Research Report

pSURF-2, A Modified BAC Vector for Selective YAC Cloning and Functional Analysis

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ABSTRACT

A modified bacterial artificial chromosome (BAC) vector, pSURF-2, adapted for the selective subcloning of yeast artificial chromosome (YAC) sequences was constructed. DH10B-U, a pyrF *derivative of the highly transformable* E. coli *strain DH10B was also constructed and used for the detection of Ura$^+$ recombinants carrying DNA linked to YAC right arms. The vector's properties were illustrated in two main ways.* (i) *An intact 25-kb YAC containing a mouse tyrosinase minigene was cloned into pSURF-2. Appropriately spliced tyrosinase RNA was detected by reverse transcription (RT)-PCR in extracts of cells transiently lipofected with the cloned YAC.* (ii) *Cells expressing human cystic fibrosis transmembrane conductance regulator (*CFTR*) from an integrated pSURF-2 recombinant containing a cDNA expression cassette were selected using the hygromycin-resistance (HyTK) marker of the vector and characterized by RT-PCR and immunoprecipitation. The unique I-*Sce*I site and HyTK marker of pSURF-2 are designed to facilitate subsequent functional studies of cloned DNA.*

INTRODUCTION

Yeast artificial chromosomes (YACs; Reference 5) have been key tools for the physical mapping, cloning and sequencing of complex genomes (7). Consequently, there is a large resource of well-characterized YACs containing known genes and other sequences of interest. P1 cloning vectors (33), P1 artificial chromosomes (PACs; Reference 31) and bacterial artificial chromosomes (BACs; Reference 11) were developed to combine the benefits of large (100–300 kb) insert cloning, approaching that which is provided by YACs, with the advantages of using *Escherichia coli* as a host and the relative robustness of supercoiled plasmid DNA. Significantly, YACs, BACs and PACs, unlike cosmids or multicopy plasmid vectors, have the capacity to carry the entire coding and regulatory sequences of most eukaryotic genes. With the emphasis now switching to functional studies, the challenge is to develop appropriate tools and strategies for the exploitation of these resources.

Linked selectable markers greatly facilitate the manipulation of cloned DNA in transfection and transgenic studies. Some YAC libraries are derived from vectors already containing a suitable marker (32); in others, markers can be inserted by retrofitting, a process that harnesses the homologous recombination machinery of yeast (24). The technical difficulties of working with YACs has prompted much effort into developing analogous ways of retrofitting BACs and PACs (19). Often, because the YAC resource is larger and better characterized, a sequence of interest is known to reside on a YAC and not on a BAC or PAC. In these cases, it would be preferable to subclone the DNA from YAC to BAC or PAC for ease of further manipulation rather than screening a BAC or PAC library de novo.

To address these concerns, here we describe the construction and use of pSURF-2 and DH10B-U (a *pyrF* host) for YAC subcloning and functional studies. The vector pSURF-2 retains the properties of the original BAC vector with the addition of a mammalian selectable marker, a unique cleavage site in the backbone and a blunt-end cloning site.

We set out to demonstrate the utility of pSURF-2 for YAC subcloning by attempting the more stringent task of cloning an entire YAC. We circumvented the problems of typical YACs of working with the larger DNAs by using YRC-Tyr, a 25-kb YAC containing the mouse tyrosinase minigene with arms derived from the vector pYAC-RC (Andreas Schedl, personal communication; Reference 15). YRC-Tyr differs from the 35-kb YAC YtTEL25 (28) only in that the latter has the minigene inserted in a modified pCGS996 YAC vector (32) rather than in pYAC-RC.

Virtually all YACs have an ampicillin-resistance (Ap^R) gene on the left arm and a *URA3* gene on the right arm. Both markers can be selected for in *E. coli*, the latter by complementation of a *pyrF* mutation (25). This suggested the methodology of subcloning sequences from YACs by direct selection in bacteria, an approach that makes it unnecessary to separate YAC DNA from other yeast chromosomes before ligation. This approach also ensures that recombinants contain arm DNA of known sequence, which can be used to help orient

Table 1. Subcloning YRC-Tyr in pSURF-2

Expt.[a] No.	BAL-31[b]	CFU[c]	Ura$^+$ CFUs[d]	YRC-Tyr Inserts[e]	Insert Sizes[f]
1	0.01 (0.5)	64	0	1/64	5.7
2A	0.023 (30)	264	0	0/36	-
2B	0.023 (30)	NA[g]	5 (39)[h]	2/5[i]	11.7; 12.8
3	0.0003 (5)	49	21	19/23	2 >15.0; 25.0[j]

[a]Three comparable experiments are shown. In Experiment 2, cultures of cells electroporated with aliquots of the ligation were split and plated out with either left-arm (Ap^R;2A) or right-arm selection (growth on minimal medium that lacks uracil; 2B). In the other two cases, selection was for left arm, and numbers in column 4 refer to colonies found to be uracil-independent (Ura$^+$) on subsequent screening. Cm was always included to select for the pSURF-2 backbone.

[b]Units of BAL-31 added per µg YRC-Tyr DNA and (in brackets) length of reaction in minutes.

[c]Colony forming units oftained after electroporation of DH10B-U and selection for Ap^R. Most of the colonies in Experiments 1 and 2 were found to contain an Ap^R plasmid that had contaminated the vector preparation.

[d]Number of colonies that grew on minimal plates that lacked uracil.

[e]Fraction of total clones examined by restriction digestion analysis and found to contain YRC-Tyr sequences.

