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Stable transformation of a mosquito cell line results in extraordinarily high copy numbers of the plasmid

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ABSTRACT **Stable incorporation of high copy numbers (>10,000 per cell) of a plasmid vector containing a gene conferring resistance to the antibiotic hygromycin was achieved in a cell line derived from the *Aedes albopictus* mosquito. Plasmid sequences were readily observed by ethidium bromide staining of cellular DNA after restriction endonuclease digestion and agarose gel electrophoresis. The plasmid was demonstrated by *in situ* hybridization to be present in large arrays integrated in metaphase chromosomes and in minute and double-minute replicating elements. In one subclone, ≈60,000 copies of the plasmid were organized in a large array that resembles a chromosome, morphologically and in the segregation of its chromatids during anaphase. The original as well as modified versions of the plasmid were rescued by transformation of *Escherichia coli* using total cellular DNA. Southern blot analyses of recovered plasmids indicate the presence of mosquito-derived sequences.**

The development of efficient procedures for the introduction of new genes into organisms has dramatically advanced our understanding of the molecular basis of many life processes. For example, studies of gene regulation and development in *Drosophila* have been greatly facilitated by the use of efficient transformation vectors derived from the P-element transposon (1–4). Research on other insects of medical and agricultural significance would undoubtedly benefit from similar studies. Attempts to transform mosquitoes by P-element-mediated transformation have been unsuccessful; rare integration events were attributed to illegitimate recombination that was not related to P-element activity (5–7). During the course of investigating the introduction of genes into mosquito cells, we have detected transformation events in which the plasmid DNA is organized in such a way that it resembles gene amplification of endogenous genes in other systems.

Gene amplification *in vivo* and *in vitro* can confer resistance to a wide range of drugs (8–10) and may contribute to the malignant progression in certain forms of cancer (11–15). Amplification of gene copy number is also the basis of resistance to certain pesticides in some plants (16) and insects (17). In *Culex pipiens* mosquitoes the development of high levels of resistance to organophosphate insecticides was accompanied by amplification of chromosomal esterase genes (18). Selection of methotrexate-resistant *Aedes albopictus* cells resulted in cells of altered karyotype, presumably because of amplification of the chromosomal dihydrofolate reductase gene (19). Gene amplification is frequently manifested in two forms of karyotypic abnormalities (12, 20): homogeneously staining regions or double-minute chromosomes. Both of these manifestations have now been observed in an *A. albopictus* cell line transformed stably with exogenous plasmid DNA. In one line the multiple copies of plasmid are organized in an array that resembles a chromosome.

MATERIALS AND METHODS

Recombinant DNA. The plasmid pUChshyg (Fig. 1) was constructed from the P-element vector pUChsneo (21) by replacement of the *neo* gene with the hygromycin phosphotransferase gene (*hyg*). The *hyg* gene together with the intron from the simian virus 40 small tumor antigen gene and poly(A) signal was isolated as a 2.2-kb *Hin*dIII–*Bam*HI fragment, and the hsp70 heat shock promoter was isolated as a 0.35-kb *Hin*dIII–*Xba* I fragment from pCHΔ2-3 (22). These fragments were cloned into *Xba* I–*Bam*HI-cut pUC18, thereby placing the heat shock promoter upstream from the *hyg* gene. The resulting *hshyg* transcription unit was excised from this plasmid as a 2.5-kb *Xho* I–*Sma* I fragment and ligated to the 4.2-kb *Xho* I–*Nae* I fragment of pUChsneo. The P-element inverted repeats in pUChshyg do not affect the transformation process in mosquito cells either with or without a helper construct carrying the P transposase gene (T.J.M. and M.J.K., data not shown). Heat shock does not significantly affect transformation efficiency.

