MOLECULAR AND CELLULAR BIOLOGY, Sept. 1996, p. 5117–5126 Vol. 16, No. 9
0270-7306/96/$04.00+0

Stable Episomal Maintenance of Yeast Artificial Chromosomes in Human Cells

KAETRIN SIMPSON,* AMANDA MCGUIGAN, AND CLARE HUXLEY
Imperial College School of Medicine at St. Mary's, London, United Kingdom

Received 5 February 1996/Accepted 29 May 1996

Plasmids carrying the Epstein-Barr virus origin of plasmid replication (*oriP*) have been shown to replicate autonomously in latently infected human cells (J. Yates, N. Warren, D. Reisman, and B. Sugden, Proc. Natl. Acad. Sci. USA 81:3806–3810, 1984). We demonstrate that addition of this domain is sufficient for stable episomal maintenance of yeast artificial chromosomes (YACs), up to at least 660 kb, in human cells expressing the viral protein EBNA-1. To better approximate the latent viral genome, YACs were circularized before addition of the *oriP* domain by homologous recombination in yeast cells. The resulting OriPYACs were maintained as extrachromosomal molecules over long periods in selection; a 90-kb OriPYAC was unrearranged in all cell lines analyzed, whereas the intact form of a 660-kb molecule was present in two of three cell lines. The molecules were also relatively stable in the absence of selection. This finding indicates that the *oriP*–EBNA-1 interaction is sufficient to stabilize episomal molecules of at least 660 kb and that such elements do not undergo rearrangements over time. Fluorescence in situ hybridization analysis demonstrated a close association of OriPYACs, some of which were visible as pairs, with host cell chromosomes, suggesting that the episomes replicate once per cell cycle and that stability is achieved by attachment to host chromosomes, as suggested for the viral genome. The wide availability of YAC libraries, the ease of manipulation of cloned sequences in yeast cells, and the episomal stability make OriPYACs ideal for studying gene function and control of gene expression.

Yeast artificial chromosomes (YACs) have allowed the cloning of intact functional regions of mammalian DNA, including both genes which span hundreds of kilobases and gene clusters with associated locus control regions. *Saccharomyces cerevisiae* provides an ideal host for genetic manipulation by homologous recombination, allowing both large-scale and subtle alterations to be engineered into the YAC insert or into the vector arms (10, 43). Transfer of YAC DNA into mammalian cells in tissue culture, or into transgenic mice, permits the study of gene function and control of gene expression within these regions of DNA (22). YACs also provide a system with which to study the functional components of the mammalian chromosome, such as origins of replication, telomeres, and the centromere (5, 9, 11, 31, 37).

YACs have been successfully introduced into mammalian cells by a variety of methods, including microinjection, lipofection, and spheroplast fusion (22). In the majority of cases, transfected YAC DNA is found to integrate into the host cell genome. In two cases, the YAC DNA has been found to exist as extrachromosomal elements in the mammalian cells (11, 37). In both cases, the YAC was transferred by spheroplast fusion and the extrachromosomal elements contained both yeast genomic DNA and YAC DNA, indicating that recombination had occurred. Where analyzed, the extrachromosomal elements were found to be rapidly lost from the mammalian cells during growth in the absence of selection, but the loss rate was too slow to be accounted for by dilution, suggesting that the elements were replicating. However, the observed ability to replicate could be due to either the yeast genomic or the YAC DNA which is present.

Plasmid vectors based on Epstein-Barr virus (EBV) are stably maintained extrachromosomally over long periods of time with or without selection and have been shown to replicate once per cell cycle (18, 55). The functional component of such vectors is the EBV latent origin of replication (*oriP*) (32, 54), which interacts with the viral transactivator protein EBNA-1 (32, 56) to promote episomal maintenance. *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 (32, 45). Both sets of repeats are necessary for stable extrachromosomal maintenance of plasmid DNA in human cells (45), and both bind the EBNA-1 protein (42). The dyad symmetry element is the site of initiation of episomal DNA replication (12), while the family of repeats acts as an EBNA-1-dependent enhancer of transcription (44) as well as a replication fork barrier and termination site (12). In addition, the family of repeats promotes nuclear retention by the specific binding of EBNA-1 (42), followed by nonspecific association of EBNA-1 with host cell chromosomes (20, 30).

Here we describe an extension of the *oriP*/EBNA-1 system to allow the stable maintenance of DNA cloned as YACs within the nuclei of human cells. The *oriP* domain was incorporated onto two different circular YACs by homologous recombination in the yeast *S. cerevisiae*; these OriPYACs were then introduced by spheroplast fusion into human cells expressing EBNA-1. Analysis of the resultant cell lines demonstrates that OriPYACs up to 660 kb in size are maintained as unrearranged, episomal molecules for long periods of time in the absence of selection, and stable maintenance appears to be due to association of the episomes with host cell chromosomes.

MATERIALS AND METHODS

OriPYAC construction. The linear YACs were initially circularized by using the vector pPM680 (11, 13) as previously described. Correct circularization of the 90-kb YAC occurred with a frequency of about 10% of transformants. To introduce the EBV *oriP* domain onto circularized YACs, a retrofitting vector was constructed by using plasmid pHEBo as a basis (49). pHEBo contains the *oriP*

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Genetics, Imperial College School of Medicine at St. Mary's, Norfolk Place, London W2 1PG, United Kingdom. Phone: 44 171 594 3795. Fax: 44 171 706 3272.

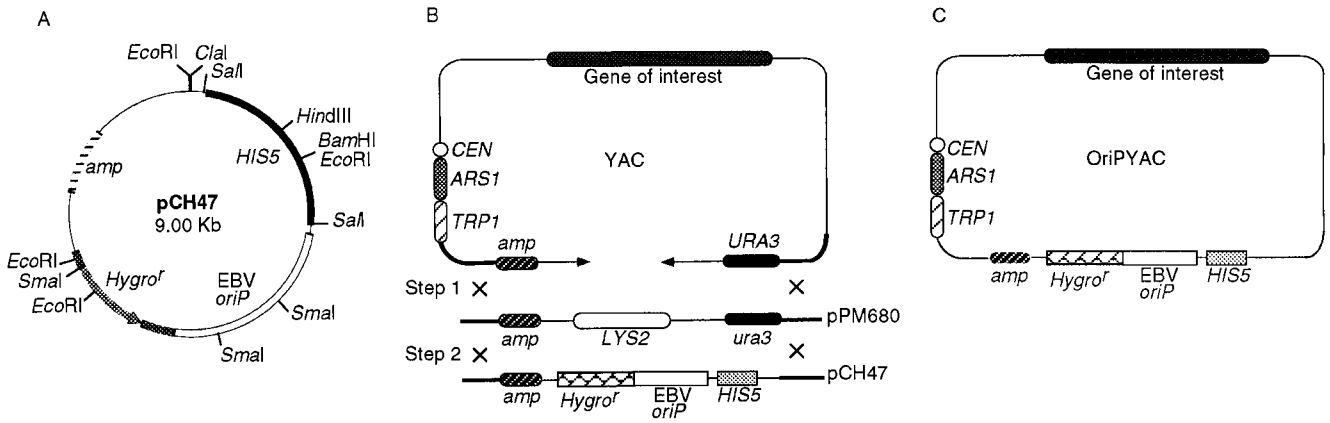

FIG. 1. OriPYAC construction. (A) The retrofitting vector pCH47. Construction is described in Materials and Methods. Selected restriction sites are shown. *HIS5* is the yeast selectable marker, *Hygro*^{r} is the hygromycin selectable marker driven by transcriptional controls from the herpes simplex virus type 1 *tk* gene, and the *oriP* region contains both the family of repeats and the dyad symmetry element. (B) Circularization of YACs and addition of the EBV *oriP* domain. The YACs were first circularized by using pPM680, which has the *LYS2* yeast selectable marker, while the *ura3* gene contains a mutation (step 1); correctly circularized YACs were then retrofitted with *oriP* and the hygromycin selectable marker by using pCH47 (step 2) as described in Materials and Methods. (C) Final OriPYAC construct carrying the yeast centromere (*CEN*), an autonomously replicating sequence (*ARS*), the *TRP1* and *HIS5* yeast markers, as well as Hygro^r and the EBV *oriP*.

domain and the coding sequences for the *hph* gene from *Escherichia coli*, flanked by promoter and polyadenylation signals from the herpes simplex virus type 1 *tk* gene, such that expression in mammalian cells confers resistance to hygromycin B. pHEBo was cut with *Bam*HI and blunt ended, and a *Cla*I linker inserted. A 2.1-kb *Sal*I fragment containing the yeast *HIS5* selectable marker gene was cloned into the *Sal*I site in pHEBo, to generate pCH47.

pCH47 was linearized with *Cla*I and transfected into the yeast strains containing circular YACs by spheroplast transformation (6). Transformants were screened to find His^+ Lys^- clones, which arose at a frequency of 4/60 for the 90-kb YAC, of which all were the correct circular molecule, and 6/36 for the 660-kb YAC, of which 4 were correct. It should be possible to carry out the circularization directly with pCH47. However, no correctly circularized clones were detected out of 100 screened for the 90-kb YAC, although the vector worked efficiently for a 40-kb YAC.

