Nucleic Acids Research, 1996, Vol. 24, No. 23 **4693–4699**

A shuttle system for transfer of YACs between yeast and mammalian cells

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Received August 20, 1996; Revised and Accepted October 17, 1996

ABSTRACT

The development of a system for shuttling DNA cloned as yeast artificial chromosomes (YACs) between yeast and mammalian cells requires that the DNA is maintained as extrachromosomal elements in both cell types. We have recently shown that circular YACs carrying the Epstein–Barr virus origin of plasmid replication (*oriP*) are maintained as stable, episomal elements in a human kidney cell line constitutively expressing the viral transactivator protein EBNA-1. Here, we demonstrate that a 90-kb episomal YAC can be isolated intact from human cells by a simple alkaline lysis procedure and shuttled back into *Saccharomyces cerevisiae* by spheroplast transformation. In addition, we demonstrate that the 90-kb YAC can be isolated intact from yeast cells. The ability to shuttle large, intact fragments of DNA between yeast and human cells should provide a powerful tool in the manipulation and analysis of functional regions of mammalian DNA.

INTRODUCTION

Shuttle systems exist which allow cloned DNA to be transferred between bacteria and yeast (1) and between bacteria and mammalian cells (2–4). Bacterial–yeast shuttle vectors have permitted the cloning of yeast genes by complementation of yeast mutants. This has included identification of genes important to the yeast cell cycle, metabolism (5), and repair and recombination systems (6) and has facilitated characterisation of *cis*-acting DNA elements such as promoters (7) and autonomously replicating sequences (ARSs) (1). Genes encoded on bacterial–mammalian shuttle plasmids have been shown to be stably expressed in mammalian cells (4) and such vectors have been used for the efficient expression of cDNA libraries (2). The *oriP*–EBNA-1 shuttle vectors allow cloning of genes by direct complementation of mutant phenotypes and have been shown to be 100-fold more efficient than vectors lacking the EBV elements in the isolation of low abundance sequences (8).

The term 'shuttle system' reflects the ability to move a vector back and forth between two hosts, hence a means of selecting the vector in both hosts is required as well as a means of propagating it in both organisms. The original *Saccharomyces cerevisiae*–*Escherichia coli* shuttle vectors, YEp and YCp (reviewed in 1) have different systems for autonomous replication in yeast. Both vectors contain pBR322 sequences, allowing replication and selection in *E.coli*, and both carry a gene for selection in yeast. Replication of YEp plasmids in yeast initiates from within the 2μm origin of replication and the plasmid is stably maintained at a copy number of 30–50 per cell. In contrast, YCp vectors contain yeast centromere sequences and an origin of replication which allows autonomous replication of the single copy plasmid in yeast cells.

Escherichia coli–human cell line shuttle vectors contain a mammalian selectable marker gene and rely on interaction between the Epstein–Barr virus latent origin of replication, *oriP*, and the viral transactivator protein EBNA-1, for stable, extrachromosomal maintenance of the plasmids in human cells (9,10). *oriP* consists of two components: a family of repeats which comprises 20 tandem copies of a 30-bp repeat, and the dyad symmetry element which contains four copies of the repeat (11,12). Both sets of repeats are necessary for stable, extrachromosomal maintenance of plasmid DNA in human cells (12), and both bind the EBNA-1 protein. The dyad symmetry element is the site of initiation of episomal DNA replication (13), while the family of repeats acts as an EBNA-1-dependent enhancer of transcription (14) as well as a replication fork barrier and termination site (13). In addition, the family of repeats promotes nuclear retention by the specific binding of EBNA-1 (15), followed by non-specific association of EBNA-1 with host cell chromosomes (16,17).

Plasmids carrying *oriP* are present as multiple copies within the human cells (2,8) permitting easy isolation by Hirt extraction (2,18), which can be followed by reintroduction into *E.coli* by transformation of competent bacterial cells. The ability to isolate the extrachromosomal DNA leads to possibilities in addition to expression cloning. For example, *in vitro* mutagenesis in *E.coli* can be followed by reintroduction of mutated clones into mammalian cells and screening for altered phenotypes. The extrachromosomal DNA can then be isolated from the human cell lines, and returned to bacteria for analysis and further manipulation, thus facilitating the functional analysis of mammalian genes and their regulatory elements.

