

Transient and selectable transformation of the parasitic protist *Trichomonas vaginalis*

(electroporation/chloramphenicol acetyltransferase/luciferase/neomycin phosphotransferase/parasite)

MARIA G. DELGADILLO, DAVID R. LISTON, KAYVAN NIAZI, AND PATRICIA J. JOHNSON

Department of Microbiology and Immunology, School of Medicine, Molecular Biology Institute, University of California, Los Angeles, CA 90095

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ABSTRACT We have developed methods to transiently and selectable transform the human-infective protist *Trichomonas vaginalis*. This parasite, a common cause of vaginitis worldwide, is one of the earlier branching eukaryotes studied to date. We have introduced three heterologous genes into *T. vaginalis* by electroporation and have used the 5' and 3' untranslated regions of the endogenous gene α -succinyl CoA synthetase B (α -SCSB) to drive transcription of these genes. Transient expression of two reporter proteins, chloramphenicol acetyltransferase (CAT) or luciferase, was detected when electroporating in the presence of 50 μ g closed-circular construct. Optimal levels of expression were observed using $\approx 2.5 \times 10^8$ *T. vaginalis* cells and 350 volts, 960 μ Fd for electroporation; however, other conditions also led to significant reporter gene expression. A time course following the expression of CAT in *T. vaginalis* transient transformants revealed the highest level of expression 8–21 hr postelectroporation and showed that CAT activity is undetectable using TLC by 99 hr postelectroporation. The system we established to obtain selectable transformants uses the neomycin phosphotransferase (*neo*) gene as the selectable marker. Cells electroporated with 20 μ g of the NEO construct were plated in the presence of 50 μ g/ml paromomycin and incubated in an anaerobic chamber. The paromomycin-resistant colonies that formed within 3–5 days were cultivated in the presence of drug and DNA was isolated for analyses. The NEO construct was shown to be maintained episomally, as a closed-circle, at between 10–30 copies per cell. The ability to transiently and selectable transform *T. vaginalis* should greatly enhance research on this important human parasite.

Trichomonas vaginalis is one of the earliest-diverging eukaryotes studied to date (1, 2). This organism, a flagellated protist belonging to the group Parabasalia, is parasitic to humans. *T. vaginalis* is transmitted from host to host by sexual intercourse and is a common cause of vaginitis worldwide. In addition to its medical importance, a number of unusual properties of trichomonads have captured the attention of scientists interested in energy metabolism, organelle biogenesis, gene transcription, drug-resistance, and pathogenesis. The appeal of *Trichomonas* from a biological viewpoint stems, in large part, from properties that reflect both its primitive nature and its parasitic lifestyle. For example, trichomonads lack two organelles typically found in eukaryotes, the mitochondrion and the peroxisome, but instead contain an organelle involved in carbohydrate metabolism called the hydrogenosome (3, 4). Interestingly, the hydrogenosome and mitochondrion appear to have evolved from a common

endosymbiont (5–8). Recent molecular analyses have also revealed two unusual features regarding gene transcription in trichomonads. All examined protein-coding genes have a conserved motif that surrounds the start site of transcription, which appears to play a critical role in transcription initiation (9). Also, trichomonads contain an atypical RNA polymerase II and transcription of protein-coding genes is α -amanitin insensitive, unlike that observed for most eukaryotes (10).

Given its evolutionary status, *T. vaginalis* could provide an excellent model system for examining the evolution of eukaryotes. Although the application of recombinant DNA technology during the last decade has greatly increased our knowledge of trichomonads, the lack of a genetic system still hinders research progress. Here we describe the development of both transient and selectable transformation assays for this important human parasite. We demonstrate the applicability of two reporter genes, chloramphenicol acetyltransferase (*cat*) and luciferase (*luc*), for studies using transient transformation. We have also established a selectable transformation procedure that allows rapid isolation of clonal populations expressing the reporter gene neomycin phosphotransferase (*neo*). The ability to use transformation to study the variety of intriguing biochemical processes presented by trichomonads promises to expand our understanding of the biology of early diverging eukaryotes.

