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Genetics

Introduction of plasmid DNA into the trypanosomatid protozoan *Crithidia fasciculata*

(paromomycin resistance/autonomously replicating sequence/integration/bacterial gene for aminoglycoside 3′ phosphotransferase I)

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ABSTRACT **_Crithidia fasciculata_ cells were treated with a plasmid (pDK96) containing pBR322 sequences, a _Leishmania tarentolae_ maxicircle autonomously replicating sequence, and the bacterial gene for aminoglycoside 3′ phosphotransferase I inserted between the yeast alcohol dehydrogenase 1 promotor and terminator sequences. Resistant colonies were selected on agar plates containing paromomycin and screened for vector DNA by hybridization. Approximately 1% of the resistant colonies contained detectable vector DNA, which was present as extrachromosomal closed circular molecules ranging in copy number from 1 to 160 per cell. The plasmids could be recovered from _Escherichia coli_ transformed to ampicillin resistance with _Crithidia_ total cell DNA. Most of the recovered plasmids were a deleted product of pDK96, which lacked the maxicircle autonomously replicating sequence and contained a unique fragment of _Crithidia_ nuclear DNA present at a low copy number in the wild-type genome. The plasmid DNA in resistant _Crithidia_ was unstable even under selective conditions and was lost within 30 cell divisions.**

The trypanosomatid protozoa represent a large class of lower eukaryotic parasitic cells that possess many advantages for the study of basic biological problems. In addition, they are of interest from the viewpoint of their adaptation to a parasitic way of life. The genomic complexity is approximately 3–15 times that of *Escherichia coli* (1–5), and a small number of permanently uncondensed chromosomes can be visualized by the technique of orthogonal field agarose gel electrophoresis (6, 7). Diploidy is indicated by an analysis of genomic DNA content and kinetic complexity (5) and also by an analysis of restriction site polymorphisms in several glycolytic genes (8). There is some indirect evidence derived from analysis of isoenzyme patterns for the existence of a sexual phase in the life cycle of *Trypanosoma brucei* (9), and there is one report of the recombination of drug-resistant markers in *Crithidia fasciculata* during growth in culture (10). However, there is no system available for classical genetic analysis of any trypanosomatid protozoan and there are no selectable vectors available for the application of recombinant techniques for gene manipulation in these organisms. The possibility of developing such a vector is demonstrated by the discovery of two endogenous extrachromosomal multicopy plasmids, one of which contains the dihydrofolate reductase gene, in methotrexate-resistant *Leishmania tropica* (11).

We have approached this problem by constructing various pBR322-based plasmids containing the bacterial aminoglycoside 3′ phosphotransferase I (Neo^r) genes as eukaryotic selectable markers (12–14) and a sequence known to function as an autonomously replicating sequence (ARS) in yeast (15) and have attempted to transform *C. fasciculata* cells by several methods. We report in this paper the successful introduction of DNA into *Crithidia* with one such plasmid construct.

MATERIALS AND METHODS

Cell Culture. The clonal strain of *C. fasciculata* maintained in our laboratory for 15 years was cultured in Kidder–Dutta (KD) defined medium (16) with gentle agitation at 25°C by subinoculation every 3 days.

Construction of Plasmids. See legend to Fig. 1.

Transformation Protocol. The cells were treated by a modified version of a DNA transformation protocol for yeast (17). Approximately 10^8 logarithmic-phase cells were harvested in a clinical centrifuge, washed in 5 ml of Hanks' balanced salt solution at 4°C, and resuspended in 1 ml of 1 M sorbitol/10 mM Bicine [*N,N*-bis(2-hydroxyethyl)glycine], pH 8.35/3% ethylene glycol. Fifty micrograms of plasmid DNA in 25 μl of water was added and the cells were incubated for 10 min at 25°C. Ten milliliters of 40% polyethylene glycol 1000 (PEG 1000)/200 mM Bicine, pH 8.35, was added with gentle mixing. Cells were kept stationary for 20 min at 25°C, harvested by centrifugation, washed twice, and resuspended in 5 ml of KD medium. These cells were transferred into 100 ml of KD medium in an Erlenmeyer flask and incubated for 17 hr at 25°C with gentle agitation. The cells were counted and concentrated by centrifugation to 10^8 cells per ml. One hundred microliters (10^7 cells) was spread on 1% KD agar supplemented with paromomycin at 300 μg/ml in a 100 × 15 mm plastic Petri dish. We observed that plating $>10^7$ cells per dish resulted in an unacceptably high background growth of cells. The agar plates were sealed with Parafilm and stored inverted for 7 days at 25°C.

