Conditionally Amplifiable BACs: Switching From Single-Copy to High-Copy Vectors and Genomic Clones

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The widely used, very-low-copy BAC (bacterial artificial chromosome) vectors are the mainstay of present genomic research. The principal advantage of BACs is the high stability of inserted clones, but an important disadvantage is the low yield of DNA, both for vectors alone and when carrying genomic inserts. We describe here a novel class of single-copy/high-copy (SC/HC) pBAC/oriV vectors that retain all the advantages of low-copy BAC vectors, but are endowed with a conditional and tightly controlled *oriV*/TrfA amplification system that allows: (1) a yield of ~100 copies of the vector per host cell when conditionally induced with L-arabinose, and (2) analogous DNA amplification (only upon induction and with copy number depending on the insert size) of pBAC/oriV clones carrying >100-kb inserts. Amplifiable clones and libraries facilitate high-throughput DNA sequencing and other applications requiring HC plasmid DNA. To turn on DNA amplification, which is driven by the *oriV* origin of replication, we used copy-up mutations in the gene *trfA* whose expression was very tightly controlled by the *araC*–P_{ara}BAD promoter/regulator system. This system is inducible by L-arabinose, and could be further regulated by glucose and fucose. Amplification of DNA upon induction with L-arabinose and its modulation by glucose are robust and reliable. Furthermore, we discovered that addition of 0.2% D-glucose to the growth medium helped toward the objective of obtaining a real SC state for all BAC systems, thus enhancing the stability of their maintenance, which became equivalent to cloning into the host chromosome.

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The progress in genomic research in the present decade relied on use of the very-low-copy bacterial artificial chromosome (BAC) vectors developed by Shizuya et al. (1992). The BAC vectors were extensively used for preparation of DNA libraries, for physical mapping, and for large-scale DNA sequencing efforts. BAC libraries from fungi (Diaz-Perez et al. 1996), plants (Woo et al. 1994; Mozo et al. 1998), mammals (Cai et al. 1995; Schibler et al. 1998), and human DNA (Kim et al. 1996; Asakawa et al. 1997) have been constructed. The main advantage of using BACs for genomic library construction was the stability of the large, very low or single-copy (SC) clones. However, the SC state of vectors and clones is also of great disadvantage, because of very low levels of DNA recovery, and consequently, reduced purity of DNA with respect to host DNA.

Our objective was to construct vectors that retain the advantages of the SC stability of the BAC clones during the maintenance phase while acquiring elements for a conditional in vivo amplification of BAC vectors and clones. Such amplification would be turned on only when high yields of DNA are required, as when preparing vector DNA for library construction or cloned DNA for sequencing. Ideally, such high yields will lower the dependence on the DNA purification. To achieve such a goal, we constructed the SC pBAC/oriV vector whose conditional, high-copy (HC) origin of DNA replication *oriV* is completely inactive in the commonly used hosts, because they do not produce the TrfA replication protein upon which replication at *oriV* depends. To supply the TrfA protein, we constructed special hosts, in which synthesis of copy-up TrfA mutant protein is very tightly controlled by the P_{ara}BAD (P_{BAD}) promoter and AraC protein. Thus, we created a system permitting conditional amplification of BAC plasmids (without or with) inserts consisting of the pBAC/oriV vector and a host supplying (only upon induction) a copy-up mutant of TrfA protein. In such a system, the pBAC/oriV clone is maintained at the SC level, but when the synthesis of the TrfA protein is induced, DNA is amplified up to 100-fold.

In our earlier preliminary studies, the *trfA* gene was under control of the inducible P_{tet} promoter (Hradecna et al. 1998; Szybalski et al. 1999), but in the present study we used the *araC*–P_{BAD} expression system, which offers additional advantages.

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RESULTS

Derivatives of the pBeloBAC 11 Vector Allowing "on Command" Amplification of BAC Clones

For construction of stable genomic libraries, Shizuya et al. (1992) developed the pBAC108L vector, whose replication mode was that of the *Escherichia coli* F factor. This pBAC108L vector is maintained at 1–2 copies per host chromosome, and its replication is stringently controlled at the level of initiation. Replication initiates at *oriS* (*ori2*), which consists of (1)

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four directly repeated sequences of 19 bp (iterons), (2) an AT-rich region, and (3) binding sites for the host DnaA protein. The RepE protein (251 residues, 29 kD), when in the monomeric form, mediates assembly of a replication complex at *oriS*. The dimeric form of RepE binds to the inverted repeats of the *repE* operator exerting autogenous repression (Komori et al. 1999). Similarly to the F plasmid, the stability of the pBAC's maintenance is ensured by the partition system consisting of ParA, ParB, and ParC elements (Mori et al. 1986).

The pBAC108L vector, however, was of limited use, because clones had to be identified by colony hybridization. To ease detection of clones, Kim et al. (1996) constructed the pBeloBAC11 vector carrying the *lacZ*α fragment for blue/white color screening based on the α-complementation of β-galactosidase (Ullmann et al. 1967; Ullmann 1992). Libraries of DNA fragments up to 300 kb prepared in this vector were shown to be rather stable, and chimeric forms were rarely found (Boysen et al. 1997). Yet another BAC derivative, the SacB-based positive-selection vector with MCS was constructed (Frengen et al. 1999) and used for constructing libraries from human, primate, canine, and murine genomes (Osoegawa et al. 1998). However, the amounts of DNA that are generated from such BAC-derived clones are usually suboptimal, especially for genetic manipulations or sequencing, because the plasmids are present at only 1–2 copies per chromosome. Moreover, preparation of large quantities of the pBeloBAC11 vector DNA, essential for library construction, is laborious and time consuming.

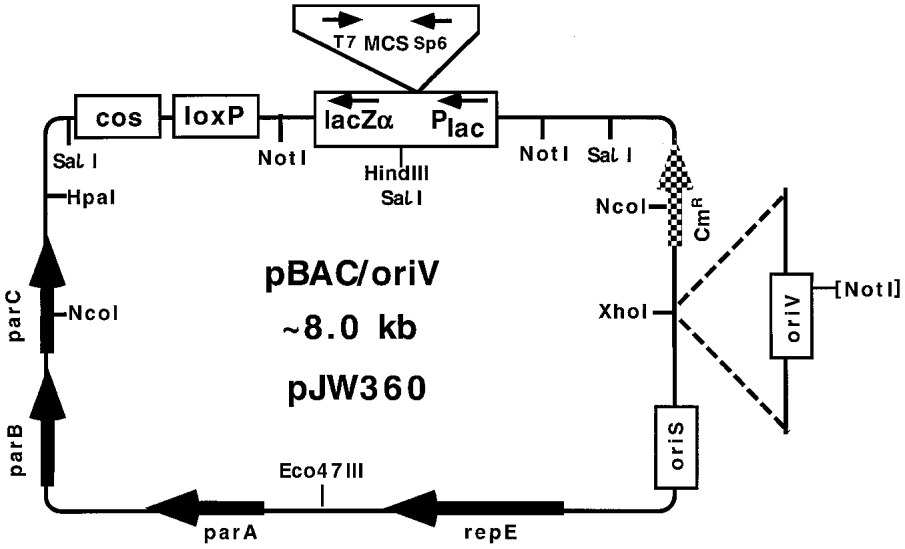

Figure 1 The pBAC/oriV vector permitting its single-copy (SC) maintenance and, alternatively, its conditional, tightly regulated DNA amplification. This new derivative of the pBeloBAC11 vector preserves most of its original specific features, including the plasmid F-derived SC maintenance system based on the *oriS–repE–parABC* genes (see Kim et al. 1996), but was equipped with a second origin of DNA replication, *oriV*, from the broad-host-range plasmid RK2 (Stalker et al. 1981). We cloned the *Not*I-less *oriV* at the *Hpa*I or *Xho*I sites, but for reasons not fully anticipated, the TrfA–*oriV*-directed DNA amplification (see Figs. 2 and 3) was the highest for *oriV* in the *Xho*I site. Four derivatives of pBAC/oriV have been constructed: (1) The pBeloBAC11/SceI/oriV (pJW408 = pBAC/oriV/SceI) vector with the I-*Sce*I recognition site at the *Hpa*I site of pBAC/oriV. (2) The pTrueBlue-BAC2/oriV (pJW406) vector, with *oriV* at the *Xho*I site. This vector features dark-blue colonies (darker than for original BACs and similarly dark as for pIndigoBAC/oriV; see below), thus allowing more accurate blue/white colony screening. It is based on pTrueBlue-BAC2 (Genomics One 1999 Catalog), which contains four additional cloning sites, as compared with pBeloBAC11, all within the specially constructed *lacZ*α segment (Slilaty and Lebel 1998). (3) The pTrueBlue-BAC2/oriV/SceI (pJW419) vector with the I-*Sce*I recognition site cloned into the *Eco*47III site of pTrueBlue-BAC2/oriV. (4) The pIndigoBAC/oriV (pJW550) vector with *oriV* at the *Xho*I site. This vector features enhanced, dark-blue-color colony screening and is based on pIndigoBAC-5 (Epicentre 2001 Catalog). Details of the construction of pBAC/oriV and their derivatives are described in Methods. [NotI] Inactivated *Not*I site.

