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Enhancer-controlled expression of the simian virus 40 T-antigen in the green alga *Acetabularia*

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Nuclei from *Acetabularia mediterranea* were isolated, microinjected with simian virus 40 (SV40) DNA and fused with cytoplasts from the same species. Various times after fusion of the injected nuclei the fusion products were screened for expression of the T-antigen by indirect immunofluorescence. One and two days after injection a bright fluorescence could be observed in the nuclei of *Acetabularia*. On the basis of this immunofluorescence we conclude that in *Acetabularia* cells the T-antigen is expressed and accumulated in the nucleus. Moreover, evidence is presented that the *Acetabularia* cell recognizes the SV40 enhancer sequence. The expression product of the SV40 DNA appears significantly earlier than the expression products of other foreign genes in *Acetabularia*. The results suggest that the well characterized SV40 can be used as a vector system for the introduction and expression of foreign genes in *Acetabularia*.

Key words: gene expression/immunofluorescence/microinjection/plant cell

Introduction

The unicellular green alga *Acetabularia* provides a useful system for studying the expression of heterologous genetic information, irrespective of its origin from plant or animal cells (Schweiger and Berger, 1979). This is true for tobacco mosaic virus (TMV) RNA (Cairns *et al.*, 1978a), mengo virus RNA (Cairns *et al.*, 1978b) and adenovirus type 2 DNA (Cairns *et al.*, 1978c), as well as for cloned plant genes, like the small subunit gene for ribulose-1,5-bisphosphate carboxylase/oxygenase from wheat and pea (Broglie *et al.*, 1983; Neuhaus *et al.*, 1983), and the zein gene (Langridge *et al.*, in preparation).

For a number of reasons simian virus 40 (SV40) has served as a model system for studies on gene function in animal cells (for review, see Tooze, 1980). During both permissive and non-permissive infections the SV40 T-antigen is expressed. A regulatory element of the SV40 genome, the so called enhancer, has been identified upstream from the initiation site for early transcription of T-antigen mRNA (Gruss *et al.*, 1981; Benoist and Chambon, 1981; Banerji *et al.*, 1981; Fromm and Berg, 1982). Enhancer elements can increase the transcriptional activity of many genes *in vivo*, relatively independent of distance and orientation with respect to the coding region (for review, see Khoury and Gruss, 1983).

An interesting question is whether the SV40 early transcriptional control elements can be used in a plant cell. A partial answer to the question might come from experiments in which the SV40 genome is expressed in *Acetabularia* and where constructions containing an intact and a defective enhancer, respectively, are used. Moreover, it is of interest to ask whether in *Acetabularia* the expression product of the early region, the T-antigen, is located in the nucleus as in animal cells.

Results

Injection of nucleic acids into *Acetabularia* cells is hampered by the thickness of the cell wall and the large vacuole with a pH of 1–2. Consequently, a suitable method has been developed for introducing foreign nucleic acids into *Acetabularia* under protective conditions. It is based on the fact that the primary nucleus of *Acetabularia* (Schulze, 1939) can be easily isolated and either re-implanted into an anucleate cell fragment (Hämmerling, 1955) or fused with a cytoplast (Primke *et al.*, 1978), and that it is feasible to microinject an isolated nucleus (Cairns *et al.*, 1978b) without impairing its stability and morphogenetic activity (Neuhaus-Url and Neuhaus, unpublished data). We used the procedure shown in Figure 1, microinjecting SV40 DNA and plasmid DNA (see below).

Expression of the SV40 genome in *Acetabularia* was monitored by screening the fixed fusion products by indirect immunofluorescence with antibody against T-antigen. A bright fluorescence was observed 24 and 48 h after fusion of a SV40 DNA injected nucleus with a cytoplast (Figures 2f,h). As early as 2 h after fusion a fluorescence, clearly distinguishable from the controls, was already detectable (Figure 2d).

A comparison of the fluorescing samples with corresponding white light images revealed fluorescence exclusively within

