High yield and stable transformation of the unicellular green alga *Acetabularia* by microinjection of SV40 DNA and pSV2neo

Gunther Neuhaus, Gabriele Neuhaus-Url,
Egon J.de Groot and Hans-Georg Schweiger

Max-Planck-Institut für Zellbiologie, Rosenhof, D-6802 Ladenburg bei
Heidelberg, FRG

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SV40 DNA and pSV2neo were microinjected into isolated nuclei of *Acetabularia mediterranea*. The injected nuclei were implanted into anucleate cell fragments of the same species. Such combinations not only survived but also formed progeny. The F₁, F₂ and F₃ generations of these combinations were analyzed. In the case of SV40-treated cells T-antigen was expressed and accumulated in the nuclei of all three generations studied as shown by indirect immunofluorescence. Nuclear exchange experiments revealed expression of the T-antigen only if a transformed nucleus but not if only a transformed cytoplasm was involved. Transformation by pSV2neo, a chimeric gene with a selectable marker was demonstrated by the induction of G-418 resistance as well as immunofluorescence. Genomic DNA was isolated from gametes, originating in cysts from the F₁, F₂ and F₃ generations of injected cells, and subjected to Southern analysis. These experiments demonstrated that both types of DNA are integrated into the host genome.

Key words: antibiotic resistance/microinjection/nuclear implantation/nuclear genome/transformation

Introduction

Attempts to transfer genes into plant cells so far have focused preferentially on the *Agrobacterium tumefaciens* Ti-plasmid (for review see Schell *et al.*, 1984) and the Cauliflower mosaic virus vector system (for reviews see Hohn *et al.*, 1982; Howell, 1982).

In previous experiments it has been shown that in the unicellular green alga *Acetabularia mediterranea* a number of genes, genomes and gene constructions respectively, are efficiently expressed. To express these genes a nucleus is isolated, microinjected and implanted into an anucleate fragment. Expression was observed using TMV RNA (Cairns *et al.*, 1978a), mengo virus RNA (Cairns *et al.*, 1978b), adenovirus type II DNA (Cairns *et al.*, 1978c), simian virus DNA (Neuhaus *et al.*, 1984), the cloned small subunit gene for the ribulose-1,5-bisphosphate carboxylase/oxygenase from wheat and pea (Neuhaus *et al.*, 1983), zein genes (Langridge *et al.*, 1985) and the neomycin resistance gene under the control of the β -tubulin promoter of *Chlamydomonas* (Weeks *et al.*, 1985).

These experiments lead to the question of whether the expression of foreign genes without vectors would be transient or stable and in the latter case whether it would be due to integration of the genes into the genome of *Acetabularia* and therefore would be retained in the succeeding generations. To answer this question nuclei were isolated from *A. mediterranea* and microinjected with SV40 DNA (Neuhaus *et al.*, 1984) or the chimeric construction pSV2neo (Southern and Berg, 1982). Advantageous

features of the two DNAs used for expression are that they contain the SV40 early regulatory sequences (Khoury and Gruss, 1983; Neuhaus *et al.*, 1984) and the pSV2neo contains a selectable marker (Southern and Berg, 1982).

The microinjected nuclei were implanted into anucleate fragments of *Acetabularia* cells. These combinations formed caps and cysts and underwent sexual reproduction. Cells of the following three generations were analyzed for expression products and DNA sequences homologous to the microinjected genes.

Results

Transformation

Nuclei isolated from cells of *A. mediterranea* at the stage of early cap formation (Schweiger and Berger, 1979) were isolated and either injected with viral SV40 DNA (Neuhaus *et al.*, 1984) or with the chimeric construction pSV2neo (Southern and Berg, 1982). The injected nuclei were implanted into anucleate apical fragments of cells of the same species and the same stage. After surgery the cells were grown until cysts formed (Schweiger and Berger, 1979). Cyst formation occurred in ~60–70% of the treated cells. Each cell containing cysts was cultured individually. Control experiments were performed by injecting either buffer solution or pBR322 into isolated nuclei, which were manipulated as described before.