Screening for Vector DNA in Paromomycin-Resistant _Crithidia_ Colonies. Individual resistant colonies were transferred to 2 ml of liquid medium containing paromomycin at 50 μg/ml and grown to a cell density of 10^6 cells per ml. The division period of the resistant cells under these conditions was 13 hr, which is ≈2-fold longer than that of the wild-type (wt) cells in drug-free medium. Cells were then lysed and total cell DNA was bound to a nitrocellulose filter using a dot blot manifold from Bethesda Research Laboratories. The filter was incubated with a mixed probe containing ^{32}P-labeled pBR322 DNA and the Neo^r gene fragment. This method could detect a single-copy sequence in total DNA from 10^6 cells, as determined by hybridization with the pDSC6 (Fig. 1*B*) single-copy probe (data not shown). Those cultures that gave a positive hybridization signal were inoculated into 100 ml of drug-containing medium and grown at 25°C for several

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Abbreviations: wt, wild-type; kb, kilobase(s); ADH, alcohol dehydrogenase; ARS, autonomously replicating sequence; KD, Kidder–Dutta.

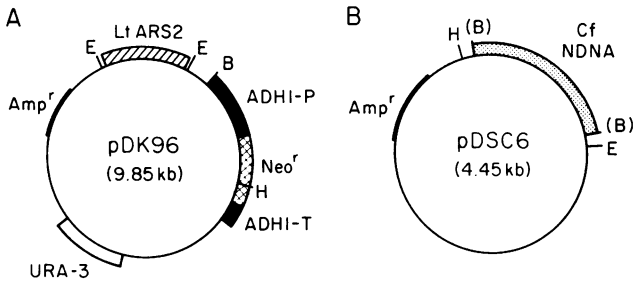

FIG. 1. Plasmids constructed for transformation experiments. (*A*) pDK96 is a 9.85-kilobase (kb) plasmid derived from the yeast shuttle plasmid YIp5 (19). LtARS2, a 1.87-kb *Sau*3A maxicircle fragment from *L. tarentolae* (20), was inserted into the *Bam*HI site of YIp5 (hatched box). Growth of this plasmid in *E. coli* resulted in a modified plasmid in which an additional *Eco*RI site was present adjacent to the LtARS2 fragment. A 1.50-kb fragment containing the yeast alcohol dehydrogenase gene 5′ (ADH1-P) and 3′ (ADH1-T) flanking regions (solid boxes) from AAH5 (21) was then inserted into the *Sal* I site. A 1.22-kb *Ava* II fragment containing the Neo^r gene (crosshatched box) from Tn*903* (22) was then inserted between the ADH1 promoter and terminator regions. (*B*) pDSC6 was constructed by the insertion of random 1- to 2-kb *Sau*3A fragments of *C. fasciculata* nuclear DNA (Cf NDNA) into the *Bam*HI site of pUC9 (23) and selection of chimeric plasmids having single-copy inserts by hybridization of genomic blots. pDSC6 contains a 1.75-kb nuclear DNA fragment (stippled box) that hybridizes to one band in genomic DNA digested with each of several enzymes. E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII.

generations. Total DNA was isolated from 10^9 cells and purified on a CsCl/ethidium bromide equilibrium gradient.

Reagents and Chemicals. Paromomycin sulfate (Humatin) was obtained from Parke-Davis. G418 was obtained from Schering. PEG 1000 was obtained from Sigma. KD medium was prepared from reagents purchased from Sigma. Restriction enzymes were purchased from Bethesda Research Laboratories and New England Biolabs and used as recommended by the manufacturers.

Gel Electrophoresis. Agarose gel electrophoresis, staining, blotting, and hybridization were performed by standard procedures (18). λ DNA digested with *Hin*dIII and bacteriophage φX-174 replicative form DNA digested with *Hae* III were used as molecular weight standards.