[f]Size in kb of inserts in YRC-Tyr recombinant plasmids.

[g]Not applicable.

[h]Thirty-nine colonies appeared after several days of incubation on minimal plates containing Cm and lacking uracil, but only five grew after subsequent streaking out.

[i]Three of the Ura$^+$ colonies could not be analyzed because of the contaminating high-copy plasmid.

[j]Seventeen of the 21 recombinants from Ura$^+$ colonies contained intact YRC-Tyr (25 kb); see text for details.

and map the inserts. The *lox* site inherited from pBAC108L and the inserted blunt-end cloning site permit pSURF-2 to be used for turbo-cloning YACs, which is, in principle, a highly efficient means of generating the required recombinants (3). Furthermore, since a natural role of the *lox*/Cre system is to recircularize the 100-kb P1 genome (27), we expected the procedure to succeed on YACs of small to moderate size.

Using the example of human cystic fibrosis transmembrane conductance regulator (*CFTR*) DNA, we present data showing that the hygromycin-resistance (HyTK) marker can be used to select permanent cell lines expressing genes subcloned into pSURF-2.

MATERIALS AND METHODS

General

Standard molecular biological methods and purification techniques for plasmid DNA were used (26). Total yeast DNA from strain AB1380 harboring YRC-Tyr was prepared by Zymolyase/sodium dodecyl sulfate (SDS) lysis (23); because of the small size of the YAC, no special steps were taken to avoid DNA shearing. P1 transduction and other manipulations of *E. coli* strains were as previously described (13,20). Oligonucleotides were synthesized using a Model 381A DNA Synthesizer (PE Biosystems, Foster City, CA, USA). *Srf*I was from Stratagene (La Jolla, CA, USA); I-*Sce*I, other restriction enzymes and T4 DNA Ligase were from Boehringer Mannheim GmbH (Mannheim, Germany).

Construction of pSURF-2

All filling-in was done with T4 DNA Polymerase (Boehringer Mannheim GmbH) in the presence of excess dNTPs (26). The 5-kb plasmid tgCMV/HyTK containing the hygromycin phosphotransferase-thymidine kinase fusion gene driven by the cytomegalovirus (hCMV) promoter/enhancer, an Ap^R gene and replication origin (14) was linearized with *Xho*I, filled in and ligated between the filled-in *Not*I sites of pBAC108L to generate multicopy plasmid pBAC-HyTK. A linker containing an I-*Sce*I site formed by annealing the oligonucleotides 5′-GTTACCGCTAGGGATAACAGGGTAATATAG-3′ and 5′-GTAACCTATATTACCCTGTTATCCCTAGCG-3′ was inserted into the unique *Bst*EII site of pBAC-HyTK to give pBCHI. A *Bam*HI linker containing an *Srf*I site formed by annealing the self-complementary oligonucleotide 5′-GATCGCCCGGGC-3′ was inserted at the unique *Bam*HI site of pBHI to give pBCHIS. pUC4KΔDra, an ampicillin-sensitive (Ap^S) derivative of pUC4K formed by deleting DNA between the *Dra*I sites at coordinates 3126 and 3837, was linearized at its unique *Fsp*I site and inserted as a blunt-ended, 3203-bp fragment into filled-in, *Not*I-cut pBCHIS to give pSURF-2.

Preparation of Yeast DNA for Cloning

BAL-31 (1500 U/mL; Boehringer Mannheim GmbH) was titrated on yeast and other DNAs. Typically, aliquots of total yeast DNA were digested with dilutions of the enzyme, with the reactions stopped by addition of 20 mM EGTA at various time intervals between 5 and 30 min. The extent of digestion was determined by 0.8% agarose gel electrophoresis. Treated yeast DNA, from time points where BAL-31 activity had just removed very high-molecular-weight DNA entrapped in the well, was found to be optimal for

cloning. A 5-min treatment of 5 µg of yeast DNA with 0.0015 U of BAL-31 gave the best results. After drop dialysis (16), the BAL-31-treated yeast DNA ends were made blunt with T4 DNA polymerase in the presence of dNTPs (38). Enzyme was heat-inactivated by a 15-min incubation at 65°C.

Table 2. Features of pSURF-2 Vector

1. A hybrid *HyTK* gene encoding both hygromycin-resistance and thymidine kinase under the control of the strong, widely expressed hCMV promoter/enhancer (29). The *HyTK* gene can be selected both for and against using hygromycin and ganciclovir, respectively (14).
2. A stuffer replication and kanamycin-resistance cassette based on the multicopy vector pUC4K (35), allowing large quantities of vector to be made easily using standard techniques.
3. *Srf*I and *Not*I cloning sites flanking the stuffer for the cloning of *Not*I, *Eag*I, *Eae*I, *Bsp*120I and any blunt-ended fragments. Efficient turbo-cloning (3) of blunt-ended fragments is made possible by the presence of the *lox* site inherited from the pBAC108L backbone (31).
4. A single 18-bp, intron-derived endonuclease I-*Sce*I recognition site, allowing unique linearization of clones.

