High frequency vector-mediated transformation and gene replacement in *Tetrahymena*

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Received August 1, 1994; Revised and Accepted October 13, 1994

ABSTRACT

Recently, we developed a mass DNA-mediated transformation technique for the ciliated protozoan *Tetrahymena thermophila* that introduces transforming DNA by electroporation into conjugating cells (1). Other studies demonstrated that a neomycin resistance gene flanked by *Tetrahymena* H4-I gene regulatory sequences transformed *Tetrahymena* by homologous recombination within the H4-I locus when microinjected into the macronucleus (2). We describe the use of conjugant electrotransformation (CET) for gene replacement and for the development of new independently replicating vectors and a gene cassette that can be used as a selectable marker in gene knockout experiments. Using CET, the neomycin resistance gene flanked by H4-I sequences transformed *Tetrahymena*, resulting in the replacement of the H4-I gene or integrative recombination of the H4-I/neo/H4-I gene (but not vector sequences) in the 5' or 3' flanking region of the H4-I locus. Gene replacement was obtained with non-digested plasmid DNA but releasing the insert increased the frequency of replacement events about 6-fold. The efficiency of transformation by the H4-I/neo/H4-I selectable marker was unchanged when a single copy of the *Tetrahymena* rDNA replication origin was included on the transforming plasmid. However, the efficiency of transformation using CET increased greatly when a tandem repeat of the replication origin fragment was used. This high frequency of transformation enabled mapping of the region required for H4-I promoter function to within 333 bp upstream of the initiator ATG. Similarly ~300 bp of sequence downstream of the translation terminator TGA of the β -tubulin 2 (BTU2) gene could substitute for the 3' region of the H4-I gene. This hybrid H4-I/neo/BTU2 gene did not transform *Tetrahymena* when subcloned on a plasmid lacking an origin of replication, but did transform at high frequency on a two origin plasmid. Thus, the H4-I/neo/BTU2 cassette is a selectable marker that can be used for gene knockout in *Tetrahymena*. As a first step toward constructing a vector suitable for cloning genes by complementation of mutations in *Tetrahymena*, we also

demonstrated that the vector containing 2 origins and the H4-I/neo/BTU2 cassette can co-express a gene encoding a cycloheximide resistant ribosomal protein.

INTRODUCTION

Tetrahymena thermophila is an useful eukaryotic model to study such diverse cellular activities as gene amplification and genome rearrangement, cell motility, surface antigen switching, stimulated exocytosis and intracellular patterning. Initial discoveries made in *Tetrahymena*, such as the identification of telomere structure (3), telomerase RNA (4), self-splicing RNA (5) and the first non-muscle molecular motor, dynein [see (6)], opened new avenues in molecular and cellular biology. In recent years, experimental analyses in *Tetrahymena* have been augmented by the development of methods for genetic transformation. Tondravi and Yao (7) first transformed *Tetrahymena* using a paromomycin-resistant version of the ribosomal RNA gene (rDNA) and subsequently this technique has been used to analyze the sequence elements involved in rDNA palindrome formation, chromosome fragmentation and DNA rearrangement (8–10). An rDNA-based circular vector has been constructed (11) and applied to mutagenic studies on the function of the RNA template of telomerase (12,13). Interestingly, *Tetrahymena* belongs to a small group of organisms in which insertion of DNA into the genome is exclusively by homologous recombination (2,14–16), allowing for highly predictable targeting of transforming DNA. Yao and Yao (15) performed the first replacement of a protein-coding gene in *Tetrahymena* using a cycloheximide-resistant ribosomal protein L29 gene to replace its endogenous counterpart. Kahn *et al.* (2) inserted a heterologous gene conferring neomycin resistance into the *Tetrahymena* H4-I gene to perform the first genomic knockout in *Tetrahymena*. Thus, gene replacement potentially offers a powerful approach to address directly the necessity and function of a cloned gene in *Tetrahymena*.

Initial transformation studies in *Tetrahymena* were performed by direct macronuclear microinjection (7,11,17). Recently, we described a method for mass transformation of *Tetrahymena* which generates transformants in large numbers by electroporation of conjugating cells (1). Here we describe the use of the conjugant electrotransformation (CET) method to develop new vectors for gene knockout/replacement and for high

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efficiency transformation mediated by high copy number vectors containing selectable non-rDNA genes.

MATERIALS AND METHODS

Strains and cell culture conditions

Strains CU428.1 *Mpr/Mpr* (mp-s, mt VII) and B2086 *Mpr⁺/Mpr⁺* (mp-s, mt II) were kindly provided by Dr P.J.Bruns (Cornell University, Ithaca). The CU428.1 strain is a heterokaryon which carries the unexpressed dominant allele for resistance to 6-methylpurine in the micronucleus but is sensitive to this drug owing to the presence of recessive alleles in the macronucleus. Both strains also contain the wildtype, paromomycin sensitive rDNA gene as well as the wildtype cycloheximide-sensitive ribosomal protein L29 (*rpL29*) gene in both macro- and micronuclei. Cells were grown to late logarithmic phase in SPP medium (18) at 30°C with agitation.