Although highly efficient, one major limitation of plasmid based bacterial–mammalian shuttle systems is the size of DNA which can be accommodated in the vector. This problem could be overcome by the use of shuttle cloning systems with a larger capacity for DNA, e.g. P1 artificial chromosome (PACs), bacterial artificial chromosomes (BACs) or yeast artificial chromosomes (YACs). The use of YACs is particularly advantageous because the

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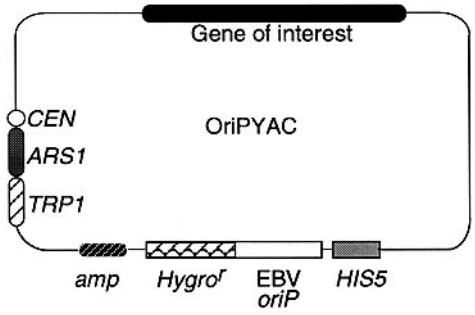

Figure 1. Structure of OriPYACs showing the cloned DNA insert, the yeast centromere (*CEN*), an autonomously replicating sequence (*ARS*), the *TRP1* and *HIS5* yeast markers, the mammalian selectable marker *Hygro*^{r} and the EBV *oriP* domain.

yeast host allows efficient characterisation and manipulation of cloned sequences by homologous recombination, prior to introduction into mammalian cells.

The inability to maintain YACs as stable, extrachromosomal elements in mammalian cells has, however, hindered the development of a large cloning capacity yeast–mammalian shuttle vector system. We have recently made circular YACs carrying the EBV *oriP* domain, termed OriPYACs, as shown in Figure 1. Two YACs of 90- and 660-kb were used in the initial study. After circularisation and addition of *oriP*, the OriPYACs were introduced into human 293 cells constitutively expressing EBNA-1 by spheroplast fusion. A total of six cell lines were generated: three from fusion with yeast containing OriPYAC90 (F90-1, -2 and -3) and three from fusion with yeast containing OriPYAC660 (F660-1, -2 and -3). All six cell lines contained replicating, extrachromosomal elements. The 90-kb OriPYAC was found to be intact and unrearranged in all of the cell lines analysed, whereas the intact form of the 660-kb OriPYAC was present in two out of three cell lines. The episomal elements were found to be highly stable, with loss rates of 1–3% per cell division in the absence of selection (19).

We demonstrate here that the OriPYACs can be isolated as intact circular elements from human cells by a simple alkaline lysis procedure and shuttled back into the yeast host. In addition, we show that it is possible to isolate OriPYAC90 intact from yeast cells by the same procedure.

MATERIALS AND METHODS

OriPYAC design and generation of human fusion cell lines containing episomal OriPYACs

A map of the final OriPYAC construct is shown in Figure 1. Construction of the OriPYACs and generation of human kidney fusion cell lines containing the OriPYACs has been described elsewhere (19). Fusion cell lines F90-2 and F90-3, containing an average of 1.3 and 18 intact copies of the 90-kb circular molecule respectively, and F660-3, containing three intact copies of the 660-kb circular molecule per cell were used for the isolation of episomal elements.

Isolation of OriPYAC episomal elements

OriPYAC episomes were isolated from the human fusion cell lines by a modification of a previously described alkaline lysis procedure (20–22). 1×10^6 cells were washed once in phosphate buffered saline (without calcium or magnesium), pelleted by centrifugation at 1000 r.p.m. and resuspended in 100 µl of lysis buffer (50 mM NaCl, 2 mM EDTA, 1% SDS, adjusted to pH 12.45 with 2 M NaOH). The cells were lysed by vortexing at highest speed for 1 min followed by incubation at 30°C for 30 min. The lysis buffer was neutralised by addition of 0.2 vol of 1 M Tris pH 7.0, 0.11 vol of 5 M NaCl and 0.01 vol of 10 mg/ml proteinase K. The cellular proteins were partially degraded by incubation at 37°C for 30 min. The cell lysates were then cooled to room temperature before extraction with 1/3 vol phenol (saturated with 0.2 M NaCl, 0.2 M Tris–HCl pH 8.0). Extraction was carried out by very gentle inversion of the tube several times. The tubes were then chilled slowly to 4°C and the phases separated by centrifugation at 5000 r.p.m. for 20 min at 4°C. The aqueous phase was recovered using a cut-off tip and 1/3 the volume of 24:1 chloroform–isoamylalcohol added. The phases were separated as before and episomal DNA precipitated overnight at –20°C in the presence of 25 µg of glycogen (in the case of OriPYAC90 elements) or 2 µg of sheared salmon sperm DNA (for purification of OriPYAC660) and 2 vol of ethanol. The DNA was recovered by centrifugation at 10 000 *g*, 4°C, for 30 min, washed once in 70% ethanol and resuspended overnight in 10 µl H_2O (for yeast transformation) or 50 µl H_2O (for γ irradiation) at 4°C.

