Stable transformation of *Drosophila* Kc cells to antibiotic resistance with the bacterial neomycin resistance gene

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By transfection with a plasmid containing the APH(3') gene under control of the HSV I thymidine kinase promoter, independent series of stably transformed *Drosophila* cells were established and grown for more than one and a half years under highly selective pressure (2 mg G 418/ml). Analysis of transformed *Drosophila* cell DNAs shows that the APH(3') gene was integrated into the genome. Neomycin phosphotransferase is constitutively expressed in transformed cells. This efficient selective system by a dominant marker makes it possible to introduce, by cotransfection, any DNA sequence of interest into the genome of cultured *Drosophila* cells.

Cell transformation (Drosophila) *G 418* *Antibiotic resistance* *Aminoglycoside 3'-phosphotransferase gene*

1. INTRODUCTION

The functional analysis of DNA sequences and of their control mechanisms requires the reintroduction of the cloned sequences (with or without modifications) into the genome either of embryos or of cultured cells.

A variety of techniques for gene transfer have been devised and the most widely used is the DNA-Ca phosphate co-precipitation procedure developed for mammalian cells by Wigler et al. [1].

With regard to *Drosophila*, the elegant method published by Spradling and Rubin [2] utilizes the natural mobility of a *Drosophila* transposon (P element) for making transgenic flies. It is an unrivalled approach for studying the tissue and stage specificities of the expression of transfected genes.

Concurrently, transformation of in vitro cultured *Drosophila* cells retains, however, several obvious advantages: under conditions of transient expression, it makes possible a direct analysis of the comparative efficiency of putative promoters and of their regulation by various stimuli [3–5]. But, the main interest of stably transformed cell lines may be the synthesis, in mass cultures, of specific and rare gene products (e.g. regulatory proteins). Such stable cell transformation requires a good selection system. Two methods have been described. One is based on the resistance to methotrexate conferred by the prokaryotic dihydrofolate reductase gene [6] and the other relies upon the use of *E. coli* guanine phosphoribosyl transferase gene as a selective marker [7]. Both remain delicate to handle, because they interfere with the synthesis or salvage pathways of nucleotides and because no strictly defined medium is available for long culturing of *Drosophila* cells.

Therefore, for *Drosophila* cells we have adapted a much simpler selective system, the efficient G 418 antibiotic resistance independently devised for mammalian cells by Colbere-Garapin et al. [8] and Southern and Berg [9]. We showed that the aminoglycoside phosphotransferase gene

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Abbreviations: HSV I, herpes simplex virus type I; TK, thymidine kinase; MMTV, mouse mammary tumor virus; LTR, long terminal repeat; kb, kilobase pairs; APH (3'), aminoglycoside 3'-phosphotransferase

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(APH(3') gene), from the bacterial transposon Tn5 [10], can confer on *Drosophila* cells a high level of resistance to the aminoglycoside antibiotic G 418. Concurrent with our experiments, Rio and Rubin [11] reported a study of *Drosophila* cell transformation by the *neo* gene linked to the *copia* LTR promoter DNA segment.

2. MATERIALS AND METHODS

2. *Plasmid vector*

We used a derivative of the pAG60 plasmid designed by Colbere-Garapin et al. [8] in which the APH(3') gene is under the control of the promoter region of HSV I TK gene. At the unique *Cla*I site (see fig. 1 [8]) one MMTV LTR sequence has been inserted by B. Devaux and M. Crepin; this plasmid is called pAG LTR X I (fig.1).

2.2. *Cell transformation*

2.2.1. *Drosophila* cell line

89K is a clone from Kc cells [12] grown in D 22 medium supplemented with 5% fetal calf serum. One day before transfection, about 10^7 cells were seeded with fresh medium into each 50 ml plastic culture flask (Nunclon).

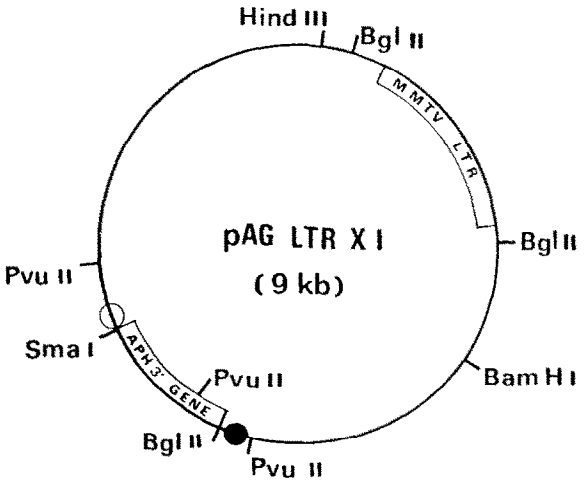

Fig.1. Structure of the pAG LTR X I plasmid. Only the restriction sites used for the analysis of transformed cell DNAs are given. The filled circle represents the TK eukaryotic promoter region, the open circle represents the TK polyadenylation site.