Cloning of YRC-Tyr

The protocol for Experiment 3 is described below; those for Experiments 1 and 2 differed in non-essential respects (Table 1). Eighty nanograms of gel-purified, dephosphorylated (calf intestinal phosphatase; Boehringer Mannheim GmbH) pSURF-2 were linearized with *Srf*I to remove stuffer and provide blunt ends, and ligated to 50 ng BAL-31/T4 DNA polymerase-treated yeast DNA in a 12-µL turbo-cloning reaction to produce predominantly linear vector-insert-vector trimers as previously described (3). After 30 min at room temperature, ligase activity was heat-inactivated by a 15-min incubation at 65°C, and 24 µL medium-salt restriction enzyme buffer (Boehringer Mannheim GmbH) containing 0.25 µg of Cre protein (a gift from Dr. Andrew Bates, Liverpool University, UK) were added to resolve the trimers into recombinant circular molecules (Figure 2A). After 30 min at 30°C, Cre activity was heat-inactivated by a 15-min incubation at 65°C, and the reaction mixture was drop-dialyzed against TE for 15 min.

Aliquots (25 µL) of electrocompetent DH10B-U cells prepared by repeated washes in ice-cold 10% glycerol as previously described (9) were transformed with 2.5-µL aliquots of the ligation mixture (using a Gene Pulser® System; Bio-Rad, Hercules, CA, USA) and plated out (after 1-h expression) on selective media (see Table 1).

PCR Amplification of Tyrosinase Minigene DNA

Primer pairs TYR1-U (5′-CAGGCAGAGGTTCCTGCCAG-3′)/TYR1-L (5′-GTGGGGATGACATAGACT-3′) and TYR2-U (5′-GAGCCTTACTTGGAACAAGCC-3′)/SV-L (5′-CTGCTCCCATTCATCAGTTCC-3′) were used to amplify a 315-bp region of exon 1 and a 515-bp region, respectively, of the 3′ end (including the simian virus 40 [SV40]-derived untranslated region [UTR]) of the tyrosinase minigene DNA (2). Aliquots (1 ng) of *Pvu*II-cleaved plasmid DNA template were amplified in 25-µL reactions using *Taq* DNA Polymerase (Boehringer Mannheim GmbH) under conditions recommended by the supplier [except for the addition of 10% dimethyl sulfoxide (DMSO) (30)] on a TRIO Thermoblock™ (Biometra GmbH, Göttingen, Germany). The program used was the following: 4-min denaturation at 92°C followed by 28 cycles of 92°C for 15 s, 55°C for 20 s and 72°C for 20 s; terminated with 5-min extension time at 72°C. PCR products were visualized on 1.8% agarose gels.

Transfections and Associated Assays

Cells were transfected with plasmid DNA complexed with LIPOFECTAMINE™ Reagent (Life Technologies, Gaithersburg, MD, USA) or DOSPER Liposomal Transfection Reagent (Boehringer Mannheim GmbH) according to the supplier's recommendations.

For reverse transcription polymerase chain reaction (RT-PCR) analysis of simian kidney endothelial COS-7 cells transiently transfected with DOSPER/pSURF-Ytyr4-β, cells were harvested 48 h after transfection, and RNA was extracted with RNAzol® (BioGenesis Ltd., Poole, England, UK). Samples were split, and cDNA was made from one aliquot by RT (cDNA Synthesis Kit; Boehringer Mannheim GmbH). Both samples were subject to RT-PCR using primers TYR1-U (see PCR Amplification of Tyrosinase Minigene DNA above) and TYR2-L (5′-ATCGCATAAAACCTGATGGC-3′; complementary to exonic sequence 3′ of the intron), which define a 722-bp region of spliced tyrosinase minigene mRNA. The reaction conditions were as recommended by the *Taq* DNA polymerase supplier (Boehringer Mannheim GmbH), except that 10% DMSO was included. The program used was the following: 3-min denaturation at 94°C followed by 35 cycles of 93°C for 30 s, 55°C for 60 s and 75°C for 90 s. PCR products were run on an agarose gel, blotted and probed with ^{32}P end-labeled TYR1-L.

Human embryonic kidney 293 cells were transfected with linearized pBAC-HyTK-CMVcftr DNA complexed with LIPOFECTAMINE and permanent cell lines established using hygromycin (Calbiochem-Novabiochem, San Diego, CA, USA) selection (14). Extracts of cell lines were analyzed by RT-PCR as described (18). The primer pairs used were S1U (5′-CTTCTGTGGACTTGGTTTCCTGATAGTCCTTGC-3′)/Q1L (5′-TCATTTTTTCCATTGCTTCTTCCCAGCAGTATG-3′) amplifying a 185-bp region of *CFTR* exons 6a and 6b, and H24A1 (5′-AAGAAGAGGTGCAAGATACAAGG-3′)/SPA1 (5′-GTGGTATGGCTGATTATGATC-3′) amplifying a 339-bp, vector-specific region from exon 24 to the SV40-derived 3′ UTR. Blots were probed with appropriate end-labeled internal oligonucleotides.

Immunoprecipitation

Protein-standardized extracts of 293 cells transiently lipofected with plasmid DNA were immunoprecipitated

with CFTR-specific antibody MATG 1104 (Transgene, Strasbourg, France) or control antibody MOPC21 (purified mouse myeloma immunoglobulin; Sigma, Poole, UK) as described (17), and the products were separated using polyacrylamide gel electrophoresis (PAGE) (Figure 5C).

RESULTS

Construction of pSURF-2

The BAC vector pBAC108L was designed primarily as an alternative to YAC cloning vectors and has no eukaryotic selectable markers (31). We have used the backbone of pBAC108L to make an enhanced pSURF-2 vector (Figure 1) with the novel features described in Table 2.

The complete DNA sequence of pSURF-2 was collated from the sequences of its constituent parts and used to produce the map shown (Figure 1).