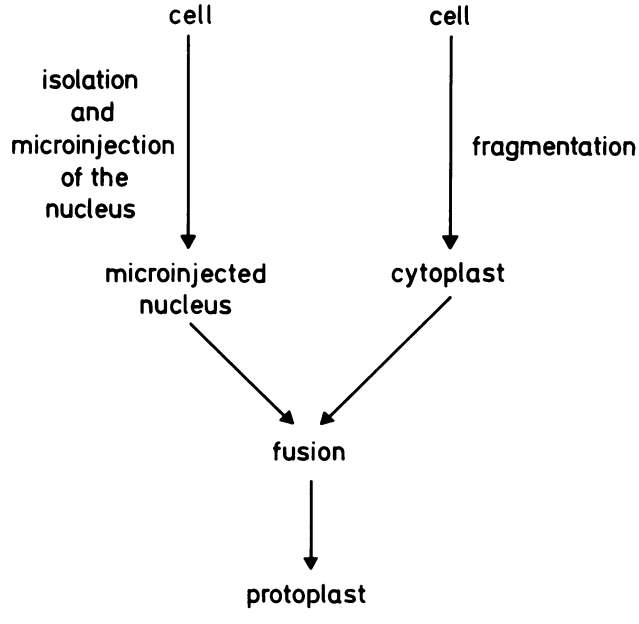

Fig. 1. Procedure used to fuse an injected nucleus with a cytoplast.

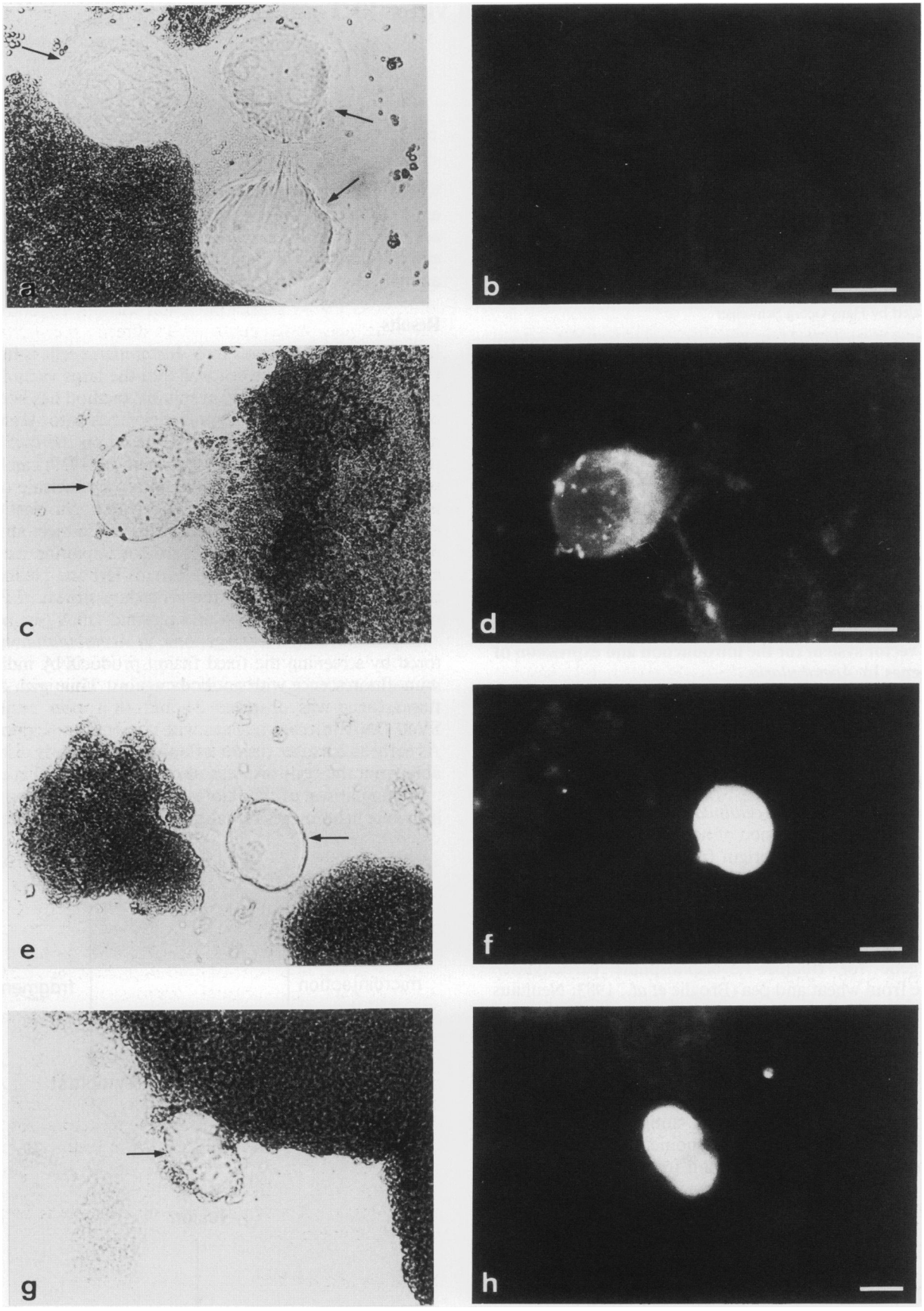

Fig. 2. Appearance of T-antigen in *Acetabularia* nuclei after microinjection of SV40 DNA. The immunoreaction was observed under u.v. light (**b,d,f,h**). In the corresponding white light images (**a,c,e,g**) the nuclei (arrows) and the surrounding cytoplasm can be easily distinguished. The fusion products were fixed and analysed immediately after fusion (**a,b**), 2 h after fusion (**c,d**), 24 h after fusion (**e,f**) and 48 h after fusion (**g,h**). All the u.v. light photographs were taken with the same exposure time. Bars = 50 μm.