Conjugant electrotransformation (CET)

To induce conjugation (19), 50 ml cultures of two strains of different mating types were spun at 1100×g for 3 min, resuspended in the same volume of 10 mM Tris-HCl buffer, pH 7.5, spun down and resuspended again as above. The cells were starved for 8–12 h at 30°C without shaking and counted. 50 ml of each mating strain at a concentration of 3×10⁵ cells/ml were mixed in a 2 liter Erlenmeyer flask. 1 ml of a 100× antibiotic/antimycotic mixture (BRL/GIBCO) was added (at final concentration of 100 units/ml penicillin G, 0.1 mg/ml streptomycin, and 0.025 mg/ml amphotericin B). The mixed cells were vigorously agitated on a lab shaker at 160 r.p.m. for 10–12 h to prevent pair formation (19). During the last 2 h of shaking, the shaker was automatically turned off and on repeatedly in 30 min intervals. Mating started after the shaker was finally turned off (18–24 h of starvation). Ten to 11 h after mating was initiated, cells were spun down at 1100×g for 5 min, washed with 50 ml of 10 mM Hepes buffer pH 7.5 (made at final concentration and autoclaved), spun down again for 3 min and resuspended in a total of 1 ml of the Hepes buffer at 3×10⁷ cell/ml. The transforming DNAs were resuspended in 125 µl of Hepes buffer prior to electroporation. In some gene replacement experiments the insert was released from the plasmid vector by restriction enzyme digestion and digested DNA purified by organic extractions and ethanol precipitation. Eighteen to 20 µg of replicative vector or 50 µg of circular or linear integrative (gene replacement) vector DNA were used. To transform, 125 µl of cells were mixed with the same volume of vector DNA, transferred into a 0.2 cm wide gap chamber and subjected to electroporation immediately using the BTX ECM 600 electroporator (BTX Inc., San Diego, CA). Unless stated otherwise, the following settings were used: voltage at 250V, capacitance at 275 µF and resistance at 13 ohms. One min after delivery of the electric pulse, the cells were resuspended in 20 ml of SPP + 1× antibiotic mix (SPPA) in a Petri plate. The original dilution (20 ml) is defined as 8× (about 8 microtiter plates are needed to plate the 20 ml of cells). Because transformation frequency was determined using the Poisson distribution (19), serial dilutions of treated cells were made to ensure that an appropriate number of positive wells is available on at least one transformant plate. Therefore, the cells were diluted to make 16×–1280× plating dilutions in SPPA. We also made an 8000× dilution plate in each experiment to monitor cell survival. One or two microtiter plates were prepared for each

dilution by plating 25 µl of cells into each well which already contained 175 µl of SPPA. In most experiments involving replicative vectors, 80–320× plating dilutions gave the optimal number of positive wells per plate to use the Poisson distribution. For gene replacement experiments, all cells from the electroporated sample were distributed at 8× dilution. Plates were subject to appropriate drug selection based on the resistance conferred by a particular transforming vector. After 12–18 h (if not otherwise indicated) of incubation at 30°C, the transformant plates were subject to selection either with 120 µg/ml of Paromomycin (either Parke-Davis or Sigma) or 15 µg/ml cycloheximide (Sigma) by adding 50 µl of 5× drug solution in SPPA to each well. The 'cell survival' plate (8000×) was selected with 15 µg/ml 6-methylpurine added as a 5× stock in SPPA. We define cell survival as the number of clones that survived electroporation and are conjugation progeny, calculated as a number of 6-methylpurine resistant clones. The CU428 strain is a heterokaryon containing the dominant resistance *MPR* gene only in its micronucleus. Therefore only conjugation progeny and not parental cells are resistant to 6-methylpurine. Plates were scored 3–5 days after addition of drugs.

Transformation vectors

The transformation vectors are shown in Figure 1. pD5H8 is an rDNA processing vector (15,17) that transforms *Tetrahymena* to paromomycin resistance at high frequency using CET (1). It contains a paromomycin resistant micronuclear ribosomal RNA gene that is excised and amplified during macronuclear development (17). pH41A is a pUC18 derivative containing a *neo* resistance gene subcloned between the flanking sequences of the histone H4-I gene (2). The H4-I/*neo*/H4-I gene confers resistance to paromomycin in *Tetrahymena*. pH4*neo* contains the H4-I/*neo*/H4-I marker (2) and a 1.9 kb fragment containing the replication origin of the *Tetrahymena* rDNA (11). pAU3 is a pUC119 derivative with a cycloheximide-resistant ribosomal protein *rpL29* gene (Pan and Blackburn, personal communication) and a tandem repeat of two replication origin fragments from the rDNA. Plasmid AUH4-12 was constructed by subcloning the *EcoRI* digested H4-I/*neo*/H4-I gene fragment from pH4*neo* into p#5, a pUC119 plasmid with a repeat of the rDNA replication origin fragments (Pan and Blackburn, personal communication). pΔ5-2 was constructed by subcloning the *EcoRV*–*EcoRI* restriction fragment of the H4-I/*neo*/H4-I gene from pH41A into the *SmaI* and *EcoRI* sites of p#5. pΔ3-2 is a pΔ5-2 derivative with a deletion in the 3' flanking sequence in the H4-I/*neo*/H4-I gene. It was constructed by digestion of pΔ5-2 with the *AflIII* restriction endonuclease, making blunt ends with T4 DNA polymerase, purifying the major plasmid fragment and re-circularizing. pΔ5-7 is a pΔ5-2 derivative with part of the 5' flanking sequence of the H4-I/*neo*/H4-I gene deleted. It was made by digestion of pΔ5-2 with the *BspHI* restriction endonuclease, and making blunt ends with T4 DNA polymerase. The digestion mixture was purified with the Magic Cleanup System (Promega) and digested again with *EcoRI* restriction endonuclease. The 2 kb *BspHI*–*EcoRI* fragment containing the H4-I/*neo*/H4-I gene with a 5' deletion was gel-purified and ligated between the *SmaI* and *EcoRI* sites in p#5.