Shuttle transfer of purified episomal DNA into *S.cerevisiae* AB1380

Purified OriPYAC90 DNA from the human fusion cell lines was used to transform the YAC library host strain *S.cerevisiae* AB1380 (23). Yeast cells were grown up in the rich medium YPD (1% yeast extract, 2% bactopeptone, 2% dextrose) to a density of ~3×10^7 cells/ml. Spheroplast transformations were carried out as described previously (24). Episomal DNA isolated from 10^6 human cells was used in each transformation reaction. An aliquot of 5 µg of sheared salmon sperm DNA was added to act as carrier and 1 mM polyamines (0.75 mM spermidine trihydrochloride–0.30 mM spermine tetrahydrochloride) (25) were included to help maintain the high molecular weight DNA intact. Transformed spheroplasts (7.5×10^7) were plated onto five 90 mm sorbitol agar plates lacking histidine.

Isolation of intact OriPYAC DNA from yeast

Yeast clones containing OriPYAC90 or OriPYAC660 were grown to a density of ~3×10^7 cells/ml. 1.5×10^9 yeast cells were then washed once in 20 ml of water and once in 20 ml of 1 M sorbitol and resuspended in 20 ml of SCEM (1 M sorbitol, 0.1 M sodium citrate pH 5.8, 10 mM EDTA). Yeast lytic enzyme (ICN) (1.3 U) was added and the cell suspension was incubated at 30°C until the yeast cells were 95% spheroplasted, ~25 min. The spheroplasts were washed twice in 20 ml of STC (1 M sorbitol, 10 mM Tris pH 7.5, 10 mM $CaCl_2$) before resuspension in 1 ml of STC. OriPYAC DNA was prepared from aliquots of 1×10^6 spheroplasts by the same alkaline lysis procedure as that described for human cells. Carrier DNA was included during precipitation of the purified OriPYAC DNA, in an attempt to reduce shearing of the DNA.

DNA preparation and analysis

Agarose plugs of high molecular weight yeast or mammalian cell DNA were prepared by modification of a previously described protocol (26). γ irradiations were carried out in a Gammacell 1000

Elite. Plugs were equilibrated twice in TE at 4°C prior to γ irradiation in 2 ml of TE. Purified episomal DNA was irradiated in solution, using the same irradiation conditions. Restriction enzyme digests of plug DNA were carried out overnight in a volume of ~200 µl. Plugs were equilibrated twice in TE at 4°C, followed by two washes for 30 min in restriction enzyme buffer on ice, prior to overnight digestion with 40–60 U of enzyme.

Pulsed-field-gel electrophoresis (PFGE) was carried out on a BioRad CHEF DRII or DRIII apparatus, using a 1% agarose gel cast and run in 0.5× TBE. Samples of irradiated DNA in solution were loaded onto a pre-chilled gel and run for 15 min without circulation of the buffer. The gels were then run at 11°C and 200 V, with the switching times given in the figure legends. Southern transfer, onto Hybond N+ membranes (Amersham), was carried out using the conditions described by the manufacturer. All prehybridisations and hybridisations were carried out in modified Church buffer (16.8 g/l $NaH_2PO_4 . H_2O$, 54.1 g/l $Na_2HPO_4 . 12H_2O$, 7% SDS, 100 µg/ml salmon sperm DNA) at 65°C. Filters were washed with 2× SSC for 5 min, followed by two washes in 0.1× SSC– 0.1% SDS for 30 min, all at 65°C. The 0.9 kb *Sma*I fragment from pHEBo was used as the *oriP* probe (27).

