Aust. J. Biol. Sci., 1977, **30**, 461–9

Fusion and Hybridization of Marsupial and Eutherian Cells II.* Fusion of Marsupial Cells

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Abstract

Marsupial cells could be fused using inactivated Sendai virus, and fusion frequencies were comparable to those for similar eutherian cell types. Established lines fused readily, primary diploid fibroblasts fused less well and lymphocytes fused poorly. The effects of varying the virus titre and the time of incubation of the fusion mixture were determined. The optimal conditions for fusion of marsupial cells to form homokaryons are described.

Introduction

The availability of marsupial × eutherian cell hybrids would widen the scope of cytogenetic and somatic genetic studies of mammalian cells. These hybrids offer some advantages over combinations of cells from different eutherian species, because of the greater evolutionary distance between eutherian and marsupial mammals. We would expect this greater divergence to have resulted in greater genetic and chromosome differences, which could be exploited for studying the genetics and biology of mammalian cells. However, many attempts to obtain marsupial × eutherian cell hybrids have been unsuccessful (Hope and Graves, unpublished data). A number of steps must be completed for the formation of viable hybrids; these steps include cell fusion, formation of functional heterokaryons, synkaryon formation, and the survival, proliferation and selection of hybrid clones.

In the first paper of this series (Graves and Hope 1977) we reported optimal conditions for the growth and cloning of marsupial cells, and described the growth of marsupial and eutherian cells in mixed culture. We observed that mixed cultures undergo a cell-sorting process, which results in the formation of homogeneous domains of marsupial and eutherian cells. However, we found no evidence of growth inhibition of either cell type, and clones of one cell type were readily obtainable using feeder layers of the other. This suggests that growth of hybrid cells would not be inhibited by the presence of excess parental cells, and indicates that one of the other problems referred to above must be responsible for the difficulties in obtaining marsupial × eutherian cell hybrids.

In this paper the fusion of marsupial cells is examined, different methods of cell fusion are assessed and optimal conditions for fusion are described.

* Part I, *Aust. J. Biol. Sci.*, 1977, **30**, 445–59.

Materials and Methods

Parent Cells

The marsupial, human, and rodent cells used are listed in Table 1.

Cell Culture

The media and culture techniques used for monolayer culture have been described in detail previously (Graves and Hope 1977). Lymphocytes were separated from defibrinated or heparinized blood (Bøyum 1974; D. Coghlan and R. M. Hope, unpublished data).

Table 1. Cell types used in the present study

Name and type of cells	Description	Obtained from	Reference
	(*a*) Marsupial established lines		
PtK2	*Potorous tridactylus*		See Graves and Hope (1977)
PtK2 BN1	*Potorous tridactylus*, TK^- [A]		See Graves and Hope (1977)
Sc9/01	*Sminthopsis crassicaudata* ♀		See Graves and Hope (1977)
CSL 235	*S. crassicaudata* ♀ pouch young	Mr D. Pye, Commonwealth Serum Laboratories, Australia (C.S.L.)	Stanley *et al.* (1975)
	(*b*) Marsupial primary diploid lines		
SG9	*Macropus giganteus*		See Graves and Hope (1977)
K2	*Macropus rufus* = *Megaleia rufa* (red kangaroo) ♂	Professor D. W. Cooper, Macquarie University, Australia (D.W.C.)	
ewe32	*Macropus robustus robustus* × *M. r. erubescens* hybrid		See Graves and Hope (1977)
OK1	*M. robustus* × *M. rufus* hybrid		See Graves and Hope (1977)
	(*c*) Marsupial lymphocytes		
	M. giganteus (eastern grey kangaroo) ♀ G56	D.W.C.	
	Pseudocheirus peregrinus (ringtail possum) ♂	D.W.C.	
	(*d*) Eutherian established lines		
EUE	Human $HPRT^-$ [A]		See Graves and Hope (1977)
HEp2	Human	CSL	
A9	Mouse $HPRT^-$		See Graves and Hope (1977)
Na	Mouse $HPRT^-$ derivative of NCTC2472		Graves (1975)
BIo	Chinese hamster TK^-, ouabain-resistant		See Graves and Hope (1977)

[A] TK^-, deficient in thymidine kinase. $HPRT^-$, deficient in hypoxanthine guanine phosphoribosyltransferase.

