High Copy Number Plasmids Compatible with Commonly Used Cloning Vectors

A number of genetic and biotechnological applications are facilitated by the ability to transform *Escherichia coli* with more than one recombinant plasmid. For example, a plasmid that carries a gene of interest may require that a *trans*-acting regulatory protein be expressed from a second plasmid in the cell to effectively regulate its expression (9,16,29). In addition, purification of multisubunit complexes, or characterization of the interaction between gene products, can also be facilitated by elevated expression of the interactive components. In *E. coli*, for example, the Ffh protein interacts with a 4.5S RNA species to form a ribonucleoprotein complex known as the signal recognition particle (SRP) (17,18). To better understand the interactions between Ffh and 4.5S RNA we sought to construct new cloning vectors that are compatible with many commonly used plasmids and that replicate at a high copy number. Although replication of CoIE1-like plasmids is dependent on DNA polymerase I and their replication is regulated by the interaction of two complementary RNA transcripts, distinct incompatibility groups have been identified (20,22). The CoIE1-like plasmid RSF1030 (10), for example, is able to co-reside with both pMB1 and p15A-derivative plasmids, as well as with non-CoIE1 vectors such as pSC101. The ori from RSF1030 was therefore selected for construction of a series of new cloning vectors.

Wild-type RSF1030 is maintained at a copy number of 15–20 per chromosome equivalent, a copy number that is comparable to pBR322 (6), pACYC177 and pACYC184 (4). To construct a cloning vector that replicates at a high copy number yet maintains its compatibility with commonly used cloning vectors, a high copy number mutant of RSF1030 was isolated. An RSF1030 derivative, pST19 (22), was converted to a high copy number mutant by selecting transformants able to form colonies in the presence of elevated levels of ampicillin and methicillin. An overnight culture of DH5α transformed with pST19 was plated onto LB agar plates (21) containing 400 µg/mL ampicillin and 400 µg/mL methicillin (31). Several colonies that arose under these selective conditions were screened using a rapid cell lysis procedure (19) to identify transformants that contained elevated quantities of plasmid DNA. A single mutant that yielded significantly more plasmid DNA than cells transformed with pST19 was selected for further analysis.

DNA sequence analysis of a 1.5 kb segment containing the *ori* from the high copy number mutant revealed a single base pair alteration located in a region that encodes the RNAII replication primer and the antisense RNAI transcript (Figure 1A). Although this mutation does not appear to have been previously reported in selections for

![Figure 1. The ori region of RSF1030. (A) The RSF1030 ori region, including the RNAI (antisense) and RNAII (replication primer) transcripts, is schematically shown. The location of the C to A transversion responsible for the elevated copy number is also indicated as it would appear on the sense strand. (B) The predicted secondary structure of the antisense RNAI molecule (20,26). The arrow indicates the result of the high copy number mutation. The stem loop structures I’, II’ and III’ are also labeled.](image-url)
high copy number mutants of CoIE1 plasmids (7), the location of this mismatch within the REF1030 ori suggests a possible mechanism for increased copy number. It is well established that the copy number of CoIE1-like plasmids is regulated by interaction between folded RNAI and RNAII molecules in a way that prevents RNAII from functioning effectively as a primer for replication (14,15,25). Initial interaction between RNAI and RNAII occurs by contact between the loop regions of the folded RNA molecules (the regions I’, II’ and III’ are shown for RNAI in Figure 1) (24). If this initial interaction is weakened then RNAII can more frequently assume a confirmation required for it to prime the initiation of four. It is reasonable to suggest that this alteration could also disrupt the II’-loop structure and, as a consequence, weaken the ability of RNAII to interact with the RNAII primer, leading to an increased frequency of initiation of plasmid replication. It is also worthy to note that, although significant homology exists between the RNAI molecule of several CoIE1-like plasmids, the high copy number mutation corresponds to a region of RNAII that is unique to RSF1030 (20).