SV40

Three subsequent generations of SV40-treated cells of *A. mediterranea* were studied using the indirect immunofluorescence technique for the detection of the T-antigen. All cells of the F₁, F₂ and F₃ generations of the two independent cell lines investigated gave a positive reaction irrespective of the developmental stage of the algae (Table I). A positive reaction was found in the nuclei of transformed cells ('transformed nuclei') similar to that observed in previous microinjection experiments (Figure 1; Neuhaus *et al.*, 1984).

To clarify the role of the nucleus and the cytoplasm in the transformation process nuclei from transformed F₁ *Acetabularia*, cells were isolated and implanted into non-transformed anucleate

Table I. Expression of the T-antigen in the F₁, F₂ and F₃ generation of SV40-transformed *Acetabularia* cells (two independent lines)

Generation	Source of nuclei (stage of development)	Analyzed nuclei (number)	Immuno- fluorescence (number)	
			+	–
F ₁	Prior to cap formation	15	15	0
	Small caps	10	10	0
	Fully developed caps	13	13	0
F ₂	Prior to cap formation	11	11	0
	Small caps	8	8	0
F ₃	Prior to cap formation	0	10	0
	Small caps	12	12	0

fragments (cytoplasm). In addition, nuclei from non-transformed cells were implanted into anucleate fragments from transformed cells. Ten days later the cells were analyzed by indirect immunofluorescence. Only combinations with transformed nuclei showed a reaction characteristic for T-antigen while non-transformed nuclei in combination with transformed cytoplasm did not give a signal with T-antigen antibodies (Table II, Figure 1d). This experiment shows that cytoplasmic inheritance is not involved in the transformation.

Cells of randomly selected SV40-transformed cell lines of *A. mediterranea* were analyzed for homology to the transforming sequences by Southern hybridization. DNA isolated from gametes either of transformed F₁, F₂ and F₃ cells or of F₁, F₂ and F₃ non-transformed control cells, was hybridized with the *Bam*HI/*Hpa*II – *Bam*HI fragment from pSVL6 as probe (Levin-

son *et al.*, 1982; Neuhaus *et al.*, 1984) covering the SV40 T-antigen and the SV40 regulatory sequences. The *Bam*HI/*Hpa*II – *Bam*HI fragment is a *Hpa*II – *Bam*HI fragment in which the *Hpa*II

Table II. Nuclear exchange experiment

Source of nuclei (cell type)	Source of recipient anucleate fragments (cell type)	Analyzed nuclei (number)	Immuno- fluorescence (number)	
			+	-
F ₁ , SV40 trans- formed	Wild type	9	9	0
Wild type	F ₁ , SV40-transformed	11	0	11

Fig. 1. Nuclei (diameter 100 μ m) of *Acetabularia* cells immunofluorescence-stained for T-antigen. Nuclei of the F₁ (a), the F₂ (b) and the F₃ (c) generation of SV40 microinjected cells of *A. mediterranea* are presented. Nuclei from F₁ generation cells were implanted into anucleate fragments from non-transformed cells and immunofluorescence-stained 10 days later (d). Control cells (wild-type) did not give a fluorescence signal (e).

Fig. 2. Maps indicating the DNA constructions and fragments (*Bam*HI/*Hpa*II-*Bam*HI; *Pvu*II-*Eco*RI) used as probes for hybridization (inner partial circle).

site was converted to a *Bam*HI site (see Figure 2; Levinson *et al.*, 1982; Neuhaus *et al.*, 1984). This conversion allows cloning of the *Bam*HI fragment into pBR322 (Figure 2).

Unrestricted DNA from transformed *Acetabularia* gametes exhibited a hybridization signal in the high molecular weight region excluding the possibility that the homologous sequences were associated with low molecular weight structures like plasmids. This result shows that the transforming sequences were integrated into the genome (Figure 3).