To overcome the above-discussed shortcomings, but retain all the advantages of conventional low-copy BAC systems, we have reengineered both the pBeloBAC11 vector and the DH10B host so as to allow "on command" amplification of the vector alone or carrying the genomic or cDNA fragments. As a suitable system to turn on the DNA amplification on command, we chose the *oriV*/TrfA replicon system of the broad-host-range RK2 plasmid (Perri and Helinski 1993). The *oriV* origin of replication consists of eight 17-bp direct repeats (iterons) that bind a monomeric form of the initiation protein TrfA (Toukdarian et al. 1996). As a source of *oriV*, we used the *Eco*RI–*Bam*HI fragment of the pSV16 plasmid (Haugan et al. 1992), which was modified to eliminate the *Not*I site (see Methods). The modified *oriV* was cloned into the PolIk-blunted (PolIk, Klenow fragment of Pol I DNA polymerase) *Xho*I site of pBeloBAC11, creating pBAC/oriV (Fig. 1). We have also cloned *oriV* into other sites of the BAC (see legend to Fig. 1). The resulting pBAC/oriV vector retains all features of pBeloBac11, including (1) stability of SC clones; (2) the MCS within the *lacZ*α to detect cloned inserts by α-complementation; (3) two *Not*I sites flanking the MCS for excision of cloned inserts; (4) two phage promoters (T7 and SP6) reading into MCS to generate RNA probes for blotting procedures and containing sequences complementary to generally used sequencing primers; (5) the *cosN* site of phage λ, enabling packaging into phage λ particles, specific labeling of λ cohesive ends used for restriction mapping, and in vitro linearization by λ terminase; and (6) a *loxP* site for linearization and/or introduction of additional DNA fragments via the Cre–*loxP* system of phage P1 (used in our novel in vivo method to retrofit existing BAC library clones with *oriV*; Wild et al. 2001a).

As the optical mapping became a tool for construction of restriction mega-maps (Giacalone et al. 2000), it also became very desirable to have a very rare and reliable restriction site on the vector for efficient and convenient linearization of clones. To meet this goal, we used the very rare restriction site, I-*Sce*I, for the intron homing endonuclease (Monteilhet et al. 1990) and constructed pBAC/oriV/SceI by cloning the *Not*I-less *oriV* fragment (see legend to Fig. 1 and Methods section) into the PolIk-blunted *Xho*I site of pBeloBAC11/SceI, provided by the F.R. Blattner laboratory (University of Wisconsin).

Color screening of clones based on insertional inactivation (between ATG and codon 7) of the *lacZ*α that encodes the α-peptide of β-galactosidase is widely used for detecting recombinant clones. However, this screening method often leads to false results, both positives (white colonies that do

not contain the insert) and negatives (blue colonies that contain the insert). To provide high-accuracy color screening, Slilaty and Lebel (1998) reengineered the *lacZ*α fragment so as to ensure detection of DNA insertions within the region that encodes amino acids 11–36 of β-galactosidase. The DNA containing this TrueBlue gene fragment was cloned into the pBeloBAC11 vector creating TrueBlue-BAC2, which claims to offer 100% accuracy in blue/white screening together with six unique cloning sites capable of accepting DNA fragments generated by >70 different restriction enzymes or obtained by shearing or sonication (Genomics One Catalogue 1999). To obtain amplifiable derivatives, we have cloned the modified *oriV* (see Methods) into the PolIk-blunted *Xho*I site of pTrueBlue-BAC2, creating the pTrueBlue-BAC2/oriV vector (see legend 2 to Fig. 1 and Methods). Furthermore, we introduced a recognition site for the I-*Sce*I meganuclease (see legend 3 to Fig. 1) into pTrueBlue-BAC2/oriV creating the pTrueBlue-BAC2/oriV/SceI vector (see legend to Fig. 1). When compared with other *lacZ*α plasmids, the pTrueBlue-BAC2/oriV vector offers another advantage by providing a much darker blue color of colonies when enough IPTG inducer (40 μg/mL) is used. Because colonies of Indigo derivative of pBeloBAC11 (Epicentre Catalogue 2000) develop a similar deep blue color, we have also cloned *oriV* into this derivative and obtained the pIndigoBAC-5/oriV vector (see (4) in legend to Fig. 1).

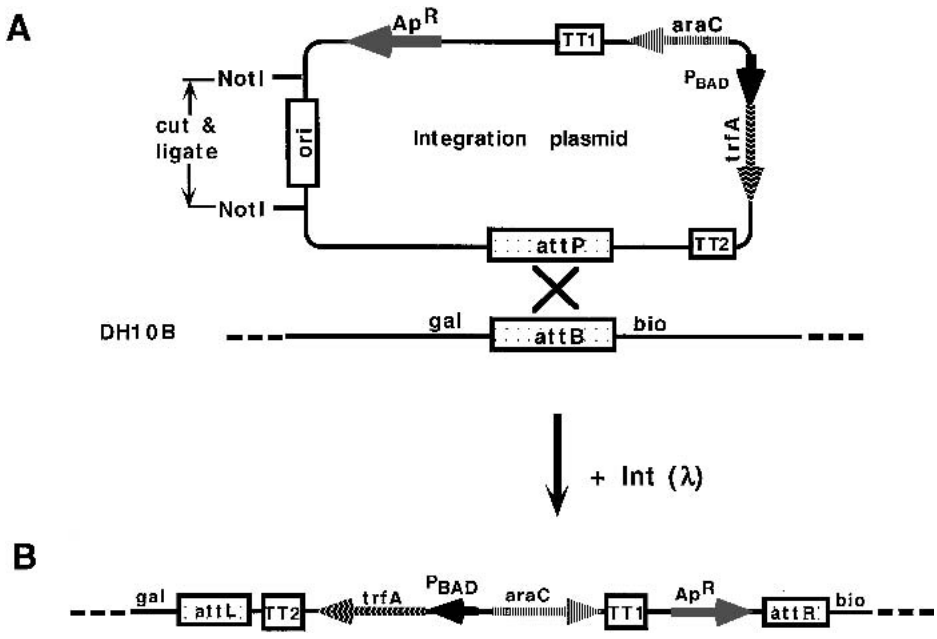

Figure 2 Construction of four DH10B-based hosts carrying a tightly regulated *trfA* gene that supplies, but only upon induction, the TrfA replication protein. (*A*) A representation of an integration plasmid and of a fragment of the host genome with the *attB* site for site-specific recombination. Four integration plasmids carrying four various *trfA* copy-up mutations have been constructed (see Table 1), as described in Methods. Each integration plasmid carries a cassette consisting of *araC*–P_{BAD} fused to the specific *trfA* gene copy-up mutant. All integration plasmids have (1) an easily removable *Not*I-flanked *ori* of plasmid pBR322, and (2) the $attP_{\lambda}$ site for site-specific integration into *attB* of the DH10B host genome, as shown below the plasmid drawing. (*B*) A diagram of the genomic segment of the host upon recombination of the *trfA*-integration plasmid. Such hosts permit conditional, tightly regulated synthesis of the TrfA protein. Experimental details on Int-mediated integration of the four plasmids into the DH10B host strains (Table 2) are described in Methods. TT1 represents the *t1* and *t2* terminators (both clockwise) from *rrnB*; TT2 represents the t_L3 (clockwise) and t_L1 (anticlockwise) terminators of phage λ.

Modified *E. coli* DH10B Hosts for On-Command Amplification of pBAC/oriV

We have chosen the *oriV*/TrfA replication system of the RK2 plasmid for "at-wish" amplification of modified pBAC vectors because this replication system is simple and requires only one RK2-encoded protein, TrfA. Replication at *oriV* depends on the RK2-encoded replication protein TrfA that binds specifically to direct repeats (iterons) at *oriV*. Specific copy-up mutations in *trfA* increase the copy number from 3- to 20-fold, as compared with the wild-type *TrfA*. We tested several *trfA* copy-up mutations (Durland et al. 1990; Haugan et al. 1992) for their effect on replication of BAC vectors that contained *oriV* (see below, "Effect of Various Copy-up Mutations in the *trfA* Gene on the Amplification of Large DNA Inserts" and Fig. 6). To tightly control expression of the *trfA* gene mutants, we used the regulatory system of the arabinose operon consisting of *araC* (encoding the AraC regulatory protein) and the P_{BAD} promoter (Guzman et al. 1995). The *ara* expression cassette was cloned into the integration vector containing the *attP* site from λ (for site-specific recombination), and the pBR322 *ori*, the latter flanked by two *Not*I sites, for its convenient removal (M. Koob, pers. comm.). The resulting pJW344 plasmid served for cloning the *trfA* copy-up mutant genes and for inserting them into the host genome (see Fig. 2, Table 1 below, and Methods for details). In the presence of inducer, L-arabinose, transcription of *trfA* mutants from the P_{BAD} promoter is turned on; whereas in the absence of inducer, the *trfA* expression is undetectable.