RESULTS

Construction of Transformation Vector. The bacterial Neo^r genes have been used to efficiently inactivate aminoglycoside compounds in several eukaryotic cell systems. The aminoglycoside antibiotic paromomycin inhibited the growth of wt *Crithidia* in defined KD liquid medium at 50 μg/ml and on KD agar at 300 μg/ml. However, spontaneous resistant colonies appeared on agar at a frequency of $\approx 10^{-7}$ after 7 days' growth and grew when transferred to liquid medium containing paromomycin.

pDK96 (Fig. 1*A*) is a YIp5 derivative that has a fragment encompassing the Neo^r gene from Tn*903* (22) inserted between regulatory regions of the yeast alcohol dehydrogenase 1 (ADH1) gene (21). This vector also contains a 1.87-kb fragment (LtARS2) from the maxicircle DNA of the related trypanosomatid, *Leishmania tarentolae*, which acts as an ARS in yeast (15). High-frequency transformation of *E. coli* (>10^6 transformants per μg) and *Saccharomyces cerevisiae* (>500 transformants per μg) to G418 resistance was obtained with pDK96 DNA (results not shown). The plasmid was stably maintained under selective conditions at a high copy number in the transformed cells of both species.

The Frequency of Paromomycin-Resistant *Crithidia* Cells in DNA-Treated Cultures Is Low but Significant. Cells treated with pDK96 plasmid DNA by a modified PEG method were screened for paromomycin resistance by agar plating after a 17-hr period of phenotypic expression in drug-free medium. Approximately 10^7 cells were plated on agar containing paromomycin at 300 μg/ml. A control culture was treated identically except for the absence of vector DNA. In all cases there was an increase in the number of resistant colonies appearing from the DNA-treated versus non-DNA-treated cells (Table 1). The colonies appearing from DNA-treated and non-DNA-treated cells were heterogeneous in size, ranging from small barely visible colonies to large colonies up to 3 mm in diameter. The small colonies did not grow out

Table 1. Plating efficiencies of *Crithidia* on paromomycin plates with and without pretreatment with pDK96 DNA

	With DNA		Without DNA	
Exp.	Plating efficiency	Number of resistant colonies / Total cells plated	Plating efficiency	Number of resistant colonies / Total cells plated
1	3.0×10^{-6}	$\frac{148}{5.0 \times 10^7}$	2.1×10^{-6}	$\frac{106}{5.0 \times 10^7}$
10	6.8×10^{-6}	$\frac{136}{2.0 \times 10^7}$	2.2×10^{-6}	$\frac{44}{2.0 \times 10^7}$
11	7.6×10^{-7}	$\frac{19}{2.5 \times 10^7}$	2.5×10^{-7}	$\frac{5}{2.0 \times 10^7}$
12	$>7.5 \times 10^{-5}$	$\frac{>1500}{2.0 \times 10^7}$	6.0×10^{-6}	$\frac{30}{5.0 \times 10^6}$
13	2.3×10^{-5}	$\frac{467}{2.0 \times 10^7}$	2.5×10^{-7}	$\frac{5}{2.0 \times 10^7}$
14	1.3×10^{-6}	$\frac{26}{2.0 \times 10^7}$	9.0×10^{-7}	$\frac{18}{2.0 \times 10^7}$

Cells were treated with 50 μg of pDK96 DNA by the PEG method (with DNA) or by the same method without DNA. Plating efficiencies were calculated by dividing the total number of colonies larger than 1 mm in diameter after 7 days' growth by the total number of cells plated. The plating efficiency of wt cells on drug-free plates is 0.3–0.5.