2.2.2. DNA transfection

The method is slightly modified from the DNA-Ca phosphate co-precipitation procedure of Wigler et al. [1]. Following a hypotonic shock (2 h in D 22 medium diluted with an equal volume of water), the medium was changed (5 ml of fresh standard medium) and then 0.5 ml of the suspension of DNA-Ca phosphate complex (containing 10–15 μg of plasmid DNA) added to the flask and allowed to sediment over the cells for 24 h.

2.2.3. Selection strategy

Owing to the low cloning efficiency of *Drosophila* cells [13], too rapid an elimination of the non-transformed cell population would leave the few transformed cells no chance to grow. So, the selective drug (G 418 Gibco) was added to the medium 2 or 3 days after transfection, and moreover, its concentration was maintained during the first 2 or 3 weeks at a level just sufficient to kill the sensitive cells. Only after this period is the final selective concentration (2 mg/ml) reached.

2.3. *DNA analysis*

High-M_r DNA was prepared from semi-confluent cells by the SDS-proteinase K method [14]. Extrachromosomal DNA from the cells and plasmid DNA were isolated according to [15] and [16], respectively. Chromosomal DNA was cleaved with restriction enzymes and, after migration in agarose gels, transferred to a nitrocellulose filter (BA 85-Schleicher and Schuell). Hybridizations were performed with nick-translated *Bgl*II-*Sma*I or *Pvu*II-*Pvu*II (as APH(3') probes) and *Bgl*II-*Bgl*II (as MMTV-LTR probe) fragments of pAG LTR X I (fig.1).

2.4. *Neomycin phosphotransferase assay*

The neomycin phosphotransferase activity was assayed following the method of Reiss et al. [17].

3. RESULTS

3.1. *G 418 resistance of transformed cells*

We found the lethal concentration of G 418 for non-transformed cells to be much higher than for mammalian cells. With our 89K clone, complete cell death is observed within 2 weeks in the presence of 600–700 μg G 418/ml. Another cell strain with a quite different genotype [18] could

survive for several weeks under a drug concentration of 1 mg G 418/ml. Consequently, we decided to use a selective concentration as high as 2 mg G 418/ml, and to maintain such a selective pressure for months.

After transfection with the pAG LTR X I plasmid DNA, we obtained in two independent series of experiments transformed cell lines (designated here I and II) which can resist such a high concentration of G 418; resistant cell line I was maintained in the presence of 2 mg G 418/ml for 18 months, which corresponds to about 70 subcultures, even if the cell growth rate is slightly lowered.

It must be noted that because of the poor adherence of *Drosophila* cells, it is difficult to determine accurately the transformation frequency; it nevertheless was estimated to be about 10^{-5}, from the number of resistant cell colonies we observed in the flasks 2–3 weeks after transfection.

3.2. *Presence of plasmid sequences (APH(3')gene and MMTV LTR sequence) in transformed cell DNAs*

In the DNA of control *Drosophila* 89K cells no sequence homologous to the APH(3') probe can be detected (fig.2A, lane 1), whereas such sequences are present in the genomic DNAs from the two transformed cell lines I and II (fig.2A, lanes 2–9). On the other hand, in cell line I, no sequence homologous to the APH(3')gene could be revealed in the circular extrachromosomal DNA prepared according to Stanfield and Helinski [15].

We note that the Southern analyses of DNAs from cell lines I and II showed great differences in the hybridization patterns obtained with the probes we used.

3.2.1. In transformed cells I

In transformed cells I, two *Pvu*II (1.3 and 0.8 kb long; fig.2A, lane 6), one *Bgl*II (11 kb; fig.2A, lane 7), one *Hin*dIII (24 kb; fig.2A, lane 8) and one *Bam*HI (17 kb; fig.2A, lane 9) fragment are detected with the *Bgl*II-*Sma*I APH(3') probe. These fragments have lengths unexpected from the physical map of the vector plasmid, except for the 1.3 kb *Pvu*II fragment.