Cell Culture and DNA Isolation. *A. albopictus* cell line C6/36 (23) was grown in Leibovitz's L-15 medium (GIBCO) supplemented with 10% fetal bovine serum at 28°C. Transformation of the C6/36 cell line with pUChshyg was performed using the Lipofectin reagent (BRL) according to the manufacturer's specifications. Twenty-four hours prior to transfection, 25-cm^2 flasks were seeded with 2×10^6 C6/36 cells. A lipofectin–DNA complex was made by incubating 10 μg of plasmid DNA with 50 μl of lipofectin reagent in 3 ml of serum-free L-15 medium for 15 min at room temperature. After rinsing the cells three times with sterile phosphate-buffered saline (PBS; 137 mM NaCl/27 mM KCl/10 mM Na_2HPO_4/1.8 mM KH_2PO_4), 3 ml of the lipofectin–DNA complex was added to each flask. Following an 8-hr incubation at room temperature, 3 ml of L-15 medium supplemented with 20% fetal bovine serum was added to each flask. After 24–48 hr, the medium was replaced with selective medium containing 300 units of hygromycin B per ml (Calbiochem). Stable transformants were selected by growth in L-15 medium supplemented with 10% fetal bovine serum and 300 μg of hygromycin B per ml. After 4–6 weeks of selection, individual clones were picked and expanded. The doubling time of these clones was about 2 days in selective medium. Clones were recovered at a frequency of 10^{-5} to 10^{-6} by this procedure. Subclones were isolated by inoculating ≈50 cells in each of several 100-mm Petri dishes. After visible colonies had formed, they were isolated by means of stainless steel cloning cylinders.

Total cellular DNA was isolated by lysing cells in 10 mM Tris·HCl/10 mM EDTA/0.6% SDS, pH 7.4. Proteinase K was added to a concentration of 0.5 mg/ml and incubated at

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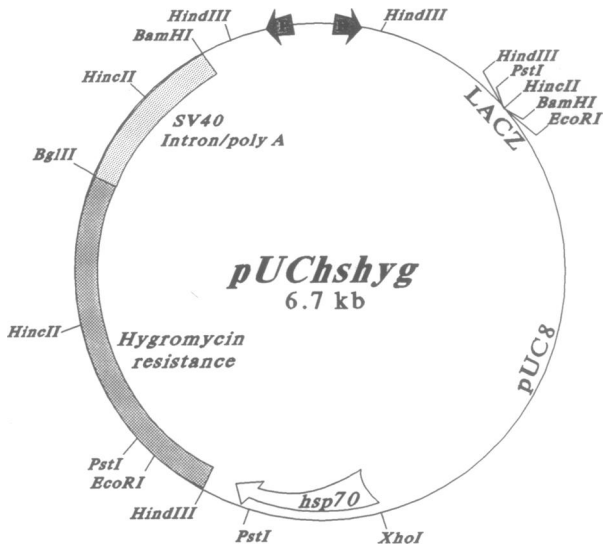

FIG. 1. Plasmid pUChshyg. The position of the *Drosophila melanogaster* hsp70 promoter and the direction of transcription are indicated by the large arrow. The hygromycin-resistance gene and the simian virus 40 (SV40) RNA processing signals are indicated by dark and light stippling, respectively. The P-element inverted repeats are indicated by short darkly shaded arrows. The pUC8 plasmid replicon is shown as a thin line. kb, Kilobases.

least 4 hr at 37°C. The lysate was extracted three times with equal volumes of phenol/chloroform (1:1) and precipitated with 2 volumes of ethanol. The DNA was dissolved in 10 mM Tris·HCl/1 mM EDTA, pH 7.4 (TE buffer), incubated with 0.1 mg of RNase A per ml at 37°C for 1 hr, phenol/chloroform extracted, ethanol precipitated, and redissolved in TE buffer. This procedure should result in isolation of chromosomal and extrachromosomal DNA. Plasmid rescues were performed by the addition of 1 μg of total DNA isolated from hygromycin-resistant C6/36 cells to *Escherichia coli* (strain HB101 or XL1-Blue) made competent via $CaCl_2$ treatment (24). Plasmids were isolated from ampicillin-resistant colonies for further characterization.