RESULTS

Isolation of intact 90-kb OriPYACs from mammalian cells

In this study, we wanted to establish whether the OriPYAC episomal elements could be easily isolated from the human fusion cell lines, and thus used as the basis of a shuttle system for transferring large DNA molecules between mammalian and yeast cells. Fusion cell lines containing the 90-kb OriPYAC rather than 660-kb OriPYAC were used first, because of the problems associated with handling very large, purified DNA.

The 90-kb episomal OriPYAC90 molecules were isolated from the human fusion cell lines F90-2 and F90-3 by a straightforward alkaline lysis procedure (21), as described in Materials and Methods, which is similar in principle to a bacterial plasmid preparation. This method involves alkali treatment to selectively denature host linear DNA, followed by shearing to allow efficient strand separation of the denatured DNA. The lysate is then neutralised and a limited protease step carried out before phenol extraction at high salt concentration (28). This method was developed for isolation of the 172-kb EBV episomal molecules from human cells. Episomal DNA purified from the human fusion cell lines was resuspended in TE containing 100 mM NaCl and the integrity of the purified episomal DNA was tested by γ irradiation followed by PFGE and hybridisation to an *oriP* probe. As can be seen from the last lane in Figure 2, the OriPYAC90 DNA purified from the human cell line gives a clear band of 90 kb after γ irradiation, suggesting that OriPYAC90 has been isolated intact from human cells.

Transfer of purified OriPYAC90 DNA from human cells into *S.cerevisiae*

We then determined whether the purified OriPYAC90 DNA could be transformed intact into yeast. Purified OriPYAC90 DNA isolated from 1×10^6 F90-2 or F90-3 cells, was used to transform the *S.cerevisiae* YAC library host strain AB1380, by a standard protocol using polyethylene glycol (PEG) (24), as described in Materials and Methods. Sheared salmon sperm DNA was included as carrier in all reactions, to reduce shearing forces and increase

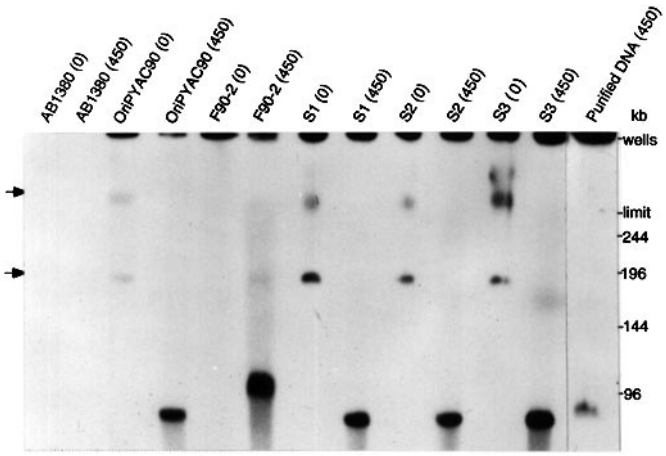

Figure 2. Analysis of *S.cerevisiae* clones transformed with OriPYAC90 episomal DNA purified from human cells. DNA prepared from non-transformed AB1380 cells, the original yeast strain carrying OriPYAC90, the human cell line F90-2, the transformation clones S1, S2 and S3, and DNA purified from the human cell line F90-2 was analysed. The DNA was treated with 0 or 450 Gy of γ irradiation, as shown in the brackets. The DNA was then resolved by PFGE with switching times of 5–15 s for 20 h, the gel was blotted, and the filter hybridised with the *oriP* probe. The locations of size markers, the limit of resolution and the wells are indicated to the right. The positions of characteristic extra bands, which probably correspond to circular forms of the OriPYAC, are indicated by arrows.

transformation efficiency. Polyamines (1 mM) were included in some transformation reactions because they have been shown to reduce shearing of high molecular weight DNA (25). However, these were found to be unnecessary to maintain molecules of this size intact. A total of 138 colonies (42 with OriPYAC90 DNA prepared from F90-2 and 96 with OriPYAC90 DNA purified from F90-3) grew on media lacking histidine. The larger number of colonies which were produced with DNA prepared from F90-3 may be a result of the higher copy number of the OriPYAC episomes (average 18 per cell) in this cell line, in comparison with F90-2 (1.3 episomes per cell) (19).