MATERIALS AND METHODS

Plasmid Construction. Three plasmids were constructed using a genomic clone of the α -succinyl CoA synthetase B (α -SCSB) gene (11) to provide 5' and 3' untranslated regions (UTRs) of 1660 and 449 nucleotides, respectively. Primers corresponding to 5' and 3' ends of the α -SCSB gene with either a *Kpn*I (5' end primer) or a *Bam*HI (3' end primer) restriction enzyme site added at their 5' ends were used in an inverse PCR to amplify the 5' and 3' α -SCSB UTRs and the pBluescript vector. These primers were 5'-GATCGGTACCTTGGATA-CAACTCTTGT-3' and 5'-GATCGGATCCATCTACTGCTTACTTTAA-3'. The 5.15-kb product of the inverse PCR, called *T. vaginalis* expression vector 1, was purified and digested with *Kpn*I and *Bam*HI. The coding regions of *cat*, *luc*, and *neo* were derived from pBLCAT3 (12), pDR100 (13), and pKm2 (14), respectively. Each plasmid was used as a template in PCR using primers with a *Kpn*I (5' end primer) or a *Bam*HI (3' end primer) restriction enzyme site added at their 5' ends. Primers used to amplify the *cat* gene were 5'-GGCCGGTAC-CATGGAGAAAAAATCACT-3' and 5'-GATCGGATCCTTACGCCCCGCCCTGCCA-3'. Primers used to amplify the *luc* gene were 5'-GGCCGGTACCATTGGAAGACGCC-AAAAAC-3' and 5'-GATCGGATCCTTACAATTTGGACTTTCC-3'. Primers used to amplify the *neo* gene were 5'-GATCGGTACCATGATTGAACAAGATGGATTG-3' and

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Abbreviations: CAT, chloramphenicol acetyltransferase; LUC, luciferase; NEO, neomycin phosphotransferase; α -SCSB, α -succinyl CoA synthetase B; UTR, untranslated region.

5'-GATCGGATCCTCAGAAGAAGACTCGTCAAGAAG-3'. The resulting PCR products were digested with *Kpn*I and *Bam*HI and ligated into *T. vaginalis* expression vector 1 (see above). The derived constructs were named α -SCSB-CAT, α -SCSB-LUC, and α -SCSB-NEO, accordingly. Plasmid DNAs were purified (Maxi Kit, Qiagen, Chatsworth, CA) and the entire 5' and 3' UTRs and roughly the first 100 nucleotides of the reporter genes were sequenced (Sequenase, United States Biochemical).

Electroporation. *T. vaginalis* strain C1 (ATCC no. 30001) was grown in Diamond's medium (15) and cells were harvested by centrifugation at $1,500 \times g$ for 10 min at 4°C. The pellet was weighed and resuspended in cold Diamond's medium to a final concentration of 1 g cells/1.5 ml medium. Three hundred microliters of the cell suspension ($\approx 2.5 \times 10^8$ cells) and closed-circular DNA constructs were electroporated using 350 volts and 960 μ Fd (unless otherwise stated) in 0.4 cm electrocuvettes using a Bio-Rad gene pulser and capacitance extender. DNA (50 μ g) was used for electroporations with both α -SCSB-CAT and α -SCSB-LUC constructs and 20 μ g of DNA was used for electroporations with the α -SCSB-NEO construct. Following electroporation with either the α -SCSB-CAT or the α -SCSB-LUC construct, cells were incubated on ice for 10 min and then diluted into 50 ml Diamond's medium and incubated for 21 hr (unless otherwise stated) at 37°C. Cells were then harvested by centrifugation. Selectable transformants were obtained from cells electroporated with the α -SCSB-NEO construct as described below.