***In Situ* Hybridization.** Metaphase chromosome spreads were prepared from C6/36 cells previously exposed to 0.1 μg of colcemid per ml (Sigma) for 3 hr. Cells were swollen in 0.075 M KCl at 37°C, fixed in methanol/acetic acid (3:1), and dropped onto cold wet slides. Slides were incubated in 70% formamide/2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate) for 2 min at 70°C to denature DNA, gradually dehydrated in a series of ethanol solutions (70%, 80%, 90%, and 100%), and air dried. Denatured spreads were hybridized for ≈16 hr at 37°C with a biotinylated probe prepared from pUChshyg by nick-translation with either biotin-14-dATP or biotin-11-dUTP (BRL). The hybridization mixture contained 2 ng of biotinylated probe per μl, 50% formamide, 2× SSC, and 0.5 μg of carrier DNA per μl. Posthybridization washes at 45°C consisted of three changes of 50% formamide/2× SSC for 3 min each, followed by three changes of 2× SSC for 2 min each, followed by three changes of PN buffer (0.1 M sodium phosphate buffer, pH 8, with 0.05% Nonidet P-40 detergent for 2 min each). The biotinylated probe hybrids were detected by application of fluorescein-conjugated avidin (Vector Laboratories). The fluorescent signal was amplified by the addition of a biotinylated goat anti-avidin antibody (Vector Laboratories) followed by addition of fluorescein-conjugated avidin. Chromosome spreads were counterstained with a 0.25 μg/ml solution of propidium iodide and/or 10 μg of 4′,6-diamidino-2-phenylindole per μl made up in a solution containing antifade (*p*-phenylenediamine dihydrochloride) to prolong the fluorescence signal. The cells were viewed by fluorescence microscopy, and chromosomes were photographed at a magnification of either ×600 or ×1000 using Kodacolor Gold 100 print film.

***In Situ* Nick-Translation.** The sensitivity of metaphase chromosomes to digestion by pancreatic DNase I was determined by *in situ* nick-translation using a procedure similar to that described by Kerem *et al.* (25). Briefly, fresh slides were treated for 10 min at room temperature with 100 μl of nick-translation mixture under a coverslip. The nick-translation mixture contained 50 mM Tris·HCl (pH 7.9), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 μg of bovine serum albumin per ml, 10 units of DNA polymerase I per ml, 20 ng of pancreatic DNase I per ml, 4 μM (each) dATP, dGTP, and dCTP, and 0.3 μM biotin-11-dUTP. The reaction was terminated by rinsing the slide in running deionized water for 1 min. Slides were then stained as described above to reveal biotin incorporation by fluorescein isothiocyanate-labeled avidin and counterstained with propidium iodide.

Flow Cytometric Cellular DNA Measurements. The cells were fixed with ethanol and stained with chromomycin A3 according to methods described previously (26). Flow cytometry was carried out using a Coulter Epics V cell sorter with the Argon ion laser tuned to 458 nm to excite the chromomycin A3 dye, which binds quantitatively to DNA. The resulting DNA per cell histograms were analyzed by a curve-fitting procedure (Multicycle; Phoenix Flow Systems, San Diego).

Southern Blot Analysis. Ten micrograms of C6/36 DNA was digested with *Hin*dIII, separated according to molecular weight by 0.8% agarose gel electrophoresis, denatured *in situ* with 0.4 M sodium hydroxide/0.6 M NaCl/0.5 M Tris·HCl, pH 7.4, and blotted onto GeneScreen*Plus* (NEN/DuPont) nylon membrane. Blots were prehybridized in 50% deionized formamide/1% SDS/1 M NaCl/10% dextran sulfate for 30 min at 42°C. The blots were then hybridized with ^{32}P-labeled probes prepared from *Hae* III fragments of rescued plasmids by extension of random oligonucleotide primers (Amersham multiprime kit). For hybridization, 4×10^5 dpm of ^{32}P-labeled probe per ml plus 100 μg of sheared, denatured herring sperm DNA per ml was added to the prehybridization mixture and incubated at 42°C for an additional 16 hr. The blots were washed twice in 2× SSC at room temperature for 5 min, twice at 65°C in 2× SSC/1% SDS for 30 min, and twice in 0.1× SSC at room temperature for 30 min. Blots were dried and exposed to x-ray film.

