The EMBO Journal vol.4 no.1 pp.167–171, 1985

A transposable P vector that confers selectable G418 resistance to *Drosophila* larvae

H. Steller[1] and V. Pirrotta[2]

European Molecular Biology Laboratory, Postfach 10.2209, 6900 Heidelberg, FRG

[1]Present address: Department of Biochemistry, University of California at Berkeley, Berkeley, CA 94720, USA
[2]Present address: Department of Cell Biology, Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 77030, USA

Communicated by V. Pirrotta

***Drosophila* larvae are rapidly killed by food containing the antibiotic G418. The bacterial gene for neomycin resistance introduced in the genome by P-mediated transformation renders larvae resistant to G418 and able to grow to fertile adults. The *neo* gene transcribed from the herpes thymidine kinase promoter gives low levels of resistance but high levels can be obtained using the *hsp70* heat-shock promoter. We have constructed a vector for P-mediated transformation which uses this finding to allow dominant selection of transformed progeny. Features of this vector also facilitate cloning and allow the rapid recovery of the inserted transposon from transformed flies. We have also constructed a cosmid vector for P-mediated transformation that incorporates the *hsp70-neo* gene.**

Key words: cosmid transposon/G418 resistance/P-transformation vectors

Introduction

The ability of P elements to transpose into sites in the genome of *Drosophila* has made them valuable as a means of reintroducing cloned sequences into the organism (Rubin and Spradling, 1982; Spradling and Rubin, 1982). P element DNA, injected into the early embryo integrates into the genome of germ line cells of the injected individual, (G0). Transformed flies are obtained from these germ cells in the following generation (G1) with a frequency that varies, in our hands, between 5 and 20%. That is, 5–20% of the survivor G0 flies give rise to at least one transformed fly in their progeny.

P elements can act as carriers of DNA sequences to be introduced in the *Drosophila* genome. The detection of transformed individuals is possible if the DNA introduced contains a gene producing an easily visible phenotype. In the case of many genes of interest, this visible selection is not available and large numbers of flies need to be individually tested by genetic or biochemical means. To solve this problem, Rubin and Spradling constructed a P-vector containing the *rosy* gene which serves as a visible marker when the injected flies carry a *rosy*$^-$ mutation (Rubin and Spradling, 1983). The *adh* gene has also been used as a marker to select transformants (Goldberg *et al.*, 1983). In this case also, mutant flies need to be used and the selection can only be imposed on the adult G1 flies.

In the present work we show that the bacterial neomycin resistance gene (*neo*) can be used to render *Drosophila* larvae resistant to the antibiotic G418. We describe P element vectors which carry the *neo* gene and include several features to facilitate their use. The *neo* gene is driven by the *hsp70* heat-shock promoter which has been shown to function in a variety of organisms from *Xenopus* to man, while G418 is toxic to yeast, fungi, algae, plant and animal cells (Jimenez and Davis, 1980; Colbere-Garapin *et al.*, 1981; Southern and Berg, 1982). We suppose therefore that the *hsp70-neo* gene would provide a good selective marker for a wide variety of organisms.

P elements introduced by microinjection have been shown to function in *Drosophila hawaiensis* (Brennan *et al.*, 1984), a species that does not normally contain them. This suggests the possibility that they may also function in the germ line of other insects, or even less related animals such as nematodes or vertebrates. The availability of a dominant selection makes this an easily testable possibility.

Results and Discussion

Sensitivity to G418

The aminoglycoside G418 is an antibiotic related to gentamycin, neomycin and kanamycin (Davies and Jimenez, 1980). In contrast to these, G418 seems to be universally toxic: prokaryotic as well as eukaryotic cells are rapidly killed by its ability to block protein synthesis.

To test the sensitivity of *Drosophila* to G418, equal numbers of Canton S flies were allowed to lay eggs on standard *Drosophila* food supplemented with various concentrations of the drug. The results, summarized in Table I show that G418 kills larvae. At low concentrations some larvae survive to give adult flies. The survivors require nearly twice the normal developmental time and remain small, with reduced pigmentation but undiminished fertility.