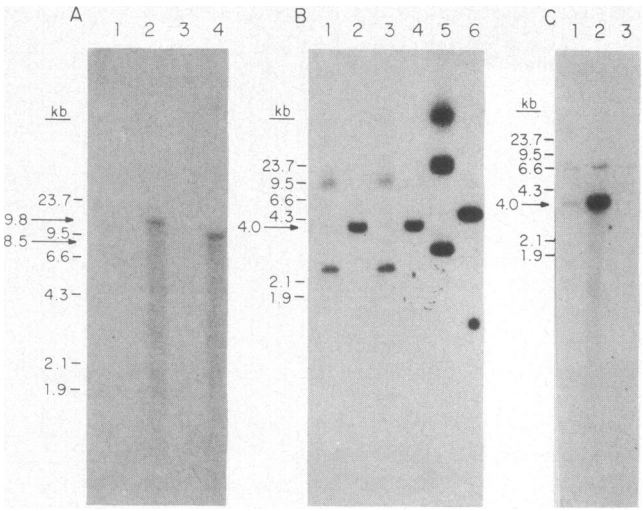

FIG. 2. Detection of vector sequences in total cell DNA from resistant *Crithidia* by Southern blot analysis. (*A*) Ten micrograms of total cell DNA from IR4 cells (lanes 2 and 4) and untreated wt cells (lanes 1 and 3) was digested with *Bam*HI (lanes 1 and 2) or *Eco*RI (lanes 3 and 4), electrophoresed on a 0.8% agarose gel, and blotted onto nitrocellulose. A mixture of ^{32}P-labeled pBR322 DNA and Neo^r fragment was used as a probe. (*B*) Blot of DNA from XR2 cells (lanes 1 and 2) and XR30 cells (lanes 3 and 4) digested with *Eco*RI (lanes 2 and 4) or undigested (lanes 1 and 3). A purified 4.0-kb plasmid (pIR48) recovered from IR4 cells was also run undigested (lane 5) or *Eco*RI-digested (lane 6) to compare the mobility of a similar-sized purified plasmid under the same gel conditions. Same probe as *A*. (*C*) Blot of DNA from XIR28 cells (lane 1), XR2 cells (lane 2), and untreated wt cells (lane 3) digested with *Eco*RI. ^{32}P-labeled pDSC6 was used as a probe.

upon subculture in drug-containing medium. The large colonies contained resistant cells that grew out upon subculture in drug-containing medium. Increasing the amount of plasmid DNA did not increase the frequency of appearance of large colonies but did increase the frequency of small colonies (data not shown), suggesting that the small colonies may represent abortive transformants.

Total DNA from a Small Proportion of Resistant Colonies Hybridizes with Vector Sequences. Resistant *Crithidia* colonies from DNA-treated cultures were screened for the presence of vector DNA sequences by dot blot hybridization. Approximately 1% of the paromomycin-resistant colonies in experiments 1, 10, 11, 12, 13, and 14 contained DNA homologous to pBR322 and the Neo^r gene fragment.

Total cell DNA was isolated from a resistant colony from experiment 1 (IR4) that showed positive hybridization to vector sequences in the dot blot analysis. wt DNA and IR4 DNA were digested with *Bam*HI or *Eco*RI and probed with a mixture of ^{32}P-labeled pBR322 DNA and the Neo^r gene fragment. The probe hybridized with a 9.8-kb *Bam*HI band and a 8.5-kb *Eco*RI band in the IR4 DNA only (Fig. 2*A*, lanes 2 and 4). These band sizes correspond to the expected fragments generated by digestion of circular pDK96 with the same enzymes.

A single 4.0-kb band hybridized to the mixed probe in Southern blots of *Eco*RI-digested total cell DNA from dot blot-positive resistant colonies obtained in experiments 10, 11, 12, and 13 (Table 2). Faint hybridization of a 20-kb *Eco*RI band and a 4.5-kb *Hin*dIII band was obtained with DNA from two resistant colonies from experiment 14 (XIV61 and XIV64, Table 2).

Vector sequences were lost in all positive cultures within 30 cell divisions in the presence or absence of paromomycin, although the resistance phenotype was stably maintained during growth in the presence of drug. The percentage of resistant cells in a culture grown in the presence of the drug was consistently 60–80%, as determined by replica-plating on drug-free and drug-containing agar. In one experiment, growth of IR4 cells in the absence of drug led to a transient decrease in the percentage of resistant cells to ≈10% after 14–30 cell divisions and then to an increase to the original 60–80% value with continued growth, although vector sequences were no longer detectable after 36 divisions (data not shown).

