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Circular YAC Vectors Containing Short Mammalian Origin Sequences Are Maintained Under Selection as HeLa Episomes

Torsten O. Nielsen,[1] **Nandini H. Cossons,**[2] **Maria Zannis-Hadjopoulos,**[2] **and Gerald B. Price**[2]*

[1]Department of Pathology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada
[2]McGill Cancer Centre, McGill University, Montreal, Quebec H3G 1Y6, Canada

Abstract pYAC*neo*, a 15.8-kb plasmid, contains a bacterial origin, G418-resistance gene, and yeast ARS, CEN, and TEL elements. Three mammalian origins have been cloned into this circular vector: 343, a 448-bp chromosomal origin from a transcribed region of human chromosome 6q; X24, a 4.3-kb element containing the hamster DHFR origin of bidirectional replication (*ori*β), and S3, a 1.1-kb human anti-cruciform purified autonomously replicating sequence. The resulting constructs have been transfected into HeLa cells, and G418-resistant subcultures were isolated. The frequency of G418-resistant transformation was 1.7–8.7 times higher with origin-containing YAC*neo* than with vector alone. After >45 generations under G418 selection, the presence of episomal versus integrated constructs was assessed by fluctuation assay and by PCR of supercoiled, circular, and linear genomic cellular DNAs separated on ethidium bromide-cesium chloride gradients. In stable G418-resistant subcultures transfected with vector alone or with linearized constructs, as well as in some subcultures transfected with circular origin-containing constructs, resistance was conferred by integration into the host genome. However, several examples were found of G418-resistant transfectants maintaining the Y.343 and the YAC.S3 circular constructs in a strictly episomal state after long-term culture in selective medium, with 80–90% stability per cell division. The episomes were found to replicate semiconservatively in a bromodeoxyuridine pulse-labeling assay for ≤130 cell generations after transfection. Furthermore, after ≤172 cell generations rescued episomal DNA could be isolated intact and unrearranged, and could be used to retransform bacteria. These versatile constructs, containing mammalian origins, have the capacity for further modification with human telomere or large putative centromere elements, in an effort to move towards construction of a human artificial chromosome. J. Cell. Biochem. 76:674–685, 2000.

Key words: artificial chromosome; autonomous replication; DNA replication; episome; human; replication origin

A human artificial chromosome (HAC) would not only provide a valuable tool for addressing difficult questions about chromosome biology, but would also create an all-human transfection vector with the capacity to carry large chromosomal regions including complete transcriptional units, from even the largest genes, for the purpose of complementation mapping, or for gene therapy [Huxley, 1994].

Methods using fragmentation of chromosomes near the centromere have been used to generate micro- or mega-chromosomes, ranging from 6–10 Mb in size [Hollo et al., 1996; Harrington et al., 1997]. However, these constructs still contain uncharacterized endogenous components with unknown structure and regulation.

Artificial chromosomes require three *cis*-acting functional components: replication origins, telomeres, and a centromere. Using the approach of assembling defined minimum sequence requirements for each element, ARS-containing *Saccharomyces cerevisiae* plasmids [Stinchcomb et al., 1979] provided the basis for the addition of TEL [Szostak and Blackburn, 1982] and CEN [Dani and Zakian, 1983] elements to complete the construction of stable yeast artificial chromosomes [Murray and Szostak, 1983]. A similar strategy proved successful for artificial chromosome assembly in

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Torsten A. Nielsen and Nandini H. Cossons contributed equally to this work.

*Correspondence to: Gerald B. Price, McGill Cancer Centre, 3655 Drummond Street, Montreal, Quebec H3G 1Y6, Canada. E-mail: facs1@med.mcgill.ca

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the fission yeast *Schizosaccharomyces pombe*, in spite of the far more complicated structure of its centromeres [Hahnenberger et al., 1989].

The first component required in our system for the assembly of a prototype human artificial chromosome is a functional human replication origin. Different techniques have permitted the identification of a limited, but rapidly increasing, number of putative and proven mammalian origins [DePamphilis, 1993; Hamlin et al., 1994]. Our group has been able to isolate large numbers of putative origins using such techniques as nascent strand extrusion [Kaufman et al., 1985] and anti-cruciform immunoaffinity purification [Bell et al., 1991]; these sequences permit short-term autonomous replication of plasmids transfected into human cells and also act as replication origins in their native chromosomal position [Wu et al., 1993b; Pelletier et al., 1999].

To use such isolated origin sequences for the construction of a first stage human artificial chromosome, they must be cloned into a circular vector, such as pYAC*neo*, which permits transfection into human cells with selection of transfected clonal subpopulations, and has the capacity for further modification to carry human-functional telomeres and putative centromere elements which could be hundreds of kilobases in size. We demonstrate that these origin-containing YAC constructs are maintained in long-term culture as independent episomal elements, and are not integrated into a host chromosome. We also show that they are maintained stably, in an unrearranged form, and undergo at least one round of semi-conservative replication.

MATERIALS AND METHODS

Molecular Cloning

pYAC*neo* is a versatile plasmid shuttle vector [Traver et al., 1989]. Because it includes the prokaryotic *Col*E1 origin and an ampicillin resistance marker from pBR322, pYAC*neo* (15,827 bp) can be grown in *Escherichia coli* as a circular plasmid. Since this vector also contains the *Saccharomyces cerevisiae* ARS1 replication origin and CEN4 centromere elements, it can alternatively be maintained as a circular yeast artificial chromosome, carrying the TRP1, URA3, and HIS3 selectable markers. Digestion of the circular plasmid with *Bam*HI removes the HIS3 gene, leaving a linear molecule capped by two 0.7-kb telomeric cassettes (originally derived from *Tetrahymena*) that are functional in budding yeast, permitting the construct to be maintained as a linear yeast artificial chromosome. In addition, pYAC*neo* carries a gene conferring resistance to the drug G418, a trait which is selectable in mammalian cells. Thus, this vector can replicate in both *E. coli* and *S. cerevisiae* and contains markers for the selection of stable bacterial, yeast, or mammalian cell transfectants.