Using partial APH(3') probes (*Pvu*II fragments corresponding either to the 3' or 5'-end of the APH(3') gene) it was possible to characterize the

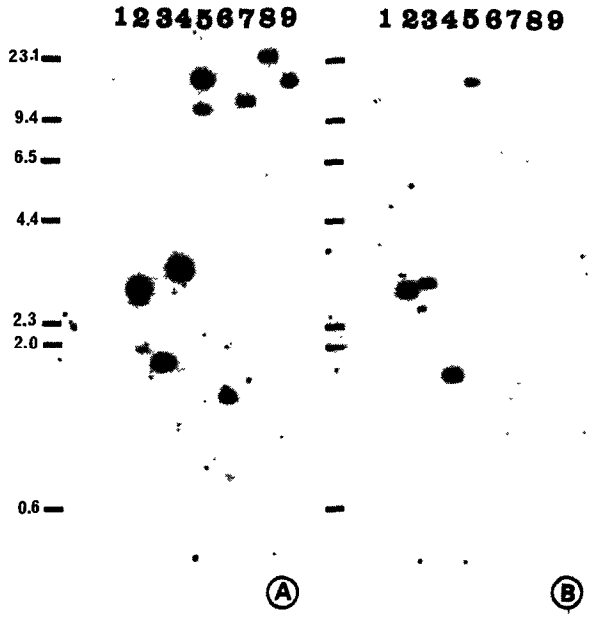

Fig.2. Analysis of DNA from G 418 resistant cell lines. High-M_r DNA was extracted from 89K cells (non-transformed or transformed cells (lines I and II)). 10 μg of DNA were cleaved with restriction enzymes and blot hybridized. Lane 1: control cells (89K clone). Lanes 2–5: resistant cells (series II) after 4 months of selective treatment. Lanes 6–9: resistant cells (series I) after 10 months of selective treatment. DNAs were digested with *Pvu*II (lanes 2,6), *Bgl*II (3,7), *Hin*dIII (4–8) or *Bam*HI (1,5,9). A, hybridization with the APH(3') probe (*Bgl*II-*Sma*I fragment) (10^8 cpm $^{32}P/\mu g$ DNA). B, hybridization with the MMTV LTR probe (*Bgl*II-*Bgl*II fragment) (4×10^7 cpm $^{32}P/\mu g$ DNA). Markers bands were produced by λ DNA *Hin*dIII digest (PL Biochemicals). Indicated sizes are in kb.

two *Pvu*II genomic fragments: the 1.3 kb fragment corresponds to the 1.3 kb plasmid fragment, whereas the 0.8 kb fragment hybridizes with the 0.5 kb probe, which indicates a rearrangement near the 5'-end of the gene.

Moreover, the absence of any sequence contained in the *Bgl*II-*Bgl*II (MMTV LTR) probe, used here as a secondary marker, confirms that a drastic rearrangement of the plasmid sequences occurred in the cell line I (fig.2B, lanes 6–9).

3.2.2. In transformed cells II

In transformed cell line II, again the lengths of the hybridizing bands after *Pvu*II (2.7, 2.6, 1.8 kb), *Bgl*III (1.6 kb), *Hin*dIII (3.1 kb) or *Bam*HI

(17,11 kb) digestions are not consistent with the insertion of a whole unmodified plasmid sequence (fig.2A, lanes 3–6). The APH(3′) sequence is now surrounded by new *Pvu*II sites and a new *Bgl*II site is present near the 3′-side of the gene. These data show that the APH(3′) coding sequence was integrated into the genome with little surrounding plasmid sequences.

In contrast with the situation found in cell line I, plasmid sequences belonging to the *Bgl*II-*Bgl*II fragment containing the MMTV LTR were observed in cell line II (fig.2B, lanes 2–6). But, again the hybridization patterns show that these sequences were rearranged.

We also compared the DNAs of cells, from line I, cultured for 4, 5 or 8 months in the presence of the antibiotic: the hybridization patterns obtained with the APH(3′) probe were found to be identical (not shown). This is indicative of a great stability of the integrated sequences, and is consistent with the idea that rearrangements took place during or immediately after transfection, and not afterwards in the course of the selective treatment. Such rearrangements, including deletions, duplications or insertions into the plasmid DNA sequence were reported in mammalian cells [19,20]. It seems that we are faced with a similar phenomenon in our transformed *Drosophila* cells, with more or less drastic alterations of the plasmid sequences.