The following steps describe construction of the hybrid H4-I/*neo*/BTU2 gene. Plasmid BB-2 contains the 3' *BamHI*–*HindIII* fragment of the BTU2 gene (20). The *DdeI* fragment containing the neomycin resistance gene and part of the H4-I gene promoter was isolated from pH41A, blunted with

T4 DNA polymerase and ligated into the *Bam*H I digested and T4 DNA polymerase blunted BB-2. The *Dde*I site is located at the TGA stop codon in the neomycin resistance gene (2) and is restored by a fill in reaction with T4 DNA polymerase. The *Bam*HI site in the BTU2 3' flanking fragment is located immediately after the TGA stop codon (20). Thus, some of the plasmid clones contained the neomycin coding sequence terminated by the TGA stop codon fused to the 3' flanking sequence from the BTU2 gene. One such clone was identified by restriction mapping. The *Hind*III fragment from this clone containing the entire 3' flanking sequence of the BTU2 and the neomycin gene coding sequence was ligated to the *Eco*RV–*Hind*III fragment of the H4-I gene promoter subcloned in KS(+) Bluescript (Stratagene). Thus, this construct (pHAB2) contains 333 bp of the H4-I promoter, the neomycin coding sequence and the entire cloned BTU2 3' flanking sequence. To reduce the size of the 3' flanking sequence in the pHAB2 construct, we took advantage of the *Nsi*I site located ~ 300 bp downstream from the TGA, beyond the polyadenylation site identified in the BTU2 gene (20). We isolated the *Nsi*I–*Eco*RV fragment of pHAB2 and cloned it into the *Pst*I and *Eco*RV sites in Bluescript KS(+) to construct p4T21 (see Fig. 1).

pH4T2 was constructed by subcloning the *Sac*I–*Eco*RV fragment of p4T21 containing the H4-I/neo/BTU2 gene into p # 5 in the *Sma*I and *Sac*I sites. pBSAUL29 contains the *rpL29* gene from pAU3 subcloned into the *Hind*III site in Bluescript KS(+). p42L29B was constructed by insertion of the *rpL29* gene from BSAUL29 into the pH4T2 vector. BSAUL29 was digested with *Sal*I and *Hha*I (*Hha*I is included to destroy the plasmid backbone sequence and facilitate gel purification of the 3.2 kb fragment of *rpL29*) and filled in with T4 DNA polymerase followed by digestion with *Sac*I. The gel purified fragment containing the *rpL29* gene was then ligated into the *Sac*I and *Sma*I sites of pH4T2 to give p42L29B. Plasmid DNA suitable for CET was prepared as described (1).

RESULTS

Optimization of electroporation conditions

We reported previously that using CET, the rDNA processing vector pD5H8 (15) transforms *Tetrahymena* conjugants at high frequency (1). We used this vector to improve conditions for electroporation and as a standard of comparison to test if the CET method could be applied to several new vectors containing non-rDNA selectable markers that transformed either by gene replacement or were capable of replication.

The effect of field strength on transformation yield was examined for two different pulse lengths: 4 ms and 1.6 ms (Fig. 2A). The maximal transformation yield for a 4 ms pulse was obtained at a field strength of 1125 V/cm. For a 1.6 ms pulse the maximal yield was at 1380V/cm. Thus, as in other cell types, shorter pulse length requires higher field strength for a maximal yield of transformants in *Tetrahymena*.

To test if lower temperature stimulates transformation in *Tetrahymena* (21), we electroporated cells that either were maintained at room temperature or were briefly exposed to cold before, after, or both before and after electroporation (Fig. 2B). The survival rate increased slightly but the transformation rate decreased dramatically if the cells were incubated 2 min on ice before electroporation. In contrast, cells incubated for 2 min on ice after electroporation exhibited only a slight decrease in transformation yield. If the cells were incubated on ice 2 min

before and after electroporation, transformation was not detected while cell survival remained unaffected. Thus, cold treatment inhibits transformation by CET in *Tetrahymena* by a mechanism that does not reduce cell survival.

In other cell types (22), electroporation works well in culture medium rather than in a simple buffer like the Hepes buffer used in our original studies (1). We tested several types of media including: SPP growth medium (18), 10 mM Tris pH 7.5 [a popular starvation medium for *Tetrahymena*, (19)], weakly conductive 0.05 M sucrose, 0.004 M CaCl₂ solution [used for electrofusion of *Tetrahymena* (23)] and the standard 10 mM Hepes buffer pH. 7.5 used previously (1). In two independent experiments, if cells were electroporated in SPP, transformation was completely suppressed while the survival rate greatly increased. The Tris medium caused 6–10-fold reduction while sucrose medium and water caused a 3–4-fold reduction in efficiency of transformation compared to 10 mM Hepes buffer (results not shown).