CAT Assay. Transformants were tested for CAT activity by resuspending cells harvested 21 hr postelectroporation in 250 μ l of 250 mM Tris, pH 7.8. Cells were lysed by three freeze-thaw cycles, alternating between incubation in dry ice/ethanol and at 37°C for 10 min each. Samples were heated to 65–67°C for 10 min to inactivate endogenous proteases in the extract. The extract was then centrifuged at $16,000 \times g$ in a microcentrifuge at 4°C for 10 min, the supernatant was collected and a 20 μ l sample was removed to determine protein concentration. The remainder of the extract was stored at –80°C. For the CAT assay, 75 μ l of the heat-treated extract was added to 75 μ l of 250 mM Tris (pH 7.8) followed by the addition of 3 μ l 14 C chloramphenicol (0.075 μ Ci, Amersham, 50–60 mCi/mmol; 1 Ci = 37 GBq) and 20 μ l 4 mM acetyl CoA (Pharmacia) and the reaction was incubated at 37°C overnight. The reaction was then extracted with 1 ml ethyl acetate. Nine hundred microliters of the upper phase was transferred to a fresh tube and dried-down using a Speed Vac. The dried sample was resuspended in 25 μ l ethyl acetate and spotted repeatedly in 5 μ l aliquots onto a TLC plate (J. T. Baker, Phillipsburg, NJ). The TLC tank was pre-equilibrated with 95% chloroform:5% methanol. Upon addition of the 20-cm plate to the tank, the solvent was allowed to run up to 5 cm from the top. Plates were then dried and exposed to x-ray film. To quantitate the data, acetylated and unacetylated spots of 14 C-labeled-chloramphenicol were cut from the TLC plate and subjected to liquid scintillation counting. The activity of the extracts was calculated and expressed as % acetylation/25 μ g protein added to the CAT assay. Protein concentrations were determined using the Bradford assay (Bio-Rad).

Luciferase Assays. Cells were assayed for luciferase activity using the luciferase Assay system from Promega with the following modifications. Cells were washed using PBS and resuspended in lysis buffer (Promega) supplemented with 50 μ g/ml leupeptin (Boehringer Mannheim) at a concentration of 7.5×10^7 cells/ml buffer. The samples were frozen at –80°C for a minimum of 1 hr and then spun at $1,500 \times g$ for 5 min. The supernatant (20 μ l) was mixed with 100 μ l of luciferase assay reagent (Promega) and light production was measured using a Monolight 2010 luminometer (Analytical Luminiscence Laboratory, San Diego).

Selectable Transformants. To obtain selectable transformants, cells were electroporated using 20 μ g of the α -SCSB-NEO construct as described above. After electroporation, the cells were resuspended in 50 ml Diamond's medium (15) and incubated at 37°C for 4 hr without drug. Paromomycin sulfate (Sigma) was then added to a final concentration of 50 μ g/ml and incubation was continued for another 17 hr. 25 ml of the culture was harvested by centrifugation at $1,500 \times g$ for 7 min, the supernatant was removed and the pellet was gently resuspended in the residual supernatant. Resuspended cells were added to 50 ml of molten modified Diamond's medium containing 0.36% agar, 10% horse serum (16), and 50 μ g/ml paromomycin at 40°C. The solution was inverted twice and plated. Plates were sealed with stretchable sealing tape (Diversified Biotech) and incubated without inversion in an anaerobic chamber for 4–5 days at 37°C to allow colony formation inside the agar. Fifty to three hundred colonies per 4 μ g α -SCSB-NEO construct per transformation can be obtained using this procedure. To assure cloning, colonies were replated. An isolated colony was then transferred into 0.5 ml liquid media containing 50 μ g/ml paromomycin with a sterile transfer pipet and incubated overnight at 37°C. The culture was scaled-up to larger volumes as cell density increased. Transformed cells can be frozen in Diamond's medium containing 10% dimethyl sulfoxide and stored in liquid nitrogen.

Analysis of Selectable Transformants. High molecular weight DNA was extracted from 100 ml cultures of transformed clones grown in the presence of 50 μ g/ml paromomycin using DNazol (GIBCO/BRL). Southern blots were prepared and hybridized with a 32 P-labeled *neo* probe as previously described (17). To determine the copy number of the NEO construct in selectable transformants, the amount of the *neo* gene (800 bp) that would represent one copy per *T. vaginalis* genome was calculated using the genome size of 2.5×10^7 bp (18). Accordingly, 32, 160, 320, 480, 640, 800, 960, and 1120 pg of the *neo* gene was used to represent 1, 5, 10, 15, 20, 25, 30, and 35 copies per genome when compared with 1 μ g total *T. vaginalis* DNA. 0.5 μ g and 1 μ g DNA from clones 1 and 2 were used for comparison. DNA samples were denatured in 0.4N NaOH for 10 min at room temperature, neutralized in 1M NH_4OAc and vacuum transferred onto nitrocellulose using a slot-blot apparatus. Blots were then hybridized and washed as before. After exposing to x-ray film, radioactive signals were quantitated using liquid scintillation counting.

