PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 164, 439–444 (1980)

Growth Potential of Sheep and Sea Mammal Cells Transformed by SV40 Early Region DNA (40892)

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Abstract. Normal fibroblasts of bearded seal, northern fur seal, sea lion, domestic sheep, and normal kidney cells of spotted dolphin and Pacific common dolphin have been transformed by fragments of SV40 DNA containing the entire early region. The transformed, T-antigen-positive cell strains grew to higher saturation densities and, except for the transformed dolphin kidney cell strains, were able, albeit poorly, to form colonies in soft agar medium. The transformed northern fur seal, spotted dolphin and Pacific common dolphin cell strains have remained diploid, the transformed sheep cells have become hypodiploid due to Robertsonian translocations, and the transformed bearded seal and sea lion cells had become hypotetraploid. Although all the transformed cells have acquired increased divisional potential and prolonged lifespan in cell culture, only the hypotetraploid cell strains of bearded seal and sea lion have evolved into continuous cell strains.

SV40 (simian virus 40) is able to transform cells of a wide range of species and tissue origin. Infection of human cells by SV40 leads to acquisition of unlimited growth potential only in the minority of the transformed clones; the majority of cells become senescent after a significantly prolonged lifespan (1–7). The cause of the limited lifespan of SV40 transformed human cells is unclear and might be a reflection of the limited lifespan of normal diploid cells *in vitro*. In addition, transfection of normal rat kidney cells with SV40 DNA fragments containing the entire early region also leads to transformation (8). We have assessed the usefulness of transfection of short term cell strains using fragments of SV40 DNA containing the entire early region in order to generate continuous cell strains, or cell strains with greatly increased divisional potential, from species in which no continuous cell lines are currently available.[1]

Materials and methods. Cells. Fibroblast strains from bearded seal (*Erignathus barbatus*), northern fur seal (*Callorhinus ursinus*), sea lion (*Zalophus californianus*), and sheep (*Ovis aries*), and a kidney cell strain of Pacific common dolphin (*Delphinus bairdi*) were kindly supplied at a passage level less than 10 by Dr. W. A. Nelson-Rees, Cell Culture Department, Naval Biosciences Laboratory, Oakland, California, and were produced with support from the National Cancer Institute under the auspices of the Office for Naval Research and the Regents of the University of California. A kidney cell strain of spotted dolphin (*Stenella plagiodon*) was obtained at the 11th serial passage from the American Type Culture Collection, Rockville, Maryland. (CCL No. 78). The cells were propagated in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and 25 μg gentamycin/ml. The transformed and the control cultures were regularly split in a ratio of 1:7 after reaching confluence (usually once or twice a week).

Preparation of SV40 DNA fragments. Unlabeled SV40 DNA mixed with 10,000 cpm of ^{32}P-labeled SV40 DNA to serve as a marker was sequentially digested with *Hae* II and *Eco* RI or *Bam* HI and *Hha* I (1 unit of enzyme/μg of viral DNA) for 60 min at 37°C in the appropriate buffer. The restriction enzymes were purchased from Bethesda Research Laboratories (Bethesda, Md.). In the sequential digestions, the enzyme

[1] These transformed cell strains are available on request to the senior author.

0037-9727/80/080439-06$01.00/0

was inactivated after the first cleavage by heating at 60°C for 10 min following the addition of EDTA to a concentration of 5 m*M*. The DNA was extracted with phenol and dialyzed against the appropriate buffer for the second enzyme cleavage.

The cleaved DNA fragments were purified by sedimentation through a 5–30% neutral sucrose gradient (w/v) for 22 hr at 10°C at 27,000 rpm in an SW41 rotor. Fractions were collected from the bottom of the tube and analyzed for the marker ^{32}P-labeled DNA by Cherenkov counting. The peak fractions of the fragments containing the early regions were pooled and the DNA further purified by electrophoresis through a 1.6% agarose slab gel (E. C. Apparatus) for 4 hr at 150 volts at 20°C in a buffer containing 40 m*M* TRIS, pH 7.8, 5 m*M* sodium acetate, and 1 m*M* EDTA. The DNA fragments were then extracted from the gel. The specific SV40 fragments used in the transfection extended clockwise from 0.144 to 0.725 map unit generated by cleavage with *Bam* HI and *Hha* I, and clockwise from 0.00 to 0.818 map unit generated by cleavage with *Eco* RI and *Hae* II (Fig. 1 and Ref. (9)).