Sendai Virus

A stock of Sendai virus, originating from the laboratory of Professor H. Harris, was propagated by inoculation of 10^3 infectivity units into the amniotic cavities of 10-day embryonated hen eggs. After incubation for 3 days at 39°C, the amniotic fluid was collected and clarified by centrifugation

at 2000 *g*. The virus was pelleted by centrifuging for 1 h at 16000 *g*, and was then resuspended in one-tenth volume of chilled Hanks balanced salt solution (BSS) (glucose-free, including 0·1% bovine serum albumin). The preparation was inactivated using β-propiolactone [Sigma; 1/400 volume, stirred at 0°C for 1 h, 37°C for 3 h, and 4°C overnight (Neff and Enders 1967)]. The infectivity and haemagglutination titres were determined (Harris *et al.* 1966) for crude, concentrated, and inactivated virus, and the inactivated preparation was diluted to a working concentration of 4000 haemagglutination units (HAU) per millilitre with Hanks BSS (glucose-free).

Fusion Techniques

(i) *Spontaneous fusion*

Mixtures of parent cells were cultivated to confluency before dissociating, plating out and fixing for assessment of fusion.

(ii) *Sendai virus-induced fusion*

Cells were fused in monolayer or suspension (Giles and Ruddle 1973).

For monolayer fusion, cells were plated at $0·5 \times 10^6$–$2·0 \times 10^6$ per Petri dish, and allowed to become confluent. The monolayer was washed twice with Hanks BSS and chilled for 20 min before removing the Hanks BSS and adding 0·5 ml of chilled Sendai virus at 500–1000 HAU/ml. The virus was allowed to adsorb for 10 min at 0°C, and was then removed; warm medium was added, and the plates were incubated at 36–37°C. Cells were then dissociated and plated at $0·25 \times 10^6$–$0·50 \times 10^6$ per Petri dish for assessment of fusion.

Cells to be fused in suspension were dissociated with trypsin–versene, washed in Hanks BSS, counted, and then concentrated to 2×10^7/ml. An equal volume of Sendai virus suspension at 1000–2000 HAU/ml was added, and gently mixed by pipetting. The mixture was kept in ice for 10 min, then incubated at 37°C, after which the cells were plated out at $0·25 \times 10^6$–$0·5 \times 10^6$ per Petri dish for assessment of fusion.

(iii) *Polyethylene glycol fusion*

Cells were plated at $1·0 \times 10^6$–$2·0 \times 10^6$ per Petri dish and allowed to attach and spread. The monolayer was washed with phosphate-buffered saline (PBS), then covered with polyethylene glycol (PEG) [10 g/10 ml of Dulbecco's modification of Eagle's medium (DME); PEG molecular weight 6000; Merck, Darmstadt, West Germany] for 10 min at 37°C. The PEG was then poured off and the film gradually diluted with warm DME. This was replaced with warm DME with 15% foetal calf serum, and the plates were incubated before transferring to Petri dishes for assessment of fusion (see Pontecorvo 1975).

Cytological Techniques

For assessment of fusion, cells growing on Petri dishes were fixed *in situ* with 3:1 (v/v) methanol–acetic acid, and then stained with Harris' alum haematoxylin as described previously (Graves and Hope 1977). Homokaryon formation by lymphocytes was assessed in Giemsa-stained, air-dry preparations of fixed cells.

Radioisotope Labelling and Autoradiography

For assessment of the frequency of spontaneous fusion, one culture of cells was prelabelled with 0·05 μCi/ml [*methyl*-^{3}H]thymidine (specific activity 5·0 Ci/mmol, Radiochemical Centre, Amersham, England) for twice the mean generation time. After mixing with unlabelled cells, and allowing fusion to proceed, cells were dissociated and plated out at $0·25 \times 10^6$ per Petri dish, allowed to attach and spread, and then fixed *in situ*. The monolayer was washed several times, then autoradiographed as described previously (Graves and Hope 1977).