RESULTS

Transformation of Mosquito Cells. Transformation of the *A. albopictus* mosquito cell line (clone C6/36) with the pUChshyg vector often resulted in transformants containing extremely high copy numbers of the plasmid. Cellular DNA isolated from one such hygromycin-resistant clone designated HShyg3 was digested with restriction endonucleases and examined by agarose gel electrophoresis. The characteristic restriction fragments of pUChshyg were detected superimposed on the genomic DNA from this clone (Fig. 2). Scanning densitometry of the photographic negative of Fig. 2 was used to estimate the copy number. The plasmid sequences comprised ≈7% of the total DNA or ≈10,000 copies per haploid genome, assuming a C value of 1.2 pg (27). The copy number did not diminish substantially when the cells were grown in the absence of hygromycin for ≈100 cell generations. Approximately half of the hygromycin-resistant lines (23 of 45) that we have characterized exhibit similarly high copy numbers of plasmid.

The state of the pUChshyg construct in the clone HShyg3 was investigated by *in situ* hybridization of biotin-labeled plasmid DNA to metaphase chromosomes of these cells (Fig. 3). The diploid chromosome number of *A. albopictus* is 6 ($2n = 6$). The continuous C6/36 line has an aneuploid karyotype (23), but the modal chromosome number is still 6. The pUChshyg probe did not hybridize to chromosomes prepared

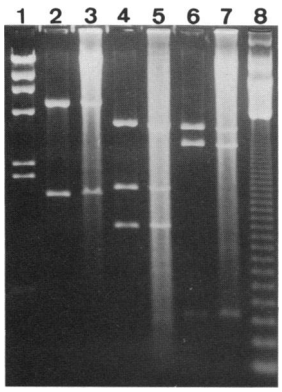

FIG. 2. Restriction enzyme digests of DNA from the pUChshyg-transformed mosquito cell line HShyg3. Cellular DNA was isolated from HShyg3, digested with restriction endonucleases, separated by electrophoresis on a 0.8% agarose gel, and stained with ethidium bromide. Lanes 1 and 8, molecular weight markers: *Hin*dIII-cut λ DNA and 123-base-pair ladder (BRL), respectively. Lanes 2, 4, and 6, pUChshyg plasmid cut with *Bam*HI, *Hin*cII, and *Pst* I, respectively. Lanes 3, 5, and 7, HShyg3 cell line also digested with *Bam*HI, *Hin*cII, and *Pst* I, respectively.

from non-transformed C6/36 cells (Fig. 3*A*); however, extensive hybridization was detected in the transformed line (Fig. 3 *B–I*). Minute and double-minute chromosomes were frequently observed (Fig. 3 *D–F*). These minute chromosomes were heterogeneous in number per cell and in size. Some of the larger minutes (Fig. 3*F*) appeared to be circular structures, and occasionally entire chromosomes appeared to be composed of the plasmid sequences (Fig. 3*I*). Large arrays of integrated plasmid sequence were detected in the chromosome arms of some cells (Fig. 3 *B, C, G,* and *H*), perhaps resulting from integration of the extrachromosomal forms into the chromosomes. Integrations were seen in centromeric (Fig. 3*G*) and telomeric locations (Fig. 3 *B* and *H*). Many of these structures resemble figures seen of mammalian cells that have undergone gene amplification events (20, 28–31). The hybridization patterns shown in Fig. 3 are from a single preparation of colcemid-treated HShyg3 cells. They illustrate the variety observed and do not reflect the relative abundance of the patterns. The most frequent pattern was double-minute chromosomes similar to those in Fig. 3 *D* and *E*.

