5054–5055 *Nucleic Acids Research, 1996, Vol. 24, No. 24*

A method for high efficiency YAC lipofection into murine embryonic stem cells

Jeannie T. Lee[1,2,*] and Rudolf Jaenisch[1]

[1]Whitehead Institute for Biomedical Research, Biology Department, Massachusetts Institute of Technology, Cambridge, MA 02142, USA and [2]Pathology Department, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA

Received September 11, 1996; Revised and Accepted November 5, 1996

ABSTRACT

We describe a modified protocol for introducing yeast artificial chromosomes (YACs) into murine embryonic stem (ES) cells by lipofection. With a decreased DNA:cell ratio, increased concentration of condensing agents and altered culture conditions, this protocol reduces the requirement for YAC DNA to a few micrograms, improves the recovery of neomycin-resistant ES colonies and increases the yield of clones containing both flanking vector markers and insert. These modifications enable generation of sufficient 'intact' transgenic clones for biological analysis with a single experiment.

Large DNA fragments carried on yeast artificial chromosomes (YACs) have been introduced into the mammalian genome (1,2). Because YACs enable cloning of hundreds of kilobases of DNA (3), YAC transgenes offer several advantages over traditional 'minigene' constructs. First, inclusion of remote *cis*-acting regulatory elements can preserve temporal, spatial and dosage information encoded at the endogenous locus (4–6). Second, YACs allow for the study of large genomic structures or regions such as alpha-centroid DNA and the mammalian X-chromosome inactivation center (7,8). Third, YAC templates are easy to genetically manipulate in yeast, thus enabling rapid generation of point mutation and deletions. Finally, YAC transgenic cell lines or mice can be used as bioreactors in the production of biotherapeutics such as immunoglobulins (9,10). For all these reasons, YAC transgenics have become valuable tools in creating mouse models for disease and vertebrate development.

To date, transfers have been achieved by lipofection into ES cells, by microinjection into mouse oocytes and by spheroplast fusion with ES cells. While spheroplast fusions have reliably yielded large numbers of transfectants with intact YAC transgenes, the incorporation of endogenous yeast genomic DNA potentially complicates intrepretation of results. Because lipofection and microinjection use purified YAC DNA, these methodologies may be preferrable for many investigators; however, the requirement for large quantities of high molecular weight DNA, variability in transfer efficiency and high frequency of transgene deletion have been major obstacles to using these techniques in many laboratories.

Here, we describe modifications of the protocol by Strauss *et al.* (6) which greatly simplify YAC transfer while enhancing lipofectant recovery. This previous protocol as well as other published protocols typically used 10–100 μg of purified YAC DNA and required screening of many lipofectants to obtain clones containing 'intact' transgenes as judged by presence of flanking vector markers and insert DNA (6,10). In our modified protocol, the requirement for YAC DNA was reduced 10–100-fold, the recovery of neomycin-resistant colonies was increased, and the yield of clones containing both flanking vector markers and insert DNA was greater.

The basic protocol is as follows.

(i) YAC DNA preparation: high quality, high molecular weight DNA was prepared by isolation from pulsed field gels. Yeast cultures were grown to stationary phase, harvested, washed once with 1 M sorbitol, and resuspended in 1 M Sorbitol, 0.2 M Na_2HPO_4/NaH_2PO_4 pH 7.4, 1 mM EDTA, 58 mM 2-mercapto-ethanol at 3×10^9 cells/ml. Cells were spheroplasted in 0.75 mg/ml zymolyase 100T (ICN) at 37°C for 30 min. Spheroplasts were mixed with SeaPlaque low-gelling temperature agarose (FMC) to a final concentration of 1% agarose and 1×10^9 cells/ml and cast in BioRad DNA plug molds. DNA plugs were then digested overnight with 0.5 mg/ml proteinase K in 0.5 M EDTA, 10 mM Tris pH 7.5 and 1% *N*-lauroylsarcosine, and dialyzed twice in 10 mM Tris pH 7.5, 20 mM EDTA, and stored in 10 mM Tris pH 7.5, 1 mM EDTA at 4°C. Genomic DNA embedded in agarose was subjected to pulsed field electrophoresis (BioRad CHEF DRII system) to separate chromosomes in the 200–1000 kb range. With ethidium bromide-stained markers as a guide, the unstained gel corresponding to the desired YAC DNA was excised and dialyzed twice in 10 mM Tris pH 7.5, 1 mM EDTA, 200 μM spermine (Sigma) and 25 mM NaCl at room temperature for 0.5 h. Using this procedure, 1–2 μg of YAC DNA have been routinely recovered from 1 ml of genomic DNA (less DNA if YAC is large and/or unstable).