Results

Fusion Index

Marsupial cells and, for comparison, some eutherian cells were tested for their ability to fuse with cells of the same type, forming homokaryons. The extent of cell fusion was expressed as the fusion index which is defined here as the percentage of nuclei which were included in fused cells, corrected for the background by subtraction

of the frequency of nuclei in multinucleate cells in control culture. The fusion indices in untreated, Sendai virus-treated, and PEG-treated cultures were compared (Table 2).

(i) *Spontaneous fusion*

The percentage of nuclei included in multinucleate cells was found to be between 1 and 5% in untreated cultures. This does not, however, represent the frequency of fusion, since most multinucleated cells probably arose by failure of cytokinesis after nuclear division. An estimate of the spontaneous fusion index was therefore obtained

Table 2. Fusion indices (%) of untreated, Sendai virus-treated and PEG-treated cells

Cell type	Spontaneous fusion	Sendai-induced: Suspension	Sendai-induced: Monolayer	PEG treated
Eutherian				
HEp2	—	64	49	100
BIo	*c.* 0·01	38	—	—
Marsupial				
PtK2	*c.* 0·01	34	25	1
SG9	<0·01	9	6	—
OK1	<0·01	10	—	—

by prelabelling one culture of PtK2 cells with [^{3}H]thymidine, fusing with an equal number of unlabelled PtK2 cells, and scoring the frequency of labelled nuclei contained in cells with at least one nucleus derived from each culture. Since the spontaneous fusion frequency was low, and cells with three or more nuclei were rare, the fusion index was obtained by doubling this quantity, as only one-half of the fusions were expected to involve both labelled and unlabelled cells. The spontaneous fusion index was extremely low for all cells tested (Table 2).

(ii) *Sendai virus-induced fusion*

It was found that marsupial cells were agglutinated by mixing with a suspension of Sendai virus, and electron microscopy showed that the virus was adsorbed to the cell surface (Fig. 1). Adsorption of Sendai virus to the surface of marsupial cells could also be demonstrated by mixing chick red blood cells with virus-treated PtK2 cells, and washing off unattached red cells (Fig. 2). Treatment with the virus also fused marsupial cells (Table 2). Figs 3 and 4 show untreated and Sendai virus-treated PtK2 cells.

(iii) *PEG-induced fusion*

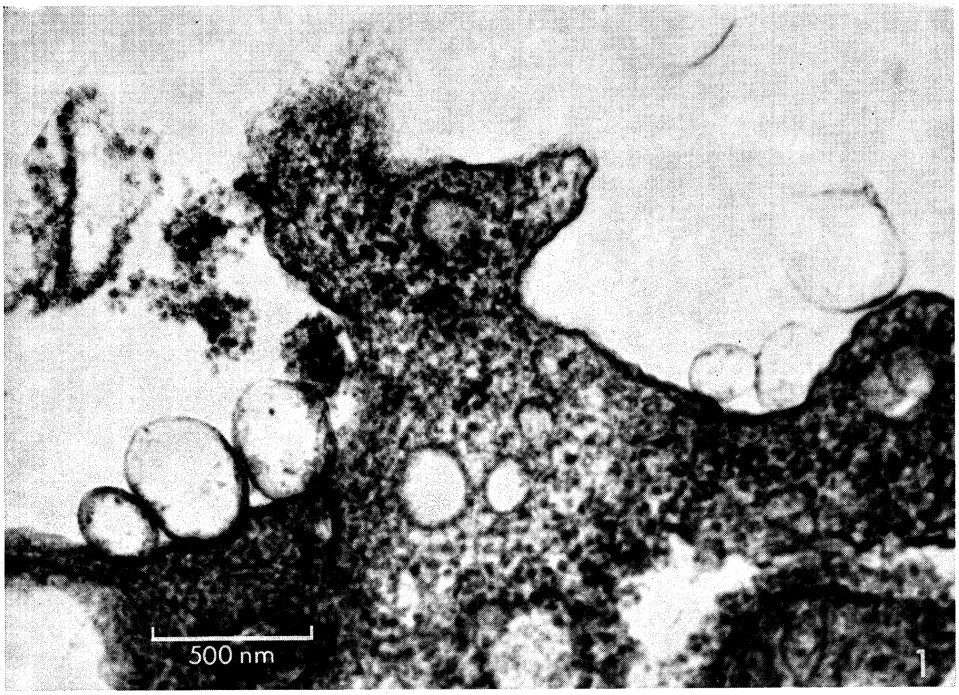
Treatment of PtK2 cells with PEG produced little or no fusion (Table 2). In comparison, cultures of HEp2 cells were completely fused, forming one giant syncytium.