Construction and Maintenance of Genomic Libraries

Methods for constructing libraries in pBAC/oriV should not differ from those in regular BACs (Cai et al. 1995; Diaz-Perez et al. 1996; Asakawa et al. 1997), other than simplifying preparation of large quantities of very pure pBAC/oriV vector. The resulting clones, however, could be transformed either into the standard DH10B, which would not allow DNA amplification, or into the DH10B/*trfA* derivatives, like JW366, that only on command, that is, when the inducer L-arabinose is added, could provide TrfA function that promotes DNA amplification. We believe that preparation of libraries directly in the latter JW366-like hosts is much more efficient than first preparing a DH10B-based library as an intermediate, and then transferring the clones into hosts with the inducible TrfA function. Such a two-step procedure is unwarranted, because libraries constructed directly in JW366-like hosts (in L-arabinose-free media) are as stable as when using *oriV*-less BACs or *trfA*-less hosts.

Table 1. List of Plasmids

Plasmid	Pertinent features	Source/Reference
pBeloBAC11	F plasmid-based very low-copy vector	Kim et al. (1996)
pBeloBAC11/SceI	pBeloBAC11 with the I–*Sce*I recognition site inserted at the *Hpa*I site	F. Blattner's lab
TrueBlue-BAC2	Accurate color selection vector	Genomics One
pBAC/oriV	pBeloBAC11 with *oriV* inserted at the *Xho*I site	Fig. 1 and Fig. 3
pCG273	pBAC/oriV containing 140-kb insert of wheat DNA	C. Gaskins
pCG274	pBAC/oriV containing 122-kb insert of wheat DNA	C. Gaskins
pCG275	pBAc/oriV containing 108-kb insert of wheat DNA	C. Gaskins
pMPM123	ColE1 *ori, araC-*P_{BAD}, Sp^R, Ap^R	Mayer (1995)
p67	pBAC/oriV containing 40-kb insert of trout DNA	C. Small and B. Bogden
p69	pBAc/oriV containing 80-kb insert of trout DNA	C. Small and B. Bogden
pJW22	pBR322 with its *ori* flanked by two *Not*I sites (formerly pANTS)	M. Koob, unpubl. (see Hasan et al. 1994)
pJW344	pJW22 containing *araC-*P_{BAD}	This paper
pJW349	pJW344 containing *araC-*P_{BAD}*-trfA*203 cassette	Fig. 2
pJW360	pBAC/oriV	Fig. 1
pJW378	pBAC/oriV containing 20 kb of *Arabidopsis thaliana* DNA inserted between two *Not*I sites	Fig. 4A; Fig. 5
pJW406	pTrueBlue-BAC2/oriV	Fig. 1, legend
pJW408	pBeloBAC11/SceI/oriV	Fig. 1, legend
pJW419	pTrueBlue-BAC2/oriV/SceI	Fig. 1, legend
pJW424	pJW344 containing *araC*P_{BAD}*trfA*254 cassette	Fig. 6
pJW457	pJW344 containing *araC*P_{BAD}*trfA*250 cassette	Fig. 6
pJW458	pJW344 containing *araC*P_{BAD}*trfA*173 cassette	Fig. 6
pJW459	pJW344 containing *araC*P_{BAD}*trfA*171 cassette	Fig. 6
pJW487	pBAC containing 100 kb of *Arabidopsis thaliana* DNA inserted between two *Not*I sites and retrofitted with *oriV*	This paper
pJW511	pBAC containing 77 kb of rice DNA retrofitted with *oriV*	This paper
pJW547	pIndigoBAC-5; phosphorylated and recircularized from the linear epicenter pIndigoBAC-5 (*Hin*dIII)	This paper; Methods
pJW550	pIndigoBAC/oriV	Fig. 1, legend

More recently, we also constructed pBAC/oriV derivatives that, in addition, carry the inducible *trfA* cassette; such plasmids can be amplified in the plain DH10B hosts. We found such pBAC/oriV/trfA plasmids perfectly stable. We used them for expression vectors (Wild et al. 2001b), but we did not evaluate them as yet for library constructions. The examples above illustrate the great flexibility of our *oriV/trfA* cloning and amplification systems.

Stability and Amplification of the pBAC/oriV Vector and Clones

Various stability controls (Fig. 3, lanes 1–7) together with the dramatic (50- to 100-fold) amplification of the pBAC/oriV vector are shown in Figure 3. The latter occurs only when the TrfA function is provided by the host (lane 8). There was no amplification when hosts do not provide the TrfA function, for example, DH10B (Fig. 3, lanes 1,2), or when the plasmid does not carry the *oriV* site (pBeloBAC11; lanes 3–5), even in the presence of the L-arabinose inducer (lane 5).

When testing various media for optimal DNA amplification, we have discovered that glucose, at 0.2%, reduces the number of BAC copies (Fig. 3, cf. lane 4 with 3, lane 7 with 6). This makes the 0.2% glucose a rather important novel tool for the maintenance of BACs in an SC state, because it prevents any undesirable rearrangements that might occur as a result of recombination between two or more plasmids present within a single host cell.

Amplification of the pBAC/oriV Clones

The DNA restriction pattern remains unchanged over many months of maintenance of the large clones and also after the entire cycle of their amplification (Figs. 4–7), as confirmed by DNA sequencing of selected clones. Amplification of the pBAC/oriV vector (Fig. 4A, lane 1 or 2 vs. 3) and the same vector carrying a 20-kb insert (Fig. 4A, lane 4 and 5 vs. 6) each in the same host, are shown in Figure 4A. Again, one can see the dramatic DNA amplification after induction of the TrfA function by L-arabinose (Fig. 4A, lanes 3,6), and the glucose-dependent reduction of copy number of uninduced pBAC/oriV plasmids, from about two to one per cell (Fig. 4A, lane 2 vs. 1, lane 5 vs. 4). We compared the extent of amplification (as assessed by a series of dilutions; Fig. 4B) in clones carrying foreign DNA inserts of 40 kb (Fig. 4B, lanes 1–5), and 80 kb (Fig. 4B, lanes 6–10). In the DH10B host expressing the *trfA203* gene (JW366), the 40- and 80-kb clones were amplified ~40-fold and ~20-fold, respectively. This host (JW366) was best suited for the amplification of clones up to 50 kb. The effect of other *trfA* mutations, more suitable for amplification of larger clones, is described below and is illustrated in Figures 6 and 7.

Regulation of Amplification of the pBAC/oriV Clones by L-Arabinose, D-Fucose, and D-Glucose

The extent of DNA amplification could be regulated either by varying concentrations of L-arabinose (Fig. 5A) or by modulating induction by other sugars (Fig. 5B,C). The optimal amplification was achieved at 0.01% of the L-arabinose (Fig. 5A, lane 2). However, lowering the concentration to 0.001% reduced the induction only slightly (Fig. 5A, lane 6). Comparison of DNA dilutions (Fig. 5A, lanes 1–5) indicates that the extent of amplification was 50- to 100-fold. Less than 0.001%