Table I. Expression of T-antigen after microinjection of SV40 DNA

Condition (injection)	Time after fusion (h)	Fusion (number)	Embedded nuclei (number)	Positive reaction (number)
None	0	22*	22	0
	24	6	3	0
Buffer	0	13*	13	0
	24	6	2	0
DNA	0	31*	23	0
	2	27	18	7
	24	25	10	8
	48	12	6	5

Differences between the number of fused and embedded nuclei are due to losses during washing of the nuclei.
*Nuclei were isolated, injected (where indicated) and embedded.

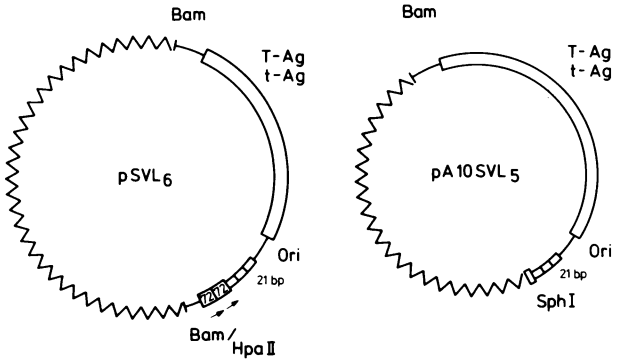

Fig. 3. Scheme of two constructions. **pSVL$_6$**: the complete early region of a viable SV40 variant 2352 (see Levinson *et al.*, 1982) was cloned into pBR322. This viable variant is identical to SV40 strain 776 except that the *Hpa*II site was converted to a *Bam*HI site. The *Bam*HI fragment comprising the early region (see Tooze, 1980, for sequence) was cloned into the *Bam*HI site of pBR322. Thus, the SV40 enhancer (72-bp repeat) is present on this clone. **pA10SVL$_5$**: the *Sph*I-*Bam*HI fragment from SV40 strain 776 was cloned into a pA10 plasmid (see Levinson *et al.*, 1982) previously also cleaved with *Sph*I and *Bam*HI. The selection of *Sph*I excludes a functional enhancer (72-bp repeat) element (Gruss *et al.*, 1981).

the nuclei (Figures 2c,d,e,f,g,h) while the surrounding cytoplasm did not fluoresce. In negative control experiments, in which only buffer solution was injected, no fluorescence could be detected in either the nucleus or the cytoplasm (Table I). No immunoreaction could be observed when nuclei injected with SV40 DNA were processed for immunofluorescence immediately after fusion (Figures 2a,b; Table I). A similar result was obtained in control experiments in which non-injected nuclei were checked for immunofluorescence (Table I). The appearance of a positive fluorescence reaction in the *Acetabularia* system depended on the time elapsed after microinjection (Table I). A significant increase of the fluorescence was observed from 2 to 24 and 48 h. The stability of fused cytoplasts was a limiting factor for experiments exceeding 2–3 days.

A comparison of two constructions, one containing the intact enhancer sequence (pSVL$_6$) and the other with an incomplete enhancer region (pA10SVL$_5$) (Figure 3) revealed that the early expression of the T-antigen depends on the complete enhancer region (Table II). These data were confirmed by injecting constructed chimeric recombinant plasmids containing a plant promoter linked to the coding sequence of SV40 T-antigen. Expression of this chimeric gene could be demonstrated but not before 100 h after injection (unpublished data).