RESULTS AND DISCUSSION

To establish a transient transformation assay for *T. vaginalis*, we prepared two constructs containing either the reporter gene *cat* or *luc* in pBluescript (Fig. 1) These genes are flanked by 1660 and 449 nucleotides of the 5' and 3' UTR, respectively, of the *T. vaginalis* α -SCSB gene (11). Additionally, DNA encoding the first 27 amino acids of α -SCSB are fused in-frame to the reporter genes. We chose to flank *cat* and *luc* genes with

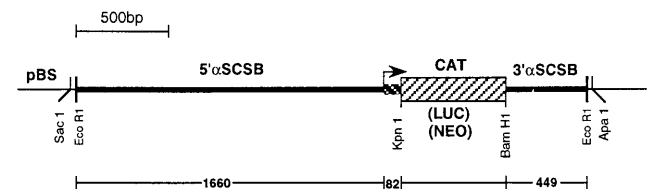


FIG. 1. Schematic diagram of the plasmids α -SCSB-CAT, α -SCSB-NEO and α -SCSB-LUC used to transform *T. vaginalis*. Sequences of the 5' and 3' ends of the α -SCSB UTRs are represented by a heavy line. \rightarrow Represents the start of the α -SCSB coding region, which is followed by the first 82 bp of the α -SCSB gene (■) fused in-frame to the reporter gene (■). The coding regions of *cat*, *neo*, and *luc* are flanked by *Kpn*I/*Bam*HI sites that were used to prepare the constructs using the pBluescript (pBS) vector. Relevant restriction enzyme sites are shown.

α -SCSB UTRs, as the α -SCSB gene is highly expressed (11) and was expected to provide a strong promoter to drive the expression of these reporter genes.

50 μ g of either the closed-circular *cat* or *luc* construct was introduced into $\approx 2.5 \times 10^8$ *T. vaginalis* cells by electroporation in Diamond's medium. The cells were then cultured in Diamond's medium for 21 hr, harvested and assayed for the presence of reporter activity. Relatively high levels of CAT activity were detectable by TLC in cells subjected to electroporation at 350 volts and 960 μ Fd (Fig. 2, lane 4). In contrast, CAT activity was undetectable in either nonelectroporated *T. vaginalis* extracts (lane 2) or *T. vaginalis* extracts prepared from cells electroporated in the absence of DNA (lane 3), showing that neither endogenous nor contaminating CAT activity is responsible for the activity detected upon electroporation with the α -SCSB-CAT construct. Furthermore, no CAT activity is detected when the cells are electroporated with a CAT construct (pBLCAT) that lacks both 5' and 3' α -SCSB UTRs (lane 5) demonstrating the requirement of flanking *T. vaginalis* DNA for gene expression. *T. vaginalis* cells electroporated with the α -SCSB-CAT construct were plated on Luria-Bertani broth and no bacterial colonies were observed, ruling-out the possibility that the observed CAT activity derives from bacterial contamination of the *T. vaginalis* cultures.

T. vaginalis cells electroporated using the α -SCSB-LUC construct likewise express high levels of luciferase activity, ranging from 40,000 to 450,000 light units (data not shown). Unlike that observed for CAT activity, luciferase activity requires the addition of the cysteine protease inhibitor, leupeptin, to a concentration of ≈ 30 mM to cell extracts to prevent proteolysis. Moreover, we found levels of luciferase to vary over 10-fold in individual experiments where the same conditions and reagents were used. For these reasons, we chose to optimize conditions for transient expression of proteins in *T. vaginalis* using the α -SCSB-CAT construct.