Subclones isolated from HShyg3 were found to exhibit a much more stable karyotype than the parental line. Twelve subclones were examined cytogenetically to determine the chromosomal nature of the plasmid sequences. Each was relatively homogeneous and showed hybridization patterns characteristic of one of the types shown in Fig. 3 *B–I*. One of these clones designated HShyg3/11 carried a single large chromosome that apparently is composed entirely of the pUChshyg plasmid (Fig. 4). To the limits of detection by *in situ* hybridization, this chromosome is the only one that hybridized to the pUChshyg probe (Fig. 4*A*) and did not hybridize significantly when probed with labeled mosquito DNA (Fig. 4*B*). Interestingly, this pUChshyg chromosome appears to have the structural elements of a normal chromosome, including a centromere, as shown by its behavior during mitosis (Fig. 4*C*). We do not attach any significance to the occasional observation of a submetacentric chromosome (Fig. 4*A*) as opposed to the more usual metacentric

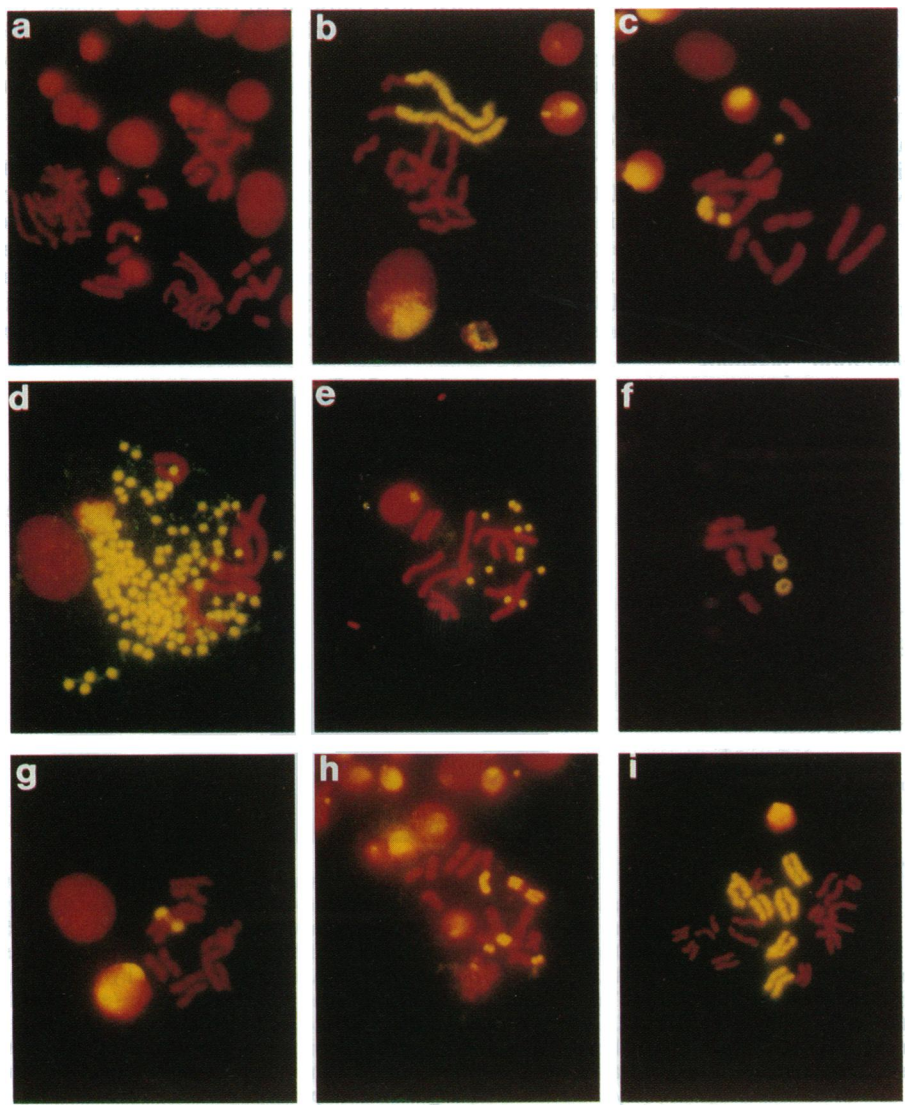

FIG. 3. *In situ* hybridization to chromosomes of the HShyg3 cell line. Biotin-labeled pUChshyg was hybridized to chromosomes and hybridization was detected by fluorescein-conjugated avidin. (*A*) Nontransformed control cells. (*B*) Hybridization to extended plasmid arrays at the ends of two mosquito chromosomes. (*C*) Integration of arrays of plasmid into mosquito chromosomes. (*D* and *E*) Cells containing different numbers of minute chromosomes composed of plasmid. (*F*) Minutes