The 15.8-kb vector pYAC*neo* (Clontech) was digested with *Eco*RI. The 1.1-kb *Eco*RI insert of plasmid S3 [Nielsen et al., 1994] was ligated directly into the dephosphorylated vector, whereas the 448-bp *Eco*RI/*Hin*cII insert of pURHc34 [Wu et al., 1993a] and the 4.3-kb *Xba*I fragment of pX24 [Burhans et al., 1990] were blunt-ended with T4 DNA polymerase before ligation into blunt-ended dephosphorylated vector. The resulting circular constructs are designated YAC.S3 (16.9 kb), Y.343 (16.3 kb), and Y.X24 (20.1 kb). The region between 9825–10520 was modified, deleting a 210-bp portion of Y.343. Each was used to transform competent *E. coli*, and ampicillin-resistant colonies were grown for large-scale plasmid preparation. The structures of the cloned constructs were then confirmed by restriction enzyme digestion.

Transfection and Culturing Human Cells

HeLa cells, passaged once, as resurrection from frozen stocks were seeded in T-25 flasks at $1 \times 10^4/cm^2$, and grown for 2 days (in α-MEM + 10% FCS) before transfection with 20 μg pYAC*neo*, YAC.S3, Y.343, or Y.X24 DNA by calcium phosphate coprecipitation. Linearized forms of pYAC*neo* and Y.343, with the *Tetrahymena* telomeric ends of the YAC vector, were produced by *Bam*HI digestion of the circular constructs, and similarly transfected. Since YAC.S3 and Y.X24 contain an extra *Bam*HI site in their inserts, they have not yet been tested in linearized form. At 2 days post-transfection, cells were switched into medium containing 400 μg/ml G418, and a further 2 days later, T-25 flasks were trypsinized, counted, and 1×10^5 cells were seeded onto 60-mm dishes. The HeLa cells were cultured in G418 until 20 days post-transfection, when dishes were scored for visible growing drug-resistant colonies. Individual colonies were picked directly from the 60-mm dishes to isolate clonal subpopulations for further analysis. Cultures were maintained in 400

μg/ml G418 during this period, and had been actively growing for at least 10 weeks (an estimated minimum of 45 doublings) between the initial transfection and the subsequent analysis of the episomal versus integrated state of the transfected constructs.

Fluctuation Assay

For each cloned transfectant cell line to be tested, cells which had been maintained in G418 were counted and 4×10^5 cells were plated into two T-80 flasks, one containing drug-free nonselective medium, and one used for maintaining the culture in G418-selective medium. In parallel, cells were seeded at a similar density onto two 24-well plates (200 mm^2/well), to allow their growth curves to be followed both in the presence and absence of drug selection. Triplicate wells were trypsinized and counted daily with a Coulter counter ZM apparatus. After 6 days of growth, both T-80 flasks were trypsinized, counted, and diluted with either G418-containing or nonselective medium to final concentrations of 5 cells/ml. Next, 200-μl aliquots were then distributed to each well of a 96-well plate (32 mm^2/well). Two plates were used for the case of cells that had been passaged in nonselective medium and were now being returned to G418). Eight days later, the number of wells containing a growing cell colony was scored under the microscope.

Demonstration of Episomal DNA

As a positive control, HeLa cells were transiently transfected with Y.X24, by calcium phosphate coprecipitation, and harvested 48 h later. Using standard methods [Strauss, 1989], total DNA from approximately 5×10^6 cells was isolated from untransfected HeLa (negative control), transiently transfected HeLa, and the cloned transfectant cell lines to be tested. DNA preparations were mixed with 1 mg ethidium bromide and 75 μg of a carrier plasmid, in this case F9, a pBluescript clone containing a 0.5-kb human genomic insert [Nielsen et al., 1994]. CsCl solution was added to a final density of 1.56 g/ml before ultracentrifugation in a VTi80 rotor, 20 h at 67,500 rpm. Using the intact and nicked carrier plasmid bands as a visible guide to the position of supercoiled (lower band) and linear and relaxed circular (upper band) DNA, the two completely resolved fractions were carefully removed by side puncture. Ethidium bromide was removed by two washes with CsCl-saturated isopropanol, and CsCl was removed by ethanol precipitation with two 70% ethanol washing steps.

Aliquots representing one-eighth of the purified DNA were used as template in two separate 50 μl PCR reactions. To amplify a 131-bp region of the *neo* gene present in the transfected constructs, but not in the native HeLa genomic DNA, primers 5′-TCA GGA CAT AGC GTT GGC T-3′ and 5′-CGT CAA GAA GGC GAT AGA A-3′, located in the *neo* gene, were used (at 0.4 μM) with a mixture of all four dNTPs (each at 0.2 mM), 1 × Taq buffer, and 1 U Taq polymerase (Pharmacia). 28 cycles were performed, each 94°C, 20-s denaturation; 50°C, 90-s annealing; and 72°C, 30-s extension; the first denaturation and the final extension steps were carried out for 5 min. To amplify a 423-bp unique region on the long arm of human chromosome 6, primers 5′-TGT GTA TGG GAC GGT AGT CA-3′ and 5′-GGA GCA AGG CAG AAC TAC TC-3′ [Wu et al., 1993b] were used at 0.25 μM, with 1.5 U Taq, for 33 cycles (each 94°C, 60 s; 50°C, 60 s; 72°C, 60 s) followed by a 5-min final extension. Products of both reactions were electrophoresed in a 1.6% agarose, 1 × TBE gel.

Bromodeoxyuridine Labeling

Actively growing stable transfectants were seeded at 2.5×10^5 per T20 flask and 2 days later were pulsed with 40 μM final concentration bromodeoxyuridine for 48 h [Frappier and Zannis-Hadjopoulos, 1987]. Low-molecular-weight DNA was extracted from cells using Hirt's extraction method [Hirt, 1967], loaded onto a neutral cesium chloride/ethidium bromide gradient, and centrifuged in a Vti 80 rotor at 65,000 rpm for 16 h at 20°C. A total of 20–24 fractions of approximately 200 μl were collected from the bottom of the gradient; 50 μl of each fraction was diluted with 50 μl of distilled water, and added to an equal volume of 0.5 N NaOH. The samples were then incubated for 10 min on ice. Each sample was then diluted with 200 μl of 0.25 N NaOH, 0.25 × SSC, and applied to the dot blot manifold according to the manufacturer's instructions.