Fig. 3 shows the levels of CAT activity obtained using different electroporation conditions. Conditions typically used to transform bacteria (between 500 and 1800 volts and 25 μ Fd) yield barely detectable CAT activity in *T. vaginalis* extracts prepared 21 hr postelectroporation. On the other hand, CAT activity ranging from 29–82% acetylation per 25 μ g protein is detected when electroporating at 350–450 volts and either 500 or 960 μ Fd. Electroporation using 350 volts and 960 μ Fd yields

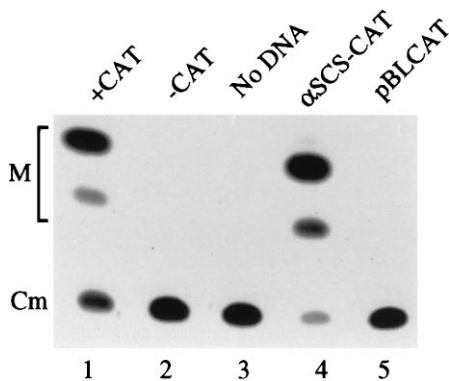


FIG. 2. CAT expression in *T. vaginalis* cells transformed by electroporation at 350 volts and 960 μ Fd. TLC demonstrating unacetylated 14 C chloramphenicol (Cm) and monoacetylated forms of the substrate (M) derived following incubation of *T. vaginalis* extracts with 14 C chloramphenicol and acetyl CoA. The following *T. vaginalis* extracts were used in CAT assays: Lane 1, nonelectroporated extracts containing 5×10 units of commercially available CAT (Pharmacia) as a positive control; Lane 2, nonelectroporated extracts without addition of commercially available CAT; Lane 3, extracts electroporated in the absence of DNA; Lane 4, extracts electroporated in the presence of the α -SCSB-CAT construct; Lane 5, extracts electroporated in the presence of the plasmid pBLCAT.

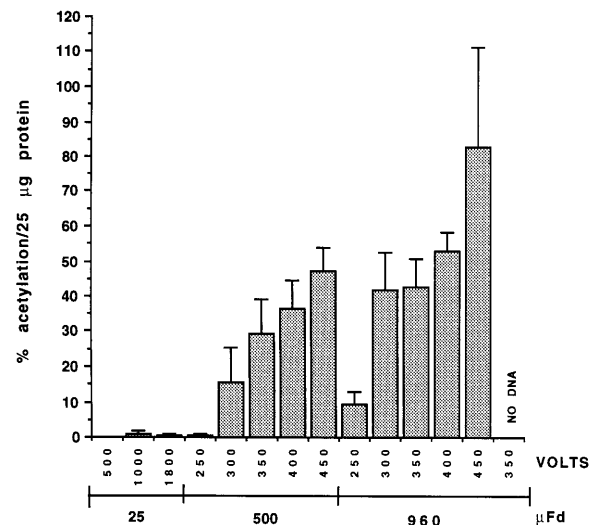


FIG. 3. CAT expression in *T. vaginalis* using various electroporation conditions. The graph shows the percent acetylation of [14 C]chloramphenicol per 25 μ g protein in CAT assays from electroporations with conditions varying from 25 μ Fd (500–1800 volts), 500 μ Fd (250–450 volts) to 960 μ Fd (250–450 volts). Except for the no DNA control, cells were electroporated using 50 μ g of the α -SCSB-CAT construct, incubated 21 hr at 37°C and then assayed for *cat* expression. The average value \pm SD for three experiments is shown.

on average 43% acetylation per 25 μ g protein. Electroporation at higher voltages (i.e., 400 or 450) results in even higher levels of acetylation, however, these conditions also lead to lower cell survival (higher kill rate) and severe cell aggregation. Taking all parameters into account, we have chosen 350 volts and 960 μ Fd as our standard electroporation conditions. Transformation experiments were conducted using $\approx 2.5 \times 10^8$ *T. vaginalis* cells resuspended in Diamond's medium prior to electroporation. However, cells electroporated in Cytomix (19) and Zimmermann's postfusion media (20) were also found to express comparatively high levels of CAT activity (data not shown).