In contrast, adult flies are relatively insensitive and begin to be affected only after 6–10 days of subsistence on food containing 1 mg/ml G418. This relative resistance allows parents to lay eggs unaffected for several days.

Transformation to G418 resistance

G418 is inactivated by the phosphotransferase encoded by the gene for neomycin resistance, *neo*, from the bacterial transposon Tn5 (Davies and Smith, 1978). In an initial construction, we isolated from plasmid pAG50 (Colbere-Garapin *et al.*, 1979) a fragment containing the *neo* gene lacking the

Table I. Survival of larvae on G418

G418 (μg/ml)	50	100	200	500	1000
survival (imagines)	10–30%	5–10%	0.1–1%	0%	0%
larval growth	++++	++++	++	+	–

Standard *Drosophila* food was melted in a microwave oven, cooled to 40°C and supplemented with G418 at different concentrations. Survival at 25°C is expressed in terms of eclosing imagines while average larval growth is indicated qualitatively.

bacterial promoter but driven by the herpes thymidine kinase (*tk*) promoter. We inserted this fragment into a P-transformation vector and injected this construction into *Drosophila* embryos. The progeny of the injected individuals was tested for ability to survive on G418. In contrast to unicellular organisms or cell cultures, antibiotic resistance of a whole metazoan organism may be more difficult to achieve since insufficiency in one essential tissue, for example the nervous system, might still be lethal. To ensure survival we would need the expression of the *neo* gene in all the cells of the developing larvae or at least in the gut cells that process the ingested food.

Using the *tk* promoter we obtained individuals resistant to 0.5 mg/ml G418 but the *neo* gene is apparently poorly expressed. The surviving larvae take several days more than control larvae to reach pupal stages and are frequently small and weak. Even at relatively low concentrations of G418 (0.5 mg/ml), transformed larvae have a high incidence of mortality and may fail to survive altogether when heterozygous for certain genetic markers or balancer chromosomes. We conclude that the *tk* promoter is not sufficiently active in *Drosophila* or at least not in all cells of the larva.

To increase the level of expression and, in particular to guarantee expression in all tissues, we replaced the thymidine kinase promoter with the *Drosophila hsp70* heat-shock promoter. We used a 456-bp *Xba-Xmn* fragment from the *hsp70* gene, containing the promoter and 206 nucleotides of untranslated leader sequence (Ingolia *et al.*, 1980) which we had previously shown to be heat inducible and expressed in all embryonic cells upon induction (Steller and Pirrotta, 1984).

To select transformed progeny, flies were allowed to lay eggs on food containing 1 mg/ml G418. The flies were then removed and the eggs were given a brief heat shock (30 min, 37°C) shortly before hatching and every third day until they climbed the walls to pupariate.

With this regimen we find that 10–20% of the injected adults give transformed progeny. The transformed larvae survive and pupariate as fast as control larvae. They appear healthy, have a low incidence of mortality even in combination with balancer chromosomes and, in general, give rise to normally fertile adults. Uninjected larvae subjected to the same heat-shock regimen die on 1 mg/ml G418 during the first instar stage.

At this concentration of G418, non-transformed larvae die very early, allowing the screening of large numbers of embryos without crowding. However, even large numbers of resistant larvae do not consume or inactivate the G418 in the food, as tested by its ability to kill bacteria or untransformed larvae.

Heat shock-induced resistance

Resistant lines were established by crossing transformed flies individually with flies carrying various balancer chromosomes and again selecting G418-resistant offspring. Genomic Southern blot hybridization of five independent lines showed that each contained a single *hs-neo* transposon integrated at a different chromosomal site. Northern blot hybridization of one of these lines showed that the transcription of the *neo* gene was undetectable at 25°C but was massively induced by treatment at 37°C for 1 h (Figure 1).