Optimal Conditions for Sendai Virus-induced Fusion

Since treatment with Sendai virus fused marsupial cells, experiments were undertaken to define the optimal conditions for fusion by this method.

(i) *Fusion in monolayer and suspension*

The fusion indices of PtK2 and SG9 cells (and human HEp2 for comparison) were found to be consistently higher when cells were fused in suspension (Table 2).



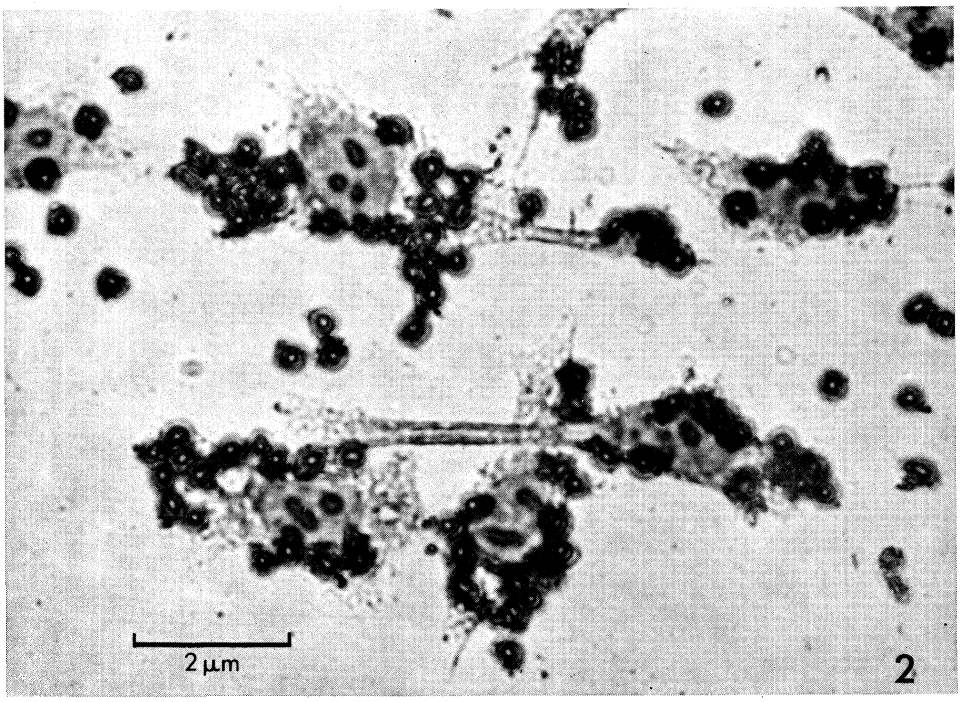


Fig. 1. Electron micrograph of *Sminthopsis* cell, showing Sendai virus particles adsorbed to the surface.

Fig. 2. Adsorption of chick erythrocytes to Sendai virus-treated potoroo cells.