*Bam*HI and *Hpa*II-digested DNA from transformed cells hybridized to the probe at a band in the 3-kbp region where one would expect the *Bam*HI/*Hpa*II-*Bam*HI fragment of the SV40 (3056 bp) (Levinson *et al.*, 1982; Neuhaus *et al.*, 1984) to be (Figure 3). Sometimes a faint band was observed in the 4-kbp region (Figure 3). Its significance is still unclear. DNA from non-transformed wild-type cells and from F₂ pBR322-injected cells were analyzed in the same way. They did not hybridize to the probe.

Dot blot analyses indicated that in the genome of transformed *Acetabularia* cells between 1 and 3 copies of the injected gene occur (data not shown).

Additional hybridization experiments were performed after digestion of DNA from transformed cells with the restriction enzyme *Bcl*I which on the basis of the nucleotide sequence is known to cut the SV40 DNA at one site. In this case three bands corresponding to 10.2, 5.2 and 3.8 kbp gave hybridization signals (Figure 3). The 10.2-kbp band corroborates the statement that the SV40 DNA is integrated into a high molecular weight structure since its size substantially exceeds the size of the SV40 genome known to be 5.2 kbp (Tooze, 1980). The size of the se-

Fig. 3. Southern analysis. Unrestricted and restricted (*Bam*HI and *Hpa*II or *Bcl*I) DNA was probed with sequences covering the T-antigens and the SV40 regulatory sequences. Unrestricted DNA (20 µg/lane) from SV40 transformants (a and b), wild-type *Acetabularia* (c) and restricted DNA of SV40 virus (0.5 ng) (d), SV40-transformed *Acetabularia* cells (30 µg) (e), pBR322 injected cell lines (30 µg) (f) and wild-type cells (30 µg) (g) were hybridized against a *Bam*HI/*Hpa*II-*Bam*HI fragment from pSVL6. DNA from two different SV40 lines corresponding to lanes a and e (h) as well as b (i) was restricted with *Bcl*I and probed as in lanes d-g.

Fig. 4. Schematic presentation of Southern analysis results. DNA from SV40 transformed F_3 generation *Acetabularia* cells was restricted with *Bam*HI and *Hpa*II or DNA from pSV2neo-transformed F_3 generation cells was restricted with *Pvu*II and *Eco*RI and probed with SV40 and pSV2neo DNA respectively both resulting in one hybridization band (a). The two enzyme combinations are known to excise the respective gene. DNA from SV40-transformed F_3 generation cells was restricted with *Bcl*I and probed with SV40 DNA resulting in three bands (b). Note that the molecular size of the 10.2-kbp band exceeds the total size of the microinjected DNA indicating that it is integrated into the genome. The 5.2-kbp band is in good agreement with the size of the microinjected SV40 DNA indicating that the SV40 DNA is repetitively integrated and tandemly arranged in the *Acetabularia* genome. DNA from pSV2neo-transformed F_3 generation cells was restricted with *Bcl*I and probed with the pSV2neo NPT II coding sequence resulting in two bands (c). *Bcl*I is known to have one restriction site on the coding sequence of pSV2neo.

Table III. Genetic analysis of SV40-transformed and wild-type cells

Crossing (type)	Analyzed nuclei (number)	Immunofluorescence			
		+		–	
		Number	%	Number	%
$C^x \times C^x$	25	25	100	0	0
$C^0 \times C^0$	30	0	0	30	100
$C^x \times C^0$	35	13	37	22	63

C^x and C^0 stand for cysts from F_1 generation-transformed and wild-type cells respectively. The cells originated in 8, 8 and 7 crossings, respectively. Out of the seven $C^x \times C^0$ crossings in only one, with three cells, was no positive reaction observed.

cond band is in good agreement with the assumption that the SV40 sequence is repetitively integrated and tandemly organized (Figure 4). The intensity of the signal of the second band as compared to the first and third band suggests that 2–3 copies are integrated into the *Acetabularia* genome.