Bacterial Retransformation Assay

Episomal DNA isolated from stable HeLa cell clones using the Hirt's extraction method was used directly to transform DH5α strain of *E. coli* as previously described [Landry and Zannis-Hadjopoulos, 1991]. Several randomly chosen

bacterial colonies were used to prepare plasmid DNA. DNA was then subjected to digestion with several restriction endonucleases, according to the manufacturer's instructions, to test the integrity of the molecule and the size of the fragments.

RESULTS

Cloning Mammalian Origins Into a YAC Vector

Three mammalian sequences previously shown to permit autonomous replication in human cells have been cloned into the *Eco*RI site of pYAC*neo* (Fig. 1; see under Materials and Methods); 343 is a 0.45-kb cDNA clone derived from a transcribed region on the long arm of human chromosome 6 [Shihab-El-Deen et al., 1993], to which in vivo origin activity has been localized by nascent strand PCR mapping [Wu et al., 1993b]. S3 is a 1.1-kb human sequence isolated by anti-cruciform immunoaffinity purification of genomic DNA, followed by competitive selection for clones possessing strong autonomous replication activity by mass transfection and in vitro replication assays [Nielsen et al., 1994]. Finally, X24 carries a 4.3-kb *Xba*I fragment from the hamster *DHFR* 3′ region and includes the predominant initiation site oriβ as indicated by multiple techniques [Burhans et al., 1990; Zannis-Hadjopoulos et al., 1994].

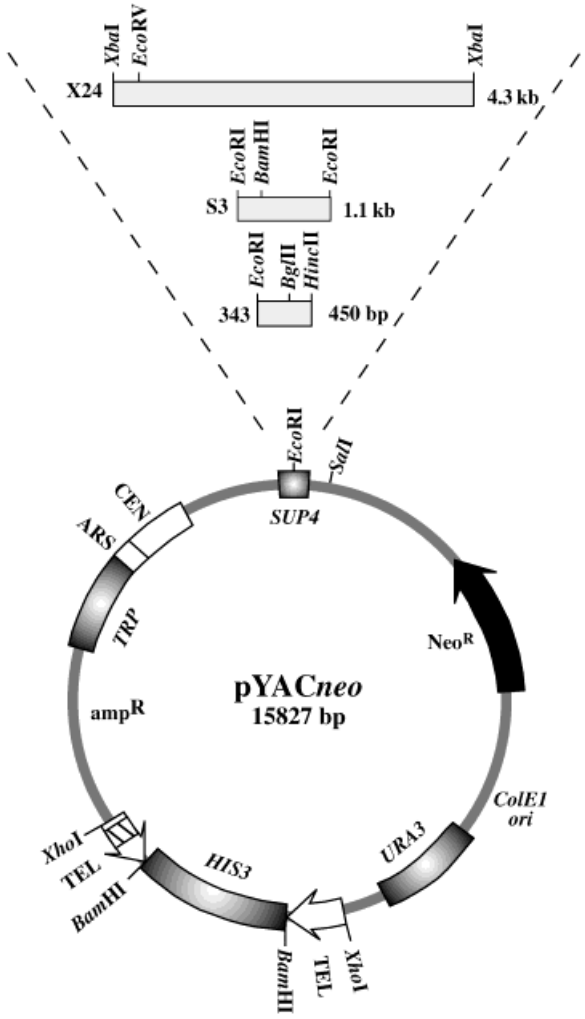

Fig. 1. Diagram of pYAC*neo* and cloned mammalian origin sequences. Prokaryotic vector sequences are indicated by a thin gray line. Yeast chromosomal components, open boxes; yeast genetic markers, shaded boxes; the mammalian-selectable marker, black arrow. Deleted portion of Y.343, cross-hatched box. Two human (343, S3) and one hamster (X24) origin of DNA replication were cloned into the *Eco*RI site of pYAC*neo*. Note restriction sites indicating the orientation of the fragments. Not drawn to scale.

HeLa Transfection Efficiency Is Higher With Origin-Containing Constructs

Since the Y.343, YAC.S3, and Y.X24 constructs remain relatively small (16.3 kb, 16.9 kb, and 20.1 kb, respectively), they can be grown in bacteria, and pure preparations can be transfected by the relatively high-efficiency calcium phosphate coprecipitation method, unlike YACs in the range of 0.1–1.0 Mb, necessitating the use of techniques such as yeast spheroplast fusion for transfecting mammalian cells. Calcium phosphate-treated, mock-transfected cells resulted in no G418-resistant colonies; pYAC*neo* vector devoid of any mammalian origin gave 98 colonies per 3×10^5 cells plated. Importantly, the test constructs YAC.S3, Y.343, and Y.X24 gave markedly more stably transfected G418-resistant colonies than the vector alone (374, 504, and 432 colonies per 3×10^5 cells plated, respectively). Correction for the relative efficiency of transfection, using cotransfection with a plasmid containing the luciferase reporter gene, yielded differences in transformation efficiency from 1.7 (YAC.S3) to 8.7 (Y.343) times, as compared with the pYAC*neo* vector alone (Fig. 2). These data suggested that the presence of a short, origin-containing insert may facilitate the maintenance of the transfected *neo* trait in human cells. Multiple experiments have further shown that linearized versions of pYAC*neo* and Y.343, produced by *Bam*HI digestion to free the T_2G_4 telomeric ends, are at least 100-fold less efficient in producing stable transfectants (data not shown).