E. coli Host Modification

We used P1 transduction (13) to make DH10B-U, a *pyrF287* derivative of the cloning host DH10B (9).

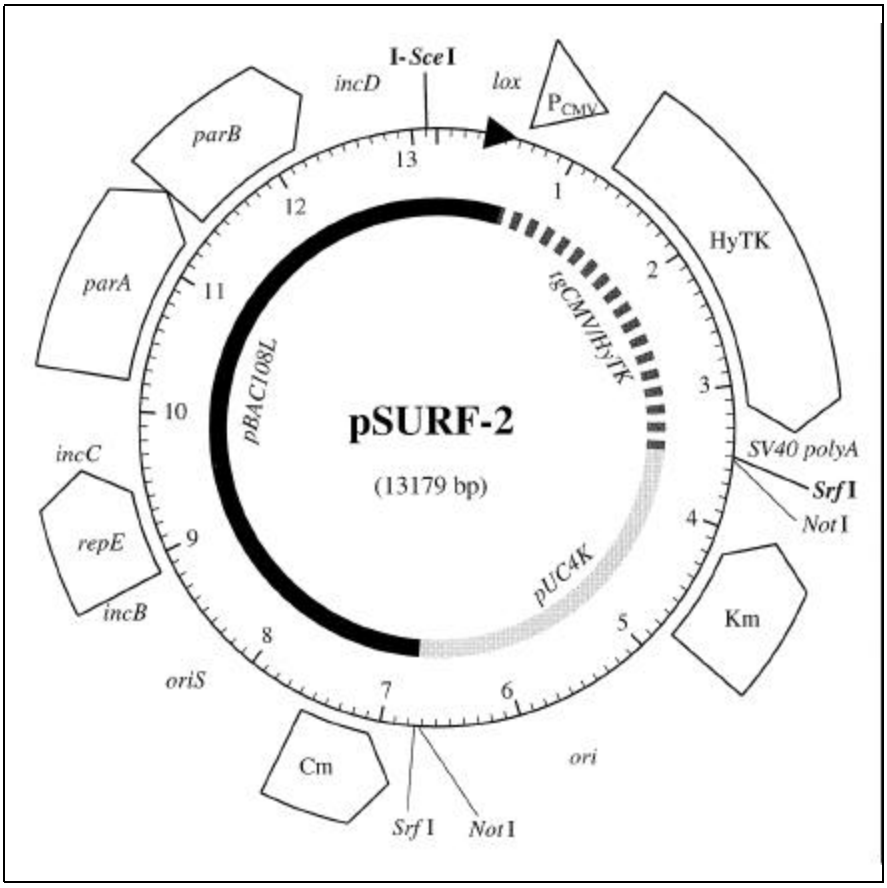

Figure 1. Map of the vector pSURF-2. The plasmid is oriented such that coordinate 1 is the fourth base of the *Sal*I site 5′ of the *cosN* sequence in pBAC108L (31). All genes are shown as open-arrowed arc segments defining their directions of transcription. The approximate extents of the three main components of the backbone are indicated by subdivisions of the inner circle as follows: the 7-kb *Not*I fragment from pBAC108L, which contains genes and sites derived from the F plasmid (*repE*, *parA*, *parB*, *oriS*, *incB*, *incC* and *incD*) necessary for replication and partitioning of recombinants, the chloramphenicol acetyltransferase (*CAT*) gene conferring Cm^R and the *lox* site needed for turbo-cloning (*pBAC108L*); the positive/negative selection cassette derived from tgCMV/HyTK containing the *HyTK* gene driven by the hCMV promoter/enhancer shown as an open triangle (P_{CMV}) and terminated by an SV40 poly(A) signal sequence (*tgCMV/HyTK*); the pUC4K-derived stuffer fragment carrying a kanamycin-resistance gene (Km^R) and multicopy origin (*pUC4K*). The *Srf*I site in the *tgCMV/HyTK* region and the I-*Sce*I site in the *pBAC108L* region (shown in bold) were inserted as short linkers (see Materials and Methods). The collated sequence of pSURF-2 is available on request or on-line (**http://www.hgu.mrc.ac.uk/Users/Christopher.Boyd/publicseqs/index.htm**).

DH10B-U will not grow on minimal medium that lacks uracil but is complemented by the yeast *URA3* gene (25), which encodes the *pyrF* orthologous enzyme (orotidine 5′-phosphate decarboxylase). The low reversion rate of the *pyrF287* allele (transduced by linkage to *trp* from strain PK0803; a gift from P. Kuempel [University of Colorado, Boulder, CO, USA]), allows selection or screening of clones encompassing the *URA3* gene contained on the right arm of YACs. DH10B-U retains the *deoR* mutation that contributes to its ability to be successfully transformed with plasmids up to at least 300 kb (31).

A *pyrF* version of XL1-Blue has been previously described (36), but this strain is DeoR$^+$ and unsuitable for cloning very large fragments.

Cloning and Analysis of YRC-Tyr

The principal difference between turbo-cloning (3) a blunt-ended DNA fragment and intact YAC DNA is that the specialized telomeric structures blocking the ends of the latter must be removed before ligation (38). Thereafter, the process is very similar (Figure 2A; Materials and Methods).