We tested several transformed lines carrying the *hs-neo* transposon in different chromosomal sites for survival of larvae on 1 mg/ml G418 with or without heat shock, compared with survival on normal food. In most cases, heat shock results in survival of up to 90% of the progeny but, depending on the site of integration, equally good survival is obtained even without heat shock. We know from other experiments using the heat-shock promoter that, while induction can result in a several hundred fold increase in transcription, the basal level of the promoter is significant (Steller and Pirrotta, in preparation). Table II summarises the results obtained with four transformed lines which represent the range of G418 resistance observed.

In some of the transformants, the *hs-neo* transposon was apparently inserted in chromosomal sites less favourable to expression. In these cases, *neo* RNA was barely detectable even after heat induction and the larvae required heat-shock treatment for good survival on G418. Since the heat-shock regimen causes no detectable developmental abnormalities, we recommend its use to avoid possible loss of these borderline cases.

The pUChsneo transformation vector

We have incorporated the *hs-neo* construction in a transformation vector called pUChsneo. Figure 2 describes its restric-

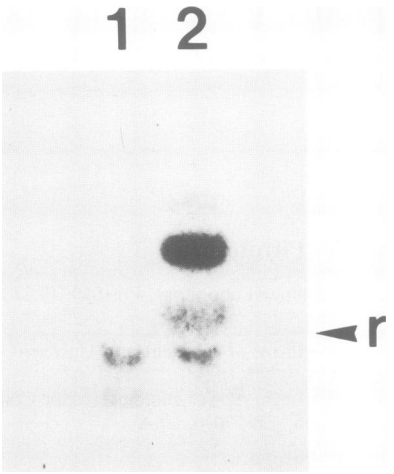

Fig. 1. Heat shock-induced transcription of the *neo* gene. Flies carrying a precursor of the pUChsneo transposon, containing a 1.6-kb *neo* fragment were homogenised without or immediately after heat shock for 1 h at 37°C. The total RNA was extracted as described by Steller and Pirrotta (1984) and one fly equivalent of RNA was electrophoresed in a formaldehyde-agarose gel, blotted onto a nitrocellulose filter and hybridised with a probe including only the *neo* gene. **Lane 1:** not heat shocked, **lane 2:** heat shocked. The size of the heat shock-induced RNA species is ~2.4 kb, consistent with termination near the 3′ P terminal repeat [200 bp *hsp70* leader + 1600 bp neo + 400 bp P′3 and P′3 end + poly(A)$^+$ tail]. The lower band seen in both lanes co-migrates with the rRNA and is probably artifactual.

Table II. Survival of transformed lines on G418

line	survivors (% flies)		
	+hs −G418	+hs +G418	−hs +G418
F1	100%	70-90%	70-90%
F2	100%	50-70%	50-70%
M1	100%	50-70%	40-60%
M6	100%	20-30%	1-5%

Approximately equal numbers of flies from four different transformed lines were allowed to lay eggs for 2 days on normal food and on food containing 1 mg/ml G418. The developing embryos were then given a heat-shock treatment for 1 h at 37°C every other day until pupariation. The yield of adult flies is given as percentage of the yield without selection.