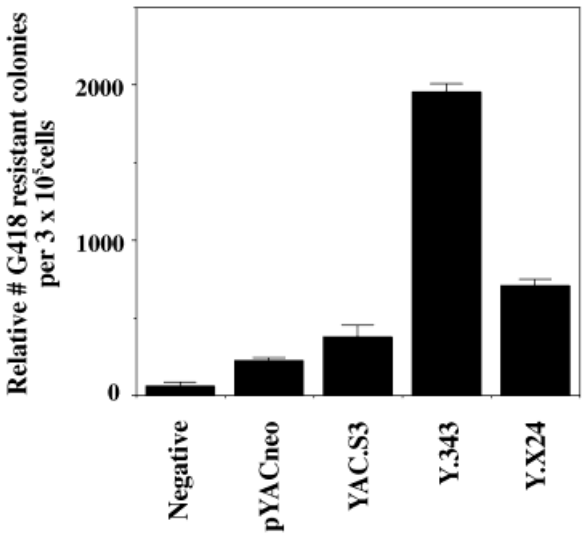

Fig. 2. Transformation efficiency of origin-containing constructs. Relative number of G418-resistant colonies, black bars. Transformation efficiency is standardized for the efficiency of transfection using co-transfection with a plasmid containing the luciferase reporter gene.

Isolated colonies were cloned and grown in G418 to select for maintenance of the transfected constructs, and spent a minimum of 67 days in culture before testing for the presence and stability of episomal *neo*-containing DNA.

Mitotic Stability of Transfected Constructs

A protocol based on the Luria-Delbrück fluctuation assay [Luria and Delbrück, 1943] permits calculation of the stability of the drug resistance marker during cell growth in nonselective medium. Stable transformants obtained in the usual fashion, through integration of the transfected marker into the host genome, maintain the drug resistance trait, even in the absence of selective pressure. However, episomally replicating DNA that lacks a functional centromere will not partition accurately at mitosis and will display a characteristic loss rate per generation.

Examples of G418-resistant clonal cell populations initially transfected with circular pYAC*neo*, YAC.S3, Y.343, or Y.X24, or with *Bam*HI-linearized pYAC*neo* or Y.343, were tested by fluctuation assay. In essence, cells were passaged from G418 into nonselective medium and allowed to grow, while in parallel, cells were also seeded onto 24 well plates to allow daily monitoring of their growth rate.

Growth rates of individual clones in selective medium as compared with nonselective medium varied (Fig. 3). Just over one-half of the tested cell lines (including YAC*neo* clone 1, YAC.S3 clone 2, Y.343 clones 1, 19, and 20) grew slightly more slowly in G418 than they did in nonselective medium, as did Y.X24 clone 1 (Fig. 3A). However, growth rates in the remaining cell lines, including Y.343 clones 2 and 11, YAC.S3 clone 1, and YAC*neo* clones 2 and 3, were retarded in G418, with a doubling time in nonselective medium from 1.5 to 1.8 times that observed in the presence of G418 (Fig. 3B). Although in some cases, cells that carried the

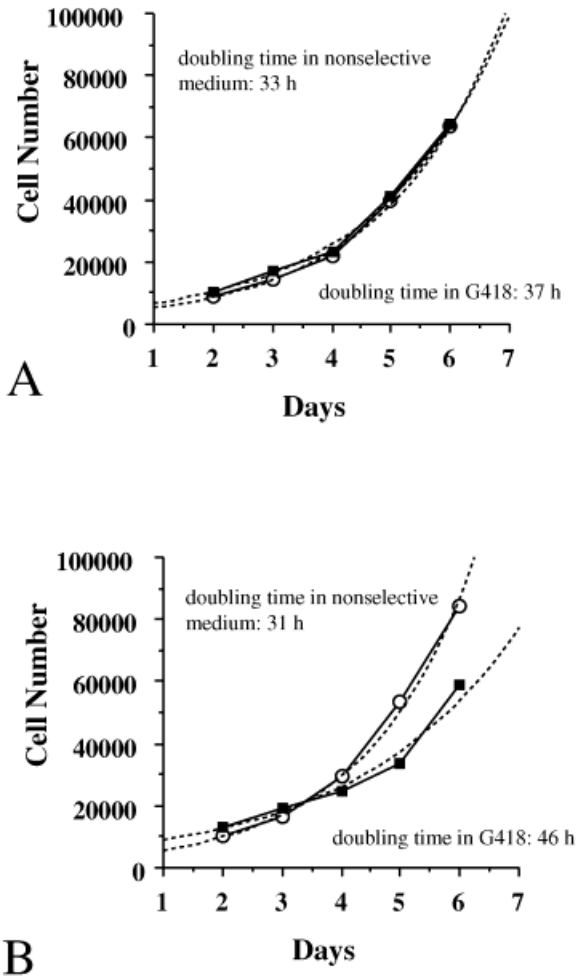

Fig. 3. Growth curves of two representative stable HeLa cell clones. **A:** HeLa cell clone Y.X24 clone 1. **B:** HeLa cell clone Y.343 clone 2. After plating in 200-mm^2 wells, cell number was determined by Coulter counts; each plotted point represents the average of triplicate wells for each day during log-phase growth. Open circles, cell number in nonselective medium; filled squares, track growth in the presence of 400 μg/ml G418. Dashed lines, exponential equation derived from regression analysis of each series of points, from which the population doubling time is determined; in all cases, the curve-fit correlation (R^2) value was better than 0.98.

construct in an episomal form grew significantly more slowly than cells containing the DNA in an integrated form (as shown by subsequent tests), this could not be used as a specific test, as a subset of integrated cell lines also exhibited slow growth in G418. The number of doublings which took place during the fluctuation period was calculated from a regression analysis of the exponential growth curves in nonselective medium.

Six days after seeding into nonselective medium, while the cells were still in log phase, the test flasks were trypsinized, diluted, and replated to determine the proportion of cells that still retained the *neo* marker. Results are shown in Table I, along with the calculated stability of the constructs in each of the subpopulations tested. Two of the HeLa clones transfected circular pYAC*neo* vector maintained the *neo* marker with a stability of 1, supporting the hypothesis that their transformation was the result of integration events. The same is true for YAC.S3 clone 2 and Y.343 clone 1-cell lines. Clones transfected with linearized constructs (Table I) have so far only displayed fluctuation results compatible with integration, in line with observations that the YAC vector's T_2G_4 telomeres are nonfunctional in human cells [Hanish et al., 1994]. However, for the rest of the HeLa cell clones transfected with the origin-containing constructs, a significant proportion of the cells in each population lost the G418 resistance trait during the nonselective fluctuation period (index of 0.8–0.9), strongly suggesting that these clonal lines carried only episomal forms of the transfected constructs. Indeed, the calculated stability of 0.8 per generation for some clones (Y.343 clone 2 and YAC.S3 clone 1) is similar to