Cell culture and spheroplast fusion. The cell line 293 EBNA-1 was obtained from Invitrogen. It is a transformed human kidney line constitutively expressing EBNA-1. The cells were routinely grown in 90% Dulbecco's modified Eagle's medium with 10% fetal calf serum and 250 μg of active G418 per ml to maintain selection for the EBNA-1 gene. Fusions were carried out essentially as described previously (11), with the following modifications. Before addition of the polyethylene glycol, 50 μl of medium lacking serum was added to the pellet of cells, and the two cell types were mixed by gentle stirring for 1 min; 500 μl of prewarmed polyethylene glycol (Boehringer Mannheim) (supplemented with 10 mM $CaCl_2$ and 10% dimethyl sulfoxide) was added, and stirring was continued for 2 min. The cells were then diluted over a 2-min period with 5 ml of serum-free medium. The mixture was pelleted by centrifugation at 130 × *g* for 5 min before the cells were seeded onto 90-mm-diameter tissue culture dishes coated with 1 μg of ProNectin F Recombinant Attachment Factor (Stratagene) per cm^2. After 24 h, the plates were washed with phosphate-buffered saline (PBS) (without Ca^{2+} or Mg^{2+}), and medium containing 200 μg of hygromycin B per ml was applied 48 h after the fusion to select for the OriPYAC molecules. Fusion lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 250 μg of G418 per ml, and 200 μg of hygromycin B per ml.

DNA preparation and analysis. Agarose plugs of cell line DNA were made by a modification of a previously published method (48). An equal volume of 2% SeaPlaque agarose in PBS was added to cells suspended at 2×10^7/ml in PBS, giving 1% agarose and 10^7 cells per ml of plug. The plugs were treated with LDS (1% dodecyl lithium sulfate, 100 mM EDTA, 10 mM Tris [pH 8.0]) at 37°C with agitation for 1 h and then with fresh LDS overnight. They were then washed twice for 2 h in NDS (0.2% lauryl sarcosine, 100 mM EDTA, 2 mM Tris [pH 9.0]) at room temperature with agitation. Plugs equilibrated in NDS were γ irradiated in a Gammacell 1000 Elite. Pulsed-field gel electrophoresis (PFGE) was carried out on a BioRad CHEF DRII or DRIII, using a 1% agarose gel cast and run in 0.5× Tris-borate-EDTA. Southern transfer was onto Hybond N+ membranes (Amersham) under the conditions described by the manufacturer. All prehybridizations and hybridizations were carried out in modified Church buffer (7) (16.8 g of $NaH_2PO_4 \cdot H_2O$ per liter, 54.1 g of $Na_2HPO_4 \cdot 12H_2O$ per liter, 7% sodium dodecyl sulfate [SDS], 100 μg of salmon sperm DNA per ml) at 65°C. Filters were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 5 min and then washed twice in 0.1× SSC–0.1% SDS for 30 min, all at 65°C.

The *oriP* probe is the 0.9-kb *Sma*I fragment from the plasmid pHEBo (49), the Ty1 probe is the 1.2-kb *Xho*I-to-*Hin*dIII fragment from pCS-X (47), the cystic fibrosis transmembrane regulator (*CFTR*) probe is a 1-kb fragment from the 3′ untranslated region of the human *CFTR* cDNA, and the hypoxanthine phosphoribosyltransferase (*HPRT*) cDNA probe is the largest *Rsa*I-to-*Msp*I fragment from pDSK1 (39). These probes were labeled by using the Megaprime DNA labeling system (Amersham), and unincorporated nucleotides were removed by using NucTrap purification columns (Stratagene).

FISH. Metaphase spreads were prepared from cell lines by standard cytogenetic techniques. Fluorescence in situ hybridization (FISH) was carried out as described previously (28), with various modifications. The probe, pCH47, was labeled by incorporation of biotin-14-dATP, using a Bionick kit (GIBCO BRL). After overnight hybridization at 37°C, the slides were washed three times for 5 min each in 65% formamide–2× SSC, five times for 2 min each in 2× SSC, and once for 5 min in 4× SSC–0.1% Tween 20. All washes were at 42°C. Probe detection was with 1 μg of fluorescein isothiocyanate-conjugated avidin for 40 min at 37°C followed by three 5-min washes at 42°C in 4× SSC–0.1% Tween 20. Amplification of the signal was with 1 μg of biotinylated anti-avidin D (Vector Laboratories) followed by three 5-min washes at 42°C in 4× SSC–0.1% Tween 20. The fluorescein isothiocyanate-avidin incubation and wash step was then repeated as described above. Host chromosomes were counterstained with propidium iodide. Slides were visualized on a Leitz Aristoplan Microscope and photographed by using Fujichrome 1600 ASA slide film.

RESULTS

Construction and transfer of the OriPYACs. Two YACs were used in this study: yW30-4, a 90-kb YAC derived from the *CFTR* region of chromosome 7 (but lacking any *CFTR* sequence) (15), and yHPRT, a 660-kb YAC containing the human *HPRT* gene from the X chromosome (23, 26). These were modified by homologous recombination in *S. cerevisiae*. The linear molecules were first circularized by using the previously described plasmid pPM680 (11, 13). *oriP* and a hygromycin selectable marker were then introduced by using the construct pCH47 (Fig. 1A and B), which is based on plasmid pHEBo (49). This generates the final circular constructs, which are referred to as OriPYACs, as shown in Fig. 1C. OriPYAC90 is derived from yW30-4, and OriPYAC660 is derived from yHPRT.

After modification, the YACs were checked to determine whether rearrangement had occurred. Treatment with γ irradiation introduces double-stranded breaks into DNA molecules, which allows identification and sizing of episomes (3). The final constructs, OriPYAC90 and OriPYAC660, were treated with γ irradiation and then analyzed by PFGE to show that they were indeed circular molecules of the expected sizes (data not shown).

The OriPYACs were transferred to human kidney 293 cells,

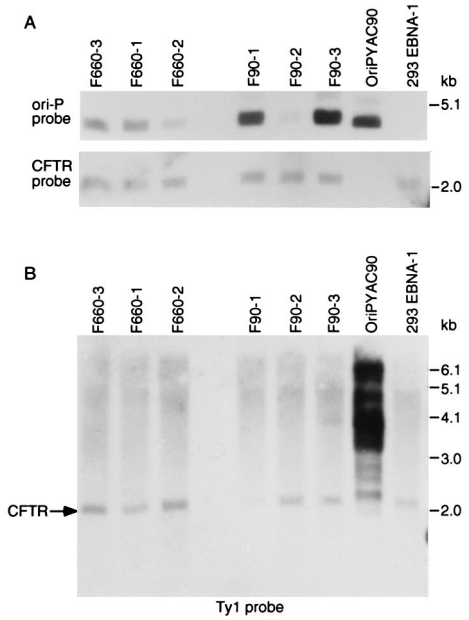

FIG. 2. DNA content of the human 293 cell lines containing OriPYACs. DNA from the cell lines F660-3, F660-1, F660-2, F90-1, F90-2, F90-3, the yeast carrying OriPYAC90, and the parental cell line 293 EBNA-1 (as indicated above each lane) was digested with *Eco*RI and resolved on a 1% agarose gel. (A) The filter was hybridized with the *oriP* and *CFTR* probes sequentially. (B) The filter was hybridized simultaneously with the Ty1 and *CFTR* probes. The *CFTR* is a positive control for loading. The positions of size markers are indicated on the right.