For genetic analysis one cyst from a F_1 -transformed cell (C^x) was cultured together with one cyst from a control cell (C^0). Since it is known that one cyst contains either (+) or (–) gametes (Hämmerling, 1944) progeny can only emerge if the two cysts represent different mating types (see Materials and methods). Other combinations included either two cysts from transformed cell lines ($C^x \times C^x$) or from non-transformed cells ($C^0 \times C^0$). Nuclei of F_3 cells, derived from the different cyst combinations, were analyzed for the T-antigen using the indirect immunofluorescence technique. Nuclei of all progeny cells obtained from $C^x \times C^x$ reacted positively, whereas none of the $C^0 \times C^0$ combinations responded to the T-antigen antibodies. The combination of a transformed and a non-transformed cyst ($C^x \times C^0$) gave positive results in 13 out of 35 cells (Table III). In one of these crossings ($n = 3$) only negative cells were observed.

pSV2neo

In previous experiments it was shown that a construction containing the neomycin resistance (neomycin phosphotransferase II; NPT II) gene from Tn5 (Haas and Dowding, 1975; Jimenez

Fig. 5. Test for G-418 resistance. A total of 30 pSV2neo (□) and 30 pBR322 (○) injected cells of *Acetabularia* were analyzed. The bars indicate where the cells were exposed to G 418. On day 12 a second round of G-418 treatment started with the survivors of the first round and with a new group of 30 control pBR322 injected cells.

and Davies, 1980) under the control of the β -tubulin promoter and terminating sequences from *Chlamydomonas* is capable of inducing resistance to the antibiotic G 418 in *Acetabularia* (Weeks *et al.*, 1985). To find out whether a similar construction containing the neomycin resistance gene and the early regulatory SV40 sequences (pSV2neo, Southern and Berg, 1982) can transform *Acetabularia* cells, nuclei of *A. mediterranea* were microinjected with pSV2neo and implanted into anucleate cell fragments. After 10–14 days these cells were analyzed for G-418 resistance in Müller's medium, containing 1 mg/ml of the antibiotic. As a control, buffer injected cells were treated simultaneously in the same way. After 6 days all control cells had died whereas 60% of the injected cells had survived. After a period of several days in antibiotic-free medium the pSV2neo-injected cells were subjected to a second treatment with G 418. Another group of control cells was simultaneously incubated

Fig. 6. pSV2neo-transformed (a) and wild-type (b) cells of *A. mediterranea*. The cells were treated twice with 1 mg/ml G 418 (see Figure 5).

Fig. 7. Antibiotic resistance of cells of the F₃ generation. Cells of the F₃ generation originating in a pSV2neo-injected cells (●) were analyzed for antibiotic resistance in the presence of 0.5 mg/ml G 418. Wild-type cells of *Acetabularia* served as controls (○).

under the same conditions. In contrast to 0% of the control cells ~ 90% of the pSV2neo-injected cells were resistant and survived the second incubation period in the presence of G 418 (Figure 5). They exhibited normal growth and morphogenesis (Figure 6).

The resistant cells were kept in the presence of 1.0 mg/ml G 418 until they started to form cysts which happened in 60% of the injected cells. From then on cysts containing cells were cultured individually in Müller's medium in the absence of the antibiotic. Cells of the F₁, as well as the subsequent F₂ and F₃ generations, were analyzed for antibiotic resistance by culturing

them in the presence of 0.5 mg/ml G 418 (Figure 7). Cells of the F₁, F₂ and F₃ generations of buffer or pBR322-microinjected cells served as controls. Morphogenesis of antibiotic resistant pSV2neo-transformed cells was normal independent of whether they were cultivated in Müller's medium or in the selection medium (data not shown). All control cells of the F₁, F₂ and F₃ generations of buffer or pBR322-treated cells died in the selection medium.

To characterize the expression product of pSV2neo the transformed cells were analyzed by indirect immunofluorescence using an antibody against the NPT II (gift from S. Lipphard). All G-418 resistant cells of the P, F₁, F₂ and F₃ generations studied gave a strong fluorescence signal with the NPT II specific antibodies (Figure 8). Attempts were made to characterize the expression product of pSV2neo by searching for the product of the NPT II reaction using two different methods (Keller *et al.*, 1985; unpublished results). In contrast to control experiments with extracts from *Escherichia coli*, containing the bacterial resistance gene, we did not succeed in identifying the reaction product with extracts from pSV2neo-transformed cells. This, however, may simply reflect a quantitative problem since, in contrast to other transformation systems where 10⁷ cells were used, in the case of transformed *Acetabularia*, the enzyme tests (Keller *et al.*, 1985) were performed on only 20 cells.