that appear to have a circular configuration. (*G*) Chromosomes with plasmid sequences either integrated into or associated with a centromeric region. (*H*) Integration of plasmid arrays at the ends of a mosquito chromosomal fragment. (*I*) Plasmid arrays organized into chromosome-like structures. (×1800.)

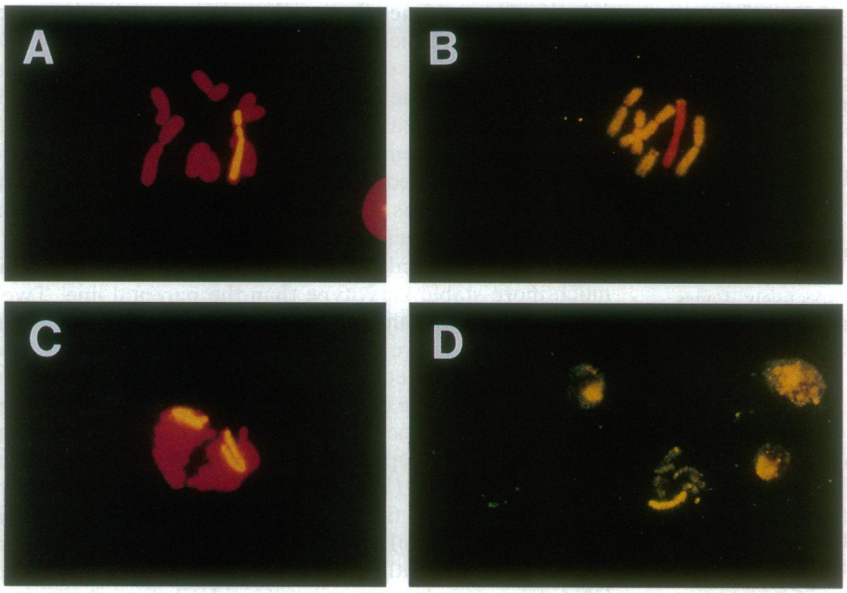

FIG. 4. *In situ* hybridization to HShyg3/11. (*A* and *C*) Hybridization with biotin-labeled pUChshyg. (*B*) Hybridization with biotin-labeled mosquito DNA. (*D*) *In situ* nick-translation on metaphase chromosomes with biotinylated nucleotides. (×1800.)

forms (Fig. 4 *B* and *C*). Flow cytometric analysis (Fig. 5) indicated that the cells contain about 20% more DNA than the untransformed C6/36 cells. This amounts to about 60,000 copies per cell. The karyotype has been stable for >1 year in culture or >200 generations. In addition to the karyotype stability as judged visually, quantitative measurements of DNA content did not change over a 6-month period. The stability of the morphology of the chromosome over a period of 1 year suggests that sequences that function as telomeres may be present as well. The DNA in the pUChshyg chromosome seems to be significantly more accessible to DNase I, as shown by heavy labeling by *in situ* nick-translation (Fig. 4*D*). Other workers have shown that DNase I sensitivity is often related to the transcriptional activity or potential activity of chromatin and that this DNase sensitivity is maintained in metaphase chromosomes (e.g., refs. 32 and 33).