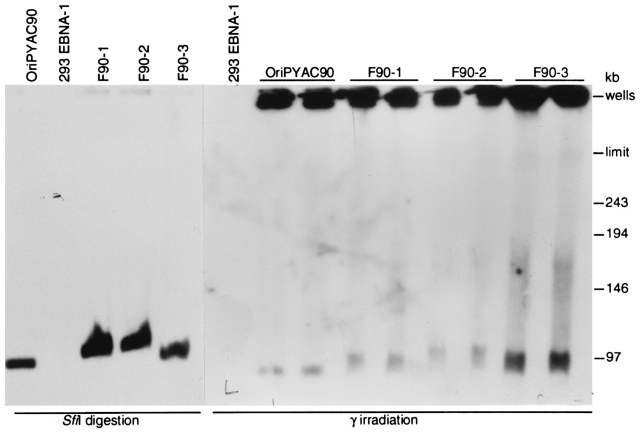

FIG. 3. Long-range analysis of cell lines containing OriPYAC90. High-molecular-weight DNA from yeast containing OriPYAC90 and from the cell lines 293 EBNA-1, F90-1, F90-2, and F90-3 (as indicated above each lane) was treated with *Sfi*I or γ irradiation as indicated at the bottom. Samples were run on the same pulsed-field gel with switching times of 5 to 15 s for 20 h. After Southern blotting, the filter was hybridized with the *oriP* probe. The section of the filter with the *Sfi*I digestions was exposed overnight, while the portion with irradiated samples was exposed for 3 days. The positions of the size markers, the wells, and the limit of resolution are indicated on the right. The characteristic shift between mammalian and yeast DNA as a result of the increased complexity of the mammalian genome is apparent.

constitutively expressing EBNA-1, by fusion with yeast spheroplasts. Cell lines F660-1, F660-2, and F660-3 were derived from fusion with OriPYAC660, while F90-1, F90-2, and F90-3 were from fusion with OriPYAC90. All fusion cell lines are clonal in origin. The frequency of colony formation was approximately one positive colony from 5×10^6 human cells fused. This is lower than that obtained when YACs are introduced into rodent cell lines such as LA-9, which gives a frequency of about 1 to 10 colonies from 10^6 cells fused (23).

YAC but not yeast genomic DNA is present in the fusion cell lines. Initial analysis of DNA content of the six fusion lines with various probes is shown in Fig. 2. After *Eco*RI digestion, a single band hybridizes with the *oriP* probe, identical in size to that seen in the YAC, indicating that YAC DNA has been transferred to the human cells as a result of the fusion reaction (Fig. 2A). The same blot was hybridized with both the *CFTR* control probe and a probe for the yeast repetitive element Ty1, which is present in about 30 copies spread throughout the yeast genome. Although there are multiple, strong bands with the Ty1 probe in the lane containing the yeast strain carrying OriPYAC90, no Ty1 DNA was detected in any of the fusion cell lines (Fig. 2B) (there is some background signal, but this is also present in the negative control lane). This finding indicates that little or no yeast genomic DNA has been maintained in the human lines along with the OriPYAC DNA.

OriPYACs of 90 kb are maintained as unrearranged, circular extrachromosomal elements. A restriction enzyme map of OriPYAC90 was not available, but digestion with *Sfi*I was found to linearize the circular YAC, producing a band of 90 kb (Fig. 3). DNA from the three fusion cell lines F90-1, F90-2, and F90-3 was digested with *Sfi*I, and the fragments were resolved by PFGE. Hybridization to an *oriP* probe demonstrated 90-kb fragments in all three cell lines, suggesting that OriPYAC90 has been transferred intact in each case (Fig. 3). However, digestion with a restriction enzyme does not distinguish between tandem integrations of the OriPYAC and maintenance as episomal elements. Gamma irradiation at the correct dose can be used to introduce a single, double-strand break into circular molecules (3), thus providing a straightforward way of distinguishing between these two possibilities. In all three OriPYAC90 cell lines, treatment with γ irradiation resulted in a band of 90 kb (Fig. 3), indicating that the OriPYAC DNA was being maintained as unrearranged episomes. Linearization by γ irradiation is never 100% efficient, and some molecules, which could be circular or integrated into the chromosomes, remain trapped in the wells.

OriPYACs of 660 kb are maintained episomally but undergo a higher frequency of rearrangement. Gamma irradiation was carried out to determine whether the OriPYAC660 molecules were episomal. Irradiation of F660-3 revealed a single band of 660 kb which was absent in the unirradiated control lane (Fig. 4A), suggesting that the OriPYAC660 is intact and episomal. The 660-kb band was detected both with an *HPRT* probe and with an *oriP* probe (Fig. 4A), indicating that both of these regions are present on the extrachromosomal molecules.

A band of 660 kb was also apparent in F660-1 (Fig. 4A), but in addition two smaller bands were visible (not shown) on longer exposure, indicating that although the molecules are episomal, deleted forms as well as the unrearranged 660-kb molecule are present. Irradiation of F660-2 gave small bands of around 100 and 150 kb (Fig. 4B). These molecules are sufficiently small that they enter the gel without prior irradiation and require high doses (300 to 500 Gy) for linearization (Fig. 4B).

F660-3 contains episomal OriPYACs, 660 kb in size, which appear to be unrearranged. Long-range restriction mapping was carried out to determine the degree of rearrangement of the OriPYAC660 episomes. A previously described map of the circular form of yHPRT contained no single-cutting enzymes (11), and so characterization was carried out with the rare-cutting enzymes *Sfi*I, *Sst*II, *Bss*HII, and *Sal*I. *Sfi*I digestion

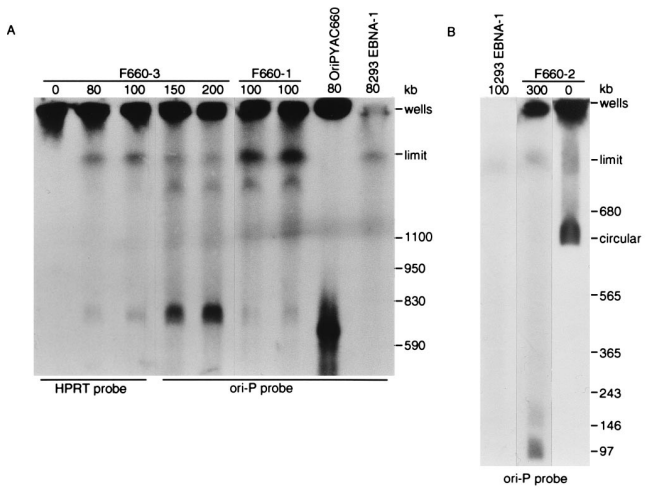

FIG. 4. Gamma irradiation of the OriPYAC660 episomes. (A) High-molecular-weight DNA was prepared from the cell lines F660-3 and F660-1, yeast cells carrying OriPYAC660, and the cell line 293 EBNA-1 as indicated above the lanes. The DNA was treated with 0, 80, 100, 150, or 200 Gy of gamma irradiation as indicated above each lane, separated on a pulsed-field gel with 60- to 120-s switching for 24 h, and blotted. The first three lanes were hybridized with the *HPRT* probe, whereas the other lanes were hybridized with the *oriP* probe. (B) DNA from the cell line 293 EBNA-1 treated with 100 Gy and from F660-2 treated with 300 and 0 Gy (as indicated above each lane) was separated on a pulsed-field gel with 45-s switching for 24 h, blotted, and probed with the *oriP* probe. The positions of the size markers, the wells, and the limit of resolution are shown on the right of each panel. The circular molecules present in F660-2 are also indicated because they run anomalously on pulsed-field gels.

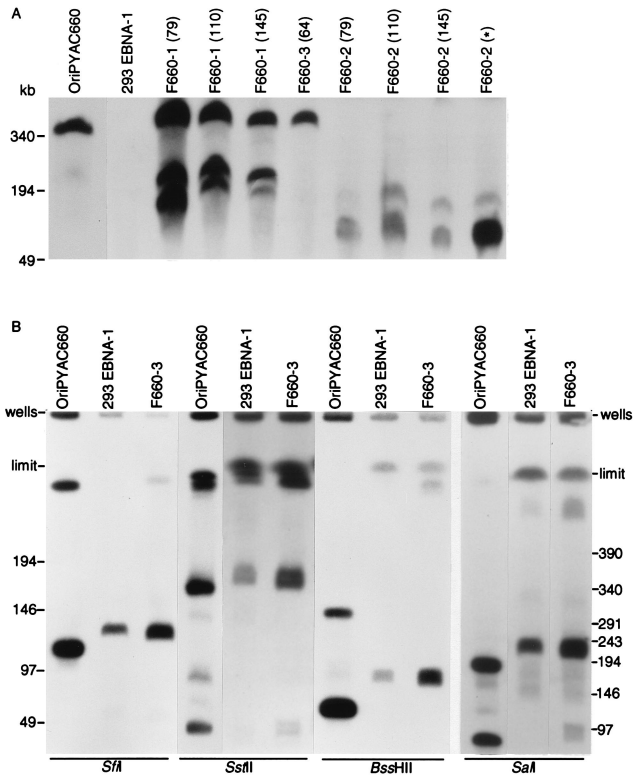

FIG. 5. Long-range restriction analysis of cell lines containing OriPYAC660. (A) DNA from yeast carrying OriPYAC660 and the cell lines 293 EBNA-1, F660-1, F660-3, and F660-2 after various times in selection was digested with *Sfi*I and resolved on a pulsed-field gel, and the filter was hybridized with the *oriP* probe. The number in parentheses after each cell line indicates the number of days of growth in selection from the time of fusion. (*) indicates cells grown out of selection for 150 days before selection was reapplied for 14 days. The pulsed-field gel was run with 45-s switching for 20 h. (B) DNA from yeast carrying OriPYAC660 and the cell lines 293 EBNA-1 and F660-3 was digested with *Sfi*I, *Sst*II, *Bss*HII, and *Sal*I, resolved on a pulsed-field gel (12-s switching for *Sst*II, *Sfi*I, and *Bss*HII and 25-s switching for *Sal*I; 20 h), blotted, and probed with the *HPRT* cDNA probe. The positions of the size markers, the wells, and the limit of resolution are shown at the sides.

followed by hybridization to an *oriP* probe resulted in a 350-kb fragment spanning the vector region of OriPYAC660 (Fig. 5A). This fragment alone was observed for the cell line F660-3 (Fig. 5A), which would correspond to the intact molecules seen with gamma irradiation. F660-1 however, contained smaller fragments in addition to the 350-kb band, while F660-2 contained only *Sfi*I fragments of less than 350 kb (Fig. 5A). In each case, the fragments observed upon *Sfi*I digestion appeared to correspond to the episomes seen after gamma irradiation; F660-3 seemed to contain intact 660-kb episomes, F660-1 contained intact and also deleted forms, and F660-2 contained only deleted versions of the OriPYACs.