Initially, we sought to turbo-clone YRC-Tyr (Figure 2B) by using the origin-free, 1-kb *Sca*I-*Sma*I fragment of pBSlox (3) as the minimal *lox*-containing vector moiety and relying on the Ap^R gene and replication origin on the YAC left arm for recombinant plasmid selection. Using yeast DNA that had been treated with 0.01 U of BAL-31 per µg for 30 min, two plasmids, plox-Ytyr2 (13.2 kb) and plox-Ytyr5 (17.2 kb), were isolated. Restriction mapping confirmed that each plasmid contained approximately the left half of YRC-Tyr. Many much smaller recombinants were also isolated; this emphasized the importance of titrating BAL-31 digestion.

Later, *Srf*I-cut pSURF-2 was used as turbo-cloning vector, and selection made for chloramphenicol-resistance (Cm^R) together with Ap^R or uracil independence (Table 1). The data for Experiments 1 and 2 suggest that if exposure to BAL-31 is excessive, recombinants with small inserts of truncated YRC-Tyr are recovered. Only when the amount of enzyme was reduced to 0.0003 U per µg (Experiment 3) were large numbers of recombinants recovered. Here, of the 49 colonies obtained, 21 were Ura$^+$. Two of the Ura$^-$ colonies were examined and found to contain plasmids with partial (ca. 75%) YRC-Tyr inserts. It was clear from restriction analysis that 17 Ura$^+$ colonies contained plasmids with complete YRC-Tyr inserts; both orientations of insert were observed, and the banding patterns indicated that no rearrangements had occurred. Examples of each orientation, pSURF-Ytyr4 and pSURF-Ytyr6, were examined further. Figure 3A shows sample restriction patterns of these plasmids compared with those of the partial YRC-Tyr subclone plox-Ytyr5. The integrity of the tyrosinase

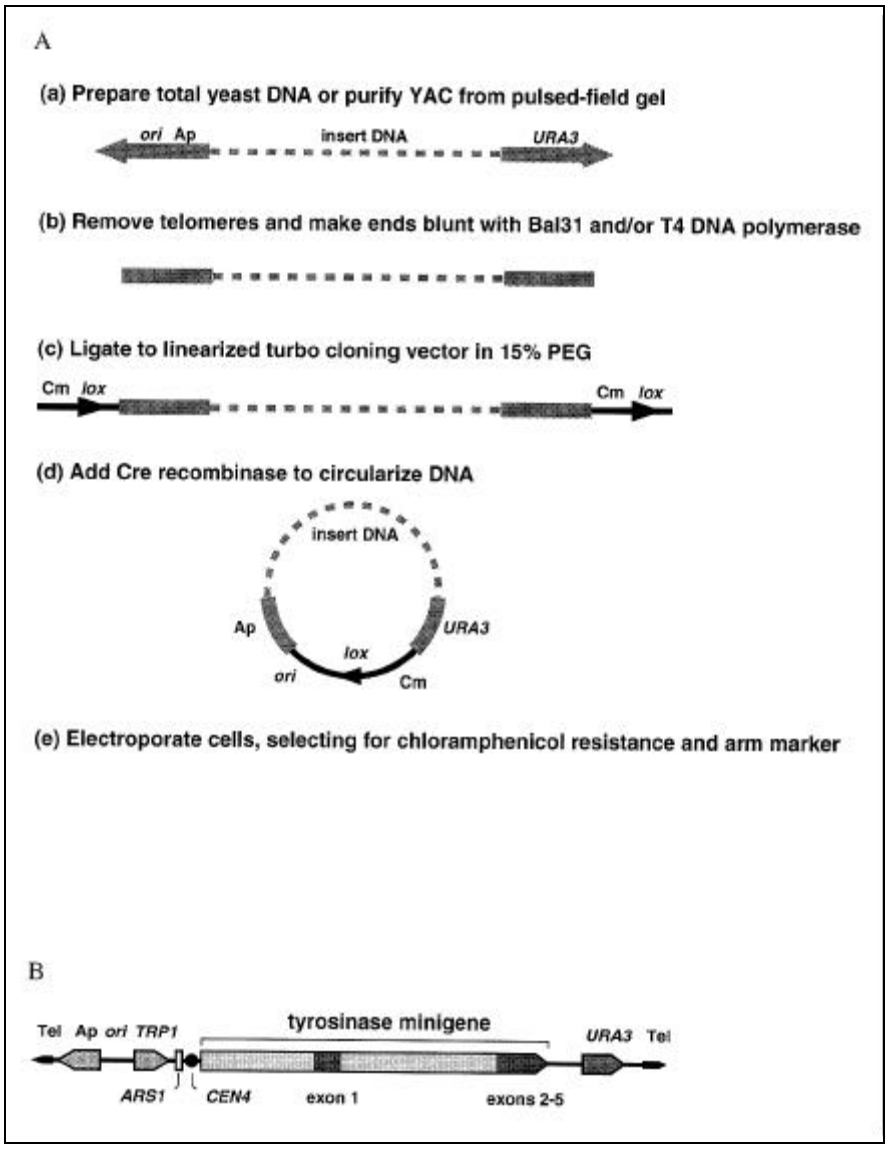

Figure 2. YAC cloning. (A) Strategy for turbo-cloning YACs: (a) total yeast or purified YAC DNA is treated with BAL-31 nuclease and T4 DNA polymerase to remove chromosome end structures and create blunt ends; (b) dephosphorylated vector is added in excess and ligated to the yeast DNA in the presence of 15% polyethylene glycol (PEG) to create trimers; (c) Cre resolves appropriate trimers into circles; (d) DH10B-U is electroporated with aliquots and recombinants containing both YAC left-arm and vector sequences selected using Cm and Ap; and (e) colonies are screened for the presence of the YAC right-arm marker *URA3* by their ability to grow on minimal medium that lacks uracil. (B) Schematic diagram of 25-kb YAC YRC-Tyr. The tyrosinase minigene is shown as a long, shaded arrow, indicating direction of transcription with exons highlighted. Markers are: telomere (Tel); ampicillin-resistance gene (Ap); bacterial origin of replication (*ori*); yeast phosphoribosyl anthranilate isomerase gene (*TRP1*); yeast autonomous replication sequence (*ARS1*); centromere (*CEN4*); yeast orotidine 5′-phosphate decarboxylase gene (*URA3*).