Transformation by replacement vectors

In yeast, the frequency of homologous DNA recombination is stimulated by linearization of a vector within or close to, homologous sequences (24). To determine the effects of linearization on transformation yield with replacement vectors,

Figure 1. Structure of plasmid vectors used to transform *Tetrahymena* by CET. Linearized forms of circular plasmids are shown. E, *Eco*RI; V, *Eco*RV; A, *Afl*III; B, *Bsp*HI; S, *Sal*I; C, *Sac*I restriction sites. The arrowhead in the replication origin fragment marks the direction of transcription from the rRNA promoter sequence that is also present on the rDNA origin fragment. However, the origin fragments used to construct vectors other than pD5H8 contain a point mutation in the rRNA promoter (marked by an asterisk) that inactivates its function (11).

we transformed cells in the presence of 50 μ g of either uncut or linearized replacement vector pH4IA. The H4-I/neo/H4-I gene has been shown to recombine into the H4-I gene locus resulting either in gene replacement (H4-I gene knockout) or integration of the insert into the 5' region flanking the H4-I gene (2). The linear vector was prepared by digestion with *Eco*RI restriction endonuclease which releases the insert from the plasmid and creates ends that are homologous to the histone H4-I gene except for very small regions derived from the vector polylinker at the very ends of the insert (see Fig. 1) In three independent

Figure 2. Effect of field strength, pulse length and cold treatment on transformation efficiency by CET. Cells were electroporated in the presence of 18 μ g of pD5H8. (A) Effect of field strength using a 1.6 ms pulse (obtained by a combination of 50 μ F capacitance and 24 ohms resistance) and a 4 ms pulse (275 μ F and 13 ohms). The maximal efficiency, for 4 ms pulse was 1125 V/cm (250 V/0.2 cm setting) and for 1.6 ms was 1380 V/cm (325V/0.2 cm setting). Data shown are from a single experiment. In two other experiments using 4 ms pulse, maximal transformation yield was obtained using 250V. (B) Effect of cold treatment on transformation yield and cell survival. Cells were incubated either at room temperature (RT) or incubated for 2 min on ice before (B), after (A) or before and after (B+A) pulse treatment. Both the transformation efficiency and the cell survival are expressed as the fraction of results obtained at room temperature. No transformants were obtained in B+A. Data shown are from a single experiment. The inhibitory effect of cold on transformation was also found in another independent experiment (not shown).

experiments, the linear construct transformed with six fold higher efficiency compared to the circular vector (56 and 9 transformants were obtained with linear and intact vector respectively). In all cases analyzed, transformation occurred by homologous recombination. By Southern blotting we detected the two types of events previously observed using microinjection (2): gene replacement, and integration of the insert into the 5' flanking region of the H4-I gene. We also observed a previously unreported event of insert integration into the 3' flanking region of the H4-I gene (results not shown). In all cases (n=6) only the insert DNA was found and not the vector backbone as was also shown by Kahn *et al.* (2) using microinjection. One of the analyzed transformants obtained using undigested plasmid DNA also was found to be a result of gene replacement without retention of vector sequence.

These studies demonstrated that CET can be used instead of microinjection to perform gene replacement/knockout in *Tetrahymena* and that linearization of the targeted DNA increases the transformation yield. However, also as shown earlier for microinjection-mediated transformation (15), linearization is not required for gene replacement using CET. Surprisingly, even when intact plasmid is used, only homologous DNA without vector sequence is integrated into *Tetrahymena* genome.

Effect of rDNA replication origin(s) on transformation frequency

The plasmid containing the H4-I/neo/H4-I gene (pH4IA) transforms with a very low frequency (Table 1) compared to an rDNA processing vector. Low frequency of transformation is expected for a vector transforming by recombinative integration, which is a rare event. To determine if the presence of a replication origin affects transformation yield using CET, as in yeast (25), we tested three constructs with 0, 1 or 2 fragments containing the replication origin of the *Tetrahymena* ribosomal gene. As a control, we used the pD5H8 processing rDNA vector. No significant increase in transformation efficiency was observed if the H4-I/neo/H4-I vector contained a single replication origin

Table I. Summary of transformation of *Tetrahymena* by CET using different plasmids

Plasmid	Number of expts.	Yield ($\times 10^3$) ^a	Survival ($\times 10^3$) ^b	Percent transformed ^c	Efficiency ^d	Fraction of pD5H8 standard efficiency ^e
pD5H8	15	20.3 $\pm$ 5.8	506 $\pm$ 315	4.5 $\pm$ 3.4	1118 $\pm$ 889	1.00
pH4IA	2	0.052 $\pm$ 0.045	166 $\pm$ 187	0.059 $\pm$ 0.004	4 $\pm$ 3.9	0.0037 $\pm$ 0.0016
pH4neo	2	0.097 $\pm$ 0.1	119 $\pm$ 96	0.069 $\pm$ 0.029	3.3 $\pm$ 2.7	0.0064 $\pm$ 0.0045
pAUH4-12	3	8.9 $\pm$ 5.2	82 $\pm$ 39	9.7 $\pm$ 3.3	285 $\pm$ 137	0.66 $\pm$ 0.32
pAU-3	4	30.5 $\pm$ 24	365 $\pm$ 138	7.6 $\pm$ 4	2138 $\pm$ 436	1.45 $\pm$ 0.35
pAS-2	6	9.2 $\pm$ 8.7	352 $\pm$ 113	2.8 $\pm$ 2.7	458 $\pm$ 436	1.00 $\pm$ 0.34
pAS-2	3	0	360 $\pm$ 372	0	0	0
pAS-7	3	0	291 $\pm$ 195	0	0	0
pH4T2	9	26.5 $\pm$ 18	602 $\pm$ 284	6.2 $\pm$ 5.2	1085 $\pm$ 936	0.80 $\pm$ 0.29
p4T21	2	0	1181 $\pm$ 103	0	0	0
p42L29 (pmr)	2	7.9 $\pm$ 2.5	410 $\pm$ 441	11.5 $\pm$ 18.1	415 $\pm$ 142	0.62 $\pm$ 0.56
p42L29 (cy)	2	1.4 $\pm$ 1.2	410 $\pm$ 441	0.7 $\pm$ 0.85	70.9 $\pm$ 59.3	0.06 $\pm$ 0.03