Table II. Effect of the presence and absence of the intact enhancer sequence of SV40 on the expression of the T-antigen 24 h after fusion

Construction	Enhancer	Total number	Immunofluorescence +	Immunofluorescence –
pSVL$_6$	+	15	9	6
pA10SVL$_5$	–	15	0	15

Discussion

Three interesting results were obtained from these experiments. (i) Microinjected SV40 DNA is expressed in the *Acetabularia* system as judged from the appearance of immunofluorescence after immunoreaction with an antibody against the T-antigen. Attempts to detect directly specific transcriptional products have so far failed, presumably because of the low pH and the extremely high RNase activity in the homogenate (Schweiger, 1966). The expression of SV40 DNA corroborates previous results showing that adenovirus 2 DNA is expressed in *Acetabularia* (Cairns *et al.*, 1978c). The data indicate that transcriptional control signals from at least two animal viruses can be recognized by the transcriptional apparatus of *Acetabularia*. (ii) In contrast with the expression of other injected DNAs (Cairns *et al.*, 1978c; Neuhaus *et al.*, 1983), the expression of SV40 DNA can be detected as early as 2 h after microinjection. This difference cannot be explained by the fact that in most other experiments the injected nucleus was combined with the cytoplasm by implantation into an anucleate cell rather than by fusion with a cytoplast, because in implantation experiments SV40 DNA was expressed as early as 3 days after implantation, while with the other genes the specific fluorescene did not appear until 100 or more hours. Therefore, the substantially faster appearance of the immunoreaction is attributed to the function of the control region of the SV40 genome. This control region contains, among other structural features, a 72-bp tandem repeated sequence, which plays a significant role in animal cells as an enhancer of transcription of the SV40 genome, as well as of foreign genes (for review, see Khoury and Gruss, 1983). The experiments performed using plasmid constructions, with an intact and a defective enhancer, respectively, (Table II) suggest that this enhancing element of SV40 is recognized by the transcriptional apparatus of *Acetabularia*. In any case the transcriptional and translational apparatus of *Acetabularia* must be able to recognize heterologous signals. (iii) In *Acetabularia*, a unicellular plant, the SV40 T-antigen is detected exclusively in the nucleus as is the case in infected animal cells (Tegtmeyer *et al.*, 1975; Fareed and Davoli, 1977; Elder *et al.*, 1981; Shen *et al.*, 1982). The underlying mechanism of this accumulation in the nucleus remains to be determined. However, it has to be emphasized that the accumulation of the expression product in the nucleus does not necessarily mean the correct and complete expression of the T-antigen. The results presented indicate the feasibility of developing a vector system for plant cells on the basis of the early promoter region of SV40.

Materials and methods

Cells of *Acetabularia mediterranea* were grown as previously described (Schweiger *et al.*, 1974,1977; Schweiger and Berger, 1979). Nuclei with a diameter of ~100 μm were isolated from cells at the developmental stage of early cap formation and were stored in nucleus buffer (Sandakchiev *et al.*, 1973) on ice during manipulation. Microinjection into the isolated nucleus was performed by means of two micromanipulators (Leitz) following the method

of Cairns *et al.* (1978b) with the following modification. The micromanipulators were equipped with a holder for a microcapillary. The holding capillary had a blunt tip with an o.d. of 70–80 μm and an i.d. of 20–30 μm. The isolated nucleus was held by smooth suction at the tip of the holding capillary. The injection capillary (special capillaries for microelectrodes; Hilgenberg, FRG) had an inner filament (Ø 150 μm), an o.d. of 1500 μm and an i.d. of 750 μm. The injection capillary was pulled to a tip diameter <1 μm (vertical puller; Apparatebau Müller, FRG). It was filled from the back with the solution to be injected. The injection capillary was connected to a Hamilton 1-ml microsyringe by teflon tubing. The syringe was fitted to a motor driven microdispenser (Type Unita 1, B.Braun, Melsungen, FRG).

A minimum pressure was used to establish a continuous slow outflow. The volume of injection, ~80 pl was approximated by measuring time and diameter of a drop injected into oil under elevated pressure. Approximately 10^7 supercoiled viral SV40 DNA molecules were injected under microscopic (Invertoskop, Zeiss) control at a maximum concentration of 1 mg/ml in 50 mM NaCl, 50 mM Tris-HCl buffer, pH 7.8. Control experiments were performed by injecting buffer solution.

Injected nuclei were fused in 1:1 diluted Müller's medium (Primke *et al.*, 1978) with cytoplasts obtained from fragmented cells of *A. mediterranea* (Gibor, 1965) in the developmental stage of early cap formation. More than 70% of the nuclei were stable and functional after the fusion process (Figure 1) (Schweiger *et al.*, 1974; Cairns *et al.*, 1978b). Such fusion happened spontaneously with almost 100% efficiency and did not depend on the addition of fusion supporting agents (Primke *et al.*, 1978).