The high copy number RSF1030 mutant was modified to introduce a variety of features to convert it to a series of versatile cloning vectors. Using standard techniques of recombinant DNA (19), a 1.5 kb BsrYI fragment containing the ori from the high copy pST19 mutant was ligated to a 2.3-kb BglII fragment from pWSK29 and pWSK30 (28), creating pDHA29 and pDHA30, respectively (Figure 2). These plasmids impart resistance to high levels of ampicillin (AmpR, 100 µg/mL). Derivatives of pDHA29 and pDHA30 were also constructed that impart resistance to different antibiotics. The chloramphenicol (CamR, 20 µg/mL) derivatives pDH29 and pDHC30 were constructed by replacing 0.7 kb DraI fragment containing the β-lactamase gene (bla) from pDHA29 and pDHA30 with a similar sized BsaAI-BstUII fragment encoding cat from pSU23 (1), respectively. pDH29 and pDHC30 are kanamycin resistant derivatives (KanR, 30 µg/mL) and were constructed by replacing bla on pDHA29 and pDHA30 with a 1.3 kb Stul fragment encoding the KanR determinant (apt) from pBSK8 (23), respectively. As shown in Figure 2, all vectors carry the multiple cloning site and lacZα from pBluescriptII to facilitate screening of recombinant plasmids by blue/white colony color on X-gal. The vectors also carry f1ori(+) for isolation of single-stranded DNA.

To characterize the cloning vectors, plasmid copy number determinations were made by a modification of the technique described by Wegrzyn et al. (30). DH5α (Life Technologies, Gaithersburg, MD, USA) was transformed with pDHA29, pBR322 or pBluescriptIIKS(+) as described (11) and then co-transformed with the pSC101-derivative plasmid pWSK129 (28). Cultures were grown at 30°C to saturation in the presence of antibiotics, and the cells were lysed by treatment with alkaline lysis solution (19). Plasmid DNA was resolved directly from the cell lysates by electrophoresis through a 0.8% agarose gel. Following staining with ethidium bromide, the gel image (Figure 3) was captured and analyzed by a Gel-Doc™ 2000 imaging system (Bio-Rad Laboratories, Hercules, CA, USA) using pWSK129 DNA as an internal standard. Copy number determinations were made by comparing the relative fluorescence of pDHA29 with that of pBR322 and pBluescriptIIKS(+) the copy number of pDHA29 was observed to be 150–200 copies/chromosome, a value somewhat elevated over pUC- and pBluescript-derivative plasmids (5). Similar copy numbers were also observed for the CamR and KanR derivative plasmids.

The new cloning vectors were shown

Figure 2. High copy number RSF1030-derivative cloning vectors. Features of the plasmids include a number of unique restriction enzyme recognition sites within the lacZα region; antibiotic resistance markers imparting resistance to Amp, Cam and Kan; f1ori(+) and the ori region of the RSF1030 high copy number mutant.

Figure 3. Copy number determination of high copy number cloning vectors. Plasmid DNA samples were resolved in duplicate and includes pBR322 (lanes 1 and 2); pUC18 (lanes 3 and 4); pDHA29 (lanes 5 and 6); pACYC184 (lanes 7 and 8); DNA mass ladder (Life Technologies) showing the ratio of the size (kb) and quantity of DNA (ng) in each band (lane 9). Arrow 1 indicates the location of pWSK129 and arrow 2 shows the location of the co-resident plasmids. Isolation of plasmid DNA and copy number determinations were performed as described in the text.
to be compatible with pBR322, pBlue-ScriptI, pACYC184 and pWSK129. Different pHΔ- vectors were transformed into E. coli DH5α that had previously been transformed with other cloning vectors encoding different antibiotic resistances. After culturing the transformants in the absence of antibiotic selection overnight (representing approximately 20 generations), cells were plated onto media to select for the RSF1030-derivative transformants. Colonies were subsequently replica plated onto media to screen for the antibiotic resistance encoded by the co-resident plasmid. A typical result indicated that 98% of the population retained both the high copy number plasmid and the second cloning vector. In contrast, plasmids with known incompatibilities showed a nearly complete loss of one of the plasmids. In a typical experiment, only 12% of a population retained two incompatible plasmids when cultured overnight without antibiotics. Two RSF1030-derivatives with different antibiotic resistance markers were also shown to be incompatible with each other (data not shown).