Figure 2 shows analysis of three randomly chosen yeast clones which were positive by this genetic selection. S1 and S2 were generated by transformation with episomal DNA purified from F90-2, S3 by transformation with DNA purified from F90-3. Agarose plugs of high molecular weight DNA from AB1380, the original OriPYAC90 yeast clone, the human cell line F90-2 carrying OriPYAC90, and transformation yeast clones S1, S2 and S3 were treated with γ irradiation and resolved on a PFG. Hybridisation to an *oriP* probe demonstrated the presence of a 90-kb band after irradiation of all samples except non-transformed AB1380 cells, suggesting that OriPYAC90 has been transferred intact to all yeast clones analysed. The slight shift in mobility between yeast and mammalian bands has been observed frequently (29,30) and is due to the 200-fold greater complexity of the mammalian genome in comparison with the yeast genome. OriPYAC90 is sufficiently small that the circular forms of the YAC can resolve into the gel under certain PFGE conditions, the positions of these circular molecules are indicated by arrows. Analysis of six further positive yeast transformation colonies by gamma irradiation demonstrated that OriPYAC90 was intact in nine out of nine clones (data not shown).

The yeast clones were further analysed with the rare cutting restriction enzyme *Sst*II, to determine whether the DNA was

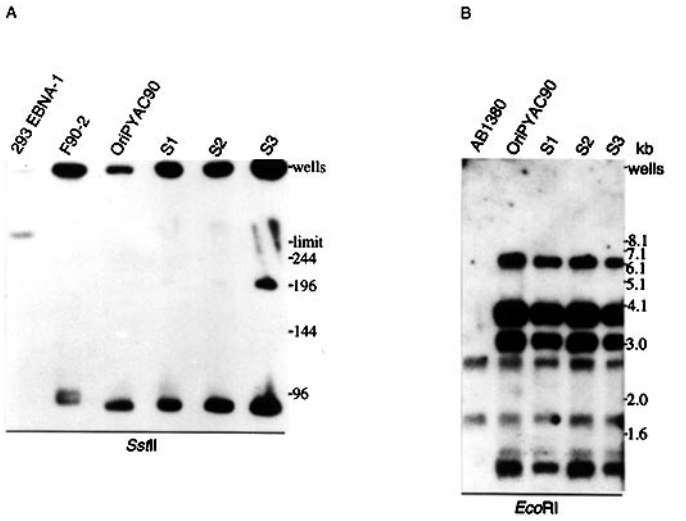

Figure 3. Analysis of transformation clones by restriction enzyme digestion. (**A**) DNA prepared from the 293 EBNA-1 cells, the human cell line F90-2, the original yeast carrying OriPYAC90 and yeast transformation clones S1, S2 and S3 (as indicated above each lane) was digested with *Sst*II, resolved by PFGE, with switching conditions of 5–15 s for 20 h, blotted and hybridised with the *oriP* probe. The positions of size markers, the limit of resolution and the wells are indicated to the right of the figure. Digestion of S3 appears to be partial, as a circular form of the molecule (at ~196 kb) is apparent. (**B**) DNA from non-transformed AB1380 cells, the original yeast clone carrying OriPYAC90 and the transformation clones S1, S2 and S3 was digested with *Eco*RI, resolved on a 1% agarose gel, blotted and hybridised with total human DNA. The positions of size markers are indicated to the right.

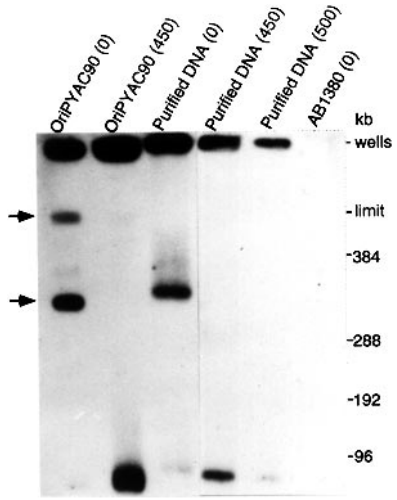

Figure 4. Purification of OriPYAC90 from yeast cells. Yeast carrying OriPYAC90 were spheroplasted and DNA was prepared by alkaline lysis as described in Materials and Methods. Agarose plugs of DNA prepared from yeast cells carrying OriPYAC90 and non-transformed AB1380 cells, and DNA purified from yeast carrying OriPYAC90 (as indicated above the lanes), were treated with γ irradiation (as shown in brackets) and resolved by PFGE with a switching time of 25 s for 20 h. The filter was hybridised with the *oriP* probe. The positions of size markers, the limit of resolution and the wells are indicated to the right. The intact circular forms of OriPYAC90, which resolve into the gel under these conditions, are indicated by arrows.