(ii) Embryonic stem (ES) cell lipofection: in preparation for lipofection, 1 ml agarose blocks containing 300 ng of purified YAC DNA were combined with 4 μg/ml low-molecular weight poly-L-lysine (Sigma) and then melted at 68°C for 10 min and digested with 1 U β-agarase (New England Biolabs) per 100 μl block according to the manufacturer's specification. In the author's experience, incomplete agarose digestion significantly reduced lipofection efficiency. Thus, agarasing should be allowed to proceed at least 4 h. From this point on, all manipulation of DNA was done with a wide-bore pipette tip to reduce mechanical shear.

*To whom correspondence should be addressed at: Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02138, USA. Tel: +1 617 258 5207; Fax: +1 617 258 6505; Email: jlee@wi.mit.edu

Table 1. Comparison of results achieved by various protocols for introducing YACs into ES cells

YAC, size (reference)	Input DNA (μg)	Lipofection efficiency: YAC-containing NeoR colonies/μg	No. clones with L & R YAC arms ('intact')	Combined yield: no. 'intact' clones/100 μg
modified protocol:				
Y116, 450 kb (8)	1.5	7	5 out of 14 (35%)	330
750 kb YAC[a] (J.T. Lee, unpublished)	2	4.5	6 out of 9 (66%)	200–300
300 kb YAC (A.Chess, unpublished)	1.5	8	2 out of 12 (16%)	100–150
Y22, 150 kb (6)	40–100	0.5–1.0	7 out of 35 (20%)	7–14
APP-8, 650 kb (4)	0.1	20–30	3 out of 23 (13%)	200–300
J1.3P, 85 kb (10)	8	15[b]	3 out of 15 (20%)	40

[a]Because of smearing of YAC DNA in the 500–900 kb range in the preparative pulsed field gel, this YAC was concentrated 2–3-fold by the Amicon Centriprep-100 system at 500 *g* prior to lipofection.
[b]This was a co-lipofection of 8 μg of YAC DNA and 4–8-fold molar excess of a neomycin resistance plasmid. 1221 neomycin-resistant colonies were screened and 15 were found to contain J1.3P DNA.

Cationic lipid DOTAP (BoehringerMannheim) (30 μg) was added, mixed with a pipette, and incubated at room temperature for 45 min, after which 1/10 vol of 10× OptiMEM (Life Technologies) was added. The DNA complex was then mixed gently with 2 ml of cell suspension containing 10^7 dispersed ES cells prepared as follows. ES cells were grown in standard ES media (DME, 15% fetal bovine serum, 0.1 mM amino acids and 0.1 mM β-mercaptoethanol, 500 U/ml LIF) to 70% confluence, dispersed by trypsinization, and resuspended in 1× OptiMEM supplemented with 1000 U/ml LIF. The DNA–cellular mixture was incubated at 37°C for 5 h without disturbance in a tissue culture incubator. DNA-treated ES cells were then combined with 10 ml of standard ES medium and plated on day 14 embryonic feeder cells. Drug selection was begun at 24 h and the media changed daily thereafter. Drug-resistant lipofectants became visible as colonies between days 8 and 18 after selection.