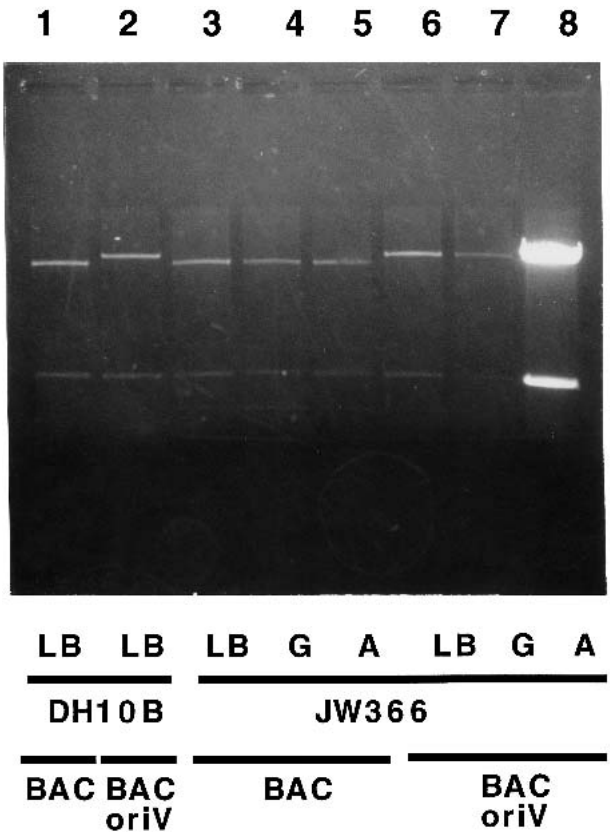

Figure 3 Maintenance and amplification of the pBAC/oriV vector: effects of the host, glucose, and L-arabinose-induced synthesis of the TrfA protein. The DH10B host and its derivative JW366, containing the *araC–*P_{BAD}*–trfA203* cassette at the λ *attB* site (Table 2), were transformed either with pBeloBAC11 (BAC) or pBAC/oriV (see Table 1). Transformants were grown in the Luria-Bertani medium (LB), LB + 0.2% D-glucose (G) or LB + 0.01% L-arabinose (A). After 5 h of growth, a 4.5-mL volume of each culture was centrifuged and the DNA was prepared using Wizard columns (Promega). All lanes (0.8% agarose gel) show two *Nco*I fragments of plasmid pBeloBAC11, either without (lanes *1*, *3–5*) or with inserted *oriV* (pBAC/oriV in lanes *2*, *6–8*). Successful DNA amplification is seen only in lane *8*, whereas lanes *1–7* represent various controls. (Lane *1*) pBeloBAC11 in the DH10B host grown in LB; (lane *2*) pBAC/oriV in the DH10B host grown in LB; (lanes *3–5*) pBeloBAC11 in the JW366 host grown in LB, LB + 0.2% G or LB + 0.01% A, respectively; (lanes *6–8*) pBAC/oriV in the JW366 host grown in LB, LB + 0.2% G or LB + 0.01% A, respectively. Whereas 0.2% G reduces the plasmid number to one per cell (lane *7* vs. *6*), induction with A amplifies DNA up to 100-fold (lane *8* vs. *6*). The induced high-copy (HC) replication of pBAC/oriV provides an ample amount of vector DNA for construction of libraries.

of L-arabinose resulted in progressively lower DNA amplification (Fig. 5A, lanes 7–9).

The inhibition of the L-arabinose-induced DNA amplification by D-glucose or D-fucose is shown in Figure 5, B and C, respectively. Glucose showed a very sharp transition between noninhibitory (0.1%) and very inhibitory (0.18–0.2%) concentrations (Fig. 5B). Significantly, at concentrations just below 0.1%, glucose enhanced the growth of the host and the final yield of the amplified DNA. Fucose blocked quite effectively the induction of DNA replication at concentrations above 0.01% (Fig. 5C, lanes 6,7). Modulation of DNA replication was mainly applicable for expression vectors that were based on the pBAC/oriV SC/HC plasmids (Wild et al. 2001b).

Effect of Various Copy-Up Mutations in the *trfA* Gene on the Amplification of Large DNA Inserts

Because the *trfA203*-expressing host was most effective in amplification of clones smaller than 50 kb, we evaluated several other copy-up mutants of *trfA* for their ability to amplify larger DNA inserts. As shown in Figure 6, three of the *trfA* copy-up mutants were somewhat more effective than *trfA173* in the amplification of the pBAC/oriV 108-kb clone. Amplification of this 108-kb clone host was less effective in the *trfA203*-bearing host than that shown in lane 4. On the other hand the amplification of smaller (20- to 40-kb) clones was better in hosts carrying the *trfA203* mutation than in some other hosts listed in Figure 6 (systematic comparative results are not shown here).

BAC amplification in the commercial derivatives of DH10B, into which we have integrated cassettes expressing various *trfA* copy-up mutations, is shown in Figure 7. The availability of such commercial hosts is of advantage for various high-throughput uses, especially for large genome sequencing. Moreover, commercially available competent cells of such hosts are more efficiently transformed by electroporation (see below) and thus are more suitable for high-throughput constructions of libraries.

Efficiency of Transformation

Efficient transformation is crucial for cloning large (>30-kb) DNA fragments. The DNA of such clones is introduced into cells by electroporation, the efficiency of which depends both on the DNA used for electroporation (its quality and size) and on the recipient bacteria. We have investigated here the effects of *oriV* presence and its various locations on the plasmid, of plasmid and insert size, of *trfA* mutations in the host, and of the method of host cell preparation. We first compared plasmid pBeloBAC11 and its pBAC/oriV derivative using as recipients strains DH10B and JW463, the latter carrying the *araC–*P_{BAD}*–trfA250* cassette at *att*λ. In all four experiments, the number of transformations obtained (Cm^R colonies), that is, 1–5 × 10^6 CFU/μg DNA, was similar. To be sure that in transformation experiments we were using equivalent amounts of pBeloBAC11 or pBAC/oriV (pJW360) DNA, each plasmid was digested with *Nco*I and various DNA aliquots were run on the gel to estimate DNA concentrations. When evaluating the commercially available Electrocomp GeneHogs and Electrocomp GeneHogs(*trfA*) (constructed by us and listed in Invitrogen 2001 Catalog, p. 53, and 2002 Catalog No. T 1060-01) as recipients for electroporation of pBeloBAC11 and pBAC/oriV DNA, we found that the number of transformants was 1 or 2 orders of magnitude higher compared with regular DH10B and JW463 hosts.

Libraries constructed in pBeloBAC11 usually contain DNA inserts of 100–200 kb. Therefore, it was important to test the efficiency of transformation using pBAC/oriV carrying large DNA inserts. To obtain such clones, we retrofitted existing pBeloBAC11 clones with *oriV* (using an in vivo procedure, outlined by Wild et al. 2001a). The resulting plasmids, pJW487 (pBAC/oriV with a 100-kb *Arabidopsis thaliana* DNA insert) and pJW511 (pBAC/oriV with a 77-kb rice DNA insert), were used to transform Electrocomp GeneHogs, DH10B, and JW463 cells. The number of transformants obtained with either plasmid was 0.5–1 × 10^5 CFU/μg DNA when GeneHogs