TABLE I. In Vivo Stability of Transfected Constructs by Fluctuation Assay

Host cell	Transfection	No. of growing colonies on 96-well plates after fluctuation		No. of generations[c]	Stability per division[d]
		Selective (G418[a])	Nonselective[b]		
HeLa	YAC*neo* clone 1	57	44	3.2	1.0
HeLa	YAC*neo* clone 2	42	47	2.8	1.0
HeLa	YAC*neo* clone 3	58	69	2.8	0.9
HeLa	YACS3 clone 1	36	64	2.9	0.8
HeLa	YACS3 clone 2	26	28	3.0	1.0
HeLa	Y.343 clone 1	20	14	3.2	1.0
HeLa	Y.343 clone 2	26	56	3.4	0.8
HeLa	Y.343 clone 11	23	38	3.8	0.9
HeLa	Y.343 clone 20	28	59	6.3	0.9
HeLa	Y.X24 clone 1	18	28	3.5	0.9
HeLa	Linear YAC*neo* clone 1	12	14	3.3	1.0
HeLa	Linear Y.343 clone 1	37	38	3.3	1.0
S. cerevisiae[e]	Circular ARS plasmid				0.7
S. cerevisiae[e]	Linear ARS plasmid				0.8
S. cerevisiae[e]	CEN-containing YAC				0.9–0.999
S. pombe[e]	Circular ars plasmid				0.7
S. pombe[e]	Cen-containing YAC				0.98–0.999
Any host cell	Integrated DNA				1.0

[a]G418 = number of wells containing growing colonies, after test clone cells, grown in nonselective medium for six days, were returned to G418 selective medium while plating at one cell per well onto 96-well plates.

[b]Nonselective = number of wells containing growing colonies, after test clone cells, grown in nonselective medium for 6 days, were kept in nonselective medium while plating at one cell per well onto 96 well plates.

[c]No. of generations = the number of cell divisions that took place during the 6-day nonselective fluctuation period, as assessed by daily counts of parallel cultures.

[d]Stability per division (x) refers to the chance, following each cell division, that a daughter cell will inherit the selectable marker and is calculated from the following relation:

$$x^g = \%PE \text{ (selected with G418)}/\%PE \text{ (unselected)}$$

where g = number of cell generations; %PE = percent plating efficiency = no. of colonies on 96-well plate/96. Results are shown to one significant figure, where 1.0 is the maximum stability possible.

[e]Data from Murray and Szostak [1983] and Hahnenberg et al. [1989]. Stability of centromere-containing yeast artificial chromosomes increases as a function of size.

that obtained by others during construction of *S. cerevisiae* and *S. pombe* yeast artificial chromosomes, using equivalent plasmids that carried a functional origin but lacked a centromere (Table I). Results obtained from the transformation efficiency and fluctuation assay experiments indicated that the Y.343-containing HeLa cell clones were the most promising in terms of their mitotic stability and replication characteristics. Further studies thus focused on these clones.

Rescue of Replicating Episomal DNA

Episomal DNA isolated from two of the Y.343 HeLa cell clones by Hirt's extraction [Hirt, 1967] was shown to correspond in size to the original input plasmid (Fig. 4A). Digestion of the episomes with *Dpn*I will differentiate between plasmid that has replicated at least once (*Dpn*I resistant) and unreplicated DNA (*Dpn*I sensitive). Y.343 episomes subjected to digestion in this fashion showed *Dpn*I resistance (Fig. 4B, lanes 1, 2), while a control pBluescript plasmid grown in bacteria showed complete digestion of monomer and dimer versions of form I DNA (Fig. 4B, lane 3). These results indicated that the construct was being maintained intact in an episomal form and was undergoing at least one round of DNA replication.

Instability of Episomes in the Absence of Drug Selection

The reduced stability of the selective trait and its maintenance as an episome predicts that the sequence would most probably be lost in the absence of selection or integration, due to unequal segregation of episomes lacking a centromere. The ultimate instability of the episomally maintained *neo* marker in Y.343 clone 2 was thus tested by repeating the fluctuation assay, but this time using a 46-day period of nonselective growth. No G418-resistant colonies were detectable and Southern blot analysis also confirmed the loss of the selective marker under nonselective conditions (data not shown).

HeLa Cells Maintain Human Origin-Containing YACs as Episomes

In an effort to confirm previous observations that the DNA was being maintained in episomal form, and to compensate for low sensitiv-