So, our data show that the APH(3′) gene was integrated into the genomes of the resistant cell lines I and II, but in different ways. Another difference between these two transformed cell lines is the copy number per haploid genome of the APH(3′) gene; by reconstitution experiments [21] (not shown) it was estimated to be close to one in cell line I and one or two in line II, which is in agreement with the Southern blotting experiments shown in fig.2A.

3.3. *Expression of the APH(3′) gene: characterization of neomycin phosphotransferase (NPT) activity*

The correlation between resistance to the antibiotic G 418 and the presence of the APH(3′) gene in the cell genome is confirmed by the demonstration that NPT activity cannot be detected in the original 89K clone (fig.3A, lane 2) whereas NPT activity is revealed in *Drosophila* transformed cells (fig.3A, lanes 1,3,5 and B, lanes 1,2).

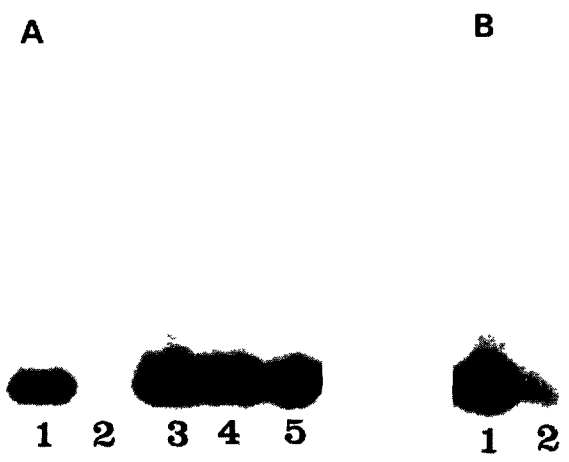

Fig.3. Neomycin phosphotransferase assay. 225 μg of total proteins from each cell extract were analysed according to Reiss et al. [16]. A. Lane 2: control 89K cells. Lanes 1,3,4,5: G 418 resistant cells (series no.I). Lanes 1,3,4: 3 independently subcultured cells grown with 2mg G 418/ml. Lane 5: resistant cells grown for 3 weeks without G 418. B. Comparison of enzymatic activities in the two resistant cell lines, in presence of G 418. Lane 1: series no.I (after 1 year of selective treatment). Lane 2: series no.II (after 4 months of selective treatment). (Panels A and B originate from the same blot, so the control is the same in A and B.)

From the blotting experiment according to Reiss et al. [17] the apparent M_r of the enzyme synthesized in *Drosophila* cells was found to be roughly the same as that of the prokaryotic form expressed in a kanamycin-resistant bacterial strain (containing the plasmid pSC 105 [22] (not shown). This differs from the situation observed in some mammalian cells, where oligomeric forms of the enzyme seemed to be present [8,17].

In the absence of the selective drug, and even after a 3 week period of culture without G 418, the enzymatic activity estimated from the quantity of radioactive phosphorus ranges from 1439 to 1679 cpm/spot, except in lane 2 (no activity in the control). Although both are resistant to the same drug concentrations, line II displays a lower NPT activity (147 cpm) than line I (1075 cpm) (fig.3B).

4. CONCLUSION

Opposed to others' experiments in which the transfected gene was linked to a *Drosophila* promoter sequence (hsp 70, *copia* LTR) [3,5,6,11], our experiment used the APH (3′) gene under control of the heterologous HSV I TK promoter [8]. But, because of the existence of rearrangements in the vicinity of the APH(3′) sequence, it cannot be excluded that the *neo* gene might be under control of *Drosophila* endogenous sequences rather than the TK promoter. If this is the case, control of the APH(3′) gene by randomly selected endogenous sequences could explain the great difference of APH(3′) activity in lines I and II. Yet, it remains to be explained how our transformed cells, containing 1–2 copies of *neo* gene, have a higher resistance level to G 418 than those obtained by Rio and Rubin [11], in which arrays of 10–20 copies of the gene were present.

In all events, we show that the APH(3′) gene conferring the G 418 resistance was successfully adapted to 89K *Drosophila* cells. The high level of resistance to the antibiotic is correlated with the insertion of the APH(3′) gene in the genome of the cells and with the expression of the gene, as demonstrated by the existence of NPT activity in resistant cells. This is a simple and efficient selective system which may allow the introduction of any DNA sequence of interest into the genome of *Drosophila* cells.

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