otherwise unrearranged. DNA from the human cell line 293 EBNA-1, the human fusion cell line F90-2, the original yeast clone carrying OriPYAC90, and the yeast clones S1, S2 and S3 was digested with *Sst*II, which is known to linearise OriPYAC90. The digestion products were resolved on a PFG and blotted, and the filter was hybridised to an *oriP* probe, as shown in Figure 3A. Digestion of the yeast clones with *Sst*II yielded a band of 90 kb, identical in size to that seen after digestion of the original OriPYAC90.

In addition, DNA from non-transformed AB1380 cells, the original yeast clone carrying OriPYAC90 and the yeast clones S1, S2 and S3 was digested with *Eco*RI and the digestion products resolved on a 1% agarose gel. The gel was blotted and the filter was hybridised with total human DNA, to produce a fingerprint of the yeast clones, as shown in Figure 3B. All three transformation clones demonstrated identical sets of bands to those seen in the original yeast clone carrying OriPYAC90.

Taken together, the gamma irradiation, *Sst*II and *Eco*RI fingerprinting data suggest that the 90 kb OriPYACs have not undergone rearrangements during shuttling between human and yeast cells.

Isolation of intact OriPYAC90 DNA from yeast cells

Transfer of YAC DNA into mammalian cells by spheroplast fusion is convenient and does not impose the same size constraints as lipofection and microinjection. However, some cell types, particularly human cell lines, appear to be refractory to spheroplast fusion. In addition, gene therapy and *in vivo* transgenic applications of OriPYACs would require introduction of purified DNA. Therefore, we wanted to determine whether OriPYAC90 DNA could be isolated intact from yeast cells.

Conventional purification from low melting point agarose gels is difficult, because circular molecules run anomalously on PFGE and bands cannot generally be seen under UV light after ethidium bromide staining. Also, isolation of episomal DNA by alkaline lysis is more difficult from yeast than from mammalian cells, due to the high concentrations of degradative enzymes in yeast cells. Yeast carrying OriPYAC90 were first spheroplasted and the alkaline lysis procedure was then carried out as described for the human cells. Carrier DNA was included in both precipitation and resuspension of the OriPYAC DNA, in an attempt to reduce nicking or degradation of the circular molecules. The purified OriPYAC DNA was then analysed by γ irradiation and PFGE.

Figure 4 shows analysis of OriPYAC90 purified from yeast cells. In the unirradiated sample of purified OriPYAC DNA, most of the DNA is in the circular, supercoiled form of the molecule. A very small amount of linearised DNA is also present, suggesting that some degree of nicking of the circular molecule has occurred during the isolation. Treatment with γ irradiation results in a band of 90 kb for both yeast carrying OriPYAC90 and the purified OriPYAC90 DNA.

The preparations of OriPYAC90 DNA from both human and yeast cells contained a small amount of additional, non-hybridising material which was visible on the ethidium bromide stained PFG. This may be the result of carry-over of denatured linear chromosomal DNA from the single phenol–chloroform extraction step. Alternatively, the contaminating material may correspond to RNA and mitochondrial DNA which are co-purified with the episomal elements (28). It may be possible to prepare relatively large quantities of highly purified OriPYAC episomal DNA by scaling up the procedure and by applying further purification