coding sequences was checked by two separate PCR analyses (Figure 3, B and C). This confirmed in particular that plox-Ytyr5 lacked the 3′ coding and UTR sequences of the minigene as predicted for a partial subclone lacking 9 kb from the right end of YRC-Tyr.

It was possible to correlate these and many other restriction digest patterns of pSURF-Ytyr4 and pSURF-Ytyr6 with those predicted from the collated sequence of the pSURF-2 vector and existing maps and partial sequence of YRC-Tyr, arbitrarily assuming that the latter had telomere repeats totaling 280 bp (38) at each end. Figure 4 shows pSURF-Ytyr4 with YRC-Tyr oriented as determined by this analysis. .

Transient Transfection Studies Using Cloned YRC-Tyr

We used transient transfection to confirm that the cloned YRC-Tyr was expressible. To monitor the efficiency of transient transfection, we turbocloned (3) the *lacZ* gene from the pCMVβ vector (CLONTECH Laboratories, Palo Alto, CA, USA) on a filled-in, 4.5-kb *Pst*I fragment into filled-in, *Not*I-linearized pSURF-Ytyr4. The 40-kb plasmid pSURF-Ytyr4-β was isolated from a blue colony selected on plates containing Ap and Cm, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) and was found by restriction analysis to have the *lacZ* gene in the opposite orientation to the tyrosinase minigene (Figure 4). The transient transfection frequencies (0.1%–10%) attained by pSURF-Ytyr4-β were similar to those obtained with pCMVβ as measured by X-gal staining in murine melanocyte (Melan-C), murine fibroblast (3T3) and simian kidney endothelial (COS-7) cells, indicating that the large size was not a barrier to efficient transfection.

RT-PCR was performed on RNA isolated from COS-7 cells transiently transfected with pSURF-Ytyr4-β (Figure 5A). At 10 to 1 liposome:DNA ratios in each of two experiments, an mRNA-specific band of the correct size was seen, indicating that the tyrosinase gene was being appropriately transcribed and spliced. No hybridizing band was seen in RNA isolated from nontransfected cells (data not shown). However, attempts to observe complementation of the albino phenotype of Melan-C melanocytes (37) using transfected pSURF-Ytyr4 failed, suggesting that expression of the gene from the construct is insufficient or not sustained during transient transfection.

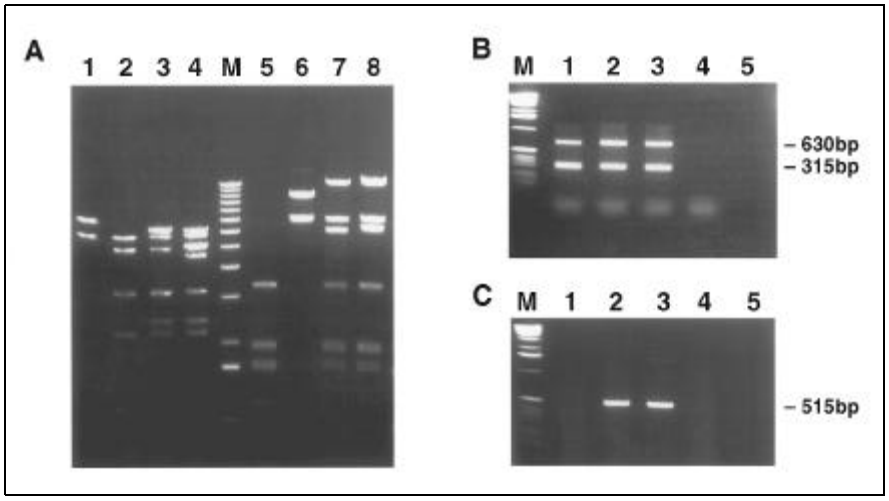

Figure 3. Agarose gel analysis of recombinant plasmids plox-Ytyr5, pSURF-Ytyr4 and pSURF-Ytyr6 containing YRC-Tyr sequences. In each panel, M represents the 1-kb size marker (Life Technologies). (A) Restriction digests. Lanes 1–4 are *Eco*RV digests of pSURF-2, plox-Ytyr5, pSURF-Ytyr4 and pSURF-Ytyr6, respectively. Lanes 5–8 are *Sal*I + *Pvu*II double digests of the same plasmids. Several bands below 1 kb were visible in all but lane 6 on shorter runs. (B) PCR using primer pair TYR1-U/TYR1-L. Lanes 1–3 had plox-Ytyr5, pSURF-Ytyr4 and pSURF-Ytyr6 as template; lanes 4 and 5 were template-free and primer-free controls, respectively. All three plasmids produce the 315-bp band, indicating the presence of 5′ exon sequence. (The 630-bp band is an unexplained PCR artifact.) (C) PCR using primer pair TYR2-U/SV-L: lane order as in Panel B. Only pSURF-Ytyr4 and pSURF-Ytyr6 produce the 515-bp band, indicating the presence of tyrosinase minigene 3′ coding and UTR sequence.