We believe that significant modifications in our protocol include the use of high concentration LIF during ES cell lipofection (maintaining cells in the undifferentiated state), the use of serum-less OptiMEM, lower concentration of input YAC DNA, and proportionally more condensing agent (spermine, poly-L-lysine). ES cells lipofected with no LIF or lesser amounts did not look as healthy as those with high LIF concentration (ES cells began to differentiate). The use of OptiMEM instead of DMEM in the DNA mixture also appeared to improve ES cell morphology following lipofection. Notably, the use of more condensing reagents resulted in formation of visible precipitates in the DNA–cell mixture, although this did not seem to negatively affect lipofection.

This protocol has been used successfully to introduce YAC 116, a 450 kb clone containing the murine X-inactivation center (8; Table 1). In a typical experiment using 1.5 μg of purified YAC DNA, 14 neomycin-resistant colonies were obtained from lipofecting 5×10^7 cells. Pulsed field and conventional Southern analyses indicated that five clones contained both vector arms, NEO (retrofitted into the URA3 arm) and TRP1, as well as intact copies of the *Xist* gene. As published elsewhere (8), these clones also contain all necessary elements required for appropriate timing and level of *Xist* expression. An additional two clones contained truncations that retained the neomycin arm and *Xist* insert DNA but deleted the TRP1 vector arm. The remaining seven clones sustained deletions of a significant amount of insert DNA.

We have also successfully introduced a second X-linked YAC of 750 kb into ES cells. While larger YAC size was expected to negatively influence the success rate, our work with the 750 kb clone yielded surprisingly comparable results (Table 1). Not only was the lipofection efficiency high, but 66% of clones analyzed contained both TRP1 and NEO as well as insert DNA. This protocol is being used presently by other laboratories. In one independent laboratory, a 300 kb YAC containing olfactory receptor loci was introduced into ES cells with a similar success rate, with a combined yield of 100–150 $NEO^+TRP1^+insert^+$ clones per 100 μg input DNA (Andrew Chess, Whitehead Institute, personal communication). Transgenic ES lines generated with this protocol have contributed extensively to chimeras, including to the germline (8; J. T. Lee, unpublished work; A. Chess, personal communication).

Table 1 summarizes the results achieved with our protocol compared with those of others. Since the rate-limiting step for successful lipofection is purification of high molecular weight DNA, the overall efficiency is normalized to amount of input DNA used. Note that our modified protocol yielded a lipofection efficiency 20-fold over that of Strauss *et al.* (6). Furthermore, a workable number of 'intact' clones can be generated in a single experiment (protocols requiring 10–100 μg input YAC DNA require many days of lipofection). Since 'intact clones' have represented 35–66% of clones generated, our protocol potentially expedites isolation of transgenic lines for biological analysis. In our experience, transgene copy numbers from 1 to >20. Analysis to date indicates that lipofection yields single integration sites at random loci in the genome. These sites have included pericentromeric, subtelomeric and central regions of at least six distinct chromosomes (J. Lee, unpublished observations).

ACKNOWLEDGEMENTS

We thank Andrew Chess for sharing and discussing results prior to publication. This work was supported by the Howard Hughes Postdoctoral Fellowship for Physicians granted to J.T.L., by the Pathology Department at the Massachusetts General Hospital where J.T.L. is a fellow, and by a grant from the National Institutes of Health/National Cancer Institute (R35-CA44339) to R.J.

REFERENCES

1 Capecchi,M.R. (1993) *Nature*, **362**, 205–206.
2 Lamb,B.T. and Gearhart,J.D. (1995) *Curr. Opin. Genet. Dev.*, **5**, 342–348.
3 Burke,D. *et al.* (1987) *Science*, **236**, 806–812.
4 Lamb,B.T. *et al.* (1993) *Nature Genet.*, **5**, 22–30.
5 Schedl,A. *et al.* (1993) *Nature*, **362**, 258–261.
6 Strauss,W.M. *et al.* (1993) *Science*, **259**, 1904–1907
7 Larin,Z. *et al.* (1994) *Hum. Mol. Genet.*, **3**, 689–695.
8 Lee,J.T. *et al.* (1996) *Cell*, **86**, 73–84.
9 Green,L.L. *et al.* (1994) *Nature Genet.*, **7**, 13–21.
10 Choi,T.K. *et al.* (1993) *Nature Genet.*, **4**, 117–123.