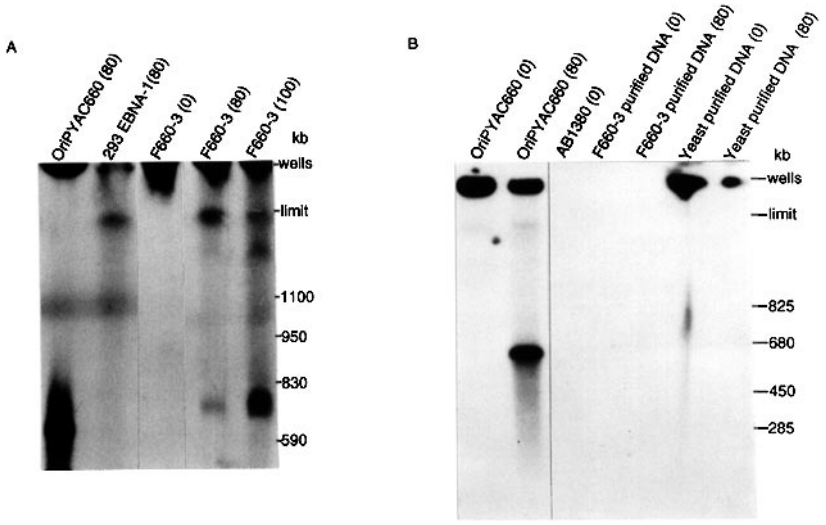

Figure 5. Purification of OriPYAC660 from human and yeast cells. (**A**) High molecular weight DNA prepared from yeast cells carrying OriPYAC660, the parental cell line 293 EBNA-1 and the fusion cell line F660-3 was treated with 0, 80 or 100 Gy of γ irradiation, as indicated above the panel. The DNA was separated on a pulsed-field gel with 60–120 s switching for 24 h and blotted. The filter was hybridised with the *oriP* probe. The positions of size markers, the limit of resolution of the gel and the wells are indicated to the right of the figure. (**B**) OriPYAC660 was isolated from yeast cells or from the human cell line F660-3 as described in Materials and Methods. Yeast carrying OriPYAC660, AB1380 and the purified DNA samples were treated with γ irradiation (shown in brackets), resolved by PFGE with a switching time of 60–90 s for 21 h and hybridised with the *oriP* probe. The positions of size markers, the limit of resolution and the wells are indicated to the right.

steps such as ethidium bromide–caesium chloride (CsCl) centrifugation, followed by either density gradient centrifugation in CsCl or glycerol gradient centrifugation (28). Alternatively, the episomal DNA could be further purified from chromosomal DNA using an affinity column (21).

OriPYAC660 could not be isolated intact from human fusion cell lines or from yeast cells

In order to see whether these methods could be applied to a 660-kb molecule, in addition to the 90-kb one, we attempted to isolate OriPYAC660 from yeast and human cells, by the same alkaline lysis procedure. Carrier DNA was included during precipitation of the isolated episomal elements, in order to reduce shearing forces, and both polyamines and high salt (100 mM NaCl) were included during resuspension of the DNA. The DNA was irradiated, resolved on a PFG and hybridised to an *oriP* probe (Fig. 5). The human cell line F660-3 contains intact, 660 kb episomal elements (Fig. 5A) which are present at a copy number of approximately three per cell (19), but no signal was seen from DNA purified from this cell line (Fig. 5B). The very large size of the OriPYAC may contribute to the inability to purify intact episomes.

The yeast genome is 200-fold less complex than the mammalian genome so purification of OriPYAC660 was also attempted from yeast cells. DNA purified by alkaline lysis from yeast cells carrying OriPYAC660 hybridised strongly to the *oriP* probe (Fig. 5B). However, although some DNA appears to remain trapped in the wells, as would be expected if the OriPYAC660 molecule has been isolated intact, a band at 660 kb is not apparent after irradiation.

Inability to purify 660-kb molecules may be due to technical difficulties in keeping such large DNA molecules intact, or the method may not be suitable for the isolation of such large molecules. Shearing of the circular DNA at high pH prior to phenol extraction may result in loss of the OriPYAC DNA with the linear, host cell genomic DNA. Standard methods for enrichment of small, circular viral and plasmid molecules from mammalian cells, e.g. Hirt extraction (18), have been shown to be unsuitable for the isolation of molecules as large as the EBV, resulting in considerable losses of DNA (31). Similarly, methods designed for isolation of the 172-kb EBV genome may be unsuitable for extraction of much larger circular molecules.