(ii) *Sendai virus strain*

The frequencies of fusion of PtK2 and Sc3F cells (and, for comparison mouse A9 cells) were determined after treatment with two independently isolated strains of Sendai virus at 500 HAU/ml. One was derived from a strain obtained from Professor H. Harris, Oxford, and the other originated in Melbourne (supplied by D. Pye, Commonwealth Serum Laboratories). Little difference was observed in the ability of these strains to fuse eutherian or marsupial cells (Table 3).

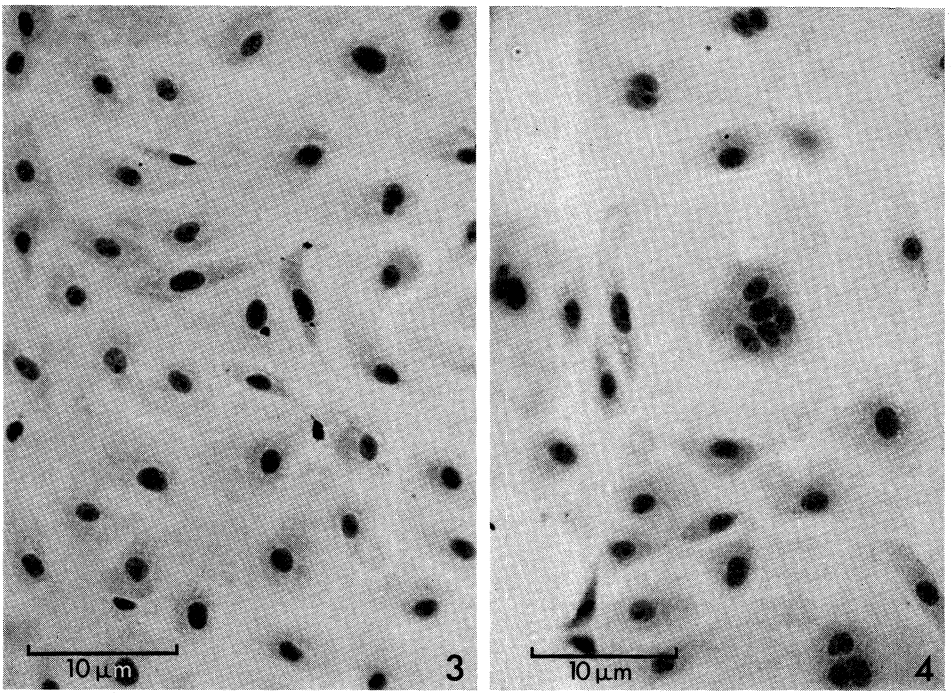


Fig. 3. Untreated culture of potoroo cells.
Fig. 4. Sendai virus-treated culture of potoroo cells.

(iii) *Sendai virus titre*

PtK2 or SG9 cells were fused in suspension (at concentrations of 10^7 cells/ml and using an incubation time of 1 h) with a range of virus titres from 0 to 4000 HAU/ml. The results are shown in Fig. 5*a*; maximal fusion was obtained at relatively low titres (400 HAU/ml). At higher virus titres the fusion index declined, and the proportion of cells which reattached to the culture vessel also decreased, especially for the SG9 cells.

(iv) *Time of incubation at* 37°C

Previous experiments with eutherian cells have shown that this is an important variable (Graves, unpublished data). PtK2 or SG9 cells were treated with Sendai virus at 1000 HAU/ml, incubated for 10 min at 0°C, and then incubated for different times at 37°C. Fig. 5*b* shows that maximal fusion was obtained at 1 h. After this

the fusion index remained unchanged; however, the frequency of larger mutinucleate cells (which do not yield viable hybrids) increased, and the frequency of reattachment decreased, especially for the SG9 cells.

Table 3. Fusion indices (%) of cells after treatment with different strains of Sendai virus

Cell type	Source of virus	
	Oxford	Melbourne
Eutherian		
A9	32	29
Marsupial		
Sc3F	56	64
PtK2	30	27

(v) *Fusion of different marsupial cells*

Table 4 shows the fusion indices for different marsupial and eutherian cell types after suspension fusion with Sendai virus at 500 HAU/ml and incubation for 1 h at 37°C. Established marsupial cell lines fused readily, with frequencies comparable to those for established eutherian cell lines, whilst diploid marsupial fibroblasts and marsupial lymphocytes fused relatively poorly.