Analysis with the methylation-sensitive enzymes *Bss*HII and *Sst*II followed by hybridization to an *oriP* probe resulted in the appearance of larger fragments in the cell lines than in OriPYAC660 (data not shown). This is probably due to methylation around the *oriP* region in human cells, a characteristic that has been noted for the latent viral genome (46).

As F660-3 appeared (by gamma irradiation and *Sfi*I digestion and hybridization with the *oriP* probe) to contain intact episomes, we carried out further analysis to determine whether the OriPYAC660 molecules are unrearranged in the vicinity of the *HPRT* gene. *HPRT* is located in the middle of the OriPYAC, separated from the *oriP* domain by 245 kb on one side and 330 kb on the other. Analysis was complicated by the presence of the endogenous *HPRT* gene in the human 293 EBNA-1 cell line and by methylation of some sites in the human cells but not in yeast cells. Since methylation is not a complete or uniform process, different regions of DNA are methylated to different extents. However, some of the *HPRT* signal in F660-3 must derive from the episomes, because irradiation followed by *HPRT* hybridization demonstrated that this gene is present on the episomal molecules (Fig. 4A).

*Sfi*I digestion of OriPYAC660 followed by hybridization to the *HPRT* cDNA probe results in a band of approximately 120 kb, with a larger band near the limit of resolution. Both the parental 293 EBNA-1 cells and F660-3 cells contain fragments of around 120 kb; these appear larger than the band in the YAC, but this is probably due to a shift in migration between the yeast and the mouse genomic DNA on PFGE. This phenomenon, which has been observed frequently (11, 14), results because there is only 0.5% as much yeast DNA electrophoresing. F660-3 also contains a band near the limit of resolution which corresponds to the band in the YAC but is not present in 293 EBNA-1. Some of the signal in the F660-3 120-kb band is almost certainly from the episomes. There are no other bands to account for the *HPRT* DNA, which from gamma irradiation is known to be present on the episomes. In addition, when a similar *Sfi*I digest was hybridized with a single-copy probe (*CFTR*), to control for loading, there was found to be 2.5 times as much *HPRT* signal in the 120-kb band from F660-3 as in the band from the 293 EBNA-1 control.

*Sst*II digestion of OriPYAC660 produces two major bands at about 40 and 170 kb (Fig. 5B). F660-3 and 293 EBNA-1 both contain the 170-kb band (again with a shift in relation to the yeast DNA). In addition F660-3, but not 293 EBNA-1, contains the 40-kb fragment. The sites around this smaller fragment

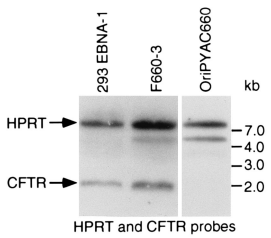

FIG. 6. Copy number of the OriPYACs. High-molecular-weight DNA from 293 EBNA-1 and F660-3 cells and from yeast cells carrying OriPYAC660 was digested with *Eco*RI, resolved on a 1% agarose gel, blotted, and probed with *CFTR* and *HPRT*. After exposure to X-ray film, the filter was completely dried and exposed to a phosphor imaging screen for 18 h. Positions of size markers are indicated on the right.

could be methylated in the 293 EBNA-1 cells and partially methylated (to account for the low intensity) in F660-3. *Bss*HII digestion of OriPYAC660 results in two fragments of approximately 50 and 140 kb. Both F660-3 and 293 EBNA-1 contain a fragment of about 80 kb. In this case, the shift between yeast and mammalian bands appears to be greater than would be expected by the effect of differential migration alone. Methylation of a site, however, could be responsible for the appearance of the larger fragment in the 293 EBNA-1 and F660-3 cell lines. *Sal*I digestion yields two bands in OriPYAC660 of about 50 and 200 kb. The larger fragment is present in both 293 EBNA-1 and F660-3 (again with a shift in migration making the fragment seem larger). The smaller fragment is present only in F660-3, further indicating that some of the signal is derived from the OriPYAC and that methylation has probably obscured this fragment in 293 EBNA-1.

Thus, the bands seen in F660-3 are all consistent with this cell line containing unrearranged 660-kb episomes. The consistent shift observed between bands derived from F660-3 and 293 EBNA-1 and those derived from yeast carrying OriPYAC660 are most probably explained by different migration of the yeast and mammalian DNA under PFGE conditions. All bands observed in F660-3 are consistent with either OriPYAC660 and 293 EBNA-1, or with the OriPYAC alone, whereas extensive rearrangements of OriPYAC DNA would be expected to result in novel fragment sizes. Where bands are held in common with the genomic copy of *HPRT*, the signal in F660-3 is more intense (confirmed by phosphor imaging), suggesting that several copies of the *HPRT* gene contributed to the bands. This is in agreement with phosphor imaging data (Fig. 6), which demonstrate an episomal copy number of 3 for F660-3, and with Fig. 4A, which shows that the *HPRT* gene is present on the episomal molecules. In addition, several of the smaller fragments present in OriPYAC660 are not seen in the 293 EBNA-1 cells; this could be due to methylation of these restriction sites in the cell line. The small fragments are, however, visible in F660-3, confirming that some of the *HPRT* signal detected is from OriPYAC660 molecules and thus indicating that the episomal DNA is not as heavily methylated as the human genomic DNA.

We were unable to determine whether the *HPRT* gene on OriPYAC660 was being expressed in the human cells, as known restriction fragment length polymorphisms (24, 25) between the introduced and endogenous versions of the gene proved uninformative. However, previous work (2, 34, 41) has demonstrated that vectors containing *oriP* can be used for expression of proteins in human cells and that the presence of *oriP* in vectors does not appear to suppress gene expression.

Episomal copy number varies between cell lines. As can be seen in Fig. 2A, the copy number of the elements varies between different fusion lines, appearing to be highest in F90-3 and lowest in F90-2. Multiple copies of episomes per cell are characteristic of EBV (38) and of EBV-based vectors (34, 50), although it is not clear how multiple copies become established. We have already shown that the episomes in F660-3 contain the *HPRT* gene and appear to be intact. Thus, to determine the copy number of the elements, we compared the *HPRT* signal, after *Eco*RI digestion, in F660-3 with that in the parental 293 EBNA-1 cells, using a single-copy probe from the *CFTR* gene to control for DNA loading in the two lanes (Fig. 6). This gave an average copy number of three OriPYAC660 molecules per cell for F660-3. The copy numbers of episomal elements in the remaining cell lines were then estimated by comparing the relative intensities of the *oriP* bands in Fig. 2A, again controlling for differences in loading between lanes with the single-copy *CFTR* probe. The episomal copy numbers were found to be 9.0 (F90-1), 1.3 (F90-2), 18 (F90-3), 5.4 (F660-1), 2.4 (F660-2), and 3.0 (F660-3). These values represent the average number of episomes per cell and include any integrated copies of the elements. It is not possible to determine the copy number from the signal obtained after gamma irradiation because this process of linearization is highly inefficient and much of the DNA (open circular molecules as well as possible integrated DNA) is trapped in the wells.

OriPYACs are stable in human cells for several months in the presence and in the absence of selection. After approximately 3 months in culture, a stability assay was carried out on the initial cell lines F90-2, F660-1, and F660-2. The doubling time of the parental 293 EBNA-1 cells was estimated in the presence of G418 and found to be 24 h. The doubling time of the fusion lines containing multiple copies of the OriPYACs was determined in the presence and absence of hygromycin; G418 was always present. The fusion cell lines were found to have doubling times of 31 h in the absence of hygromycin and 32 h in its presence.