Selection and Analysis of Permanently Transfected Cell Lines

The human *CFTR* gene under control of the human CMV promoter/enhancer was subcloned from pCMV-*CFTR* (1) on a *Not*I fragment into pBAC-HyTK, a precursor of pSURF-2 (see Materials and Methods), to produce pBAC-HyTK-CMVcftr. The recombinant was cleaved uniquely in the vector backbone with *Stu*I and transfected into human embryonic kidney 293 cells with selection for hygromycin resistance. Pools of resistant cells were cultured under selection for several weeks before analysis. Evidence of expression of the integrated *CFTR* transgene was obtained by RT-PCR (Figure 5B). Immunoprecipitation of protein extracts confirmed that mature CFTR was being produced—a band of the correct size was seen only in extracts from cells transfected by pBAC-HyTK-CMVcftr and immunoprecipitated with an anti-CFTR monoclonal antibody (Figure 5C, lane 4). Since the pSURF-2 backbone differs from that of pBAC-HyTK only by insertion of I-*Sce*I and *Srf*I sites, we infer that pSURF-2 recombinants could be used similarly to establish permanent cell lines.

DISCUSSION

We have demonstrated the feasibility of selective YAC cloning by isolating recombinant pSURF-2 plasmids containing the 25-kb YAC YRC-Tyr and confirmed that the cloned YAC expressed tyrosinase RNA in transiently transfected mammalian cells. Since RT-PCR only samples transcription, the possibility remains that full-length tyrosinase message was not being made. However, we deem this highly unlikely since the identical tyrosinase expression cassette has been shown to be functional in transgenic mice (2). In separate experiments, we showed, by the indirect measures of RT-PCR and immunoprecipitation, that the *HyTK* marker can be used to select permanently transfected cells expressing a cloned *CFTR* marker

from a cassette already known to encode functional protein (18). We infer that pSURF-2 should be useful for functional studies of cloned DNA.

A critical step in the YAC cloning procedure is the BAL-31 digestion of the yeast DNA to remove telomeric structures (Table 1). It was found esential to use the minimal amount required to cause any DNA degradation visible by agarose gel electrophoresis. It is inappropriate to apply formulae relating BAL-31 units to bp double-stranded (ds)DNA digested per minute (26) to the reactions carried out here because *(i)* there is no information on how the enzyme is affected kinetically by telomeric structures, and *(ii)* it is difficult to estimate the number of DNA ends in a yeast chromosomal preparation where no steps are taken specifically to avoid shearing. Therefore, the empirical approach is required. T4 DNA polymerase treatment alone has been reported to remove telomeric structures (38); however, our experiments using this approach were not successful.

We have shown elsewhere that pSURF-2 has a cloning capacity of at least 195 kb (8). It should be possible therefore to adapt the methodology to clone intact YACs of larger size than 25 kb. However, the risks of shearing and other damage to the DNA combined with the poorer transformability of very large molecules present considerable obstacles, and much more stringent procedures (such as those used in library construction) for the preparation and handling of DNA would need to be adopted. In the experiments described (Table 1), partial subclones of YRC-Tyr containing sequence extending from either end were obtained in attempts to clone the entire YAC. Selection for Ap^R or *URA3* expression would yield useful libraries of recombinants enriched for left-arm- or right-arm-linked insert sequences, respectively, even if no full-length clones were obtained. Cleavage at the I-*Sce*I site would facilitate physical characterization of the clones obtained.

A potential problem of subcloning large fragments from the left end of a YAC in this way is that some recombinant plasmids would be unstable due to the activity of the cloned multicopy replication origin on the arm. To counter this, we constructed a strain (DH10B-UL) that constitutively produces RNAI, the natural antisense inhibitor of replication (unpublished). Using DH10B-UL as host, the cloned YAC origin was suppressed, and the plasmid replicated solely by means of the single-copy origin of the pSURF-2 vector.

The method described here is a useful addition to the published procedures for transferring YAC sequences to *E. coli* vectors. Some harness homologous