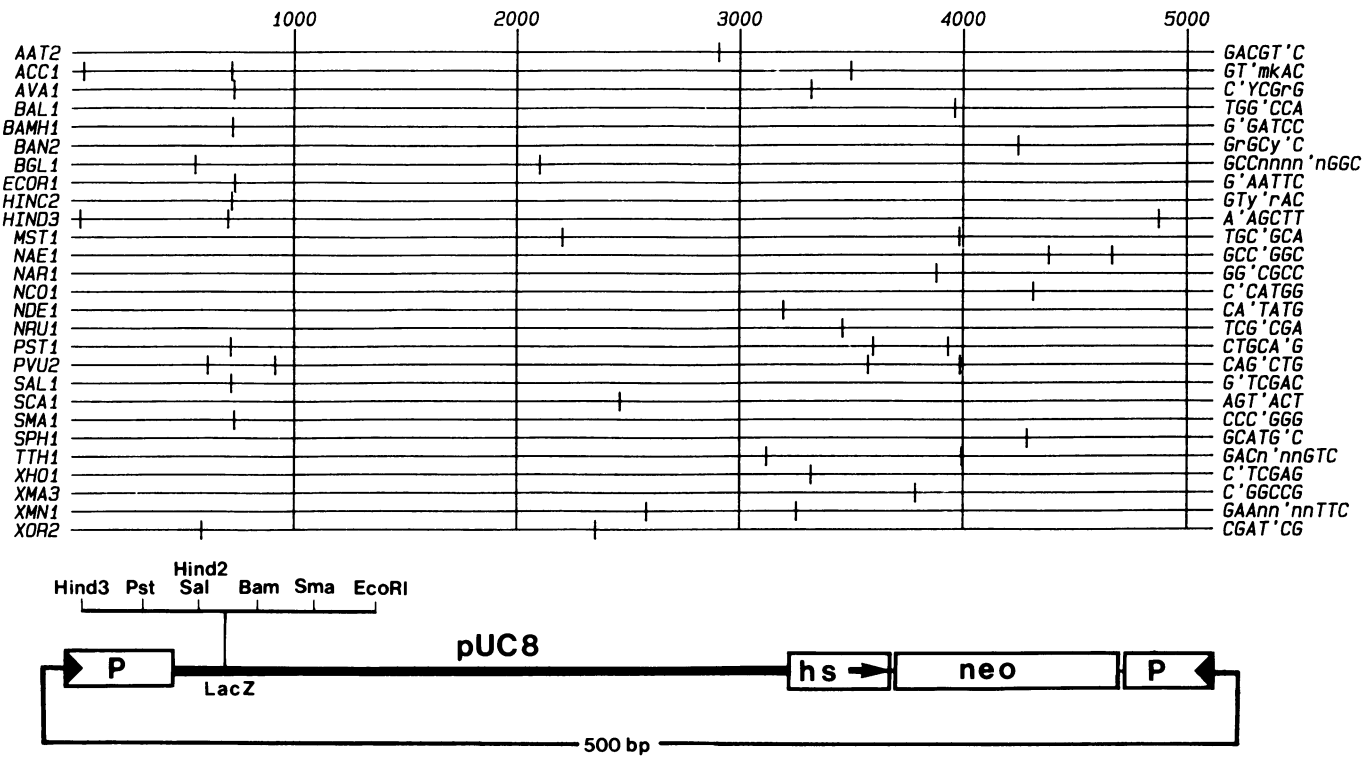

Fig. 2. The pUChsneo vector. The composition is indicated below the map showing restriction sites in the transposon. The P sequences on the left come from the 5′ end of the p6.1 P element. They are joined to the *Nar* site of plasmid pUC8. The other *Nar* end of pUC8 is attached to a 456-bp *Xba-Xmn* fragment containing the *hsp70* heat-shock promoter. The *neo* gene is in a *Bgl*II-*Sma* fragment which lacks the bacterial promoter but includes the entire coding region of the phosphotransferase. The P sequences on the right come from the 3′ end of the p6.1 P element. The two ends of the P element are connected by ~500 bp of DNA derived from the *white* locus and present in the p6.1 clone. The polylinker contains unique *Eco*RI, *Sma*, *Bam*, *Hind*II and *Sal* cloning sites. The *Hind*III and *Pst* sites cannot be used for cloning since they cut elsewhere in the transposon.