F90-2 was grown for an additional 62 days with and without selection for OriPYAC90. This provides an assay for both replication and segregation ability. As can be seen in Fig. 7A, the amount of OriPYAC90 had decreased little in the absence of selection for 62 days, indicating that the OriPYAC was segregating quite efficiently. Gamma irradiation produced a discrete band of 90 kb in all the samples (Fig. 7B), demonstrating that unrearranged, episomal forms of the YAC were still present after 62 days of growth, both in and out of selection. This result indicates that there is little tendency for the molecules to rearrange or become integrated during growth in selection and that maintenance in the absence of selection appears not to be due to integration.

Lines F660-1 and -2 were grown with and without selection for an additional 164 days, which would correspond to 123 divisions in the presence of hygromycin and 127 cell divisions in the absence of the drug. (F660-3 was generated later, and a time course was not carried out.) OriPYAC DNA was still found to be present after 164 days without selection (Fig. 8A) although at a reduced amount. The rate of loss in the absence of selection was calculated, using an exponential decay equation, from phosphor imaging of the *Eco*RI digest in Fig. 8A, using the *CFTR* hybridization as a control for loading. This analysis revealed a 3% loss per generation for F660-2 and a 1.2% loss for F660-1, or half-lives of 23 and 58 cell divisions, respectively. This stability is as good or better than values obtained for the loss of much smaller plasmids carrying *oriP*, which are lost at rates of about 2, 3, and 5% per generation (45).

The F660 cell lines were also analyzed by gamma irradiation

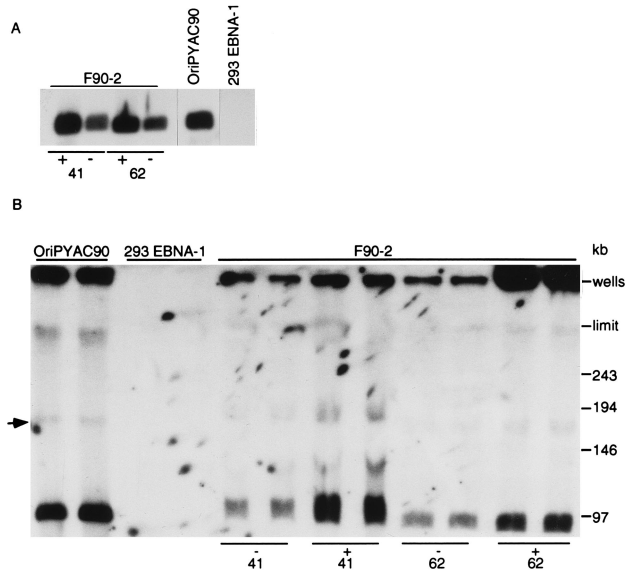

FIG. 7. Stability of the OriPYAC90 episomes in F90-2. High-molecular-weight DNA from yeast cells carrying OriPYAC90, the cell line 293 EBNA-1, and the cell line F90-2 was prepared at 41 and 62 days after the start of the stability experiment. (A) The DNA was digested with *Eco*RI, resolved on a 1% agarose gel, blotted, and probed with *oriP*. (B) DNA was γ irradiated (pairs of lanes; left lane with 400 Gy and right lane with 450 Gy) and resolved on a pulsed-field gel with 5- to 15-s switching for 20 h before being blotted and probed with the *oriP* probe. The characteristic extra bands present in both the yeast and F90-2 cells are indicated by an arrow. The number of days growth with or without selection is indicated below the lanes. +, indicates cells grown with selection; −, cells grown without selection. The positions of size markers, the wells, and the limit of resolution are shown on the right.

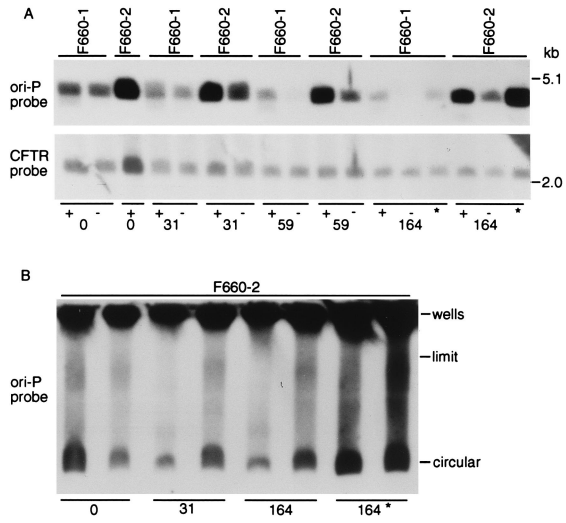

FIG. 8. Stability of the OriPYAC660 episomes. (A) Maintenance of F660-1 and F660-2 episomes without selection. The cell lines F660-1 and F660-2 were grown for 164 days (after the start of the stability experiment) with or without selection. DNA was prepared at 0, 31, 59, and 164 days (as indicated below the lanes), digested with *Eco*RI, resolved on a 1% agarose gel, and probed first with the *oriP* probe and then with the *CFTR* probe as a control for loading. +, cells grown with selection; −, cells grown without selection; ∗, cells grown without selection for 150 days before selection was reapplied for 14 days. After exposure, the filter was dried and exposed to a phosphor imaging screen; rates of loss were calculated by using the *CFTR* hybridization as a control for loading. (B) Extrachromosomal maintenance of F660-2 episomes over time in selection. High-molecular-weight DNA from F660-2 (grown in selection) was prepared 0, 31, and 164 days after the start of the stability experiment (or 79, 110, and 243 days from the fusion). 164* indicates cells grown for 150 days without selection before selection was reapplied for 14 days. The DNA was separated by PFGE with 45-s switching for 24 h. Pairs of lanes: left lane, 0 Gy; right lane, 80 Gy (80 Gy is not enough to linearize the 100- and 150-kb molecules). Under these conditions, the 100- and 150-kb circular molecules enter the gel but the two different sizes of molecule run at the same position, which is indicated to the right of the panel.

after prolonged periods of culture to determine if the OriPYACs were still episomal and whether they tended to rearrange. Irradiation of F660-3 after 64 days of growth in selection, followed by freezing, thawing, and a further 14 days growth in selection, showed the intact molecule of 660 kb (Fig. 4A), suggesting that this large molecule rearranges very infrequently and that stability is not entirely due to integration.

F660-1 was irradiated after 79 (Fig. 4A) and 128 days (not shown) in selection and gave both the 660-kb and the smaller molecules, at the same relative intensities at both time points, indicating that the smaller molecules do not have a selective advantage and that the elements do not appear to integrate. The smaller molecules seen after irradiation probably correspond to the smaller *Sfi*I fragments seen in Fig. 5A, which again do not vary in intensity after 79, 110, and 145 days growth in selection.

F660-2 gave the same-size episome (which is visible without irradiation) at 79, 110, and 243 days in selection from the time of fusion (corresponding to 0, 31, and 164 days after the start of the stability experiment) (Fig. 8B). F660-2 was also grown for 150 days (116 generations) without selection followed by reapplication of the selection for 14 days (10.5 generations) (164* in Fig. 8A and B). Under these conditions, the *oriP* signal is reamplified (Fig. 8A) and the intensity of the episomal bands increases, suggesting that episomal rather than integrated forms of the OriPYAC are amplified (Fig. 8B).

Thus, OriPYACs persist as episomes for many cell divisions in culture with little apparent integration if selection is maintained and are still present after 127 cell divisions in the absence of selection. In addition, no change in size of the molecules was observed over extended periods of growth; deletion derivatives of OriPYAC660 were observed, but these stayed the same over many cell divisions and hence probably arose early on, possibly during the fusion process.

OriPYACs associate with human metaphase chromosomes. FISH, using plasmid pCH47 as a probe, was carried out to visualize the OriPYACs on metaphase spreads and to determine whether the episomes were associated with host cell chromosomes. F90-3, F660-1, F660-2, and the parental 293 EBNA-1 line were analyzed as shown in Fig. 9. Because of the small size of the target sequence (pCH47 is 9 kb in size), the signal was amplified, which in turn leads to some background hybridization. The patterns of hybridization seen in all fusion lines were very similar and characteristic of extrachromosomal elements. The cell lines contained brightly staining elements scattered throughout the metaphase spread but always associated with host cell chromosomes. In many cases, the elements appeared as double dots of signal, which did not appear to be integrated because the double dots were often distributed either along the chromosomes or diagonally, rather than directly opposite each other on sister chromatids. Figure 9E shows the appearance of extrachromosomal elements derived from cyHPRT (a circular precursor of OriPYAC660 lacking the *oriP* domain) in a mouse LA-9 cell line which does not express EBNA-1 (11). In this case, the extrachromosomal elements are distributed among the host cell chromosomes and do not appear to show any association with the chromosomes themselves.