Rescue of Plasmids from Transformants. Although we were unable to identify unintegrated molecules by conventional gel electrophoresis or Southern blot hybridization, plasmids were rescued from these cells by transformation of *E. coli* with undigested cellular DNA. Plasmid DNA was recoverable from cell lines, including the subclone shown in Fig. 4, even after 1 year of continuous passage in culture. Since recombination-deficient (*recA*) strains of *E. coli* were used for rescue, we feel that it is unlikely that these plasmids were generated by recombination from tandem copies of the plasmid in the mosquito DNA after entering the bacteria. Although strains with two different *recA* mutations (*recA1* and *recA13*) were used, it is difficult to rigorously exclude the possibility that residual recombination activity could excise some plasmids. The majority of the recovered plasmids were indistinguishable from the original construct as determined by restriction analysis; however, some rescued plasmids yielded altered restriction patterns (Fig. 6*A*).

These altered plasmids contain the essential genes from pUChshyg (hygromycin resistance, bacterial replication origin, ampicillin resistance); however, they have an altered restriction pattern and are 20–80% larger than the original plasmid. Unique *Hae* III restriction fragments (not found in pUChshyg) were isolated from two of the variant plasmids (nos. 1 and 20, Fig. 6*A*) and used as probes in Southern blot

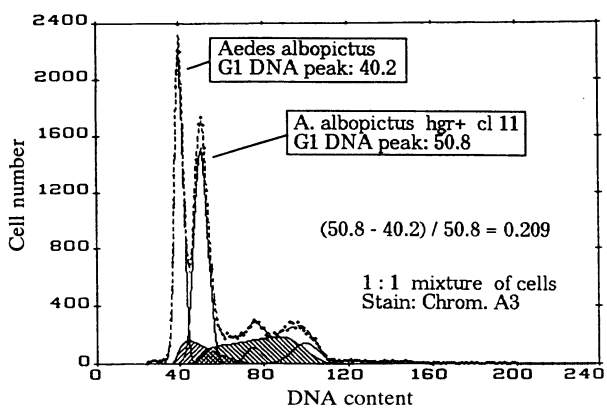

FIG. 5. Flow cytometric measurement of a 1:1 mixture of logarithmic phase C6/36 and HShyg3/11 mosquito cells. By comparison of the positions of the G_1 peaks the DNA content of the HShyg3/11 cells was determined to be ≈26% greater than that in the C6/36 cells. Thus the extra chromosome would account for 20.9% of the DNA of the HShyg3/11 cells. The shaded areas represent calculated S-phase populations for the two cell lines.