^aTotal yield of transformants per experiment.

^bNumber of clones that survived conjugation and are conjugation progeny (6-methylpurine resistant).

^cPercent of conjugation progeny transformed.

^dNumber of transformants per μ g of vector DNA (18–20 μ g of DNA/experiment).

^eTransformation efficiency expressed as a fraction of efficiency obtained with the pD5H8 vector used to transform the same batch of conjugating cells. Note that this relative efficiency is the best way to compare the different vectors because of natural variations in competence of cells for transformation (due to variable timing of pairing and age of clones).

(pH4neo, Table 1). However, the presence of a tandem repeat of replication origins stimulated transformation frequency 100-fold (pAUH4-12, Table 1).

We tested a similar double origin vector containing the cycloheximide resistant *rpL29* gene (pAU3). This vector also transformed at very high frequency. Thus, both markers, H4-I/neo/H4-I and *rpL29*, can mediate high frequency transformation when present on a double origin vector.

Localization of minimal flanking sequences of the H4-I gene required for mass transformation

The H4-I/neo/H4-I gene is flanked by 2 kb of the 5' and 1.5 kb of the 3' flanking sequence (2). To determine whether CET could be used to test the importance of H4-I flanking sequences for *neo* gene expression *in vivo*, we reduced the 5' flanking sequence to only 333 bp upstream from the ATG initiation codon on a double origin vector. This construct (pΔ5-2) transformed *Tetrahymena* at very high frequencies, close to those obtained with the pD5H8 standard (Table 1). Thus, 333 bp of upstream of the H4-I gene is sufficient to confer high frequency transformation, presumably owing to its ability to promote H4-I gene expression *in vivo*. Vector pΔ5-7 which contains only 129 bp of the H4-I gene 5' sequence repeatedly failed to transform *Tetrahymena* (Table 1). Thus, one or more sequences located between 333 and 129 bp upstream from the translation initiation codon of the H4-I/neo/H4-I gene probably is required for expression.

Figure 3. Southern blot of total DNA extracted from several pH4T2 transformants. DNA was extracted from six pH4T2 transformants (lanes 1–6), from control untransformed cells (lane 7) and control strain to which some pH4T2 plasmid was added (lane 8). Ten μ g of each DNA were digested with *SacI* and *SacI* restriction endonucleases, separated on a 0.7% agarose–TAE gel, transferred to a nylon filter, and hybridized to a total pH4T2 plasmid probe. This probe includes the H4-I/neo/BTU2 marker (1.4 kb), the replication origin fragment (2×1.9 kb) and the pUC119 sequence (3.2 kb). The *SacI* + *SacI* digestion of pH4T2 separates the vector fragment (3.2 kb) from the insert (5.2 kb) containing both the repeated replication origin and the marker gene. The 5.2 band is diagnostic for the presence of an intact plasmid in transformant DNA and this band is visible in variable amounts in all transformants but not in the untransformed strain. Bands above 5.2 kb present in transformant DNA are most likely derived by homologous recombination between the endogenous rDNA and the vector DNA resulting either in integration of the entire plasmid into rDNA or generation of an independent plasmid with a triple replication origin. These recombination events were identified using a panel of restriction endonucleases (results not shown).

We also modified the 3' flanking region of the H4-I/neo/H4-I gene by deleting most of this region and leaving only 190 bp downstream from the TGA stop codon. The sequences removed included the *in vivo* polyadenylation site of the H4-I gene (26). This construct (pΔ3-2) failed to transform (Table 1). Thus, some of the 3' flanking sequence beyond 190 bp is required for mass transformation. This result suggests that, in *Tetrahymena*, proper transcription termination/polyadenylation site(s) are required for expression on an episomal vector, as is the case in many other organisms.

Creation of a small gene cassette by replacing the 3' region of the H4-I gene with the 300 bp of the 3' region of the BTU2

We wished to create a small, selectable marker [like *URA3* in *S.cerevisiae*, see (27)] that would not transform by itself but could function on replicative plasmids and could be used to disrupt other cloned *Tetrahymena* genes in gene knockout experiments. To this end, we tested whether the 3' flanking sequence of the H4-I gene

Figure 4. Comparison of transformation efficiencies using the rDNA processing vector (pD5H8) and the double origin H4-I/neo/BTU2 vector (pH4T2). (A) The correlation curve is for results obtained using pD5H8 and pH4T2 in 17 independent experiments. (B) Effect of timing of paromomycin selection on transformation efficiency. Cells were electroporated 10 h after shaker turn off and paromomycin selection was started at different times after electroporation.