Transfection. Subconfluent cell cultures growing in 60-mm petri dishes were transfected with the SV40 DNA fragments containing the entire early region using the calcium precipitation technique (10). Each dish was infected with the genome equivalent of 1 g of SV40 DNA consisting of the early gene fragment and 10 g of unsheared salmon sperm DNA.

SV40 T-antigen detection. The cells were grown on cover slips and fixed with cold acetone. Serum from a BK tumor-bearing hamster (which cross reacts with SV40 T antigen) diluted 1:10 was allowed to react with the cells for 60 min at 37°C. The cells were then washed and reacted with FITC anti-hamster γ-globulin (Cappel, Cochranville, Pa.) diluted 1:20 for 30 min at 37°C. The cover slips were washed and mounted with 50% glycerine and examined with a Zeiss fluorescence microscope.

Growth in soft agar medium. The cells were resuspended in 0.32% agar in Eagle medium supplemented with 10% fetal calf serum and 10% tryptose phosphate broth. Two × 10 cells/ml of the 0.32% agar medium were layered over 0.7% agar medium. The cultures were fed weekly and the colony size was determined after 3 weeks by a measuring grid in the eyepiece of a Zeiss inverted microscope.

Saturation density. The numbers of cells present in confluent 35-mm dishes were counted daily during several consecutive days, while the medium was changed daily.

Karyological analysis. Exponentially growing cultures at passage 35 were incubated with colcemid (0.05 g/ml) for 2 hr. Cells were harvested and chromosome preparations were made according to standard techniques. At least 30 Giemsa-stained metaphases and five karyotypes were analyzed for each cell strain.

Results. The foci of transformed cells (local areas of refractile, round, densely piled cells) appeared 3–4 weeks after transfection, and by 6 weeks the cultures consisted only of transformed, SV40 T-antigen-positive cells (Fig. 2). No foci of transformed cells were noted in control cultures. Transformed cell strains were able to grow to higher saturation densities and except for the two transformed dolphin kidney cell strains, grew, albeit poorly, in soft agar medium (Table I).

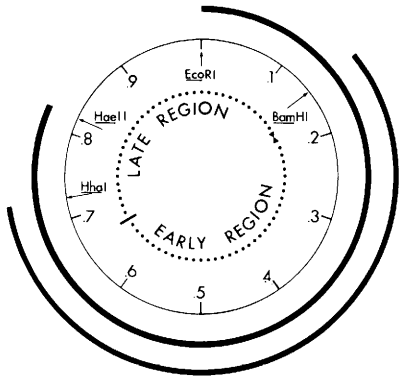

FIG. 1. Physical map of the SV40 genome. The map coordinates are oriented clockwise from the *Eco* RI cleavage site at 1.0–0.0. The early and late gene regions are indicated by the dotted lines. The segments of DNA containing the intact early region used in the transfection experiments are indicated by the solid bars outside the map. The restriction endonuclease sites used in preparing these DNAs are indicated on the physical map. For specific references to the molecular biology of SV40, see Ref. (9).