The newly constructed cloning vectors were used to express both components of the E. coli SRP in the same bacterial strain. To accomplish this, a derivative of pHΔC29 that carried ffs" was constructed. This plasmid, pHΔC29-ffs, was made by introducing a 0.6 kb 4.5S RNA from pSB832 (3) into the multiple cloning site of pHΔC29. pHΔC29-ffs was transformed into a derivative of E. coli MC4100 (21) that also carried pFfh18, a pUC18-based plasmid previously constructed in our laboratory that expresses Ffh. Control plasmids were also assembled by deleting the coding region for either ffs or ffh. pHΔC29-ffs was digested with MluI and religated to yield pHΔC29-Δffs. pFfh18Δfh was constructed by removing an EcoRV fragment to delete a significant portion of the ffh gene. Two additional plasmids were constructed by inserting ffs+, and the EcoRV deletion derivative into pBR322, yielding pFfh322 and pFfh322Δfh, respectively.

The levels of 4.5S RNA expressed from pHΔC29-ffs were determined by Northern hybridization. Cultures of MC4100 transformed with pFfh18 along with either pHΔC29-ffs or pHΔC29-Δffs were grown overnight in the presence of both Amp and Cam. Total cellular RNA was isolated from the cultures (Tri-Pure® Isolation reagent, Roche Molecular Biochemicals, Indianapolis, IN, USA) and resolved by electrophoresis on a 1.3% agarose gel under denaturing conditions (19). RNA was electrophoretically transferred to a nylon membrane (Hybond®; Amersham Pharmacia Biotech, Piscataway, NJ, USA) and probed with a 0.6 kb HindIII-BamHI ffs fragment from pSB832 that had been radioactively labeled with [32P]-dATP (HexaLabel® DNA labeling kit; MBI-Fermentas, Amherst, NY, USA). Blots were hybridized at 68°C and washed twice at room temperature before exposure by phosphorimager analysis (GS-363 Molecular Imager System™; Bio-Rad Laboratories). As shown in Figure 4 (lanes 3 and 4), the strain transformed with the pHΔC29-ffs+ plasmid yielded significantly more 4.5S RNA than the control strain where the RNA was expressed only from a single chromosomal copy of ffs (lanes 1 and 2). As noted in Figure 4, the exposure times of the filters had to be adjusted to ensure adequate resolution of both samples.

Western immunoblot analysis was then used to determine the amount of Ffh expressed from various MC4100 transformants. Cultures of different transformants were grown overnight, as described above. Samples were prepared and total cellular proteins were resolved by SDS-PAGE on a 10% gel, using a standard protocol (8). Proteins were electrophoretically transferred to nitrocellulose, and the filters were reacted with polyclonal antibodies against Ffh as described (8). The decorated proteins were visualized by addition of horseradish peroxidase-conjugated secondary antibodies and substrate (Opti-4CN™; Bio-Rad Laboratories). Although Figure 5 shows that Ffh could be detected in all samples, we observed

![Figure 4](image-url) Figure 4. Northern blot analysis of 4.5S RNA expression. RNA samples were prepared in duplicate from E. coli co-transformed with pFfh18 and pHΔC29-Δffs (lanes 1 and 2); or pFfh18Δffh and pHΔC29-ffs" (lanes 3 and 4). The location of 4.5S RNA is indicated along with the time of exposure of the blots.