Figure 5. Co-expression of the H4-I/neo/BTU2 and *rpL29* marker genes on a double origin vector. Cells were transformed with p42L29B, selected with paromomycin and tested for cross-resistance to cycloheximide (primary selection). Some clones initially selected at 120 μ g/ml of paromomycin were transferred onto medium containing paromomycin at 120, 500 or 1000 μ g/ml, allowed to grow to saturation and retested for cycloheximide resistance by replica transfer (first transfer).

could be replaced by the 3' flanking sequence from the BTU2 (beta-tubulin 2) gene (20). The 1.5 kb 3' flanking region of the H4-I gene beyond the TGA termination codon was replaced with a corresponding ~300 bp fragment from the BTU2 gene. This fragment contains the polyadenylation site of the BTU2 gene (20). This hybrid H4-I/neo/BTU2 gene was subcloned on a double origin vector to build the pH4T2 plasmid, which transformed at very high frequency (Table 1), comparable to pΔ5-2 which contains both 5' and 3' flanking sequences from the H4-I gene. Thus, the BTU2 3' flanking region can functionally substitute for the H4-I gene 3' flanking region.

In all transformants variable amounts of DNA corresponding to the structure of the original plasmid were detected (Fig 3). Thus, pH4T2 is replicated and maintained in transformants. However, we also saw a large number of DNA molecules that were derived from recombination events with the endogenous rDNA (see Fig. 3 legend). In many transformants integration into the rDNA of an entire pH4T2 plasmid occurred, as shown previously for other plasmids containing a single rDNA origin (1,2). Also DNA recombination products containing multiple fragments of the replication origin were observed. For example, in some transformants a prominent fraction of transforming DNA was found to be in the form of an independent plasmid with three replication origin fragments (results not shown). These recombination events were similar to those described earlier for a double origin rDNA somatic vector, prd4-1, and may also be explained by unequal crossing over (28).

The H4-I/neo/BTU2 hybrid gene represents an independent gene expression unit on a replicative vector. The hybrid gene contains very short (~300 bp) regions from two different *Tetrahymena* genes and it seemed reasonable to expect that this gene would not recombine with either the endogenous H4-I or BTU2 genes. To test this, we constructed a plasmid containing the H4-I/neo/BTU2 gene but no replication origin fragment (p4T21). This plasmid did not yield any transformants (Table 1). Thus, in the absence of a replication origin, the H4-I/neo/BTU2 construct can not transform, most likely because it can neither replicate independently nor integrate into the genome by homologous recombination. However, since the H4-I/neo/BTU2 does transform when present on a replicative vector such as pH4T2, it provides a convenient small selectable marker. In studies to be described elsewhere, we have shown that the linear fragments containing H4-I/neo/BTU2 inserted into other cloned *Tetrahymena* genes can be used to knock out the interrupted gene by CET.

General properties of double origin vectors

The mechanism of transformation mediated by an rDNA vector such as pD5H8 is quite different than the mechanism of transformation mediated by double origin *neo* vectors. Even though both pD5H8 and pH4T2 confer resistance to paromomycin, the biochemical basis of resistance is different. Resistance mediated by rDNA vectors is due to a mutation in the rRNA that increases the paromomycin resistance of the ribosome. In contrast, the *neo* gene encodes an enzyme that inactivates paromomycin [see (29)]. In addition, the selectable ribosomal RNA gene in pD5H8 is excised from the plasmid and amplified during macronuclear development, while the genes on the double origin vectors are not. Therefore, it seemed important to determine if conditions promoting high efficiency transformation by the rDNA processing vector and the double origin *neo* vectors are similar.

In general, the double origin vectors tested here transformed nearly as well as pD5H8. Transformation by pD5H8 is conjugation dependent and somatic cells are not transformed (1). All clones transformed by double origin vectors (with either *rPL29* or *neo*) that were analyzed were also 6-methylpurine resistant conjugation progeny ($n=31$), strongly suggesting that transformation by double origin vectors also is conjugation dependent using our methods of selection.

We found previously that there is only a brief period during *Tetrahymena* mating when cells are highly competent for transformation [see (1)]. This narrow time window, coupled with variation in the timing of conjugation events from experiment to experiment, probably explains our observation that the transformation efficiency with pD5H8 may vary by as much as 10-fold between experiments. Another factor affecting the transformation competence of mating cells is the age of conjugating clones since their last conjugation. Older clones exhibit increased incidence of macronuclear retention (30) and this phenomenon is associated with decreased transformation ability (unpublished observations). To determine if conditions affect the transformation rate of both types of vectors similarly, we plotted the efficiencies of transformation obtained with double origin *neo* vectors against those with the pD5H8 vector done at the same times (Fig. 4A). There is a good correlation between the efficiencies obtained with both classes of vectors. Thus, even though the transformation mechanisms of both types of vectors are different, the conditions that limit transformation competence appear to be similar.