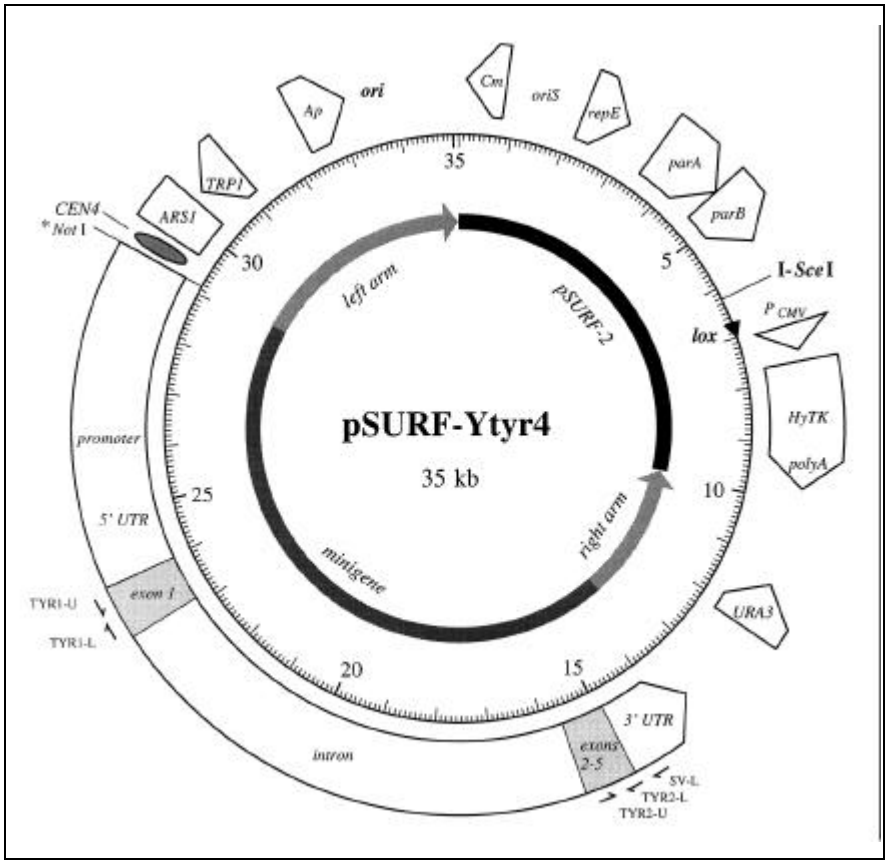

Figure 4. Map of the recombinant pSURF-Ytyr4. Clockwise from the top, the plasmid consists of (symbols as in Figure 1): pSURF-2 vector; YAC right arm containing the *URA3* gene; tyrosinase minigene in counterclockwise orientation; YAC left arm containing the centromere, autonomous replication sequence, TRP1 gene, Ap^R gene and multicopy origin. pSURF-Ytyr4-β (ca. 40 kb) is identical except for the 4.5-kb *lacZ* fragment from pCMVβ inserted at the *Not*I site shown in bold. Telomere repeats are indicated as arrows in the inner circle. Approximate positions of the five primers used in PCR analyses are shown as half-arrows parallel to the tyrosinase minigene.

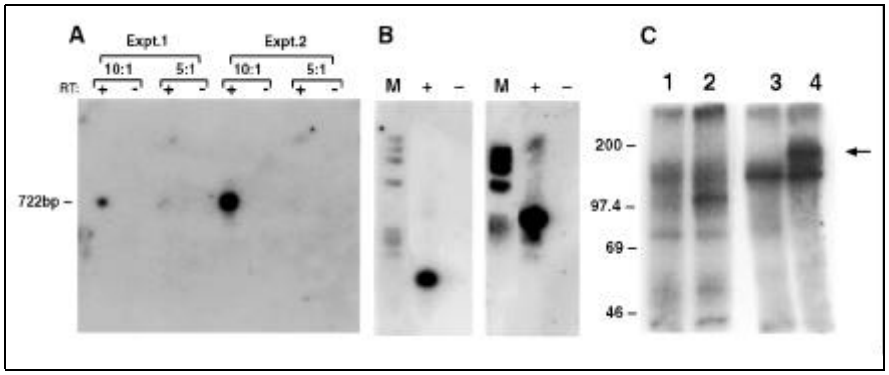

Figure 5. Expression from cloned inserts in transfected cells. (A) Autoradiograms of RT-PCR analyses of COS-7 cells transiently transfected with pSURF-Ytyr4-β. In each of two experiments, cells were transfected with 1 µg pSURF-Ytyr4-β complexed with either 5 or 10 µg DOSPER as shown. RT +, -: with or without reverse transcriptase. Specific RNA was detected only at the 10:1 ratio in both experiments. (B) RT-PCR analysis of a pool of 293 cells permanently transfected to hygromycin resistance with pBAC-HyTK-CMVcftr. Scans were generated using a PhosphorImager® (Molecular Dynamics, Sunnyvale, CA, USA). Primer pairs S1U/Q1L and H24A1/SPA1 were used for the PCRs in the left and right panels respectively. The corresponding products of 195 and 339 bp detected were also visible in ethidium bromide-stained gels (not shown). +, -: with or without reverse transcriptase; M: ϕX174/*Hae*III size marker (Life Technologies). (C) Immunoprecipitation of CFTR protein. Extracts of untransfected cells immunoprecipitated with (lane 1) MOPC21 or (lane 2) MATG 1104 antibodies; extracts of a pool of 293 cells permanently transfected with pBAC-HyTK-CMVcftr immunoprecipitated with (lane 3) MOPC21 or (lane 4) MATG 1104 antibodies. The arrow points to full-length band C form of mature CFTR protein (6) product in lane 4. Sizes in kDa are indicated at the left.

recombination in yeast to rescue YAC sequences (4,10,12), while others rely on in vitro cloning (34,36). As we have demonstrated by isolating cells permanently expressing cloned *CFTR* cDNA (Figure 5, B and C), the pSURF-2 system has the advantage that recombinants can be immediately transferred to the genomes of eukaryotic cells by selection for hygromycin resistance after unique cleavage. In certain cases, it might be possible to select permanently transfected cell lines carrying episomal circular recombinants if they happen to contain functional eukaryotic replication origins (21).

Our use of pSURF-2 for the directed subcloning of fragments up to 195 kb (8) gives us confidence that the vector retains the capacity for the construction of large-fragment genomic libraries. Our experience suggests that a similar strategy involving turbo-cloning insert DNA [prepared by limited shearing

(22) and T4 DNA polymerase polishing] into *Srf*I-cut pSURF-2 can be an efficient alternative to conventional library construction methods. The same approach could be used to generate sublibraries of large YACs.

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