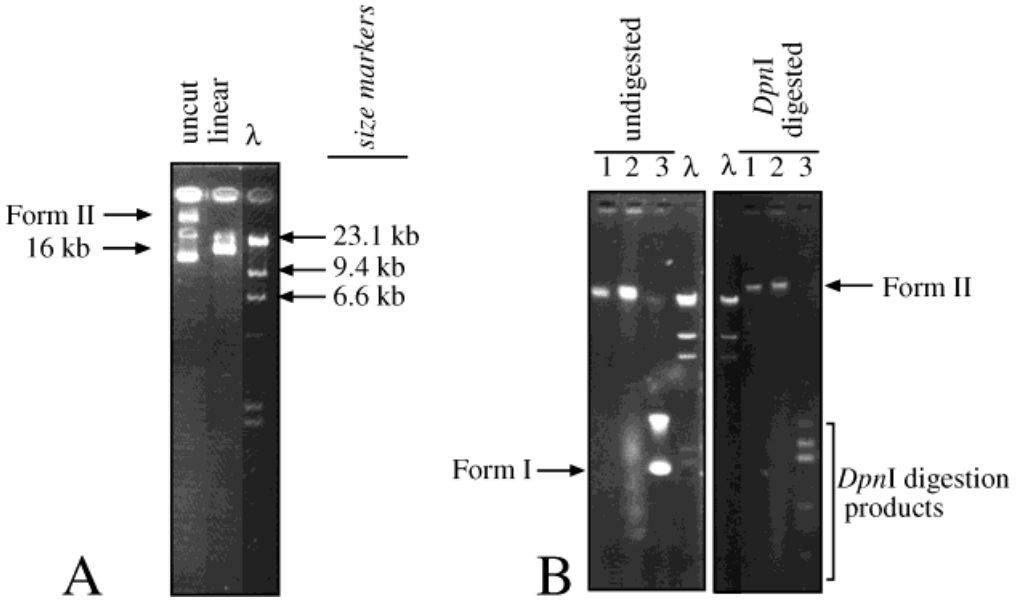

Fig. 4. Isolation of episomal DNA from stable HeLa cell clones. **A:** Agarose gel electrophoresis of Y.343 input DNA used to transfect HeLa cells. Uncut represents uncut Y.343 plasmid DNA. Positions of migration of different forms of DNA are indicated at the left-hand side with arrows. Linear represents Y.343 plasmid DNA linearized by digestion with *Sal*I; λ, molecular weight markers; sizes indicated at the right-hand side with arrows. **B:** Isolation and *Dpn*I digestion of low-molecular-weight DNA from stable HeLa cell clones approximately 45 cell generations after transfection with Y.343. Low-molecular-weight DNA was extracted using Hirt's extraction method (Hirt, 1967). 1, DNA from stable HeLa cell clone Y.343 clone 11; 2, DNA from stable HeLa cell clone Y.343 clone 20; 3, pBluescript DNA (500 ng). The second panel represents the same DNAs but digested with 1 unit of *Dpn*I for 1 h at 37°C.

ity of Southern analyses, several clones were subjected to more stringent analyses by polymerase chain reaction (PCR). Total DNA was isolated from HeLa cell clone Y.343 clone 2 (and YAC.S3 clone 1 for comparison). CsCl/ethidium bromide density-gradient ultracentrifugation was used to separate episomal from genomic DNA in HeLa cells [Radloff et al., 1967] by resolving covalently closed circular DNA from linear (form III) DNA and nicked (form II) circular DNA, which intercalate more of the buoyant dye. An unrelated carrier plasmid preparation with 50% nicked and linear forms served as a visible guide to the position of the lower (supercoiled episomal) and upper (predominantly linear genomic) bands in the density-gradient. DNA fractions prepared in this fashion from the HeLa subclones, as well as from positive and negative controls, were analyzed by PCR, using primers from the *neo* gene (Fig. 5).

DNA from normal, untransfected HeLa cells did not yield a PCR product (only the carrier plasmid is visible), as these cells do not carry a *neo* gene. A polyclonal population of HeLa cells, transfected with Y.X24, 48 h before DNA preparation, contained large quantities of PCR template in both the lower (intact circular) and upper (nicked or damaged circular, or integrated) CsCl gradient bands. However, the test clones yielded a product from only the lower, episomal DNA fraction, indicating that the transfected Y.343 constructs, as well as YAC.S3, were being maintained as covalently closed circular episomes during long-term culture in selective medium (96 days for Y.343 clone 2; 81 days for YAC.S3 clone 1).

The rightmost lanes show, through the use of primers directed at a unique genomic locus, that the linear genomic DNA segregates exclusively to the upper CsCl band; thus, the lower band is free from contaminating genomic DNA, and if a copy of the construct had integrated, it should have been detected in the upper band fraction.

Analysis of cell lines by fluorescent in situ hybridization (FISH) confirmed the presence of

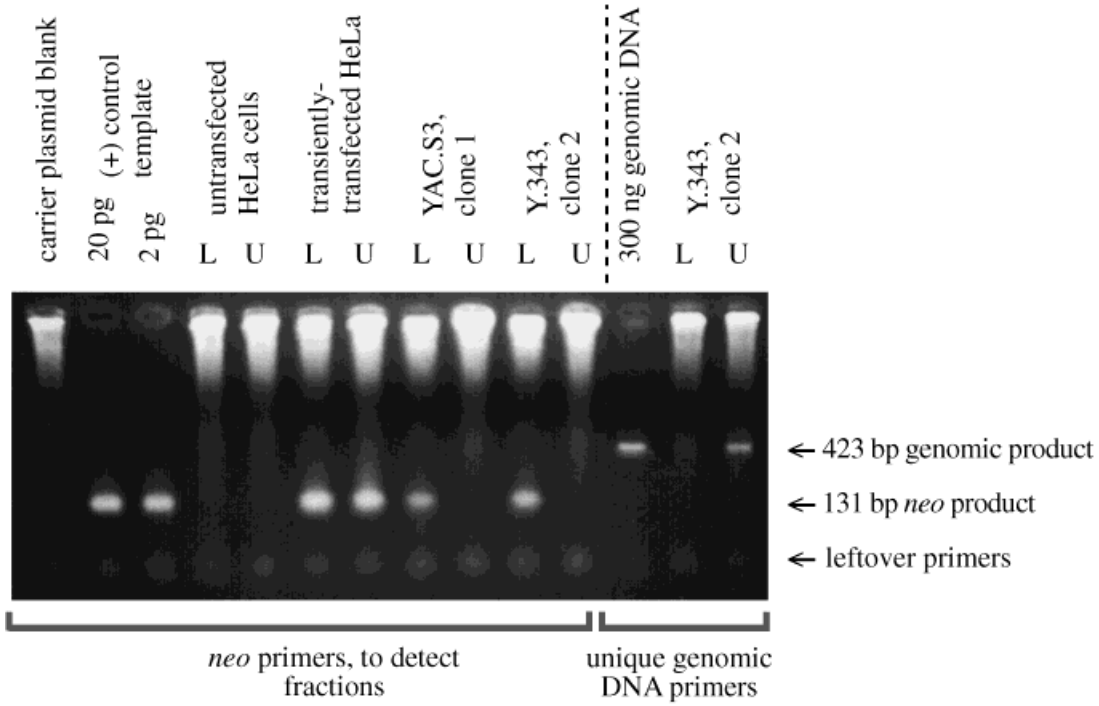

Fig. 5. Polymerase chain reaction (PCR) analysis of supercoiled episomal and linear genomic DNA fractions. Total DNA from HeLa subcultures was fractionated by cesium chloride/ethidium bromide ultracentrifugation, using a carrier plasmid as a guide to the position of the lower band (L) containing supercoiled circular DNA, and the upper band (U) containing the linear and relaxed circular forms of DNA. Fractions were tested for the presence of the transfected constructs by PCR amplification using a primer pair directed against the *neo* marker; positive control template is a pure Y.X24 plasmid sample. The position of linear genomic chromosomal DNA was confirmed in a separate amplification, shown in the three rightmost lanes, using primers that recognize a unique region on human chromosome 6; the positive control is total genomic DNA isolated from the ME180 human cell line, ATCC HTB 33.

the plasmids as nonintegrated episomes [Cossons et al., 1997].