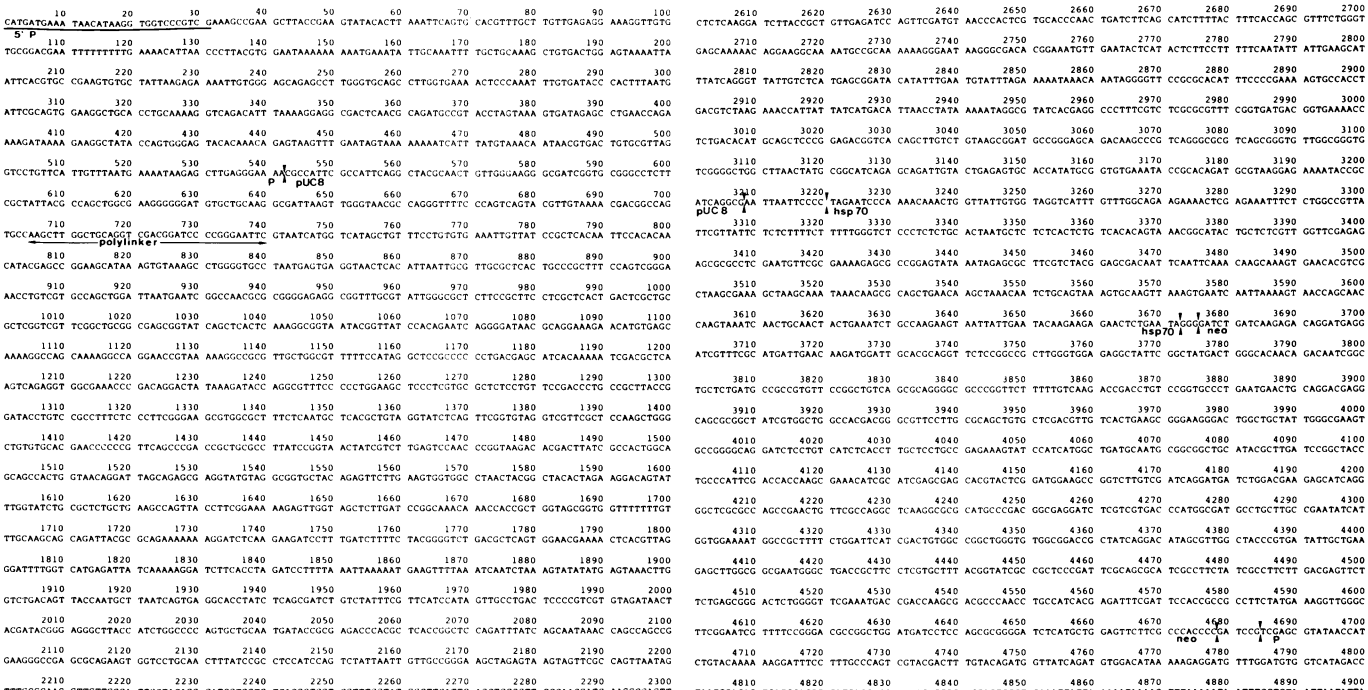

Fig. 3. Sequence of the pUChsneo transposon. The sequence starts from the 5′ end of the P element and does not include the 500 nucleotides joining the two P ends. The double arrowheads indicate beginnings and ends of each constituent block and the terminal repeats of the P element are underlined. The P element sequences were deduced from O'Hare and Rubin (1983), the *hsp70* sequence from Ingolia *et al.* (1980), the *neo* sequence from Beck *et al.* (1982) and the pUC8 sequence from Vieira and Messing (1982).

tion map and its constitution while Figure 3 gives the predicted nucleotide sequence of the transposon deduced from its components. Note that the *neo* gene in this construction renders bacteria carrying pUChsneo resistant to 10 μg/ml kanamycin. This is not due to transcription starting from the *hsp70* promoter, which is inactive in bacteria, but presumably to read-through from a plasmid promoter.

In contrast to the Carnegie vectors (Rubin and Spradling, 1983), pUChsneo includes the plasmid replicon and ampicillin resistance gene within the P terminal repeats. As a consequence of this design, these sequences also become integrated in the genome of a transformed fly, allowing the recovery of the inserted DNA by the 'plasmid rescue' method (Perucho *et al.*, 1980). Genomic DNA of the transformed fly is digested with an enzyme that does not cut in the transposon, diluted and ligated under conditions that allow circularisation of the fragments. The ligation mixture is then used to transform *Escherichia coli* hosts. This technique, described in more detail by Steller and Pirrotta (in preparation), is useful to isolate the sequences flanking the insertion site, to analyse the inserted transposon for rearrangements, mutations etc.