Figure 9 is intended as a qualitative demonstration of association of multiple OriPYAC episomes with host cell chromo-

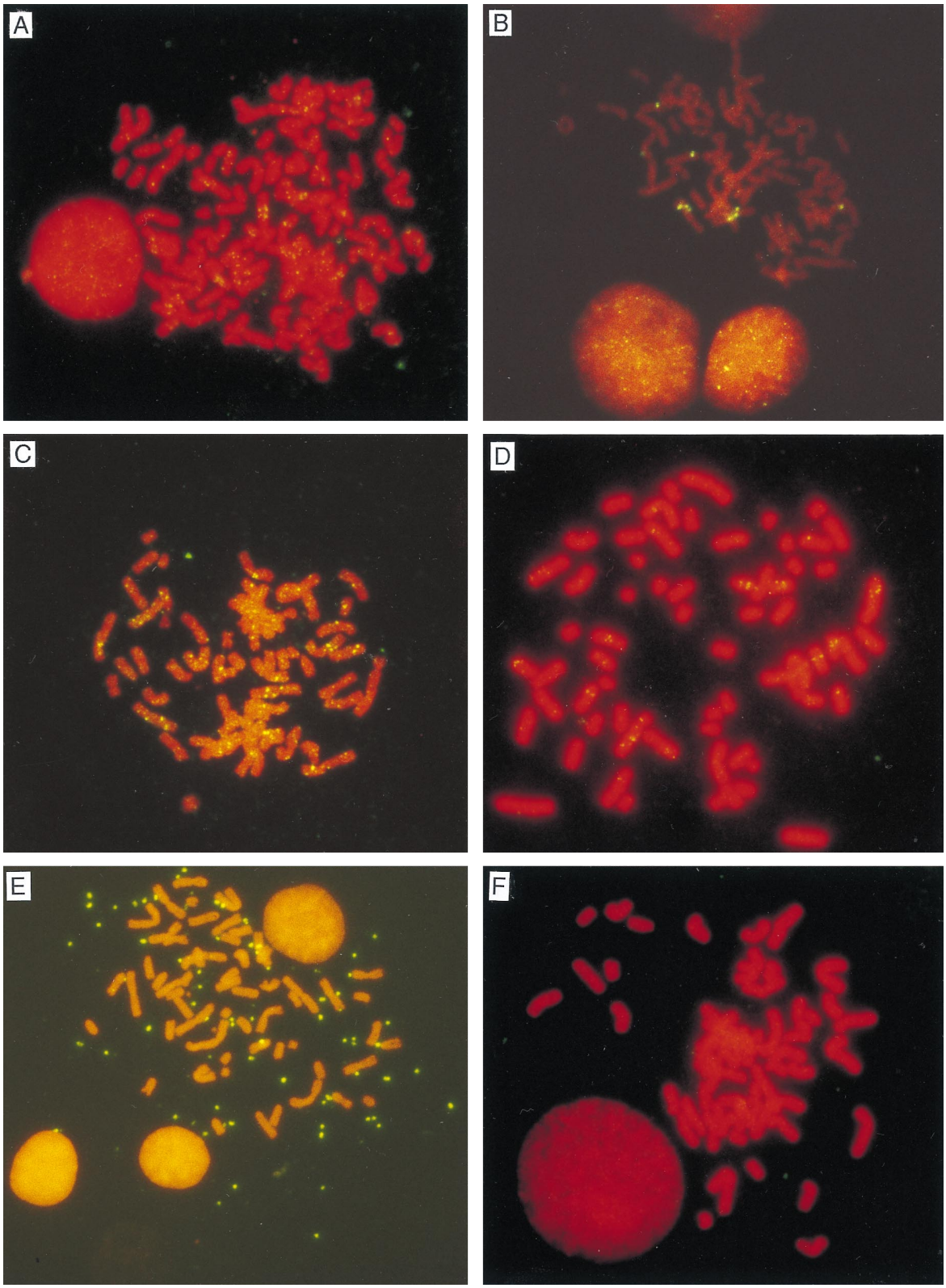

FIG. 9. FISH analysis of the OriPYAC fusion cell lines. (A to D and F) Metaphase spreads were probed with pCH47 (green), and the signal was amplified. (E) Total yeast DNA probe (green) with no amplification. Chromosomes are counterstained with propidium iodide. (A) F90-3 after approximately 105 days (79 generations) in selection; (B) F660-1 after approximately 60 days (45 generations) in selection; (C) F660-2 after approximately 60 days (45 generations) in selection; (D) F660-2 after approximately 220 days (165 generations) in selection; (E) metaphase spread from a mouse LA-9 fusion clone containing cyHPRT, a circular precursor of OriPYAC660 which lacks the *oriP* domain (11); (F) 293 EBNA-1 (negative control).

somes. The metaphase spreads chosen contain many elements and are not representative of the average copy number within the cell lines. It is not possible to accurately determine the copy number of the episomes from the FISH data because of the variable intensity of the signal from the elements. However, it is clear that the copy number of elements is not the same in all cells within a cell line but rather varies from zero to about 100. Such a distribution has been noted for other EBV-based plasmids, in which cells within a line show variable numbers of episomes (50).

DISCUSSION

The plasmid vectors described here allow the modification of linear YACs to form circular molecules carrying the EBV *oriP*, a region which in association with the viral transactivator EBNA-1 confers segregation and replication on circular molecules. The resulting OriPYACs can be transferred by spheroplast fusion into human cells expressing EBNA-1 and are then maintained as circular, extrachromosomal elements. A 90-kb molecule was found to be unrearranged in three of three cell lines, whereas a 660-kb molecule was unrearranged in one of three lines. These elements are stable over prolonged growth in culture; in the presence of selection they do not appear to vary in size or to integrate for over 8 months (180 generations) from the time of fusion, and they are retained as extrachromosomal elements for at least 5.5 months (127 generations) in the absence of selection, being lost exponentially at a rate of approximately 1 to 3% per generation.

Previous experiments in which YAC DNA carrying a mammalian selectable marker has been transferred by fusion into mammalian cells have generally led to the isolation of cell lines in which the YAC DNA is integrated into the mammalian chromosomes. Variable amounts of yeast genomic DNA are usually observed at the same site of integration as the YAC (27, 35, 40). There have been only two documented cases of extrachromosomal elements which have arisen after introduction of a YAC into mammalian cells (11, 37). In one of these studies, we transferred cyHPRT (a circular precursor form of OriPYAC660 which lacks the *oriP* domain) into mouse LA-9 cells, which do not express EBNA-1 (11). Extrachromosomal elements were observed but were present in only a minority of cell lines and contained yeast genomic DNA in addition to YAC DNA. The elements appeared to replicate quite efficiently, still being present after the cell lines had been expanded, suggesting that sequences present on the YAC or yeast genomic DNA may function as origins of replication in the mouse cells. However, when selection for cyHPRT was dropped, the elements were rapidly lost, indicating that they segregated very poorly (90% loss in 19 days); if selection was maintained, they tended to integrate, explaining why only a minority of cell lines appeared to contain extrachromosomal elements.

Attempts to stabilize YACs as extrachromosomal elements, or artificial chromosomes, in mammalian cells by addition of the functional elements of mammalian chromosomes have so far proved unsuccessful. Alphoid DNA is present at all the human centromeres (33, 53), binds the centromeric protein CENP-B (36), and is located at the functional centromere of deleted Y chromosomes (4, 52). Introduction of YACs containing alphoid DNA from the Y chromosome resulted in some characteristic centromeric features such as cytological constrictions at the site of integration and anaphase bridges (31). However, this putative centromere DNA did not lead to segregating extrachromosomal elements (17, 31). Addition of human telomeric sequences to both arms of a YAC led to human telomere formation with about 10% efficiency in CHO K1 cells, but no extrachromosomal elements with two functional telomeres were isolated (51).

Here we have achieved stable extrachromosomal maintenance of circular YACs up to 660 kb in size by exploitation of the EBV *oriP*–EBNA-1 interaction. The episomes carrying *oriP* in an EBNA-1-containing cell line behave quite differently from those formed by cyHPRT (the same YAC but lacking *oriP*) in mouse cells not expressing EBNA-1. The OriPYACs formed episomes in six of six cell lines, they were lost very slowly during growth out of selection and were maintained extrachromosomally for up to 8 months of growth in selection. Although it is possible that differences between the cell lines contribute to differences in extrachromosomal behavior, it would seem that the *oriP*–EBNA-1 interaction is essential for stable, episomal maintenance of the OriPYAC episomes.

It has previously been demonstrated that the replication origin and nuclear retention properties of the *oriP* domain may be functionally separable, though both require EBNA-1. The family of repeats confers nuclear localization, while the dyad symmetry element is the origin of replication. Plasmids containing just the family of repeats do not replicate effectively but give a transient drug resistance phenotype over about 2 weeks, with no stable transformants after approximately 4 weeks (45), probably because of prolonged retention of unreplicated DNA in the cell nucleus (30). Introduction of random fragments of human DNA over about 10 kb into these plasmids complements the ability to replicate, producing plasmids which replicate and segregate as efficiently as those which contain the intact *oriP* region (30). In these molecules, it appears that the *oriP* domain is necessary for nuclear retention, while replication initiates from within the mammalian sequences (29). Furthermore, any fragment of mammalian DNA larger than about 10 kb was able to replicate in this assay (21).