The Copy Number of Vector Sequences Varies in Different *Crithidia* Cultures. To estimate the copy number of the sequences hybridizing to the vector probe in DNA from resistant cells, a plasmid was constructed (pDSC6, Fig. 1*B*) that would hybridize to both pBR322 sequences and a single-copy fragment from *Crithidia* nuclear DNA. An example of this method for determining copy number is shown in Fig. 2*C*, lanes 1 and 2. *Eco*RI-digested genomic DNA from XIR28 and XR2 cells was probed with ^{32}P-labeled pDSC6 DNA. The 6.6-kb band represents hybridization of the nuclear DNA fragment in pDSC6 to the corresponding single-copy fragment released from nuclear DNA. Densitometric analysis of the autoradiograph indicated that the 4.0-kb pBR322-positive

Table 2. Size and relative abundance of plasmids recovered from total cell DNA of resistant *Crithidia* cells treated with pDK96 plasmid DNA

Exp.	*C.f.* clone	Amp^r *E. coli* colonies*	Number tested	Plasmid size, kb	*C.f.* nuclear DNA relative hybridization intensity†	*Eco*RI band size, kb
1	IR4	20	10	9.8, 4.0	Low copy	9.8, 4.0
10	XR2	900	5	4.0	High copy	4.0
10	XR30	900	5	4.0	High copy	4.0
11	XIR28	3	3	4.0	Low copy	4.0
12	XIIR19	1	1	4.0	No hybridization	
12	XIIR21	1	1	4.0	No hybridization	
12	XIIR118	1	1	4.0	No hybridization	
13	XIIIR78	45	6	4.0	Medium copy	4.0
14	XIVR61	0	—	—	Low copy	20.0
14	XIVR64	0	—	—	Low copy	4.5‡

C. f., Crithidia fasciculata.

*Approximately 20 μg of total cell DNA was used to transform *E. coli* to Amp^r.

†Approximately 10 μg of total cell DNA was digested with *Eco*RI and probed with the mixture of nick-translated pBR322 DNA and the Neo^r fragment. Approximate copy number values were based on the relative intensity of the *Eco*RI band signal migrating at the indicated molecular weight.

‡DNA from XIVR64 was digested with *Hin*dIII.

band in XIR28 is present at 1 copy per cell, whereas the same band in XR2 is present at ≈160 copies per cell.

Vector Sequences Are Maintained on an Extrachromosomal Plasmid. To test for the presence of extrachromosomal plasmid DNA in the resistant cells, aliquots of total cell DNA were used to transform *E. coli* to ampicillin resistance. Transformation of *E. coli* with IR4 DNA yielded 20 Amp^r colonies, 10 of which were tested for the presence of vector DNA by Southern blots. Five of these *E. coli* colonies contained a 9.8-kb plasmid and 5 contained a 4.0-kb plasmid (Table 2), both of which contained sequences homologous to pDK96. Restriction enzyme analysis of the 9.8-kb plasmid revealed it to be similar to pDK96 (data not shown).

In all other cases (XR2, XR30, XIR28, XIIR19, XIIR21, XIIR118, XIIIR78), only a 4.0-kb plasmid was recovered (Table 2). The number of *E. coli* Amp^r colonies was proportional to the hybridization intensity of the 4.0-kb band when the digested total cell DNA was probed with vector sequences (Table 2). When undigested total DNA from XR2 and XR30 was probed with vector sequences in a Southern blot analysis, two bands hybridized, suggesting the presence of closed circular and relaxed forms of an extrachromosomal circular molecule (Fig. 2*B*, lanes 1 and 3). Furthermore, the component of cellular DNA that hybridized with vector sequences comigrated with the upper and lower bands in a CsCl/ethidium bromide equilibrium gradient, again implying an extrachromosomal localization (data not shown).

The 4-kb Plasmid Contains Sequences from pDK96 and *Crithidia* Nuclear DNA and Lacks the Maxicircle ARS. To further characterize the 4.0-kb molecule obtained from the majority of *Crithidia* transformants, a partial restriction map was constructed of the plasmid pXR30 recovered from XR30 *Crithidia* cells (Fig. 3). The 4.0-kb plasmids recovered from three other independent *Crithidia* transformants exhibited similar restriction maps. A blot hybridization of digested pXR30 DNA with specific fragments of the original transforming plasmid, pDK96 (Fig. 3), showed homology with probes specific for the Neo^r, ADH1, and pBR322 sequences but a lack of homology with the LtARS2 sequence. A region of ≈1.5 kb of the pXR30 molecule exhibited no homology to the pDK96 sequences. The pXR30 plasmid was unable to confer resistance to G418 to either *E. coli* or yeast.