![Figure 5](image-url) Figure 5. Western blot analysis of Ffh expression. Ffh protein was detected from cells transformed with different combinations of plasmids, as indicated above the blot. Lane 1, molecular weight markers with the corresponding values given in kd. Lanes 2–5 represent expression of Ffh from pUC18 derivative plasmids while lanes 6–9 are from pBR322 derivatives, described in the text. The location of the Ffh protein is also shown.
that significant overproduction of the protein occurred only when 4.5S RNA was also expressed at elevated levels in the cell (compare Figure 5, lanes 2 and 3). Strikingly, the levels of Ffh expressed from pFfh18 alone were not significantly higher than that seen when Ffh was expressed from the chromosome (Figure 5, lanes 3 and 5). Similar results were also seen when Ffh was expressed from a pBR322-derivative plasmid (Figure 5, lanes 6–9). This result is consistent with a previous report that the stability of Ffh is dependent on the SRP and so significant overproduction of 4.5S RNA (12). Apparently, Ffh is unstable when not assembled into the SRP and so significant overproduction of the protein actually requires increased expression of 4.5S RNA.

In conclusion, the pDH-vectors have been constructed that replicate at high copy numbers and are compatible with commonly used cloning vectors. Derivatives of these vectors have been used to produce both components of the E. coli SRP and likewise should be useful to increase expression of a number of gene products in combination with products expressed from a number of other cloning vectors.

REFERENCES

Tapan Som for plasmids and P.C. Tai for antibodies. This is journal paper No. J-18212 of the cellular pathways involved in as valuable models for the exploration in culture. Since then, they have served derivitively easy to grow, quantifying the genesis of human tumors (1). Adenoviruses have been studied for considerable inaccuracies. One of the most widely used assays for quantifying viral titers is the cytopathic effect (CPE) assay (6). CPE assays rely on the ability of viruses to cause cells to detach from a monolayer or undergo lysis. Generally considered the most convenient assay and accurate enough for most needs, this assay can be performed on a wide variety of human cell lines.

Here, we describe a variation of the CPE assay using an inexpensive protein stain and spectrophotometric analysis, which gives an accurate and reproducible quantitation of viral titer that is less subject to investigator bias. Virus is harvested from infected 293 or other cells by centrifuging both floating and trypsinized cells at 500 g for 5 min and then resuspending the pellet in 20 mM Tris, pH 7.4. Infectious virus is released by rapidly freezing (in dry ice) and thawing the cell suspension 3x, removing cellular debris by centrifugation at 500 g for 10 min and placing aliquots of the supernatant at -70°C until needed.

We describe here the titration of wild-type adenovirus type 5 (Ad5) and the Ad5 replication defective mutant dl 312, which contains a deletion of much of the E1a coding region (5). However, this technique is equally applicable to any lytic virus or viruses capable of causing detachment from the monolayer. We seeded 3 x 10^4 293 cells into each well of a 96-well plate and left them to attach in 100 µL of DMEM + 0.5% FBS at 37°C, 10% CO2. Serial dilutions of the virus inoculum are made in a further 100 µL of DMEM + 0.5% FBS and added to the culture when cells have attached. Infected cells are incubated for 3 days at 37°C, 10% CO2 (although this time should be determined empirically, depending on the cell type and virus used), and the media and dead or floating cells are then removed by aspiration.

Cell loss (CPE) is determined by measuring changes in total protein concentrations in each well. Protein concentration is obtained by adding 200 µL of BCA™ protein assay reagent (Pierce Chemical, Rockford, IL, USA) to each well, followed by gentle shaking. This reagent contains 0.1 M NaOH and components of the Biuret reaction allowing solubilization of the cell membrane, release of cellular proteins and formation of a purple color proportional to the protein concentration. The plate is agitated for 1 min, the color is allowed to develop by incubating the plate at 37°C and absorbance ascertained by spectrophotometry at 570 nm on an Anthos microtiter plate reader. The average absorbance of several blank wells (containing no cells) is sub-