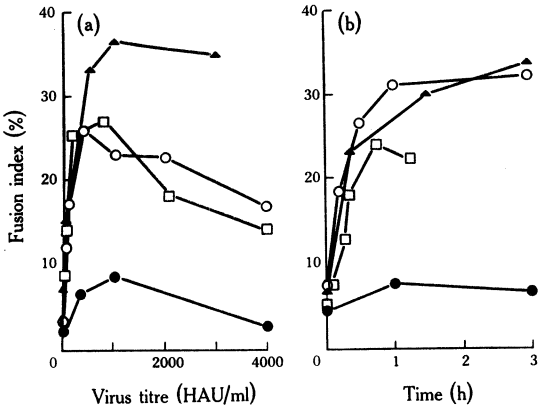


Fig. 5. Fusion of marsupial and eutherian cells (*a*) by different Sendai virus titres and (*b*) after different times of incubation at 37°C. ▲ BIo. □ CSL 235. ○ PtK2. ● SG9.

Discussion

Of the fusion methods tested, Sendai virus-induced fusion was by far the most successful. Sendai virus receptors are present on the membranes of cells from many different mammalian species (Okada 1969), and are evidently present also on the surface of marsupial cells. Sendai virus was found to promote fusion of marsupial cells at frequencies comparable to those for eutherian cells of similar type. Permanent strains could be fused with very high frequencies (up to 66%), whilst diploid fibroblasts fused less well, and lymphocytes fused very poorly. Okada and Tadokoro (1963) described a similar ranking of permanent and malignant cell strains and diploid fibroblast lines, and these workers were unable to detect any fusion of lymphocytes.

Optimal conditions for Sendai virus-induced fusion of marsupial cells were generally found to be similar to those found by us, and described by other workers

for eutherian cells. A low concentration of virus (400–1000 HAU/ml) produced near-maximal fusion and little cell death of marsupial cells, as well as of Chinese hamster and human cells. Velasquez *et al.* (1971) reported a maximal fusion index at low virus titres, and Okada and Tadokoro (1963), although they found a further increase in fusion index at higher titres, observed that cell degeneration also increased. Maximal yields of eutherian cell hybrids, too, were obtained by fusion with Sendai virus at about 500 HAU/ml (Coon and Weiss 1969; Klebe *et al.* 1970). The fusion index of marsupial cells, like that of the eutherian cells also studied here, increased with the time of incubation at 37°C, up to 1–3 h, but little advantage was gained by prolonging incubation beyond 30 min, because of the deleterious effect this had on viability (as judged by the proportion of cells which reattached to the culture vessel). Marsupial cells could be fused in a monolayer, as well as in suspension, but the fusion index was consistently lower.

Table 4. Average fusion indices (%) for marsupial and eutherian cells after Sendai virus treatment

Cell type	Average fusion index	Cell type	Average fusion index
Eutherian established lines		Marsupial primary diploid lines	
HEp2	71	K2	14
EUE	61	ewe32	12
BIo	38	OK1	10
A9	35	SG9	9
Na	30	Marsupial lymphocytes	
Marsupial established lines		Ringtail possum	< 5
Sc9	66	Grey kangaroo	< 5
Sc3F	64		
PtK2 BN1	38		
PtK2	34		

Although PEG produced little fusion of PtK2 cells, some recent results (A. MacGregor, personal communication) indicate that modifications of the PEG method may be very successful for fusion of *Sminthopsis* cells.