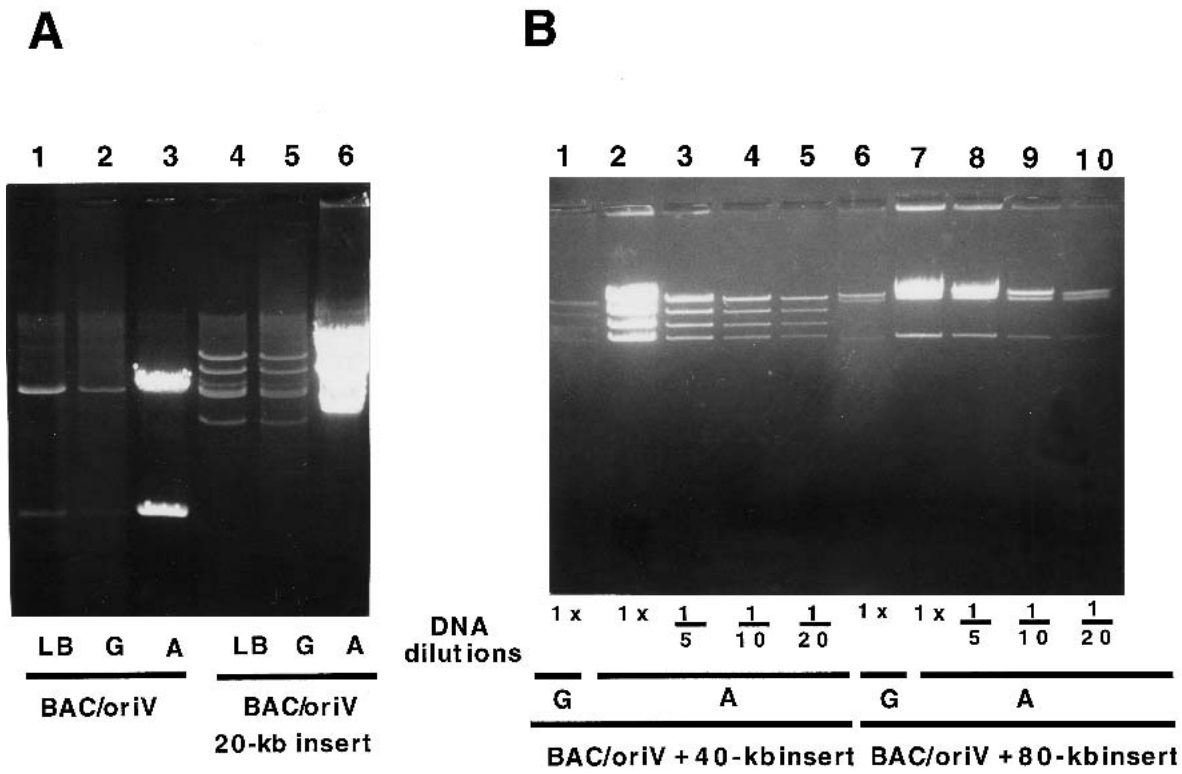

Figure 4 Maintenance and amplification of pBAC/oriV that carries DNA inserts of various length. (*A*) Comparison of amplification of pBAC/oriV vector, with or without a 20-kb insert. After 5 h of growth, cells from the 4.5-mL volume of the culture were collected, and DNA was phenol-extracted, precipitated with 70% ethanol, digested with *Nco*I (lanes *1–3*) or *Sal*I (lanes *4–6*), and run on an 0.8% agarose gel. (Lanes *1–3*) Strain JW371 carrying pBAC/oriV grown in the LB medium (LB), LB + 0.2% D-glucose (G) or LB + 0.01% L-arabinose (A), respectively (two bands are analogous to those in Fig. 3); (lanes *4–6*) strain JW378 carrying pBAC/oriV with the 20-kb insert grown, respectively, in LB, LB + 0.2% G or LB + 0.01% A. (*B*) Assessment of amplification by diluting of the amplified DNA of pBAC/oriV clones containing 40-kb or 80-kb inserts. Growth conditions, DNA analysis after *Sal*I digestion, and abbreviations are as described for *A* and in Methods. Numbers below the lanes indicate the fold of DNA dilution prior to *Sal*I digestion (results were similar for dilutions made after digestions and are not shown here). (Lane *1*) Uninduced strain JW389 carrying pBAC/oriV with the 40-kb insert grown in LB + G; (lanes *2–5*) induced strain JW389 grown in LB + A; (lane *6*) uninduced strain JW390 carrying pBAC/oriV with the 80-kb insert grown in LB + G; (lanes *7–10*) induced strain JW390 grown in LB + A. The DNA in lanes *1*, *2*, *6*, and *7* is undiluted. In lanes *3–5* and *8–10*, the DNA was diluted, as specified below the lanes.

were used and 1×10^4 CFU/μg DNA when electroporating into DH10B and JW463 competent cells. These results confirm that the presence of either the *oriV* or the *trfA* cassette had no effect on the efficiency of transformation, even under conditions when such efficiency coordinately decreased owing to the large size of the plasmid, or increased when using the commercially prepared recipient cells.

DISCUSSION

The present study describes important and useful improvements to the conventional low-copy BAC vectors that are a major workhorse of large genomes projects.

The most important feature of BAC clones is their stability resulting from their very low copy number. We showed here that the copy number can be lowered even further by the addition of glucose (0.2%) to the growth medium, both for conventional BACs and our pBAC/oriV. The real SC state should improve stability of maintenance of BAC libraries by reducing the opportunity for intracellular recombination between clones.

For practical applications like library construction and sequencing, ample amounts of pure DNA are required. Therefore, we developed the conditional *oriV*–TrfA DNA amplification system, which permits easy and prompt 30- to 100-fold increases in the amount of DNA for (1) preparing the pBAC/oriV vectors for constructing libraries, or (2) high-throughput DNA sequencing of clones. Obviously, clones that have undergone the amplification process should not be used for clone maintenance, but only for biochemical procedures, including sequencing or gene expression. Furthermore, our system offers great advantages for the purification of pBAC/oriV clones free of host DNA, usually a rather laborious procedure. The large size of BAC clones and the probability that their DNA would become sheared along with the contaminating host genomic DNA make DNA purification of traditional BACs even more difficult. Amplification of BACs using our *oriV*–TrfA system enriches the BAC clones 30- to 100-fold, thus enhancing by a similar factor the purity of BAC DNA.

Further improvements include the following: (1) Incorporation of the I-*Sce*I site into pBAC/oriV derivatives creates clones more suitable for the optical mapping (Giacalone et al. 2000). Our vectors pBAC/oriV/SceI and pTrueBlue-BAC2/oriV/SceI were designed for that purpose. (2) Introduction of the *lacZ*α derivatives into pBAC/oriV allowing a deeper blue color development (pIndigoBAC-5/oriV), together with more reliable blue/white screen for successful cloning (pTrueBlue-

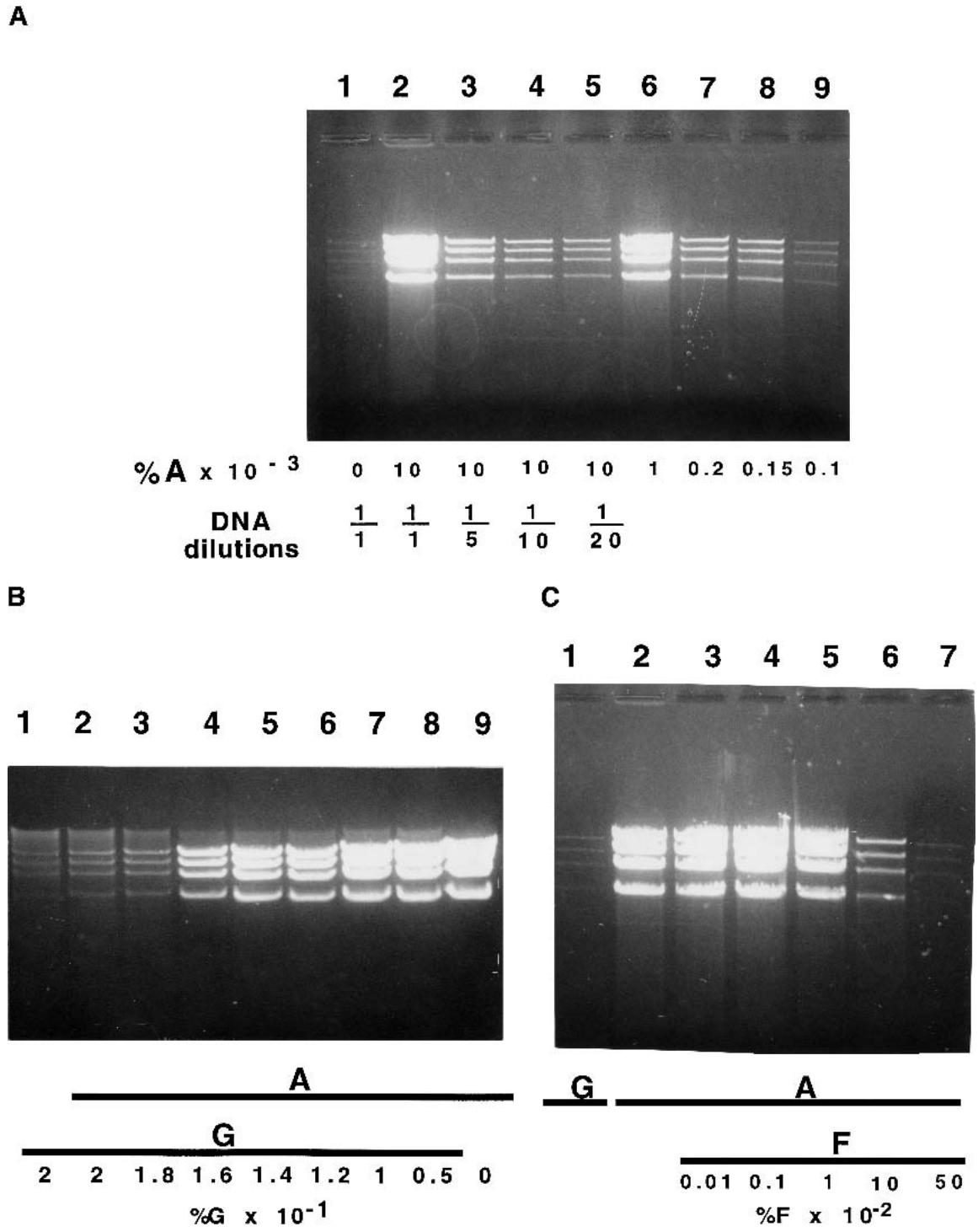

Figure 5 Effects of L-arabinose, D-glucose, and D-fucose on the amplification of the pBAC/oriV with a 20-kb insert. (*A*) Induction by L-arabinose (A). Strain JW378 (pBAC/oriV + 20-kb insert) was grown in LB medium (LB) supplemented with various concentrations of A. Induction, DNA extraction, and digestion were performed as described in Methods and in the legend to Figure 4A (lanes *4–6*). (Lane *1*) No inducer present in LB; (lanes *2–5*) LB + 0.01% A. (Lane *2*) An undiluted DNA sample was run; (lanes *3–5*) DNA samples were diluted 5-, 10-, or 20-fold, respectively, prior to the *Sal*I digestion; (lanes *6–9*) LB supplemented with 0.001%, 0.0002%, 0.00015%, or 0.0001% A, respectively. By comparing the DNA bands in lanes *1* and *5*, we estimate that DNA amplification was ~80-fold. (*B*) Inhibition of amplification by D-glucose (G). Strain, experimental design, and abbreviations are as in description of *A*. (Lane *1*) No A added, LB + 0.2% G; (lanes *2–8*) LB was supplemented with 0.01% A and with 0.2, 0.18, 0.16, 0.14, 0.12, 0.1, or 0.05% G, respectively; (lane *9*) LB supplemented only with 0.01% A. (*C*) Inhibition of amplification by D-fucose (F). Strain, experimental panel design, and abbreviations are as in description of *A*. (Lane *1*) LB + 0.2% G only; (lanes 2–7) LB + 0.01% A, supplemented with none, 0.0001%, 0.001%, 0.01%, 0.1%, or 0.5% F, respectively.