It will be of interest to determine the upper size limit of OriPYAC molecules which can be isolated intact by the method described in this paper. In addition, it may be possible to use adaptations of this protocol which reduce shearing of the DNA, such as lysing the cells at high pH (12.45) with stirring, followed by adjustment of the pH to ~8.5 prior to phenol extraction (32,33) to isolate the 660 kb molecules intact.

DISCUSSION

The system described here allows DNA cloned as YACs to be shuttled between human and yeast cells. We have previously transferred circular YACs containing the EBV *oriP* domain from yeast cells into a human kidney cell line by spheroplast fusion. The OriPYACs were found to be maintained as stable, episomal elements in all fusion cell lines analysed (19). Here we show that episomal OriPYACs of 90 kb can be isolated intact from the human cells by a straightforward alkaline lysis procedure. Ability to isolate OriPYAC DNA by this method demonstrates that the OriPYACs are being maintained as covalently closed, supercoiled molecules within the human cells, as has been demonstrated for the latent viral genome (34,35). The isolated episomal elements were then reintroduced by yeast spheroplast transformation into the *S.cerevisiae* YAC library host strain, AB1380 (23), where they were found to be maintained intact and unrearranged in nine out of nine clones analysed.

The OriPYAC shuttle system has two major advantages over conventional EBV-based bacterial–human shuttle systems; ease of manipulation of the cloned DNA by homologous recombination, and large cloning capacity. *Saccharomyces cerevisiae* is an excellent host for manipulation of DNA by homologous recombination

allowing fragmentation (36–38), generation of internal deletions (39,40), mapping of exons (41), integration of yeast or mammalian selectable markers (42–44) and introduction of defined mutations via gene replacement (45,46).

The large cloning capacity of YACs allows them to carry both intact genes and their long range controlling elements which give full levels of controlled expression. YAC DNA has been successfully introduced into mammalian cells by a variety of methods including microinjection, lipofection and spheroplast fusion (reviewed in 47,48). In most cases the YAC DNA has given full levels of gene expression, proportional to the copy number of the YAC and independent of the position of integration in transgenic mice (44,49,50). YACs have also been used to complement mutations in mammalian cells (51,52) and have recently been used in the localisation of a hereditary disease gene to a 500-kb interval (53).

The OriPYAC system should give the same high levels of controlled expression and could be used to directly clone genes by complementation of mutant phenotypes. OriPYACs spanning the critical region for a particular gene could be introduced into cell lines demonstrating the mutant phenotype. OriPYACs could then be isolated from cells showing complementation and reintroduced into *S.cerevisiae* for further manipulation, such as exon trapping or cDNA selection.

We also demonstrate here that the 90-kb OriPYAC can be isolated intact from yeast cells which should allow extension of the OriPYAC system to applications where spheroplast fusion cannot be used. Successful spheroplast fusion has only been described for two human cell lines (19,54) and is clearly not suitable for many applications. Purification of the OriPYAC DNA from yeast will permit introduction of OriPYACs into mammalian cells by other methods, including lipofection, which are applicable to a wide variety of cell types and which can be used for *in vivo* gene therapy applications (55–57).

An EBV-based system of gene therapy would have the advantage of long term maintenance of the DNA, without the risk of mutagenesis by random integration. In addition, the large insert size of OriPYACs should permit efficient expression of the introduced gene. Such an approach may be particularly suitable for syndromes which can be corrected through the release of diffusible therapeutic factors from lymphoblastoid cells, such as haemophilia A or B, or chronic granulomatous disease. EBV is carried latently in the lymphoblastoid cells (asymptomatically) by >90% of the world-wide adult human population (58) and recent work has demonstrated that a helper-dependent mini-EBV vector can stably transduce B lymphoblastoid cells from a Fanconi anaemia group C patient (59). The mini construct showed *in vitro* correction of the FA phenotype, with episomal expression persisting with a half-life of 30 days.

The ability to transfer OriPYACs between human cells and yeast thus provides the basis for a large cloning capacity yeast/human shuttle vector system which should be of use in gene therapy applications as well as for functional analysis of large fragments of mammalian DNA.

ACKNOWLEDGEMENTS

We thank B. Griffin, D. Huertas and A. McGuigan for helpful suggestions. K.S. is a Wellcome Trust Prize Student. This work was partly supported by CF Trust grant PJ387.

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