Circular Episomes Replicate Semiconservatively In Vivo

Increased transformation efficiency, *Dpn*I resistance, expansion of the HeLa cell lines containing the episomal YACs and perpetuation of these, for over 100 generations in some cases, indicated that the episome was replicating in vivo. To confirm this, pulse labeling with 40 μM bromodeoxyuridine for 48 h was performed on selected clones, at approximately 63–133 cell generations. The results obtained with Y.343 clone 20 at 133 cell doublings are shown in Figure 6A. Both heavy-light (HL) and heavy-heavy (HH) DNA were detected [Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991; McWhinney and Leffak, 1990], indicating that the episome was replicating semi-conservatively, and had undergone at least one round of replication. The amount of unreplicated (LL) DNA was approximately 2.5 times lower than that for HH DNA, which banded at a refractive index of between 1.410–1.413, in agreement with previous reports [McWhinney and Leffak, 1990; McWhinney et al., 1995].

Episome Rescue and Retransformation

To confirm the integrity of the replicating episome, low-molecular-weight DNA was isolated from HeLa cell clone Y.343 clone 20 by Hirt's extraction [Hirt, 1967], at 172 cell generations after transfection. The DNA was then used to transform DH5α *E. coli* cells and plasmid DNA was isolated from several independent, randomly chosen colonies. Digestion of the DNA, and separation by agarose gel electrophoresis, indicated that there was no difference in the episomal DNA by comparison to the original transfected plasmid (Fig. 6B), suggesting that the DNA had not undergone any rearrangements in vivo.

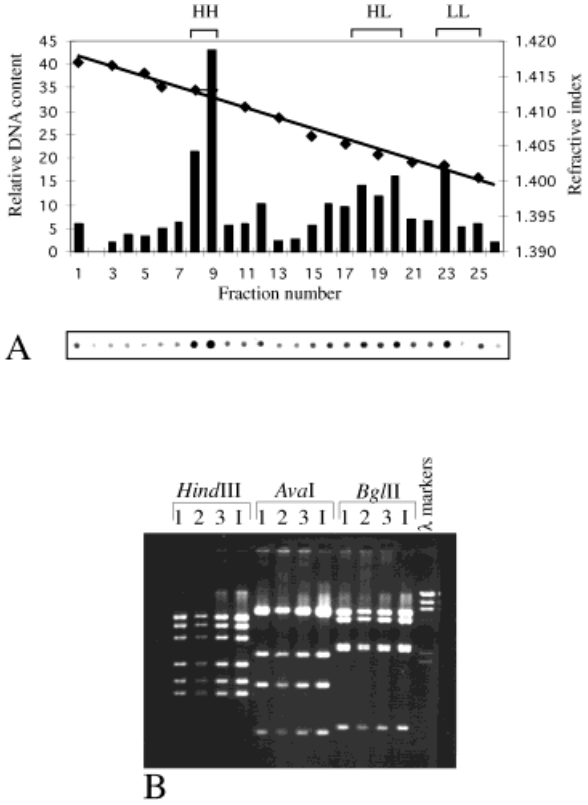

Fig. 6. Bromodeoxyuridine in vivo labeling and rescue of stable HeLa cell clones. **A:** Bromodeoxyuridine incorporation into nascent DNA. Cells growing in log phase were pulsed with 40μm bromodeoxyuridine for 48 h before harvesting low-molecular-weight DNA by Hirt's extraction, and loading onto a neutral cesium chloride gradient. Approximately 200μl fractions were collected, with fraction 1 representing the bottom of the gradient (x-axis). Refractive indices (filled diamonds) were measured using a refractometer (Y2 axis) and samples from each fraction were loaded onto a dot-blot manifold and hybridized with pYAC*neo* probe. The amount of DNA as quantified by PhosphorImage analysis is shown by the solid black bars (Y1 axis). The signal obtained from dot-blot analysis is indicated in the panel below the graph. **B:** Ethidium bromide-stained gel of plasmid DNA obtained from bacteria transformed with episomal DNA isolated from stably transfected HeLa cell clone Y.343 clone 20. **Lanes 1–3**, plasmid DNA isolated from three independent, randomly chosen bacterial clones, transformed with low-molecular-weight DNA isolated from HeLa cell clone Y.343 clone 20 approximately 172 cell doublings after transfection. I indicates the original input Y.343 plasmid DNA used to transfect the cells. Molecular weight sizes are indicated by the position of the markers in the far right lane (λ markers). Sizes (in kb): 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.6.

DISCUSSION

We have examined the long-term stability of three mammalian origin sequences cloned into a pYAC*neo* vector and transfected into HeLa cells, by calcium phosphate co-precipitation. The efficiency of stable transfection was increased by 1.7 to 8.7 times by the presence of an origin sequence, in line with previous observations using a cloned portion of the human 5′ c-*myc* origin [McWhinney and Leffak, 1990]. This is in contrast to the 1,000-fold increase in yeast transfection efficiency conferred by ARS elements [Stinchcomb et al., 1979]. However, *S. cerevisiae* has very little capacity for illegitimate recombination and integration of nonho-

mologous DNA [Schiestl and Petes, 1991]; thus, the magnitude of this increased efficiency is inflated by a very small denominator (i.e., the very poor transforming capacity of the vector alone in yeast) and cannot be directly compared with the increased transfection efficiencies of the equivalent mammalian constructs in human cells, where integration of vector devoid of an origin occurs comparatively frequently through illegitimate recombination.