Appearance of SV40 T-antigen was detected by indirect immunofluorescence. Different times after fusion the fusion products were transferred to a microscope slide and smeared. Usually the nucleus could be easily recognized in the smear. In contrast with the method used by Cairns *et al.* (1978b), in this study the smears were first dried before they were fixed. Each washing step was shortened from 1 h to 15 min. The fixed samples were treated with a polyclonal anti-serum from hamster after dilution 1:10 with buffer (0.43 M NaCl/0.1 M Tris-HCl buffer, pH 7.4) for 30 min. Fluorescein conjugated anti-hamster antibody from goat was used at a dilution of 1:75 with the same buffer as above. The samples were treated for 30 min in darkness. After staining and washing the samples were embedded in polyviol (Wacker Chemie).

Microscopical examination was performed by a Zeiss microscope (Axiomat) fitted with a fluorescence attachment. Photographs were prepared on Fujichrome 400.

$pSVL_6$ is a construction which contains the complete early region of a viable SV40 variant (2352; Levinson *et al.*, 1982) and pBR322 (Figure 3). The second plasmid, $pA10SVL_5$ carries a *SphI-Bam*HI fragment from the SV40 strain 776. This insert contains a non-functional enhancer (Gruss *et al.*, 1981) and is cloned into pA10 (Figure 3) (Levinson *et al.*, 1982).

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References

Banerji,J., Rusconi,S. and Schaffner,W. (1981) *Cell,* **27**, 299-308.
Benoist,C. and Chambon,P. (1981) *Nature,* **290**, 304-310.
Broglie,R., Coruzzi,G., Lamppa,G., Keith,B. and Chua,N.H. (1983) *Biotechnology,* **1**, 55-61.
Cairns,E., Sarkar,S. and Schweiger,H.G. (1978a) *Cell Biol. Int. Rep.,* **2**, 573-578.
Cairns,E., Gschwender,H.H., Primke,M., Yamakawa,M., Traub,P. and Schweiger,H.G. (1978b) *Proc. Natl. Acad. Sci. USA,* **75**, 5557-5559.
Cairns,E., Doerfler,W. and Schweiger,H.G. (1978c) *FEBS Lett.,* **96**, 295-297.
Elder,J.T., Spritz,A.R. and Weissman,S.M. (1981) *Annu. Rev. Genet.,* **15**, 295-340.
Fareed,G.C. and Davoli,D. (1977) *Annu. Rev. Biochem.,* **46**, 471-522.
Fromm,M. and Berg,P. (1982) *J. Mol. Appl. Genet.,* **1**, 457-481.
Gibor,A. (1965) *Proc. Natl. Acad. Sci. USA,* **54**, 1527-1531.
Gruss,P., Dhar,R. and Khoury,G. (1981) *Proc. Natl. Acad. Sci. USA,* **78**, 943-947.
Hämmerling,J. (1955) *Biol. Zentralblatt,* **74**, 545-554.
Khoury,G. and Gruss,P. (1983) *Cell,* **33**, 313-314.
Levinson,B., Khoury,G., Vande Woude,G. and Gruss,P. (1982) *Nature,* **295**, 568-572.
Neuhaus,G., Neuhaus-Url,G., Schweiger,H.G., Chua,N.H., Broglie,R., Coruzzi,G. and Chu,N. (1983) *Eur. J. Cell Biol. Suppl.,* **2**, 30.
Primke,M., Berger,S. and Schweiger,H.G. (1978) *Cytobiologie,* **16**, 375-380.
Sandakchiev,L., Niemann,R. and Schweiger,H.G. (1973) *Protoplasma,* **76**, 403-415.
Schulze,K.L. (1939) *Arch. Protistenk.,* **92**, 179-225.
Schweiger,H.G. (1966) *Planta,* **68**, 247-255.
Schweiger,H.G. and Berger,S. (1979) *Int. Rev. Cytol. Suppl.,* **9**, 11-14.
Schweiger,H.G., Berger,S., Kloppstech,K., Apel,K. and Schweiger,M. (1974) *Phycologia,* **13**, 11-20.
Schweiger,H.G., Dehm,P. and Berger,S. (1977) in Woodcock,C.L.F. (ed.), *Progress in Acetabularia Research,* Academic Press, NY, San Francisco, London, pp. 319-330.
Shen,Y.M., Hirschhorn,R.R., Mercer,W.E., Surmacz,E., Tsutsui,Y., Soprano,K.J. and Baserga,R. (1982) *Mol. Cell. Biol.,* **2**, 1145-1154.
Tegtmeyer,P., Schwartz,M., Collins,J.K. and Rundell,K. (1975) *J. Virol.,* **16**, 168-178.
Tooze,J., ed. (1980) *DNA Tumor Viruses. Molecular Biology of Tumor Viruses Part 2,* published by Cold Spring Harbor Laboratory Press, NY.

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