Levels of *cat* expression were examined from 2–165 hr postelectroporation to determine the optimal time for harvesting cells. CAT activity was found to peak between 8–21 hr postelectroporation (Fig. 4). Two percent acetylation (per 25 μ g protein) was observed at 8 hr, rising to a maximum of 32%

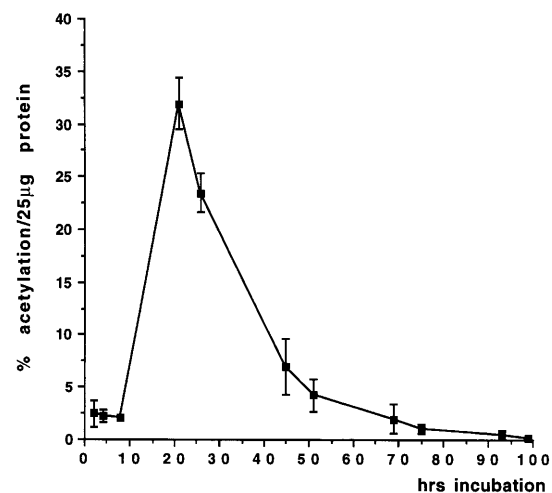


FIG. 4. Time course for CAT activity in *T. vaginalis*. Cells were electroporated, subcultured into fresh medium every 24 hr and harvested at various times postelectroporation and assayed for CAT activity. The results are the average \pm SD of an experiment done in triplicate.

at 21 hr. Activity drops to 23.5% by 26 hr and by 51 hr posttransformation only 4% acetylation is observed. Between 90–99 hr posttransformation, CAT activity becomes undetectable using TLC. Cells harvested at later time points were passed into fresh medium and were healthy prior to being tested for CAT activity. Thus, loss of CAT activity between 90–99 hr is not due to cell death but probably results from turnover of the CAT protein and loss of the plasmid from the cells. A similar time course experiment using cells transformed with the α -SCSB-LUC construct also showed that LUC activity peaks between 8–21 hr postelectroporation (data not shown).

To increase the sensitivity of *T. vaginalis* transformation methods, allowing a broader range of biological properties to be studied, and to ultimately develop methods for knocking-out genes in this parasite, we have also established a system to obtain selectable transformants using *neo* as a selectable marker. The sensitivity of nontransformed *T. vaginalis* cells to paromomycin sulfate was found to be 50 μ g/ml when cells are grown either on plates or in liquid medium. Under these conditions, no growth was observed on plates or cultures incubated up to 10 days. A construct containing the *neo* gene flanked by the α -SCSB UTRs (Fig. 1) was introduced into *T. vaginalis* as described for the α -SCSB-CAT construct, except that only 20 μ g of DNA was used in the electroporation. Transformants were plated and incubated in an anaerobic chamber. After 3–5 days of selection, colonies were replated to ensure clonality. Two of the resulting colonies (clones 1 and 2) were cultivated in liquid medium and total DNA was prepared to confirm the presence and to determine the physical structure of the α -SCSB-NEO construct. Restriction enzyme digestion and Southern blot analysis of total DNA reveals that the α -SCSB-NEO construct is maintained in the closed-circular form originally introduced into the cells (Fig. 5). Digestions of the DNA derived from clone 1 with four enzymes that cut only once in the construct (see Fig. 1) always result in detection of a linear band the size of the construct (\approx 5800 bp) upon Southern blotting and hybridization with a *neo* gene probe (lanes 1–4). Likewise fragments of the predicted size are observed when digesting with enzymes that cut at two locations

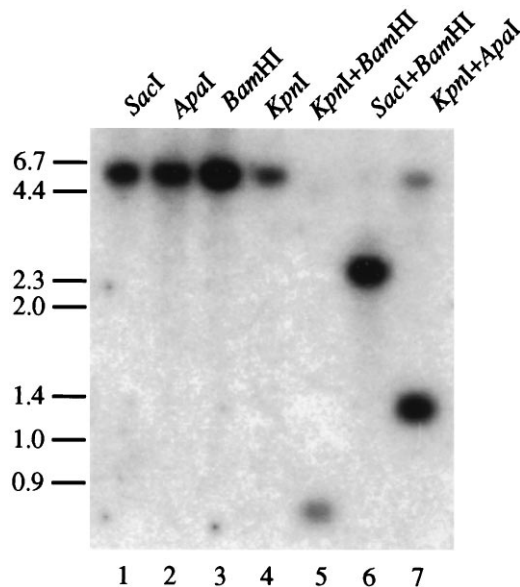


FIG. 5. Southern blot analysis of total *T. vaginalis* DNA from clone 1, transformed with the α -SCSB-NEO construct and selected using 50 μ g/ml paromomycin. DNA (1 μ g) was digested with a single restriction enzyme, as indicated (lanes 1–4) or a combination of two restriction enzymes, as indicated (lanes 5–7), and hybridized with a 32 P-labeled *neo* gene probe. See Fig. 1 for restriction enzyme map of the construct.