Stable transfectants also showed alterations in growth characteristics in the presence of the selective drug G418, with growth rates increasing from 1.1 to 1.8 times under selection. This is similar to the 1.3 times increased growth rate seen in *ori*PYAC clones containing the *ori*P domain of Epstein-Barr virus (EBV) transfected into 293 cells and selected with hygromycin [Simpson et al., 1996].

Results obtained from the fluctuation assay demonstrated an overall mitotic stability ranging from 0.8–0.9, indicating that the selectable *neo* marker was being lost in the absence of selection, and thus most probably episomal in form. These results are similar to those obtained in yeast, where mitotic stabilities of around 0.7 were seen with equivalent constructs, and also comparable to results obtained in mammalian cells, transfected with episomally maintained origin-containing constructs. Previous studies on plasmids containing the replication origin of the human c-*myc* gene have also shown instability in the absence of selection, with a loss rate per generation of 5% in HeLa cells [McWhinney and Leffak, 1990]. In this system, it was also found that the plasmid was maintained in an extrachromosomal form, with no evidence of integration, for >300 cell generations. By contrast, Featherstone and Huxley [1993] demonstrated a relatively high loss rate of 85–94% for a 660-kb YAC construct containing the *hprt* gene, during a 17–19-day nonselective period. However, further work on 293 cells, transfected with YACs containing the EBV *ori*P domain (oriPYAC), and kept under nonselective conditions for up to 164 days, showed an apparently higher mitotic stability, with a loss rate per generation of between 1.2–3%, estimated using exponential decay from phosphorimage analysis of an *Eco*RI digest [Simpson et al., 1996].

Previous studies on EBV constructs have shown that episome maintenance may be stabilized by the presence of certain genetic elements, such as a nuclear retention signal [Krysan et al., 1989], and further work on the *ori*PYAC constructs, described above, demonstrated that removal of the nuclear retention element from the EBV origin of DNA replication significantly decreased the stability of the episome [Simpson et al., 1996]. However, all work to date has been performed on viral sequences that may have different requirements in terms of nonintegration/stability.

In addition, *ori*PYACs were shown to associate with host cell metaphase chromosomes and it was proposed that attachment to the chromosomal scaffold, by virtue of an *ori*P-EBNA-1 interaction, could be involved in increasing the stability of *ori*PYAC episomes in the absence of selection [Simpson et al., 1996]. Although there do not appear to be nuclear retention signals in our sequences, preliminary experiments that we have performed have indicated that the presence of a matrix attachment region (MAR) sequence may, in fact, act in a similar way by stabilizing the maintenance of the construct as an episome and enhancing the segregation properties [Cossons et al., 1997].

In the clones used in this study, copy number ranged from approximately 30 per cell (Y.343 clone 2, data not shown) to much lower copy numbers, which could only be detected by PCR (YAC.S3 clone 1). These levels were similar to those in yeast [Murray and Szostak, 1983], in mouse cells transfected with *hprt*-containing YACs (1–50 copies per cell) [Featherstone and Huxley, 1993], and in human (293) cells transfected with *ori*P-containing YACs (1.3–18 copies per cell) [Simpson et al., 1996]. This apparent amplification results from inaccurate partition of plasmids at mitosis and reaches an equilibrium in long-term culture above some threshold value needed for sufficiently stable maintenance and cell growth under selection. High copy numbers cannot confer a mitotic stability greater than 70–80%, which requires the presence of an element (e.g., a centromere) directing accurate partition.

Stringent analysis by PCR detected several cell lines with the transfected DNA in a purely episomal form, with no apparent integration into the host genomic DNA. FISH analyses confirmed these data [Cossons et al., 1997]. Furthermore, constructs remained in an episomal state even after 45–172 cell doublings, and analysis of low-molecular-weight DNA extracted from the stable HeLa cell clones indi-

cated that the DNA was *Dpn*I resistant, and corresponded in size to the original transfected plasmid. We further demonstrated that the episomal DNA could be extracted, and used to transform bacteria. Restriction analysis of plasmid DNA after extraction from the transformed bacteria, indicated that the DNA was still in its original unrearranged form. These results are consistent with those reported previously for *ori*P-containing episomes after long-term culture [Simpson et al., 1996].

Subsequent bromodeoxyuridine labeling of stable cell clones confirmed that the episome was replicating efficiently, and at least once per 48-h period. Similar results were obtained by McWhinney and Leffak [1990], who transfected HeLa cells with a plasmid containing the c-*myc* origin of DNA replication and found very low levels of residual unreplicated (LL) DNA, but approximately equal amounts of DNA that had replicated either once (HL) or twice (HH).

The overall stability of YAC.S3 and Y.343 episome maintenance during growth in nonselective medium is comparable to that obtained with autonomously replicating yeast plasmids (Table I), which constituted the first step in the construction of yeast artificial chromosomes. Several Y.343 HeLa cell clones have been demonstrated to be capable, in a G418-selected human cell system, of persisting for several months in a purely episomal form.

Vectors capable of long-term persistence in mammalian cells have been constructed by others, but these rely on viral origins of DNA replication, e.g., a murine plasmid based on a defective polyomavirus [Gassman et al., 1995], a "human artificial episomal chromosome" that carries the EBV latent origin, *ori*P [Sun et al., 1994], and other *ori*P-containing constructs [Simpson et al., 1996]. While capable of acting as excellent gene vectors in vitro, such constructs are not suitable for studying mammalian chromosomal origin biology, because they require the presence of viral *trans*-activating proteins for their replication.

The data described demonstrate that small circular YAC vectors containing short mammalian origin sequences are able to exist as stable autonomously replicating episomes in long term culture in human cells without the need for viral replication proteins. The modified YAC vectors described can be grown in bacteria or yeast, can be easily transfected by calcium phosphate coprecipitation, and also provide a completely defined construct. They are sufficiently versatile to allow addition of cloned human telomeres and of large putative centromeric blocks with or without intrinsic replication origins. The next step in this approach to constructing a human artificial chromosome is to replace the T_2G_4 *Tetrahymena* telomeres with mammalian-functional T_2AG_3 telomere cassettes. The resulting linear acentromeric chromosome will represent an appropriate cloning vector for the isolation of a minimal mammalian sequence with centromeric function.

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