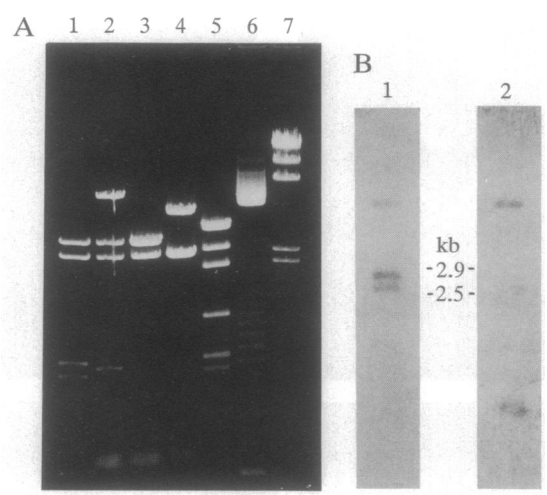

FIG. 6. (*A*) Ethidium bromide-stained gel containing four variant plasmids rescued from HShyg3 mosquito cells. Lane 1, pUChshyg cut with *Hin*dIII. Lanes 2–5, variant plasmids designated nos. 1, 6, 20, and 23 also digested with *Hin*dIII. Lane 6, 123-base-pair ladder (BRL). Lane 7, *Hin*dIII-cut λ DNA. (*B*) Southern blot hybridizations of *Hin*dIII-cut C6/36 DNA. Unique *Hae* III fragments (not found in pUChshyg) were isolated from rescued plasmids, nos. 1 and 20, ^{32}P labeled, and hybridized to Southern blots of *Hin*dIII-cut C6/36 DNA. Lane 1 was hybridized with a fragment from plasmid 1 and lane 2 was hybridized with a fragment from plasmid 20.

hybridizations with DNA isolated from nontransformed C6/36 cells (Fig. 6*B*). Specific bands were identified in the mosquito DNA that hybridized to the plasmid DNA. Thus, both of these new plasmids acquired sequences from the mosquito genome. The rescued plasmids, nos. 1, 6, 20, and 23 (Fig. 6*A*), were used to transform C6/36 cells to determine if the presence of mosquito sequences would increase the frequency of transformation. No increase was found.

DISCUSSION

Stable transformation of the *A. albopictus* C6/36 cell line with plasmids carrying the hygromycin-resistance gene as a selectable marker often results in transformants containing $>10^4$ copies of the plasmid. The plasmid is present in double-minute chromosomes and integrated arrays. Transformation of *D. melanogaster* cells with plasmid occasionally results in transformants containing several hundred to perhaps several thousand copies of the plasmid (34, 35). The factors that governed the copy number of the plasmids in the *Drosophila* transformants are not understood (36). Similarly, the reason for the development of extremely high copy number mosquito cell transformants is not known. Clearly, it is not obligatory for the survival of the cells under the selection conditions imposed because a number of stable transformants of C6/36 cells have been isolated that have only one to a few copies per cell, as shown by Southern blot hybridization (data not shown). The construction of the plasmid pUChshyg does not seem to be unusual. Plasmid derivatives of pUChshyg have been constructed that lack the simian virus 40 sequences and the P-element inverted repeats, and these have been shown to give high copy number transformants in which the plasmid bands are visible on gels in the total DNA stained with ethidium bromide. Thus, the development of high copy numbers is not due to unusual sequences in viral DNA or transposable element DNA.

The rescue of plasmid DNA by transformation of *E. coli* with undigested total cellular DNA was also unexpected, since this suggests that free monomer plasmid copies may be maintained in the cell line along with the minute chromosomes and integrated arrays. Whether these monomers are capable of autonomous replication or are present because of recombination events in the mosquito cells that release them from the minute chromosomes and the arrays is not known. Extrachromosomal circular DNAs having sequences homologous to chromosomal genes have been detected in other eukaryotic systems and are presumably generated by recombination events involving chromosomal DNA (37, 38). The rescue of plasmids carrying mosquito DNA suggests that these must have arisen as a result of recombination between plasmid and chromosomal DNA. Characterization of the junctions between plasmid and cellular DNA should provide insight into recombination mechanisms in these cells.

These mosquito sequences may be useful for development of transformation systems for mosquitoes. For example, a moderately repeated genetic element that increases the frequency of gene amplification in human cells has recently been described (39), and a human DNA fragment with centromere function has been isolated (40). The apparent stability of the *hyg* chromosome in the subclone described in Fig. 4 suggests that it may have acquired sequences that allow stable propagation of this chromosome. These sequences may represent cellular replication origins, centromeres, or telomeres, all of which would be expected to contribute to the stability of extrachromosomal elements or perhaps artificial chromosomes.

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