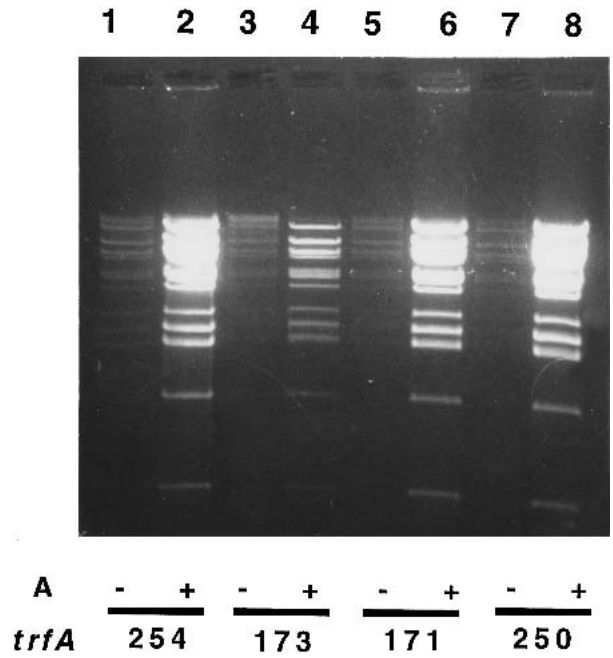

Figure 6 Effect of copy-up mutations in the *trfA* gene on DNA amplification. All host strains carry the same pBAC/oriV plasmid with a 108-kb DNA insert (pCG275). Growth conditions and the induction of TrfA synthesis by L-arabinose (A) are described in Methods. DNA samples prepared by phenol extraction and ethanol precipitation were digested with *Sma*I and run on a 0.6% agarose gel. (Lanes *1,3,5,7*) LB medium (LB); (lanes *2,4,6,8*) LB + 0.01% A. (Lanes *1,2*) Strain JW439 containing the *trfA254* mutation; (lanes *3,4*) strain JW499 containing the *trfA173* mutation; (lanes *5,6*) strain JW500 containing the *trfA171* mutation; (lanes *7,8*) strain JW501 containing the *trfA250* mutation. Amplifications (lanes *2,6,8*) of plasmid + 108-kb insert are estimated to be up to 30-fold.

BAC2/oriV), and developing methods for the positive selection of clones, prepared either by restriction enzyme digestion or random sheer, that will soon be available for construction of libraries based on our pBAC/oriV vectors. (3) Both the pBAC/oriV vectors (see Fig. 3) and clones (Wild et al. 2001a) are as stable as the commonly used BACs. They should be even more stable when maintained as a single copy, in the presence of 0.2% glucose. (4) Our hosts, which contain the tightly regulated *trfA* cassette, are as easily transformed as the *trfA*-less parental hosts. Our *trfA* hosts are now commercially available in highly electro-competent forms (e.g., strains Stbl2–*trfA*, Life Technologies; GeneHogs–*trfA*, Invitrogen), and TransforMax EPI300 (Epicentre). (5) The extent of the L-arabinose-induced DNA amplification can be easily modulated by the appropriate concentrations of glucose (with a sharp transition in the 0.1%–0.2% range). This is illustrated in Figure 5B. (6) Our system is specially designed for high-throughput sequencing of both ends of cloned fragments. At present, this is probably the most effective approach for sequencing of large genomes.

An addition to the improvements listed above is the reduction in plasmid size as mediated by the Flp/*FRT* excision system (see Szybalski et al. 1999). In this mode of amplification, only the cloned fragment and *oriV* are excised and replicated.

We are in the process of preparing genomic libraries, using our pBAC/oriV vectors to evaluate the stability of clones and the effectiveness of DNA amplification. Moreover, several libraries have been constructed by commercial laboratories, including Research Genetics (at present Invitrogen), using our pBAC/oriV–TrfA systems, and we were informed that, if anything, they are superior to regular BAC libraries as far as the ease of construction and maintenance are concerned (M. Ragland, pers. comm.). The amplification of clones yielded ample amounts of DNA, similar to the results reported here, and the fidelity of DNA sequencing was high, as expected.

The plasmids and hosts are available from the authors, unless they are or will become available from commercial or other sources.

METHODS

Bacterial Strains and Media

The *E. coli* strains used in this study were mostly derivatives of strain DH10B (see Table 2), widely used for the preparation of genomic libraries. Bacterial cultures were routinely grown with shaking in Luria-Bertani broth (LB medium) at 37°C, unless different temperatures are indicated. Antibiotics were added at following concentrations: ampicillin (Ap), 50 μg/mL; chloramphenicol (Cm), 12.5 μg/mL; and spectinomycin (Sp), 30 μg/mL.

DNA Manipulations

Standard media, buffers, and DNA techniques were used (Sambrook et al. 1989). DNA from small plasmids was purified using the Wizard DNA Purification System (Promega), whereas DNA from plasmids carrying inserts >20 kb was extracted by alkaline lysis, followed by the phenol–chloroform treatment and precipitation with 70% ethanol. We routinely prepared DNA from 4.5 mL of overnight culture. Purified DNA was resuspended in 40–50 μL of TE buffer.

Construction of pBAC/oriV Derivatives

As a source of *oriV*, we cloned the *Eco*RI–*Bam*HI fragment of the pSV16 plasmid (Durland et al. 1990) into the same sites of plasmid pUC19, resulting in pJW32. The 617-bp *oriV* sequence contains the *Not*I site preceding the eight iterons required for replication (Stalker et al. 1981). We have shown that truncation of 92 bp from the 5′ end of this *oriV* fragment does not affect replication directed by *oriV* when cloned into the *Xho*I site of pBeloBAC11. Therefore, pJW32 was digested with *Not*I + *Bam*HI, and the 0.5-kb fragment was gel-purified, blunted with PolIk (Klenow fragment of DNA polymerase Pol I), and ligated to pBeloBAC11 (that was digested with *Xho*I, blunted with PolIk, and dephosphorylated with alkaline phosphatase). The resulting plasmid, pBAC/oriV, is shown in Figure 1. To obtain pBAC/oriV/SceI, the *oriV*-containing fragment was prepared as described above and cloned into the *Xho*I site of pBeloBAC11/SceI (gift from F.R. Blattner's laboratory, University of Wisconsin), as described above for pBAC/oriV (see legend to Fig. 1). The pTrueBlue-BAC2/oriV vector (pJW406; Table 1) was constructed in a similar manner (see legend to Fig. 1) by cloning the *oriV*-containing fragment into the *Xho*I-digested pTrueBlue-BAC2 plasmid (1999 Catalogue of Genomics One). To obtain the TruBlue-BAC2/oriV/SceI vector (pJW419), a 0.5-kb *Eco*RI–*Sal*I fragment containing the recognition sequence for I-*Sce*I was prepared from pSCM522 (Monteilhet et al. 1990), blunted with PolIk, gel-purified, and ligated to *Eco*N47III-digested and dephosphorylated pTrueBlue-BAC2/oriV (JW406), resulting in pTrueBlue-BAC2/oriV/SceI (JW419). To construct pIndigoBAC/oriV, a commercially available linearized plasmid, pIndigoBAC-5 (*Hin*dIII-Cloning Ready from Epicentre Technologies), was phosphorylated and religated to reconstruct circular pIndigoBAC-5. This vector