in the construct (lanes 5–7), indicating no DNA rearrangements have occurred as would be predicted upon integration of the construct into the *T. vaginalis* genome. The same results are obtained upon analysis of the DNA from clone 2 (data not shown). These data demonstrate that the α -SCSB-NEO constructs isolated from *T. vaginalis* clones 1 & 2 are maintained as episomally closed-circular plasmids. Furthermore, when total DNA isolated from clone 1 was used to transform *E. coli*, a plasmid that is indistinguishable by restriction enzyme analysis from the original α -SCSB-NEO construct used to transform *T. vaginalis* was retrieved (data not shown). Also, Northern blot analysis of total RNA prepared from *T. vaginalis* clones 1 and 2 revealed the presence of a \approx 850 nucleotide RNA when hybridized with a *neo* probe, consistent with the size predicted for a *neo* mRNA (data not shown). In preliminary experiments, we have also been able to select transformants of *T. vaginalis* using hygromycin phosphotransferase as a marker and 100 μ g/ml hygromycin in the selection procedure (data not shown).

To determine the copy number of the α -SCSB-NEO construct in paromomycin-resistant clones 1 and 2, either 0.5 μ g or 1 μ g of total DNA isolated from the clones was slotted onto nitrocellulose and hybridized with the *neo* probe (Fig. 6). Varying amounts of the *neo* gene were also slotted onto the blot, corresponding to the amount of *neo* DNA that would be present per μ g of total DNA if there were 1, 5, 10, 15, 20, 25, 30, or 35 copies of the construct per cell. Quantitation of the hybridization signals shown in Fig. 6 indicates that clone 1 harbors 10 copies of the construct per cell, whereas clone 2 contains between 24–30 copies per cell.

The ability to transform *T. vaginalis* now provides the means to directly test the function and properties of a variety of molecules *in vivo*. Currently, we are using this new technology to study organelle biogenesis, drug-resistance and gene transcription. These transformation assays should provide invaluable tools for advancing our knowledge of the basic biology and pathogenesis of trichomonads, as similar methods developed in the past few years for other protozoan parasites (19–28) have done.

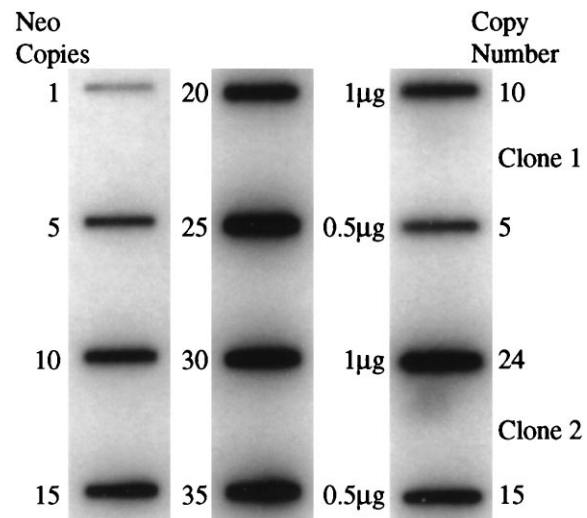


FIG. 6. Quantitation of the copy number of α -SCSB-NEO construct copy number per cell. DNA from *T. vaginalis* clones 1 and 2, transformed with the α -SCSB-NEO construct and selected using 50 μ g/ml paromomycin was slotted onto nitrocellulose and probed with a 32 P-labeled *neo* gene fragment. The amount of DNA equivalent to 1–35 copies of the α -SCSB-NEO construct per cell when compared with 1 μ g total *T. vaginalis* DNA was slotted in the left two lanes. *T. vaginalis* DNA (1 and 0.5 μ g of total) from clones 1 and 2 was slotted in the far-right lane. Copy number was calculated by counting the radioactivity in each slot using a liquid scintillation counter.

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