The plasmid replicon, which consists of the pUC8 vector (Vieira and Messing, 1982) in its entirety, includes also the *lacZ* fragment and polylinker cloning sites which facilitate the identification of insertions on plates containing X-gal (5-bromo-4 chloro-3-indolyl-β-galactopyranoside). Because of the presence of *Hind*III sites in the P sequences, and of *Pst* sites in the *hs-neo* sequence, these two enzymes cannot be used for cloning although their sites are present in the polylinker.

The G418 selection is well suited for mass screening. By eliminating untransformed individuals at a very early stage, it allows the transformed ones to survive in uncrowded conditions. Because G418 is a powerful antibiotic, it also protects the food from contamination with mold or bacterial growths. Most importantly, it does not require the use of mutant strains for the selection of transformants. As a result, it could be applied to other *Drosophila* species for which little or no genetics may be available, to other insects and, in principle, to any other organism in which P-mediated transposition can be made to occur.

A cosmid transposition vector carrying hs-neo

P-mediated transposition allows the testing of a cloned DNA fragment for its biological activity. If large genomic regions could be tested by this method, chromosomal walks in search of a gene of interest could be undertaken even in the absence of any detailed molecular information or any means to identify the gene other than its biological function. We have constructed a cosmid vector whose cloning site is flanked by the P terminal sequences. Genomic cosmid clones constructed with this vector can be used directly for P mediated transformation.

As a starting point we took cos4, a cosmid vector previously used by Pirrotta *et al.* (1983). Cos4 contains three λ*cos* sites for historical reasons, although only two are essential to its design. To this vector, by a series of steps detailed in Materials and methods, we added the 3′ P end from Carnegie-1 and the 5′ P end from p6.1 (Rubin and Spradling, 1983). This yielded cos-P, a cosmid vector with a unique *Bam*HI site for cloning fragments generated by partial *Sau*3A digestion, bracketed by the P ends. As reported elsewhere (Haenlin *et al.*, in preparation), we have used a cosmid clone isolated in this vector to introduce a 43-kb DNA fragment

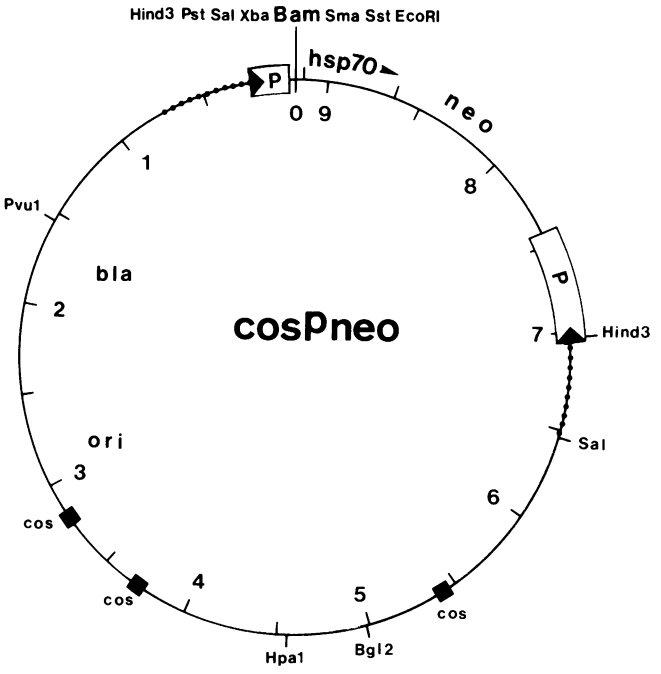

Fig. 4. The cosPneo cosmid vector. The λ cos sites are indicated by small black boxes. The dotted region represents sequences from the *white* locus flanking the insertion site of the P element in clone p6.1. The P sequences are shown boxed in and *bla* indicates the β-lactamase gene. The *hsp70* promoter is directed away from the polylinker site and transcribes the *neo* gene. The *Hind*III, *Pst* and *Sal* sites cannot be used for cloning but the *Xba*, *Bam*, *Sma*, *Sst* and *Eco*RI sites are unique.

containing the K10 female sterility locus into the genome of homozygous K10 flies, thus restoring fertility.