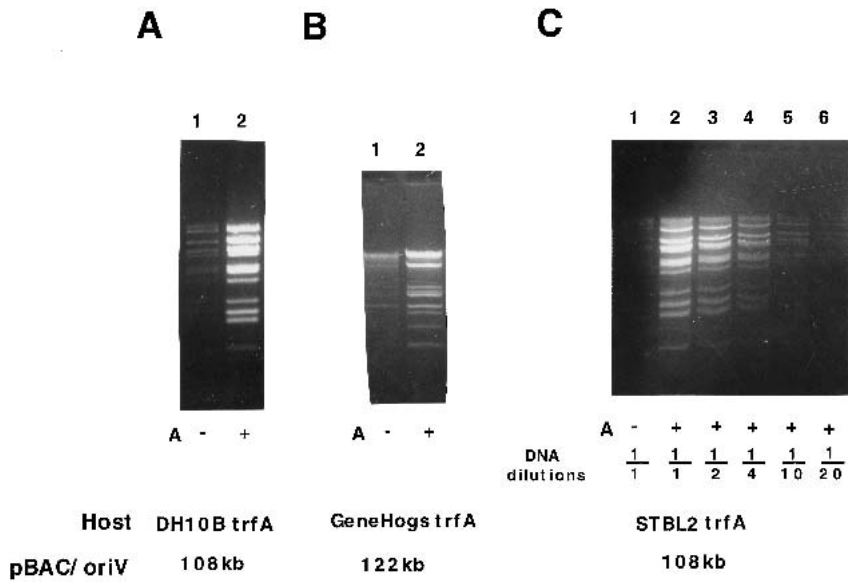

Figure 7 Amplification of the DNA of pBAC/oriV clones carrying (*A,C*) 108-kb or (*B*) 122-kb inserts of foreign DNA, when propagated in the DH10B host and in two commercial hosts, GeneHogs (Invitrogen) and Stbl2 (Life Technologies, presently Invitrogen). All three host strains contain the *araC*–P_{BAD}–*trfA254* cassette at their $attB_{\lambda}$ site. Growth conditions and induction of TrfA synthesis by L-arabinose (A) are described in Methods. DNA samples prepared by phenol extraction and ethanol precipitation were digested with *Sal*I (*A,C*) or with *Sma*I (*B*) and run on a 0.6% agarose gel. (*A*) Amplification of pBAC/oriV carrying a 108-kb insert (pCG275) in the JW427 host (*trfA254*; see JW439 in Table 2). (Lane *1*) LB medium (LB); (lane *2*) LB + 0.01% A. (*B*) Amplification of pBAC/oriV carrying a 122-kb insert (pCG274) in JW480 (GeneHogs *trfA254*). (Lane *1*) LB; (lane *2*) LB + 0.01% A. (*C*) Amplification of pBAC/oriV carrying a 108-kb insert (pCG275) in JW526 (Stbl2 *trfA254*). (Lane *1*) LB; (lanes *2–6*) LB + 0.01% A. (Lanes *3–6*) DNA preparations were diluted 1/2, 1/4, 1/10, and 1/20, respectively. Comparison of lanes *1* and *6* indicates an ~30-fold amplification of the plasmid with the 108-kb DNA insert.

was digested with *Sca*I + *Stu*I, and the smaller fragment (1.75 kb) was replaced with the *oriV*-containing *Sca*I–*Stu*I fragment (2.2 kb) from pJW360 (see Table 1).

Construction of Plasmids That Deliver TrfA

To secure very tight regulation of TrfA synthesis, we chose the regulatory system of the *ara* operon. A cassette containing the regulatory gene *araC*, the P_{BAD} promoter, and the Sp^R gene was obtained from pMPM123 (Mayer 1995) as a 4-kb *Kpn*I–*Sac*I fragment. Upon blunting with PolIk, the fragment was ligated to the integration vector pJW22 (Table 1), which was digested with *Eco*RI + *Hin*dIII, blunted with PolIk, and dephosphorylated with alkaline phosphatase. The resulting pJW344 plasmid was used for cloning of the *trfA* mutant genes. The *trfA203* mutation (Haugan et al. 1995) was retrieved as a 1.2-kb *Eco*RI–*Pst*I fragment from pRD110-34, blunted by PolIk, and ligated to pJW344, which was digested with *Xba*I, blunted, and dephosphorylated, creating pJW349. The 1-kb *Eco*RI–*Pst*I fragment containing a *trfA254* copy-up mutation (Durland et al. 1990) was blunted and cloned into the *Hin*cII site within the MCS of pUC19. The *Xba*I–*Hin*dIII fragment of this clone was ligated with pJW344 (digested with the same enzymes), resulting in pJW424. Plasmids pJW457, pJW458, and pJW459 were constructed by cloning *Eco*RI–*Pst*I fragments carrying *trfA250* (Durland et al. 1990), *trfA173*, and *trfA171* (Haugan et al. 1995), respectively, into pJW344 (digested with the same two enzymes). All four integration plasmids were used to create *trfA*-expressing hosts, as described in the next section.

Site-Specific Recombination into the *attB* Site in the Host Genome

The integration plasmids listed in Table 1 were inserted into the *attB* site in the *E. coli* genome by site-specific recombination, using the Int/*att* system of phage λ. The Int-producing pINT-ts plasmid (Hasan et al. 1994), in which the Cm^R marker replaced Ap^R, carries a gene encoding a heat-sensitive mutant of the pSC101 Rep protein; therefore, these ts plasmids are easy to eliminate at elevated temperatures. Preparation of competent cells carrying pINT-ts (ensuring delivery of Int) was as described by Hasan et al. (1994). The DNA fragment destined for integration was cloned into the pJW22 carrying the phage λ *attP* site, the Ap^R gene, MCS, and pBR322 *ori*, the latter flanked by two *Not*I sites. After digestion with *Not*I, the fragment containing cloned DNA, but missing the pBR322 *ori*, was gel-purified, recircularized using *Not*I–*Not*I ligation, and transformed at 30°C into competent DH10B cells already carrying pINT-ts (Hasan et al. 1994). Transformants were grown in LB + Ap for 2–3 h, then transferred to 37°C for overnight growth. Serial dilutions of these cultures were plated on LB + Ap plates and incubated at 42°C. Single colonies were tested for growth on LB + Ap and LB + Ap + Cm at 42°C. Colonies unable to grow on LB + Ap + Cm must have lost the pINT-ts Cm^R plasmid and were selected as *trfA* integrants.

Induction of TrfA Synthesis

Overnight cultures grown in LB supplemented with the appropriate antibiotics were used to inoculate fresh cultures that were grown in the same medium to A_{590} = 0.2–0.3; then L-arabinose (A) inducer was added to the final concentration of 0.01%. Parallel uninduced cultures were grown in LB or LB + 0.2% D-glucose (G); because G reduces the pBAC/oriV copy number to one. Cultures were grown for an additional 4–5 h before the cells were harvested and the DNA was extracted.

Additional methods are described in the legends of the individual figures.

ACKNOWLEDGMENTS

We thank F.R. Blattner (University of Wisconsin) for providing the pBeloBAC11/SceI plasmid; D. Helinski's (UCSD) and S. Valla's (University of Trondheim) laboratories for the plasmids carrying the *trfA* gene with copy-up mutations and plasmids with *oriV*; M.P. Mayer for the pMTM123 plasmid carrying *araC*–P_{ara}BAD; F. Schomburg from R.M. Amasino's lab (University of Wisconsin) for the T1024 and T20K24 clones of *Arabidopsis thaliana* DNA; C. Small and R. Bogden from Genomex for the pBAC/oriV clones carrying inserts of trout DNA; C. Gaskins from Invitrogen for pBeloBAC11 with inserts of wheat DNA; and O. Azzam for Messing's (Rutgers University) clone of rice DNA. We thank Barbara Hunter-Sandor for the expert editing.