We occasionally had experiments in which the transformation efficiency with pH4T2 was much lower than with pD5H8. These experiments are seen in Figure 4A as points significantly above the correlation curve. We noted that in these experiments drug selection was started later than usual. To further study this phenomenon, we transformed cells with pH4T2 or pD5H8 and started drug selection at different times after electroporation (Fig. 4B). For pD5H8, the transformation yield improved with increasing time between electroporation and the onset of selection as shown earlier (1). Surprisingly, the opposite effect was observed for transformation with pH4T2. The maximal yield of transformants was obtained when selection was initiated at the earliest time point after electroporation. This observation suggested that cell concentration at the onset of selection may affect the transformation yield with pH4T2. This hypothesis was confirmed by a subsequent experiment in which cells transformed with pH4T2 were plated at different cell concentrations, all of which were treated with drug at the same time. The transformation yield obtained for pH4T2 decreased with increase in cell concentration (results not shown). We also measured the maximal cell concentrations reached in microtiter wells under selective conditions by cells transformed with either pH4T2 or pD5H8. The pH4T2-transformed cells reached 3-fold lower maximal cell density than cells transformed with pD5H8 in paromomycin-containing medium (2.4 ± 0.5 versus $7.2 \pm 0.7 \times 10^5$ cells/ml). In contrast, pH4T2-transformed cells and pD5H8-transformed cells grew to similar high densities in drug free medium. Similar, low maximal cell densities in medium containing drug were observed in cultures transformed with gene replacement vectors containing either H4-I/neo/H4-I or H4-I/neo/BTU2 marker (results not shown). However, in drug, strains transformed with a *neo* gene under control of both 5' and 3' flanking sequences from the BTU1 gene grew to high densities characteristic of pD5H8 transformed cells (results not shown).

Thus, the lower transformation rate at higher densities is correlated with inability of cells transformed with the H4-I/*neo* marker (with either the H4-I or BTU2 3' flanking region) to grow to high density in drug medium. These results strongly suggest that the observed decrease in transformation yield is due to a specific pattern of *neo* gene expression under control of the histone H4-I promotor which has been shown to be cell-cycle regulated and transcribed much less in non-growing (starved) cells than in growing cells (31). This potential problem can be solved by early drug selection (as shown in Fig. 4B) or by incubation of cells at lower (room) temperature to reduce growth prior to selection (results not shown).

Two marker genes (H4-I/*neo*/BTU2 and *rpL29*) can be expressed on the same double origin vector

pH4T2 is the smallest vector constructed so far which mediates high frequency transformation. We wished to test if this vector could be used as a vehicle for expression of other genes, a useful precondition for constructing libraries to be used to clone genes by complementation of mutant function. We subcloned the cycloheximide-resistant *rpL29* gene on the pH4T2 vector downstream of and in the same transcriptional orientation as the H4-I/*neo*/BTU2 cassette. Cells were electroporated in the presence of this construct (p42L29B) and selected with either cycloheximide or paromomycin. Transformants were obtained using both types of selection, indicating that both markers are expressed (Table 1). However, the number of transformants obtained with paromomycin was 5–6-fold higher than with cycloheximide selection. Thus, direct selection based on the H4-I/*neo*/BTU2 gene is more efficient than the *rpL29* dependent selection even though both are on the same p42L29B plasmid. Selected clones were tested for cross-resistance. 29% of the paromomycin-resistant primary transformants (n=55) were resistant to cycloheximide. In contrast, all cycloheximide-resistant clones were cross-resistant to paromomycin. The paromomycin-resistant clones were replica transferred twice (10–15 generations each transfer) in medium containing the same low paromomycin concentration (120 µg/ml) and retested for cycloheximide resistance. 67 and 98% (n=54) become resistant to cycloheximide after the first and second transfer, respectively. Next we tested whether cells transferred only once on plates containing increased paromomycin concentrations (120, 500 or 1000 µg/ml) would show increased levels of resistance to cycloheximide, presumably due to increased plasmid copy number/expression. As shown in Figure 5, there is a decrease in the number of clones able to grow on higher paromomycin concentration when transferred directly from the lowest concentration. It should be noted, however, that nearly all clones survived at up to 1000 µg/ml of paromomycin when serial transfers to medium containing paromomycin at smaller concentration increments are used (results not shown). The fraction of doubly resistant clones increases in response to increased paromomycin selection pressure, and nearly all cells transferred on 1000 µg/ml of paromomycin become cross-resistant to cycloheximide. Thus, the level of cross-resistance to cycloheximide increases in response either to additional growth at low paromomycin concentration or to growth at increasing paromomycin concentrations. Because nearly all clones eventually became resistant to both drugs, in nearly all cases the transformation was most likely due to the maintenance of the entire plasmid bearing both markers. However, we can not conclude that both marker genes were actually expressed on the

vector plasmid itself. It is also possible that the 3.2 kb *rpL29* gene recombined with its chromosomal homologue and that only the chromosomal copies are functional. Regardless of the exact mechanism of cycloheximide resistance, these results demonstrate that a pH4T2 plasmid can be used to clone and express other genes in *Tetrahymena*.

DISCUSSION

As in yeast (25), transformation frequencies obtained with integrative type vectors are low in *Tetrahymena*, and the transformation rate is greatly increased by inclusion of fragments containing a known replication origin. However, unlike in yeast, a single *Tetrahymena* origin did not increase transformation frequency significantly; a tandem repeat of rDNA origins was required to stimulate the transformation rate. These results are consistent with observations made by Yu and Blackburn, (11) who reported that a circular rDNA vector with a single origin-containing fragment transformed exclusively by homologous recombination while presence of a second origin fragment promoted plasmid replication.