The ability of the OriPYACs to replicate efficiently is demonstrated by the continued presence of episomes for more than 8 months (approximately 180 generations) after fusion, with little apparent evidence of integration. In addition, FISH analysis demonstrated that some of the elements appeared in pairs on the metaphase chromosomes. These double dots probably indicate that the episome has replicated once in the preceding S phase, as has been shown to occur for the latent viral genome (16, 19). Double dots of signal are also seen with double minute elements, which replicate once per S phase, in cancer cell lines (8). The presence of single dots, however, prevents firm conclusions from being drawn about the timing of episomal replication. Replication of OriPYAC90, which is approximately half the size of the viral genome, could be expected to occur efficiently if *oriP* alone was used as the origin. However, the apparently efficient replication of OriPYAC660, nearly four times as large as the latent viral genome, suggests that replication may initiate from human sequences present on the YAC rather than from within *oriP*. Given the large size of the OriPYACs, it is highly probable that they both contain sequences which can be used as origins (29, 30). In addition, extrachromosomal elements containing cyHPRT and yeast genomic DNA, but lacking the *oriP* domain, appeared to replicate efficiently in mammalian cells (11). Thus, we propose that the *oriP*–EBNA-1 interaction is probably not necessary for replication of the OriPYAC episomes.

In contrast, the *oriP*–EBNA-1 interaction appears to be essential for nuclear retention and episomal maintenance. The FISH data provide strong evidence of association of the OriPYACs with host cell metaphase chromosomes, in a manner similar to that demonstrated for the latent viral genome (20) and for other vectors carrying the *oriP* domain (2). This is

in direct contrast to the extrachromosomal elements which formed after introduction of cyHPRT (no *oriP*/EBNA-1 present) and which were seen to be scattered among the mammalian chromosomes, with no obvious chromosomal association (Fig. 9). Attachment to the chromosomal scaffold may explain the increased stability of the OriPYAC episomes in the absence of selection. Segregation of the OriPYAC elements with the host cell chromatids at mitosis would ensure that the episomes are partitioned between daughter cells, in much the same way as has been proposed for the latent virus (20). The observed variation in episomal copy number between metaphase spreads within each cell line suggests that partitioning of the elements is not exact. However, this passive method of episomal segregation, while not matching the strictly controlled segregation of endogenous eukaryotic chromosomes, does appear to be effective, allowing the persistence of molecules significantly larger than the viral genome over prolonged periods of culture.

EBNA-1 is also known to have a nuclear localization signal spanning amino acid residues 379 to 386 (1). Binding of the protein to the *oriP* domain of the OriPYAC may efficiently target movement of the DNA to the host cell nucleus after the fusion event. This would reduce the potential for recombination and could explain the absence of yeast genomic DNA in the cell lines. In contrast, extrachromosomal elements generated after introduction of cyHPRT contained variable amounts of yeast genomic DNA.

The *oriP*/EBNA-1 system has recently been used to create human artificial episomal chromosomes (50) by cloning large, random fragments of human DNA into a vector containing *oriP*, followed by lipofection of high-molecular-weight DNA into EBNA-1-expressing human cells. However, the average insert size of this library was around the size of the latent viral genome at just 150 kb, and because all of the DNA was human, it was difficult to determine whether rearrangements had occurred. The system has also been used for expression of genes in human lymphoblastoid cells; in this case, the half-life of the approximately 160-kb elements was about 30 days (2), which is very similar to our findings.

One approach toward an understanding of the DNA sequences necessary for mammalian chromosome structure and function is to build up an artificial model which can be maintained as a stable, extrachromosomal element within the nuclei of mammalian cells. We have shown that circular YACs carrying a nuclear retention system can be maintained as multicopy, relatively stable extrachromosomal elements within the nuclei of human cells. Replacing the *oriP* region with functional centromeric DNA would be expected to give similarly stable elements maintained at one copy per cell, while introduction of telomeric sequences might allow the molecules to be maintained as linear elements. In addition, the postulated arrangement of the OriPYAC on the nuclear scaffold may provide a system for the study of the DNA elements necessary for chromatin loop organization and higher-order chromosome structure.

There are several advantages to application of the *oriP*/EBNA-1 system to YACs. Regions of DNA up to several megabases in size can be cloned, allowing the isolation of large genes or gene clusters intact. *S. cerevisiae* provides an ideal host for characterization and manipulation of cloned sequences prior to reintroduction into mammalian cells. In addition, the wide availability of YAC libraries makes isolation of YAC clones carrying a particular gene relatively straightforward. Thus, OriPYACs could be used to analyze control of gene expression and the effects of long-range controlling elements, as well as permitting studies of gene function by complementation of mutant phenotypes. These OriPYACs may also be of use in gene therapy, as problems due to integration would be avoided and gene expression could be under normal control.

ACKNOWLEDGMENTS

We thank B. Sugden, T. Featherstone, and R. Williamson for their contributions to this work.

This work was supported in part by MRC grant 9227430. K.S. was supported by a Wellcome Trust Prize Studentship.

REFERENCES

1. **Ambinder, R. F., M. Mullen, Y.-N. Chang, G. S. Hayward, and S. D. Hayward.** 1991. Functional domains of Epstein-Barr virus nuclear antigen EBNA-1. J. Virol. **65:**1466–1478.
2. **Banerjee, S., E. Livanos, and J.-M. H. Vos.** 1995. Therapeutic gene delivery in human B-lymphoblastoid cells by engineered non-transforming infectious Epstein-Barr virus. Nat. Med. **1:**1303–1308.
3. **Beverley, S. M.** 1989. Estimation of circular DNA size using γ-irradiation and pulsed-field gel electrophoresis. Anal. Biochem. **177:**110–114.
4. **Brown, K. E., M. A. Barnett, C. Burgtorf, P. Shaw, V. J. Buckle, and W. R. A. Brown.** 1994. Dissecting the centromere of the human Y chromosome with cloned telomeric DNA. Hum. Mol. Genet. **3:**1227–1237.
5. **Brown, W. R. A.** 1989. Molecular cloning of human telomeres in yeast. Nature (London) **338:**774–776.
6. **Burgers, P. M. J., and K. J. Percival.** 1987. Transformation of yeast spheroplasts without cell fusion. Anal. Biochem. **163:**391–397.
7. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81:**1991–1995.
8. **Cowell, J. K.** 1982. Double minutes and homogeneously staining regions: gene amplification in mammalian cells. Annu. Rev. Genet. **16:**21–59.
9. **Cross, S. H., R. C. Allshire, S. J. McKay, N. I. McGill, and H. J. Cooke.** 1989. Cloning of human telomeres by complementation in yeast. Nature (London) **338:**771–774.
10. **Duff, K., A. McGuigan, C. Huxley, F. Schulz, and J. Hardy.** 1994. Insertion of a pathogenic mutation into a yeast artificial chromosome containing the human amyloid precursor protein gene. Gene Ther. **1:**70–75.
11. **Featherstone, T., and C. Huxley.** 1993. Extrachromosomal maintenance and amplification of yeast artificial chromosome DNA in mouse cells. Genomics **17:**267–278.
12. **Gahn, T. A., and C. L. Schildkraut.** 1989. The Epstein-Barr virus origin of plasmid replication, *oriP*, contains both the initiation and termination sites of DNA replication. Cell **58:**527–535.
13. **Garza, D., J. W. Ajioka, D. T. Burke, and D. L. Hartl.** 1989. Mapping the *Drosophila* genome with yeast artificial chromosomes. Science **246:**641–646.
14. **Gnirke, A., T. S. Barnes, D. Patterson, D. Schild, T. Featherstone, and M. V. Olson.** 1991. Cloning and *in vivo* expression of the human GART gene using yeast artificial chromosomes. EMBO J. **10:**1629–1634.
15. **Green, E. D., and M. V. Olson.** 1990. Chromosomal region of the cystic fibrosis gene in yeast artificial chromosomes: a model for human genome mapping. Science **250:**94–98.
16. **Gussander, E., and A. Adams.** 1984. Electron microscopic evidence for replication of circular Epstein-Barr virus genomes in latently infected Raji cells. J. Virol. **52:**549–556.
17. **Haaf, T., P. E. Warburton, and H. F. Willard.** 1992. Integration of human α-satellite DNA into simian chromosomes: centromere protein binding and disruption of normal chromosome segregation. Cell **70:**681–696.
18. **Haase, S. B., and M. P. Calos.** 1991. Replication control of autonomously replicating sequences. Nucleic Acids Res. **19:**5053–5058.
19. **Hampar, B., A. Tanaka, M. Nonoyama, and J. G. Derge.** 1974. Replication of the resident repressed Epstein-Barr virus genome during the early S phase (S-1 period) of non-producer Raji cells. Proc. Natl. Acad. Sci. USA **71:**631–633.
20. **Harris, A., B. D. Young, and B. E. Griffin.** 1985. Random association of Epstein-Barr virus genomes with host cell metaphase chromosomes in Burkitt's lymphoma-derived cell lines. J. Virol. **56:**328–332.
21. **Heinzel, S. S., P. J. Krysan, C. T. Tran, and M. P. Calos.** 1991. Autonomous DNA replication in human cells is affected by the size and the source of the DNA. Mol. Cell. Biol. **11:**2263–2272.
22. **Huxley, C.** 1994. Transfer of YACs to mammalian cells and transgenic mice, p. 65–91. *In* J. K. Setlow (ed.), Genetic engineering. Plenum Press, New York.
23. **Huxley, C., Y. Hagino, D. Schlessinger, and M. V. Olson.** 1991. The human HPRT gene on a yeast artificial chromosome is functional when transferred to mouse cells by cell fusion. Genomics **9:**742–750.
24. **Igarashi, T., H. Ikegami, H. Yamazaki, and M. Minami.** 1990. *Bam*HI restriction fragment length polymorphisms for hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene of carriers and controls of HPRT deficiency in Japan. Acta Paediatr. Jpn. **32:**12–15.