Table 2. List of Strains

E. coli strains	Pertinent genotype
DH10B[a]	*F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80dlacZΔM15ΔlacX74 endA1 recA1 deoR Δ(ara,leu)7697 araD139 galU galK nupG rpsL*
JW366	DH10B, with pJW349 (*trfA*203) integrated at the *attB* site; see Figs. 2 and 3
JW371	JW366 [pBAC/oriV]
JW378	JW366 [pBAC/oriV+20-kb insert (pJW378)]
JW389	JW366 [pBAC/oriV+40-kb insert (p67)]
JW390	JW366 [pBAC/oriV+80-kb insert (p69)]
JW427	DH10B with pJW424 (*trfA*254) integrated at the *attB* site; see Table 1 and Fig. 2
JW439	JW427 [pBAC/oriV+108-kb insert (pCG275)]
JW450	JW427 [pBAC/oriV+122-kb insert (pCG274)]
JW461	DH10B with pJW458 (*trfA*173) integrated at the *attB* site; see Table 1 and Fig. 2
JW462	DH10B with pJW459 (*trfA*171) integrated at the *attB* site; see Table 1 and Fig. 2
JW463	DH10B with pJW457 (*trfA*250) integrated at the *attB* site; see Table 1 and Fig. 2
JW467	JW461 [pJW378]
JW468	JW462 [pJW378]
JW471	JW461 [pBAC/oriV+140-kb insert (pCG273)]
JW472	JW462 [pBAC/oriV+140-kb insert (pCG273)]
JW480[b]	GenHogs (Invitrogen) with pJW424 (*trfA*254) integrated at the *attB* site, see Table 1 and Fig. 2
JW499	JW461 [pBAC/oriV+108-kb insert (pCG275)]
JW500	JW462 [pBAC/oriV+108-kb insert (pCG275)]
JW501	JW463 [pBAC/oriV+108-kb insert (pCG275)]
JW526[c]	Stbl2 cells (Life Technologies/Invitrogen) with pJW424 (*trfA*254) integrated at the *attB* site; see Table 1 and Fig. 2
JW550	JW463[pIndigoBAC/oriV]

[a]The *E. coli* DH10B strain is described in Invitrogen Catalog 2002, p. 229.
[b]The GeneHogs strain is described in Invitrogen Catalog 2002, p. 230.
[c]The Stbl2 strain is the *gal⁺ lon⁻* parental strain of Stbl4 described in Invitrogen Catalog 2002, p. 234.
Brackets indicate plasmid, as carried in the host strain. Plasmid designations (see Table 1) or mutant numbers are in parentheses. Δ( ), deletion (followed by deleted genes, in parentheses, and number).

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REFERENCES

Asakawa, S., Abe, I., Kudoh, Y., Kishi, N., Wang, Y., Kubota, R., Kudoh, J., Kawasaki, K., Minoshima, S., and Shimizu, N. 1997. Human BAC library: Construction and rapid screening. *Gene* **191:** 69–79.

Boysen, C., Simon, M.I., and Hood, L. 1997. Analysis of the 1.1-Mb human α/δ T-cell receptor locus with bacterial artificial chromosome clones. *Genome Res.* **7:** 330–338.

Cai, L., Taylor, J.F., Wing, R.A., Gallagher, D.S., Woo, S.S., and Davis, S.K. 1995. Construction and characterization of a bovine bacterial artificial chromosome library. *Genomics* **29:** 413–425.

Diaz-Perez, S.V., Crouch, V.W., and Orbach, M.J. 1996. Construction and characterization of *Magnaporthe grisea* bacterial artificial chromosome library. *Fungal Genet. Biol.* **20:** 280–288.

Durland, R.H., Toukdarian, A., Fang, F., and Helinski, D.R. 1990. Mutations in the *trfA* replication gene of the broad-host-range plasmid RK2 result in elevated plasmid copy numbers. *J. Bacteriol.* **172:** 3859–3867.

Frengen, E., Weichenhan, D., Zhao, B., Osoegawa, K., van Geel, M., and de Jong, P.J. 1999. A modular, positive selection bacterial artificial chromosome vector with multiple cloning sites. *Genomics* **58:** 250–253.

Giacalone, J., Delobette, S., Gibaja, V., Ni, L., Skiadas, Y., Qi, R., Edington, J., Lai, Z., Gebauer, D., Zhao, H., et al. 2000. Optical mapping of BAC clones from the human Y chromosome *DAZ* locus. *Genome Res.* **10:** 1421–1429.

Guzman, L.-M., Belin, D., Carson, M.J., and Beckwith, J. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J. Bacteriol.* **177:** 4121–4130.

Hasan, N., Koob, M., and Szybalski, W. 1994. *Escherichia coli* genome targeting, I. Cre-*lox*-mediated in vitro generation of *ori⁻* plasmids and their in vivo chromosomal integration and retrieval. *Gene* **150:** 51–56.

Haugan, K., Karunakaran, P., Blatny, J.M., and Valla S. 1992. The phenotypes of temperature-sensitive mini-RK2 replicons carrying mutations in the replication control gene *trfA* are suppressed nonspecifically by intragenic *cop* mutations. *J. Bacteriol.* **174:** 7026–7032.

Haugan, K., Karunakaran, P., Tondervik, A., and Valla, S. 1995. The host range of RK2 minimal replicon copy-up mutants is limited by species-specific differences in the maximum tolerable copy number. *Plasmid* **33:** 27–39.

Hradecna, Z., Wild, J., and Szybalski, W. 1998. Conditionally amplifiable inserts in pBAC vectors. *Microbial Comp. Genom.* **3:** 58.

Kim, U.-J., Birren, B.W., Slepak, T., Mancino, V., Boysen, C., Kang, H.-L., Simon, M.I., and Shizuya, H. 1996. Construction and characterization of a human bacterial artificial chromosome library. *Genomics* **34:** 213–218.

Komori, H., Matsunaga, F., Higuchi, Y., Ishiai, M., Wada, C., and Miki, K. 1999. Crystal structure of a prokaryotic replication initiator protein bound to DNA at 2.6 Å resolution. *EMBO J.* **18:** 4597–4607.

Mayer, M.P. 1995. A new set of useful cloning and expression vectors derived from pBlueScript. *Gene* **163:** 41–46.

Monteilhet, G., Perrin, A., Thierry, A., Colleaux, L., and Dujon, B. 1990. Purification and characterization of the in vitro activity of I-*Sce*I, a novel and highly specific endonuclease encoded by a group I intron. *Nucleic Acids Res.* **18:** 1407–1413.

Mori, H., Kondo, A., Ohshima, A., Ogura, T., and Hiraga, S. 1986. Structure and function of the F plasmid genes essential for partitioning. *J. Mol. Biol.* **192:** 1–15.

Mozo, T., Fischer, S., Shizuya, H., and Altmann, T. 1998. Construction and characterization of the IGF *Arabidopsis* BAC library. *Mol. Gen. Genet.* **258:** 562–570.

Osoegawa, K., Woon., P.Y., Zhao, B. Frengen, E., Tateno, M., Catanase, J.J., and de Jong, P.J. 1998. An improved approach for construction of bacterial artificial chromosome libraries. *Genomics* **52:** 1–8.

Perri, S. and Helinski, D.R. 1993. DNA sequence requirements for interaction of the RK2 replication initiation protein with plasmid origin repeats. *J. Biol. Chem.* **268:** 3662–3669.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular cloning: A laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schibler, L., Vaiman, D., Oustry, A., Guinec, N., Dangy-Caye, A.L.,

Billault, A., and Cribiu, E.P. 1998. Construction and extensive characterization of a goat bacterial artificial chromosome library with threefold genome coverage. *Mamm. Genome* **9:** 119–124.
Shizuya, H., Birren, B., Kim, U.-J., Mancino, V., Slepak, T., Tachiiri, Y., and Simon, M. 1992. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. *Proc. Natl. Acad. Sci.* **89:** 8794–8797.
Slilaty, S.N. and Lebel, S. 1998. Accurate insertional inactivation of *lacZ*α: Construction of pTrueBlue and M13TrueBlue cloning vectors. *Gene* **213:** 83–91.
Stalker, D.M., Thomas, C.M., and Helinski, D.R. 1981. Nucleotide sequence of the region of the origin of replication of the broad host range plasmid RK2. *Mol. Gen. Genet.* **181:** 8–12.
Szybalski, W., Wild, J., and Hradecna, Z. 1999. Conditionally amplifiable BAC vector. US Patent No. 5,874,259 [revised].
Toukdarian, A.E., Helinski, D.R., and Perri, S. 1996. The plasmid RK2 initiation protein binds to the origin of replication as a monomer. *J. Biol. Chem.* **271:** 7072–7078.
Ullmann, A. 1992. Complementation in β-galactosidase: From protein structure to genetic engineering. *BioEssays* **14:** 201–205.
Ullmann, A., Jacob, F., and Monod, J. 1967. Characterization by in vitro complementation of a peptide corresponding to an operator-proximal segment of the β-galactosidase structural gene of *Escherichia coli*. *J. Mol. Biol.* **24:** 339–343.
Wild, J., Hradecna, Z., and Szybalski, W. 2001a. Easy conversion of single-copy BAC libraries into conditionally high-copy pBAC/oriV clones. *The 2001 Molecular Genetics of Bacteria & Phages Meeting*, July 31–August 5, 2001, p. 163. Madison, WI.
———. 2001b. Single-copy/high-copy (SC/HC) pBAC/oriV novel vectors for genomics and gene expression. *Plasmid* **45:** 142–143.
Woo, S.-S., Jiang, J., Gill, B.S., Paterson, A.H., and Wing, R.A. 1994. Construction and characterization of a bacterial artificial chromosome library of *Sorghum bicolor*. *Nucleic Acids Res.* **22:** 4922–4931.

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