To test for a possible nuclear DNA origin of the nonhomologous 1.5-kb region, pXR30 DNA was radioactively labeled and used to probe genomic DNA digests of wt *Crithidia* (Fig. 4). Hybridization to a small number of bands released by each enzyme digest suggested that the pXR30 DNA contains a genomic sequence that is present at a low copy number in wt cells. Three of four 4.0-kb plasmids obtained from independent transformations hybridized to the same genomic bands as pXR30 (data not shown).

The pXR30 Plasmid Is Capable of Transfecting *Crithidia* but Is Less Efficient than pDK96. pXR30 plasmid DNA was used to transform wt *Crithidia* by the standard PEG method. Of five independent experiments (≈500 paromomycin-resistant colonies), only 1 colony contained vector sequences by dot blot hybridization, and quantitation of a genomic DNA blot indicated that the vector sequence was present at a single copy per cell. The restriction map of a plasmid recovered from these cells by transformation of *E. coli* was identical to that of pXR30.

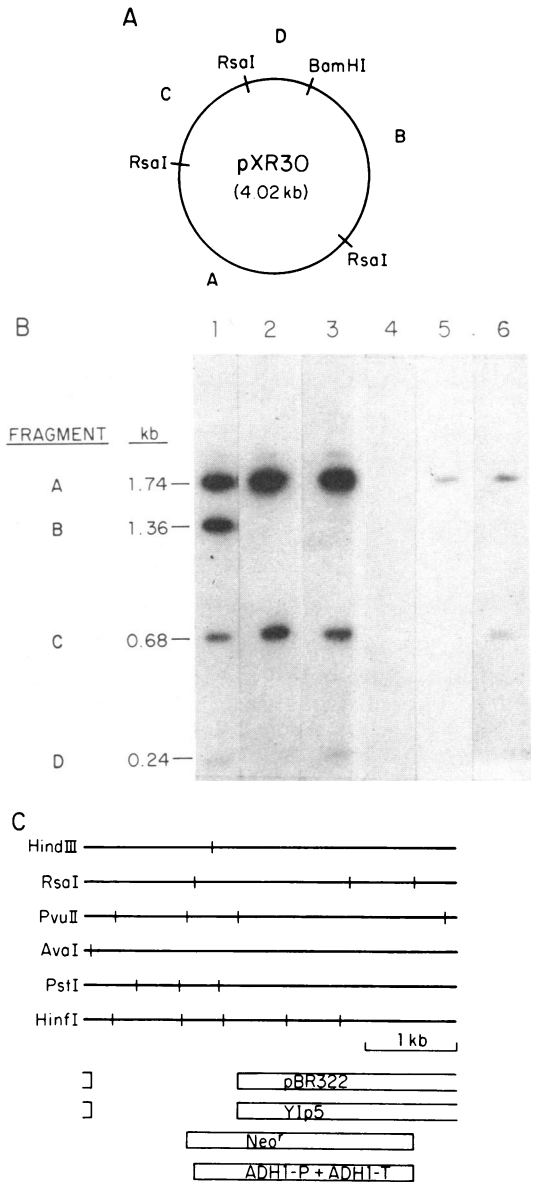

FIG. 3. Hybridization analysis of pXR30 DNA. (*A*) Map of *Rsa* I and *Bam*HI sites in pXR30 DNA. (*B*) Southern blot analysis of pXR30 DNA digested with *Rsa* I + *Bam*HI. ^{32}P-labeled probes were pXR30 (lane 1), YIp5 (lane 2), pBR322 (lane 3), LtARS2 (lane 4), Neo^r (lane 5), and ADH1-P and ADH1-T (lane 6). (*C*) Summary of additional hybridization results with pXR30 DNA. The upper portion is a partial restriction map of *Eco*RI-linearized pXR30 DNA. The open boxes below the map span restriction fragments that hybridize with the indicated ^{32}P-labeled probe. There was no hybridization with a LtARS2 probe.