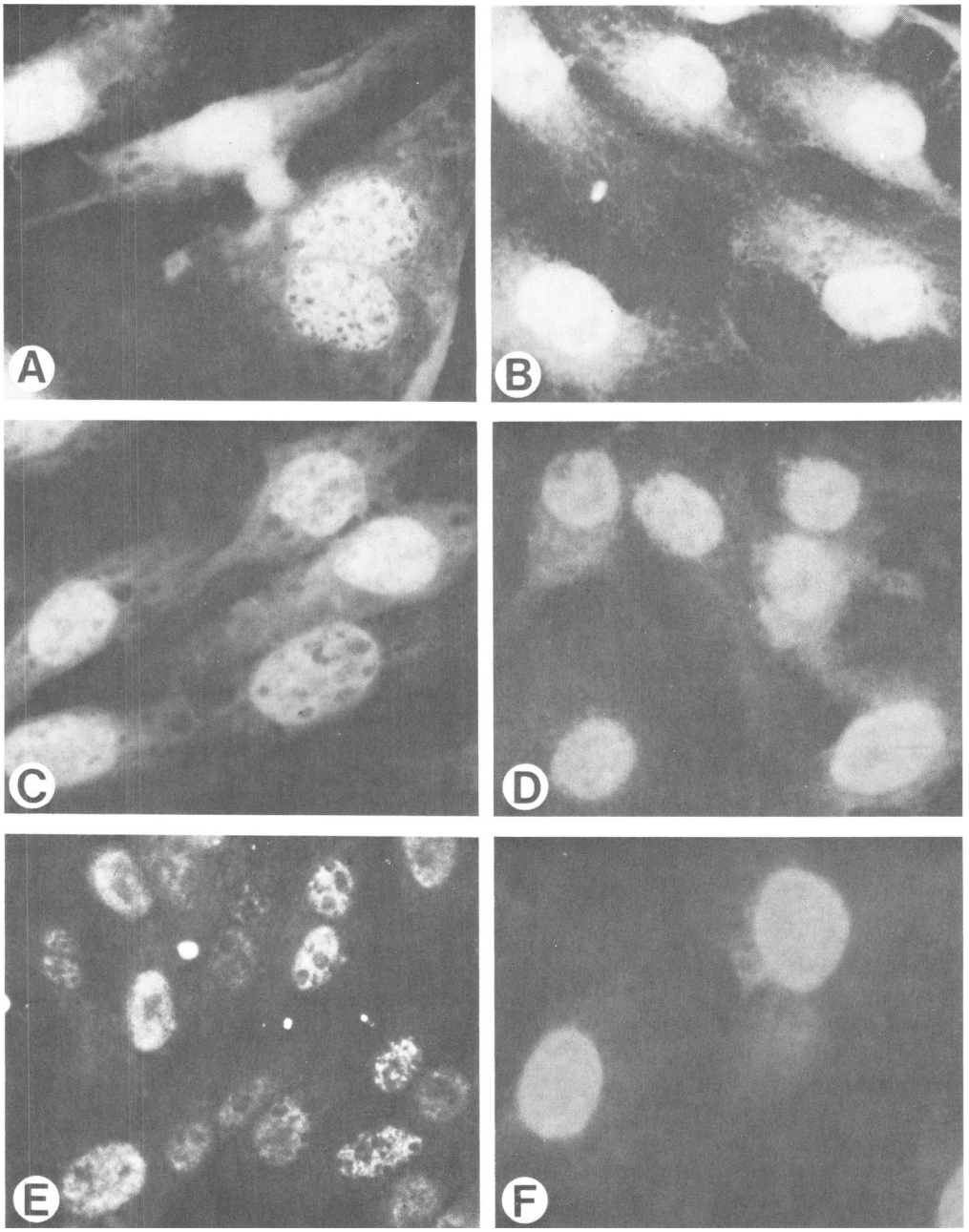

FIG. 2. Immunofluorescent detection of SV40 T antigen expression in nuclei of domestic sheep and sea mammal cells (325×). (A) Domestic sheep (OalBSV), (B) northern fur seal (30F73MuSV), (C) bearded seal (EbSV), (D) sea lion (ZcluSV), (E) spotted dolphin (SplkSV), and (F) Pacific common dolphin (DdlkSV).

The karyotypes of the transformed cells were analyzed (11–14). The transformed northern fur seal and dolphin cells were diploid, the transformed sheep cells were hypodiploid due to Robertsonian translocations and the transformed bearded seal and sea lion cells were hypotetraploid (Table I). One chromatid break was seen in

