression of the copepod green fluorescent protein (copGFP) will enable the transfection efficiency of both reagents to be quantitated via immunofluorescence [22]. The relative intensity of individual cells can also be quantitated to determine protein expression levels; this should supplement the measurement of protein levels of different cell lines using Western blotting with a polyclonal anti- *Pontellina plumata* antibody, which is available from Santa Cruz Biotechnology. Purification of the GFP protein could also be accomplished by subjecting total proteins extracts from each cell line to size exclusion chromatography in the presence of ultraviolet light. This would allow for the specific recovery of the GFP protein. Also, this plasmid can be transfected into a variety of cell lines to determine if transfection efficiency, plasmid uptake levels, transcription and translation of copGFP are consistent within a given cell line and between different cell lines for both Lipofectamine 3000 and EndoFectin Max.

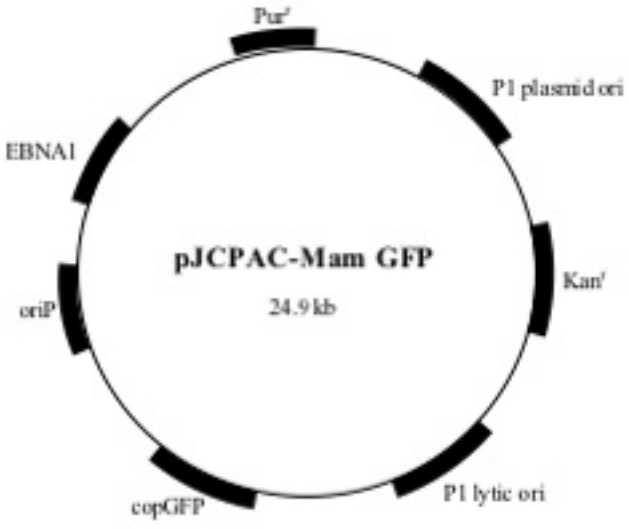

Figure 4. The PAC copGFP Plasmid. The copGFP gene is under transcriptional control of the cytomegalovirus promoter.

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