The occurrence of pSV2neo-homologous sequences in the genome of transformed *Acetabularia* cells was analyzed under the conditions described for SV40. A radioactively labelled *Pvu*II–*Eco*RI fragment from pSV2neo containing the NPT II gene and the regulatory sequences of SV40 served as a probe (Figure 2; see also Figure 4a). This probe hybridized to a high molecular weight component of the undigested genomic DNA of transformed *Acetabularia* cells, indicating that microinjected pSV2neo DNA was integrated in the genome and had replicated. Restriction of the DNA from transformed *Acetabularia* cells with *Pvu*II–*Eco*RI resulted in the appearance of a band in the 2.6-kbp region (Figure 9). From the sequence of the construction *Pvu*II and *Eco*RI digestion was expected to produce a 2.4-kbp fragment. DNA from non-injected wild-type cells and from F₂ pBR322-injected cells treated in the same way did not hybridize at all. Hybridization of *Bcl*I-restricted DNA from two different

Fig. 8. Immunofluorescence analysis of the F_3 generation of a pSV2neo-transformed cell line. Indirect immunofluorescence was performed with antibodies against the NPT II. Cytoplasmic droplets from transformed (a, b) and wild-type cells (c, d) were observed under white light (a, c) and under fluorescing (b, d) conditions ($\times 250$).

Fig. 9. Southern analysis. Unrestricted (a and b) and *PvuII*–*EcoRI* restricted (c–e) DNA was probed with a pSV2neo fragment. Unrestricted DNA (20 μ g per lane) from wild-type *Acetabularia* (a) and pSV2neo-transformed cells (b) and the *PvuII*–*EcoRI* fragment from the pSV2neo plasmid (0.2 ng) (c), restricted DNA (30 μ g) from pSV2neo-transformed cells (d) and pBR322-injected cells (e) were probed to a *PvuII*–*EcoRI* fragment of pSV2neo. DNA (35 μ g per lane) from two different (lane f; lanes g and h) pSV2neo-transformed F_3 cell lines was restricted with *BclI* and probed with the same fragment as above.

Table IV. Genetic analysis of pSV2neo-transformed and wild-type cells

Crossing	Analyzed nuclei (number)	Resistance			
		+		–	
		Number	%	Number	%
C ^x × C ^x	300	277	92.3	23	7.6
C ^o × C ^o	200	0	0	200	100
C ^x × C ^x	490	105	21.4	385	78.5

C^x and C^o stand for cysts from F₁ generation-transformed and wild-type cells respectively.

Table V. Detailed analysis of crossing experiments between wild-type cells and pSV2neo transformants

C ^x × C ^o	Analyzed cells (number)	Resistance			
		+		–	
		Number	%	Number	%
1	50	0		50	100
2	47	0		47	100
3	50	0		50	100
4	50	0		50	100
5	50	0		50	100
6	43	20	47	23	53
7	50	18	36	32	64
8	50	24	48	26	52
9	50	26	52	24	48
10	50	17	34	33	66

C^x and C^o stand for cysts from F₁ generation-transformed and wild-type cells respectively.

lines of pSV2neo-transformed F₃ generation cells, with the *PvuII*–*EcoRI* fragment from pSV2neo, resulted in two bands with the molecular sizes of 25.0 and 8.0 and 9.5 and 8.3 kbp respectively (Figure 9; see also Figure 4c). Since in both cases the bands exceed the size of the pSV2neo (5.6 kbp) it can be concluded that the pSV2neo must be integrated into high molecular size DNA and at different sites in the two lines. The fact that only two bands appeared indicates that, in contrast to the SV40-transformed line, the transforming pSV2neo sequence is integrated as a single copy. The copy number per *Acetabularia* genome is in the range of 1–3 copies as estimated by dot blot analysis (data not shown).