We then introduced the *hsp70-neo* gene into cos-P to obtain cosPneo. A map of cosPneo indicating the constituent elements is shown in Figure 4. Note that the *Hind*III, *Pst* and *Sal* sites in the polylinker cannot be used for cloning because they are not unique. However the *Xba*I, *Bam*HI, *Sma*I, *Sst*I and *Eco*RI sites are unique. To avoid possible interference with the expression of cloned genes, the *hsp70* promoter is directed away from these cloning sites. Like pUChsneo, cosPneo also renders bacteria resistant to kanamycin, presumably due to transcription read through from the plasmid region.

Embryos injected with cosPneo yield progeny transformed to G418 resistance. Although we have not yet attempted transformation with cosmid clones obtained with this vector, we conclude that cosPneo is fully active both as a transposon and as a selectable marker.

Materials and methods

The G418 used in these experiments was from a preproduction batch of the Plough-Schering Co. and kindly supplied to us by A. Falaschi and G. Della Valle. It can be obtained commercially from Gibco. Standard *Drosophila* food was supplemented with G418 by melting it in a microwave oven, cooling to 40°C and adding G418 at a final concentration of 50 – 1000 μg/ml. The G418 in the food remains active for at least 2 weeks when stored at 5°C.

Microinjection procedures were slightly modified from those described by Steller and Pirrotta (1984). The principal difference was that the DNA solution was injected in the posterior quarter of the embryo. Plasmid transposon DNA was co-precipitated with helper plasmid and resuspended in injection buffer (5 mM KCl, 0.1 mM sodium phosphate, pH 6.8) at a final concentration of 500 μg/ml of transposon and 100 μg/ml helper DNA. As helper we used pπ25wc (kindly supplied by G. Rubin) or phsπ, a construction containing the P coding region transcribed by the *hsp70* heat-shock promoter (Steller and Pirrotta, in preparation). Both helpers lack an intact P terminal repeat and are unable to integrate.

Construction of vectors

The construction of the pUChsneo plasmid went through a number of intermediates used for other purposes. The essential steps in the construction are briefly summarised as follows. The 5′ P end comes from clone p6.1, up to the *Xmn* site at position 538 in the P sequence (O'Hare and Rubin, 1983). The 3′ P end comes from the same clone, starting from the *Xho* site. The sequences flanking the P element in p6.1 come from the *white* locus. They were joined together by ligating the two *Sal* sites present in p6.1 and then partially deleted by treatment with *Bal*31. This eliminated the *Sal*I site and the *Eco*RV site and reduced the *white* sequences to ~500 bp.

The entire pUC8 sequence (Vieira and Messing, 1982), starting from the *Nar*I site was attached to the *Xmn* site of the 5′ P end. The *hsp70-neo* gene was constructed from a 456-bp *Xba-Xmn* fragment containing the *hsp70* promoter and 206 bp of the transcribed leader (Ingolia *et al.*, 1980), ligated through a *Bam* linker to a *Bgl*II-*Bam* fragment containing the coding region but not the promoter of the *neo* gene from the bacterial Tn5 transposon (Jorgensen *et al.*, 1979). The *neo* gene was subsequently reduced by excising the *Sma-Bam* fragment at its 3′ end which does not contain *neo* coding sequences.