We consistently observed relatively large amounts of independent plasmid in cells transformed with double origin vectors. However, Kahn *et al.*, (2) observed relatively large amounts of a single origin *neo* vector maintained in clones transformed by microinjection of macronuclei in vegetatively growing cells. Thus, a single origin present on a circular vector may be sufficient for replication when introduced into the macronuclei of vegetative cells. However, the *Tetrahymena* rDNA replication origin is unique because it is involved not only in replication of rDNA during each generation of vegetative growth, but also in developmentally regulated amplification from a single copy of the micronuclear rDNA during macronuclear development [recently reviewed in (32)]. The CET method delivers DNA into conjugating cells during the early stages of macronuclear genome formation, when rDNA amplification occurs (1). As it is amplified during macronuclear development, rDNA is converted into a large palindromic dimer (33). Thus, to compete efficiently with an endogenous palindromic rDNA for *trans*-acting replicative factors, vectors transforming by CET may require two replication origins.

Using the frequency of transformation as an assay, we were able to map the *cis*-acting sequences present in the H4-I flanking region that are required for CET and presumably for H4-I gene expression. The critical sequence element(s) is (are) located between 129–333 bp upstream from the translation start site of the H4-I gene. Interestingly, a conserved CCAAT element identified by Brunk and Sadler (34) is present in this region. We also show that the *neo* gene flanked by H4-I regulatory sequences requires sequences more than 192 bp beyond the TGA termination codon for expression. Thus, CET should be useful in further studies on sequences involved in gene expression in *Tetrahymena*, about which little is known.

We constructed a small hybrid H4-I/*neo*/BTU2 gene that should find general application as a convenient selectable marker. This marker does not transform *Tetrahymena* unless subcloned onto a replicative vector. H4-I/*neo*/BTU2 can also function as a convenient 'disruption cassette' to perform gene knockouts in *Tetrahymena* by inserting it into any cloned *Tetrahymena* gene which can then be targeted to the homologous locus. We have already used the H4-I/*neo*/BTU2 cassette to perform genomic knockouts of a number of *Tetrahymena* genes (unpublished

observations). All transformants were due to correct targeting, demonstrating the absence of background of recombination events involving either the H4-I or the BTU2 locus.

H4-I/neo/BTU2 should also help to clone sequences involved in initiation of replication [analogous to ARS sequences in *S.cerevisiae* (25)] if they exist in *Tetrahymena*. To isolate putative *Tetrahymena* ARS sequences one can construct a shotgun-type library using *Tetrahymena* genomic DNA in a vector containing the H4-I/neo/BTU2 gene as the selectable marker. Because integrative transformation with H4-I/neo/BTU2 is essentially undetectable, any transformant isolated after introduction of such a library into *Tetrahymena* by CET will likely be due to episomal maintenance of a plasmid containing a *Tetrahymena* ARS sequence.

The high efficiency of CET should allow for new applications. We showed that the pH4T2 vector can be used to transfer and express another gene. Although the mechanism of the coexpression of *rpL29* gene on the H4-I/neo/BTU2 plasmid has not yet been determined, preliminary observations suggest that the level of expression of *rpL29* is higher using pH4T2 than when inserted into the rDNA 3' non-transcribed spacer on the pD5H8 vector. For example using CET, we were unable to select transformants by direct cycloheximide selection of cells transformed with a pD5H8 derivative containing the *rpL29* gene (unpublished observations) while such transformants were readily obtained using a pH4T2 derivative carrying the *rpL29* gene (Table 1). However, for unknown reasons the number of transformants obtained using the *rpL29* marker subcloned on the H4-I/neo/BTU2 vector (p42L29B) was significantly lower than with the double origin vector containing only the *rpL29* gene as a marker (pAU3) and only 1/3 of the p42L29B transformants initially selected using the *neo* gene were cross-resistant to cycloheximide. It is possible that the level of expression of *rpL29* is attenuated because it is located downstream of the H4-I/neo/BTU2 marker.

Further development of appropriate shotgun-type libraries using vectors described here should allow cloning of genes by complementation of mutant phenotype, an approach that has contributed to the rapid development of a select group of organisms (eg. *E.coli*, *S.cerevisiae*) into major tools of modern molecular and cellular research. The pH4T2 vector we developed is much smaller than the pD5H8 rDNA vector used previously (1) and still transforms at very high frequency. However, further work is needed to determine if the pH4T2 cloning capacity is larger than pD5H8. Although the smaller size of pH4T2 makes it easier to handle, it contains a direct repeat of the replication origin fragment which is difficult to maintain in *E.coli* and may limit the cloning potential. However, pH4T2 provides a starting point to construct even smaller vectors containing a smaller rDNA origin or a small non-rDNA origin, which may lead to higher cloning capacity. Phenotypic complementation in *Tetrahymena* combined with conventional *Tetrahymena* genetics would provide a powerful approach for cloning the wildtype version of genes mutated in processes that can not be explored in other systems such as ciliary motility, gene amplification and developmentally programmed genome rearrangement and sequence diminution.

ACKNOWLEDGEMENTS

We thank W.Pan, E.Blackburn, R.Kahn and C.Brunck for providing us with plasmid clones prior to publication. This work was supported by Public Health Service Grant GM26973 from the National Institute of Health.

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