25. **Igarashi, T., and S. Kamoshita.** 1990. Carrier detection of partial hypoxanthine-guanine phosphoribosyltransferase deficiency by analysis with *Bam*HI restriction length polymorphisms and oligonucleotide probes. Pediatr. Res. **27:**417–421.
26. **Imai, T., and M. V. Olson.** 1990. Second-generation approach to the construction of yeast artificial-chromosome libraries. Genomics **8:**297–303.
27. **Jakobovits, A., A. L. Moore, L. L. Green, G. J. Vergara, C. E. Maynard-Currie, H. A. Austin, and S. Klapholz.** 1993. Germ-line transmission and expression of a human-derived yeast artificial chromosome. Nature (London) **362:**255–258.
28. **Johnson, C. V., R. H. Singer, and J. B. Lawrence.** 1991. Fluorescent detection of nuclear RNA and DNA: implications for genome organization. Methods Cell Biol. **35:**73–99.
29. **Krysan, P. J., and M. P. Calos.** 1991. Replication initiates at multiple locations on an autonomously replicating plasmid in human cells. Mol. Cell. Biol. **11:**1464–1472.
30. **Krysan, P. J., S. B. Haase, and M. P. Calos.** 1989. Isolation of human sequences that replicate autonomously in human cells. Mol. Cell. Biol. **9:**1026–1033.
31. **Larin, Z., M. D. Fricker, and C. Tyler-Smith.** 1994. De novo formation of several features of a centromere following introduction of a Y alphoid YAC into mammalian cells. Hum. Mol. Genet. **3:**689–695.
32. **Lupton, S., and A. J. Levine.** 1985. Mapping genetic elements of Epstein-Barr virus that facilitate extrachromosomal persistence of Epstein-Barr virus-derived plasmids in human cells. Mol. Cell. Biol. **5:**2533–2542.
33. **Manuelidis, L.** 1978. Chromosomal locations of complex and simple repeated human DNAs. Chromosoma **66:**23–32.
34. **Margolskee, R. F., P. Kavathas, and P. Berg.** 1988. Epstein-Barr virus shuttle vector for stable episomal replication of cDNA expression libraries in human cells. Mol. Cell. Biol. **8:**2837–2847.
35. **Markie, D., J. Ragoussis, G. Senger, A. Rowan, D. Sansom, J. Trowsdale, D. Sheer, and W. F. Bodmer.** 1993. New vector for transfer of yeast artificial chromosomes to mammalian cells. Somatic Cell Mol. Genet. **19:**161–169.
36. **Masumoto, H., H. Masukata, Y. Muro, N. Nozaki, and T. Okazaki.** 1989. A human centromere antigen (CENP-B) interacts with a short specific sequence in alphoid DNA, a human centromeric satellite. J. Cell Biol. **109:** 1963–1973.
37. **Nonet, G. H., and G. M. Wahl.** 1993. Introduction of YACs containing a putative mammalian replication origin into mammalian cells can generate structures that replicate autonomously. Somatic Cell Mol. Genet. **19:**171–192.
38. **Nonoyama, M., and J. S. Pagano.** 1973. Homology between Epstein-Barr virus DNA and viral DNA from Burkitt's lymphoma and nasopharyngeal carcinoma determined by DNA-DNA reassociation kinetics. Nature (London) **242:**44–47.
39. **Nussbaum, R. L., W. E. Crowder, W. L. Nyhan, and C. T. Caskey.** 1983. A three-allele restriction-fragment-length polymorphism at the hypoxanthine phosphoribosyltransferase locus in man. Proc. Natl. Acad. Sci. USA **80:** 4035–4039.
40. **Pachnis, V., L. Pevny, R. Rothstein, and F. Costantini.** 1990. Transfer of a yeast artificial chromosome carrying human DNA from *Saccharomyces cerevisiae* into mammalian cells. Proc. Natl. Acad. Sci. USA **87:**5109–5113.
41. **Peterson, C., and R. Legerski.** 1991. High-frequency transformation of human repair-deficient cell lines by an Epstein-Barr virus-derived cDNA expression vector. Gene **107:**279–284.
42. **Rawlins, D. R., G. Milman, S. D. Hayward, and G. S. Hayward.** 1985. Sequence-specific DNA binding of the Epstein-Barr virus nuclear antigen (EBNA-1) to clustered sites in the plasmid maintenance region. Cell **42:**859–868.
43. **Reeves, R. H., W. J. Pavan, and P. Hieter.** 1990. Modification and manipulation of mammalian DNA cloned as YACs. Genet. Anal. Tech. Appl. **7:**107–113.
44. **Reisman, D., and B. Sugden.** 1986. *trans* activation of an Epstein-Barr viral transcriptional enhancer by the Epstein-Barr viral nuclear antigen 1. Mol. Cell. Biol. **6:**3838–3846.
45. **Reisman, D., J. Yates, and B. Sugden.** 1985. A putative origin of replication of plasmids derived from Epstein-Barr virus is composed of two cis-acting components. Mol. Cell. Biol. **5:**1822–1832.
46. **Saemundsen, A. K., C. Perlmann, and G. Klein.** 1983. Intracellular Epstein-Barr virus DNA is methylated in and around the *Eco*RI-J fragment in both producer and nonproducer cell lines. Virology **126:**701–706.
47. **Shalit, P., K. Loughney, M. V. Olson, and B. D. Hall.** 1981. Physical analysis of the *CYC1-sup4* interval in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **1:**228–236.
48. **Southern, E. M., R. Anand, W. R. A. Brown, and D. S. Fletcher.** 1987. A model for the separation of large DNA molecules by crossed field gel electrophoresis. Nucleic Acids Res. **15:**5925–5943.
49. **Sugden, B., K. Marsh, and J. Yates.** 1985. A vector that replicates as a plasmid and can be efficiently selected in B-lymphoblasts transformed by Epstein-Barr virus. Mol. Cell. Biol. **5:**410–413.
50. **Sun, T.-Q., D. A. Fenstermacher, and J.-M. H. Vos.** 1994. Human artificial episomal chromosomes for cloning large DNA fragments in human cells. Nat. Genet. **8:**33–41.
51. **Taylor, S. S., Z. Larin, and C. Tyler-Smith.** 1994. Addition of functional human telomeres to YACs. Hum. Mol. Genet. **3:**1383–1386.
52. **Tyler-Smith, C., R. J. Oakey, Z. Larin, R. B. Fisher, M. Crocker, N. A. Affara, M. A. Ferguson-Smith, M. Muenke, O. Zuffardi, and M. A. Jobling.** 1993. Localization of DNA sequences required for human centromere function through an analysis of rearranged Y chromosomes. Nat. Genet. **5:**368–375.
53. **Willard, H. F.** 1985. Chromosome-specific organization of human alpha satellite DNA. Am. J. Hum. Genet. **37:**524–532.
54. **Yates, J., N. Warren, D. Reisman, and B. Sugden.** 1984. A cis-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. Proc. Natl. Acad. Sci. USA **81:**3806–3810.
55. **Yates, J. L., and N. Guan.** 1991. Epstein-Barr virus-derived plasmids replicate only once per cell cycle and are not amplified after entry into cells. J. Virol. **65:**483–488.
56. **Yates, J. L., N. Warren, and B. Sugden.** 1985. Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. Nature (London) **313:**812–815.