DISCUSSION

We have shown that the pDK96 plasmid DNA, constructed from the pBR322-derived yeast plasmid, YIp5, a maxicircle ARS sequence from *L. tarentolae*, and the bacterial Neo^r gene flanked by the yeast ADH1 regulatory sequences can enter *Crithidia* cells in the presence of PEG. A transient recombinational event between pDK96 and the nuclear DNA gave rise to a smaller modified plasmid in which, in all cases tested, the maxicircle ARS sequence had been deleted and an ≈1.5-kb nuclear sequence inserted. The deleted plasmid replicated extrachromosomally, yielding from 1 to 160 copies per cell, but was unstable even under selective pressure and was lost within 20–30 cell divisions. One deleted plasmid, pXR30, still contained Neo^r sequences but was no longer active in the expression of G418 resistance in bacteria or yeast, possibly due to a rearrangement or deletion within the Neo^r gene region (data not shown). However, the pXR30 plasmid could be introduced into and recovered from wt *Crithidia* cells, although at a lower frequency than the pDK96 plasmid, suggesting that the deleted plasmid is able to replicate autonomously for a transient period independent of selection.

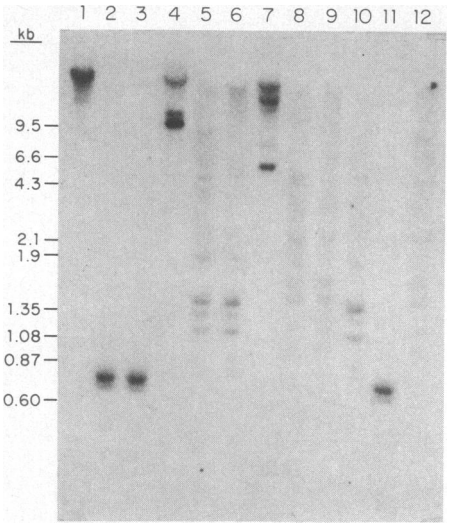

FIG. 4. The presence of pXR30 DNA sequences in *Crithidia* nuclear DNA. Ten micrograms of nuclear DNA from wt cells was digested with each enzyme, electrophoresed on a 0.8% agarose gel, blotted, and probed with ^{32}P-labeled pXR30 DNA. Enzymes used were *Eco*RI (lane 1), *Eco*RI + *Msp* I (lane 2), *Msp* I (lane 3), *Bam*HI (lane 4), *Bam*HI + *Sau*3A (lane 5), *Sau*3A (lane 6), *Hin*dIII (lane 7), *Hin*dIII + *Taq* I (lane 8), *Taq* I (lane 9), *Hin*dIII + *Sau*3A (lane 10), *Hin*dIII + *Msp* I (lane 11), and *Bam*HI + *Taq* I (lane 12).

It has not been established that the Neo^r gene is being expressed once the plasmid has entered the *Crithidia* cell. There was an increase in the appearance of paromomycin-resistant colonies after treatment with pDK96 DNA, but only a small fraction of these colonies actually contained detectable vector DNA at the time of isolation. It is possible that the Neo^r gene is transiently expressed prior to the apparently specific recombination and rearrangement of the pDK96 plasmid, giving rise to the increase in frequency of resistant colonies. Alternatively, recombination between pDK96 and the nuclear DNA may have occurred at random sites, and those plasmids that acquired a nuclear origin of replication may have been selectively replicated for a transient period independent of Neo^r expression and were detectable in a small percentage of spontaneous resistant colonies. A more useful vector may require a homologous *Crithidia* promoter to allow expression of the Neo^r gene and a homologous centromeric sequence to increase the mitotic stability of the plasmid.

The successful introduction of DNA into *Crithidia* was obtained in five independent experiments performed over a period of 8 months, but we were unable to reproduce this phenomenon thereafter. The reason for this lack of reproducibility is unknown but could be due to some uncontrolled variation in the reagents or procedures. Nevertheless, it is clear that foreign plasmid DNA can enter and replicate extrachromosomally within a trypanosomatid protozoan, a finding that may open the door for the development of a usable selectable vector system for the manipulation of genes in this important family of lower eukaryotic cells.

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