To construct the cosmid vector, the *Xho-Sal* fragment containing the 3′ P end from p6.1 was cloned in the *Sal* site of pUC8 and then excised with *Bam* + *Sal*. This cosmid vector was assembled by a three-way ligation between this fragment, a 4.7-kb *Pvu*I-*Sal* fragment from cos4 containing the *cos* sites, the plasmid replicon and part of the β-lactamase gene and a 1.5-kb *Pvu*I-*Bam* fragment from Carnegie-1 (Rubin and Spradling, 1983) containing the rest of the β-lactamase gene, the 5′ P end and part of the polylinker. The resulting plasmid was converted to cosP by partial *Pvu*II digestion and mild *Bal*31 treatment to remove the *Pvu*II site in the 5′ P fragment.

To generate cosPneo, we used the *hsp70-neo* gene which had been introduced into Carnegie-4 (Rubin and Spradling, 1983) as follows. An *Eco*RI-*Sma* fragment containing the *hsp70-neo* gene was ligated to the *Eco*RI site of Carnegie-4. The ligation was interrupted to fill in the remaining *Eco*RI ends with DNA polymerase and then resumed to join the filled in *Eco*RI site to the *Sma* end of *hsp70-neo*. The resulting clone, Ca4hsneo provided a *Hind*III fragment containing part of the 5′ P end, the *hsp70-neo* gene and the polylinker for ligation to cosP cut with *Hind*III. The resulting plasmid, with the fragment inserted in the correct orientation to regenerate a 5′ P end, is cosPneo.

Acknowledgements

We thank Spyros Artavanis for information on G418 resistance, Christa Bröckl and Helene Cambier for technical assistance. H.S. was the recipient of an EMBL predoctoral fellowship.

References

Beck,E., Ludwig,G., Auerswald,E.A., Reiss,B. and Schaller,H. (1982) *Gene*, **19**, 327-336.
Brennan,M.D., Rowan,R.G. and Dickinson,W.J. (1984) *Cell*, **38**, 147-151.
Colbere-Garapin,F., Chousterman,S., Horodriceanu,F., Kourilsky,P. and Garapin,A.-C. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 3755-3759.
Colbere-Garapin,F., Horodriceanu,F., Kourilsky,P. and Garapin,A.C. (1981) *J. Mol. Biol.*, **150**, 1-14.
Davies,J. and Jimenez,A. (1980) *Am. J. Trop. Med. Hyg.*, **29**, (5) Suppl. 1089-1092.
Davies,J. and Smith,D. (1978) *Annu. Rev. Microbiol.*, **32**, 469-518.
Goldberg,D.A., Pasakouy,J.W. and Maniatis,T. (1983) *Cell*, **34**, 59-73.
Ingolia,T.D., Craig,E.A. and McCarthy,B.J. (1980) *Cell*, **21**, 669-679.
Jimenez,A. and Davies,J. (1980) *Nature*, **287**, 869-871.
Jorgensen,R.A., Rothstein,S.J. and Reznikoff,W.S. (1979) *Mol. Gen. Genet.*, **177**, 65-72.
O'Hare,K. and Rubin,G.M. (1983) *Cell*, **34**, 25-35.
Perucho,M., Hanahan,D. and Wigler,M. (1980) *Cell*, **22**, 309-317.
Pirrotta,V., Hadfield,C. and Pretorius,G.H.J. (1983) *EMBO J.*, **2**, 927-934.
Rubin,G.M. and Spradling,A.C. (1982) *Science (Wash.)*, **218**, 348-353.
Rubin,G.M. and Spradling,A.C. (1983) *Nucleic Acids Res.*, **11**, 6341-6351.
Southern,P.J. and Berg,P. (1982) *J. Mol. Appl. Genet.*, **1**, 327-341.
Spradling,A.C. and Rubin,G.M. (1982) *Science (Wash.)*, **218**, 341-347.
Steller,H. and Pirrotta,V. (1984) *EMBO J.*, **3**, 165-173.
Vieira,J. and Messing,J. (1982) *Gene*, **19**, 259-268.

Received on 5 October 1984