These studies have revealed no special difficulties with intraspecific fusion of marsupial cells, and therefore provide no explanation for the problems we have encountered in obtaining marsupial × eutherian cell hybrids. The possibility remains that marsupial cells may not fuse with eutherian cells to form heterokaryons, or that these heterokaryons do not complete the other steps necessary for hybrid formation. The fusion data reported here should enable us to choose the most suitable cell types and the optimal conditions for obtaining marsupial × eutherian heterokaryons, and ultimately, cell hybrids. The results of our experiments into heterokaryon and hybrid formation will be reported in subsequent papers of this series.

Acknowledgments

We thank Mrs Iole Barbieri, Mrs Lorraine Billett, and Mrs Clare Greer for their expert technical assistance, and Mrs Ann Monkman for her help with the photo-

graphic work. Some of this work was performed in the Genetics Department, University of Oxford, U.K., and R.M.H. wishes to thank Professor W. F. Bodmer and his colleagues for their hospitality and advice. We wish to thank Professor D. W. Cooper and Dr D. L. Hayman for their interest and advice, as well as for their gifts of cells, and for critical reading of the manuscript. Data on the cell line CSL 235 was kindly provided by Mr A. MacGregor (C.S.L.) and the electron micrograph (Fig. 1) was supplied by Mr R. C. Hamilton (C.S.L.). This project is supported by grants to J.M.G. and R.M.H. from the Australian Research Grants Committee.

References

Bøyum, A. (1974). Separation of blood leucocytes, granulocytes and lymphocytes. *Tissue Antigens* **4**, 269–74.

Coon, H. G., and Weiss, M. C. (1969). A quantitative comparison of formation of spontaneous and virus-produced viable hybrids. *Proc. Natl. Acad. Sci. U.S.A.* **62**, 852–9.

Giles, R. E., and Ruddle, F. H. (1973). Production and characterization of proliferating somatic cell hybrids. In 'Tissue Culture. Methods and Application'. (Eds P. F. Kruse and M. K. Patterson.) pp. 475–500. (Academic Press: New York.)

Graves, J. A. M. (1975). Control of DNA synthesis in somatic cell hybrids. In 'The Eukaryote Chromosome'. (Eds W. J. Peacock and R. D. Brock.) pp. 367–79. (Australian National University Press: Canberra.)

Graves, J. A. M., and Hope, R. M. (1977). Fusion and hybridization of marsupial and eutherian cells. I. Growth of marsupial cells, and co-cultivation with eutherian cells. *Aust. J. Biol. Sci.* **30**, 445–59.

Harris, H., Watkins, J. F., Ford, C. E., and Schoefl, G. I. (1966). Artificial heterokaryons of animal cells from different species. *J. Cell. Sci.* **1**, 1–30.

Klebe, R. J., Chen, T. R., and Ruddle, F. H. (1970). Controlled production of proliferating somatic cell hybrids. *J. Cell Biol.* **45**, 74–82.

Neff, J. M., and Enders, J. F. (1967). Polio-virus replication and cytopathogenicity in monolayer hamster cell cultures fused with beta-propiolactone inactivated Sendai virus. *Proc. Soc. Exp. Biol. Med.* **127**, 260–7.

Okada, Y. (1969). Factors in fusion of cells by HVJ. In 'Current Topics in Microbiology and Immunology'. (Eds W. Arber *et al.*) pp. 102–28. (Springer: Berlin.)

Okada, Y., and Tadokoro, J. (1963). The distribution of cell fusion capacity among several cell strains or cell caused by HVJ. *Exp. Cell Res.* **32**, 417–30.

Pontecorvo, G. (1975). Production of mammalian somatic cell hybrids by means of polyethylene glycol treatment. *Somatic Cell Genet.* **1**, 397–400.

Stanley, J. F., Pye, D., and MacGregor, A. (1975). Comparison of doubling numbers attained by cultured animal cells with life span of species. *Nature (London)* **255**, 158–9.

Velasquez, A., Payne, F. E., and Krooth, R. S. (1971). Viral-induced fusion of human cells. I. Quantitative studies on the fusion of human diploid fibroblasts induced by Sendai virus. *J. Cell Physiol.* **78**, 93–110.

Manuscript received 14 April 1977