TABLE I. CHARACTERISTICS OF CONTROL AND SV40 EARLY REGION DNA-TRANSFECTED CELLS

Cells	Species	Transfected with SV40 DNA fragment	Plating Efficiency in Soft Agar		Saturation density (10^5 cells/cm^2)	Chromosomal number[a]		Pass at senesc
			Percentage	Colony size in mm		Modus	Range	
OalB	Sheep (*Ovis aires*)	—	<0.000001	—	2.04	54	—	1
OalBSV		0.14–0.73	0.04	0.18–0.78	4.55	46	41–84	4
0F73Mu	Seal (*Callorhinus ursinus*)	—	<0.000001	—	1.67	36	—	1
0F73MuSV		0.14–0.73	0.01	0.08–0.24	4.72	36	35–37	4
Eb	Seal (*Erignathus barbatus*)	—	0.000001	—	0.63	34	—	1
EbSV		0.00–0.82	0.04	0.09–0.13	2.48	64	60–69	>7
Zclu	Sea lion (*Zalophus californianus*)	—	<0.000001	—	0.85	36	—	1
ZcluSV		0.00–0.82	0.05	0.08–0.14	1.81	62	60–133	>7
Splk	Dolphin (*Stenella plagiodon*)	—	<0.000001	—	0.73	44	—	2
SplkSV		0.00–0.82	<0.000001	—	1.44	44	40–82	4
Dblk	Dolphin (*Delphinus bairdi*)	—	<0.000001	—	0.46	44	—	1
DblkSV		0.00–0.82	<0.000001	—	3.58	44	43–45	3

[a] The chromosomal number of the normal diploid cell strains was acquired from the references cited in the reference section.

Characteristics of control and SV40 early region DNA-transfected cells from domestic sheep and five species of sea mammals. The methods i
solating precise SV40 DNA map regions, and calculations of plating efficiency in soft agar, saturation density, and determination of karyot
scribed in the methods section.

1 out of 30 cells of spotted dolphin and sea lion and no breaks or gaps were present in the rest of the transformed cell strains. Although all transformed cell strains had acquired higher growth potential and prolonged lifespan, with the exception of the transformed bearded seal and sea lion cell strains, they eventually progressed into a phase of slow growth, multinucleation, cessation of growth, and finally degeneration (cell senescence). However, all cells of such terminal cultures remained SV40 T antigen positive. No upper limit for the lifespan of transformed subtetrapoloid bearded seal and sea lion cells has yet been determined. Each currently is at passage level 70 (Table I). Transfection by SV40 early region containing DNA fragments thus leads to prolonged but limited lifespan of the majority of transformed cell strains.

Discussion. The ability to prolong the lifespan of eukaryotic cells by transformation with SV40 should be useful to investigators studying cells that are difficult to maintain in cell culture. Transfecting cells with SV40 DNA containing the intact early gene but deleted in late gene sequences, eliminates a permissive or semipermissive infection with SV40 (which requires intact late gene functions). Consequently, any deleterious influence of complete SV40 replication during experimentation is avoided by the use of these cells.

The effect of SV40 transformation on specific differentiated cell functions has not been examined in this study, and may present a possible drawback to this technique. However, application of this method to Rhesus monkey trophoblast cells, isolated by reported techniques (15), has led to a T-antigen-positive cell strain *in vitro* that maintains many morphological and biochemical characteristics of normal Rhesus cytotrophoblast *in vivo* (Stromberg *et al.*, submitted for publication). In addition, others have reported the persistence of differentiated cell functions following infection with intact SV40 (16–18).

It is of interest that the only continuous cell strains arising from the SV40 transfected sea mammal cells examined in this study were subtetraploid. Abortive infection with SV40 or polyoma virus leads to T-antigen synthesis, cellular DNA replication, and chromosomal abnormalities (19–21). Analyses of the karyotype of cells stably transformed by polyoma viruses have shown, however, that it is unlikely that chromosomal changes of the type measured by metaphase chromosomes can be regarded as directly related to morphologic transformation and unlimited growth in cell culture. For example, of four hamster continuous cell lines after transformation by polyoma virus, three were near diploid and one was subtetraploid (22). Therefore, it seems unlikely that the subtetraploid number of chromosomes per se specifically contributed to the stable transformation of our two sea mammal strains which displayed extended growth potential. The transfected cell lines which underwent senescence remained SV40 T antigen positive terminally. Thus the early SV40 gene region was still stably associated with these cells and was actively being transcribed and expressed. While no quantitative studies and no detailed molecular analyses of specific T-antigen species were performed, it seems unlikely that the limited growth potential of most of these cells was due to a defect in the expression of the early SV40 gene products. It seems more likely that the defect is an inherent property of the cells themselves.

We are grateful to Doris Little for secretarial help.

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Received March 31, 1980. P.S.E.B.M. 1980, Vol. 164.