Genetic analysis of individual cysts from pSV2neo-transformed and control *Acetabularia* cells was performed as described for SV40. The combination C^x × C^x resulted in progeny of which 90% of the analyzed cells were resistant to G 418; the combination of two control cysts (C^o × C^o; cysts of buffer-injected cells) produced only non-resistant cells. In the progeny of C^x × C^o ~ 20% of the cells survived the antibiotic treatment (Table IV). A more detailed analysis of the results (Table V) revealed that five out of ten C^x × C^o crossings did not lead to antibiotic-resistant progeny while the other five resulted in ~ 50% resistant cells.

Discussion

The experiments described here demonstrate that: (i) by using the microinjection technique, cells of *Acetabularia* can easily be transformed; (ii) transformation is possible without using a vector; (iii) the yield of transformed cells is extraordinarily high; and (iv) the transformation is stable at least over three generations. This together with the remarkable size of the cell, the simple procedure available to produce anucleate fragments,

to isolate cell nuclei, to reimplant nuclei into anucleate fragments, and the fact that even after microinjection such implanted nuclei are capable of undergoing nuclear division and participating in the production of progeny, makes *Acetabularia* a favourable unicellular organism for the study of gene expression *in vivo*.

Since one cell can produce more than 10⁶ gametes, a progeny of at least 10⁶ cells or even more can be expected. This number may even be high enough for producing a gene expression product at a preparative scale.

More than 50% of the microinjected nuclei gave rise to transformed cells. This high frequency of transformants obtained by microinjection into the nucleus corresponds to similar observations on injected mammalian cells (Capecchi, 1980; Wong and Capecchi, 1985). Since some of the microinjected nuclei are probably seriously damaged by the surgery or do not survive implantation the conclusion seems to be justified that almost every successfully implanted nucleus is capable of transforming its host cell.

Another important point is the stability of the transformation. Even in the F₃ generation, without exception, all cells studied were transformed. Of course, this does not necessarily mean that the transformation is maintained over an indefinite number of generations, but it clearly shows that the integration is highly stable.

After microinjection a number of different genomes and genes are expressed in an *Acetabularia* cell (Cairns *et al.*, 1978a, b and c; Neuhaus *et al.*, 1983, 1984; Weeks *et al.*, 1985; Langridge *et al.*, 1985). Here it is shown that the transforming DNA is transferred to the progeny. This leads to the question of whether the microinjected DNA molecules are simply transferred to the progeny or whether the transforming DNA is replicated in the *Acetabularia* cell. The experiments described here leave little doubt that the microinjected DNA is indeed replicated. On average every cell is capable of forming more than 10⁶ gametes, i.e. in the third generation the DNA must be distributed over 10¹⁸ gametes. This, however, is impossible since only ~ 10⁶ molecules are microinjected.

The fact that in all cells of these three subsequent generations, without exception, the microinjected DNA is expressed indicates the presence of SV40 DNA in all cells. On the basis of the nuclear exchange experiment it can be excluded that mitochondria or chloroplasts are the site of transformation. The observation that with an appropriate probe non-restricted DNA from F₃ generation cells hybridizes in the high molecular range indicates that the microinjected DNA is integrated into the nuclear genome rather than being present as an extrachromosomal plasmid. Moreover, the fact that restriction nuclease-digested nuclear DNA from transformed cells gives a hybridization signal in one band indicates that the transforming DNA is integrated in one piece and only at one site.

Integration and replication of the transforming DNA is also indicated by the restriction analysis of pSV2neo transformed DNA using *BclI* and resulting in two bands. In the case of SV40 transformation the appearance of three bands might indicate that the transforming DNA is repetitively integrated and tandemly arranged. The number of units integrated might be between three and four. Repetitive and tandem integration of SV40 DNA has been described in animal cells (Rigby, 1979).

Crossings between cells of transformed lines and wild-type cells resulted in transformed progeny only to an extent of less than 50%. This is an interesting observation and sheds some light on the genetics of the transformation. However, it will need more detailed studies before conclusions can be drawn. The finding

that stable transformation of *Acetabularia* cells occurs after microinjection of vector-free naked DNA is remarkable. It raises the question of whether it is essential to use vector systems like Ti plasmids and virus in order to transform plant cells. As a first approach protoplasts have been used for direct gene transfer experiments (Paszakowsky *et al.*, 1984; Lörz *et al.*, 1985; Hain *et al.*, 1985).

The experiments described here are a contribution to solve one of the most challenging problems in present-day plant biology, i.e. to stably transform plant cells by simple methods and without the limitation of a narrow host range. The experiments described here were performed on the model system *Acetabularia*. Whether this technique, which works well for *Acetabularia*, may be applied to other cells, in particular those of crop plants, is a question best answered by transferring the strategy to cells from higher plants. A combination of single cell and cell fragment culture techniques (Koop and Schweiger, 1985; Spangenberg *et al.*, 1985; Spangenberg and Schweiger, in preparation), which have already been established for *Brassica napus* protoplasts, with microinjection may open an efficient method of transforming higher plant cells. Apparently this is a promising way, in particular since *B. napus* is not limited by lacking the capability to regenerate whole plants from single cells, as it is the case so far with Gramineae.

Materials and methods

Cells

Cells of *A. mediterranea* were cultured according to the method of Schweiger *et al.* (1977) and Schweiger and Berger (1979). For the microinjection and implantation experiments, cells at the stage of early cap formation were used.

Microinjection and transformation

Isolation of nuclei and the microinjection of SV40 DNA or pSV2neo into isolated nuclei of *A. mediterranea* was performed as described (Cairns *et al.*, 1978b; Neuhaus *et al.*, 1984). SV40 DNA and pSV2neo were obtained from Peter Gruss, Heidelberg. Injected nuclei were implanted into anucleate apical fragments obtained from *A. mediterranea* cells. After surgery SV40-treated cells were maintained in Müller's medium (Schweiger *et al.*, 1977), whereas pSV2neo-treated cells were grown under selective pressure in Müller's medium containing 1 mg/ml G 418. Progeny was obtained from individual cells. Therefore each transformed cell line originated in a single cell of *A. mediterranea*.

DNA techniques

Restriction endonucleases and other DNA-modifying enzymes were used as recommended by the suppliers. Other techniques were carried out following Maniatis *et al.* (1982) if not described otherwise.

Immunofluorescence

Indirect immunofluorescence analysis was performed as reported previously (Cairns *et al.*, 1978b; Neuhaus *et al.*, 1984).

Analysis of genomic DNA

Genomic DNA of *A. mediterranea* was prepared from gametes, and stored at -80°C following the method of Li-Weber (in preparation). A total of 20–30 μg of genomic DNA was digested with restriction endonucleases (Maniatis *et al.*, 1982), separated on 0.8% agarose gels, transferred to nitrocellulose filter and hybridized (Thomas, 1983). The nitrocellulose filter was dried and exposed to Kodak X-ray film for an appropriate time.

Hybridization with radioactively labelled probe (Rigby *et al.*, 1977) was performed in 50% formamide, $4 \times \text{SSC}$, $5 \times \text{Denhardt's}$, 0.2% SDS, 5% dextran sulfate, 0.1 mg/ml calf thymus DNA for 48 h at 42°C , followed by washing in 50% formamide, $3 \times \text{SSC}$, 0.1% SDS for 2–4 h at room temperature with two changes of the washing solution and in $1 \times \text{SSC}$, 0.1% SDS for 2–3 h at room temperature with three changes of washing solution. Dot blot hybridization was performed according to Thomas (1983).

Genetic analysis

Cysts of *Acetabularia* are known to produce either (+) or (–) gametes. Therefore for crossing experiments two cysts, both originating either in a non-transformed or in a transformed cell line or one originating in a transformed and the other one in a non-transformed cell line, were maintained together. After release of the gametes only one (+) and one (–) gamete could fuse. The progeny was grown

to the stage of early cap formation (Schweiger and Berger, 1979) and tested by indirect immunofluorescence and for resistance to the antibiotic G 418 respectively. F_2 and F_3 generations were obtained and tested using the same procedure.

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