

Construction of Mouse A9 Clones Containing a Single Human Chromosome Tagged with Neomycin-resistance Gene via Microcell Fusion

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Normal human fibroblasts (MRC-5 or NTI-4) were transfected with pSV2-neo plasmid DNA. Fifty G418-resistant fibroblast clones were isolated and independently fused to mouse A9 cells. The cell hybrids were selected and isolated in the medium containing G418 plus ouabain. Since micronuclei were more efficiently induced in these hybrids compared to parental human fibroblasts by colcemid treatment, the transfer of neo-tagged human chromosomes in the hybrids to mouse A9 cells was performed via microcell fusion. Two hundred A9 microcell hybrids were isolated and karyotyped. Among them, thirteen microcell clones, each containing a single human chromosome 1, 2, 5, 6, 7, 8, 10, 11, 12, 15, 18, 19 or 20 were established. Isozyme analyses confirmed the presence of each human chromosome in these A9 microcell clones. The results of Southern blot and chromosomal *in situ* hybridization analyses indicate that the human chromosomes in these clones were tagged with pSV2-neo plasmid DNA.

Key words: pSV2-neo gene — Chromosome transfer — Microcell fusion — Human mono-chromosomal library

The construction of libraries of microcell hybrids containing a single human chromosome tagged with a selectable genetic marker would facilitate human gene mapping and other genetic studies including gene dosage effects, gene deletions, trans-acting effects and complementation effects if the marked chromosome can be transferred to other mammalian cells. In our previous paper, we described the construction of mouse A9 clones which possess a single human X-autosome translocation containing hypoxanthine guanine phosphoribosyl transferase (HGPRT) locus.¹⁾ The successful microcell transfer of a single human X-autosome translocation to human tumor cells and identification of the human chromosome which carries a putative tumor-suppressor gene were reported.^{2,3)} However, the chromosomal recipient cells for the microcell transfer of these X-autosome translocations are limited to HGPRT⁻ cells. In contrast, a human chromosome tagged with a dominant selectable gene can be transferred to any cell without isolating mutant cells lacking the enzymes.⁴⁻⁶⁾ In this study, we constructed mouse A9 cells which contain a single human chromosome tagged with a dominant selectable gene, pSV2-neo gene.

We first isolated G418-resistant normal human fibroblasts after transfection of pSV2-neo plasmid DNA.

Normal human fibroblasts (MRC-5 or NTI-4) were transfected with pSV2-neo plasmid DNA by calcium phosphate precipitate methods.⁷⁾ The cells were plated at 2.5×10^5 cells per 60 mm dish one day before the transfection, and 0.5 ml of plasmid DNA ($5 \mu\text{g}/\text{ml}$) per dish was added as calcium phosphate precipitate. After incubation at 37°C for 4 h, the cells were washed once with serum-free DMEM and treated with 1.5 ml of hepes-buffered solution (140 mM NaCl, 25 mM hepes and 1.4 mM Na₂HPO₄, pH 7.1) containing 15% glycerol for 30 s. The cells were washed with DMEM and refed with fresh growth medium. After 2 days of cultivation, the cells were trypsinized and split into 3 plates (100 mm) with selective medium containing 400 $\mu\text{g}/\text{ml}$ of antibiotic, G418 sulfate (GIBCO Laboratories, Grand Island, NY). Drug-resistant colonies were formed at a frequency of about 6.5×10^{-6} treated cells. In total, 50 independent clones which grew stably in the selective medium were obtained. Direct transfer of neo-integrated human chromosome from these G418-resistant fibroblasts to mouse A9 cells via microcell fusion failed because the human fibroblasts were resistant to micronuclei induction by colcemid treatment. Therefore, we instead formed and isolated hybrids between these G418-resistant human cells and mouse A9 cells as microcell donors.

Cells (10^6) of each parental line were seeded in a 25 cm² flask and fused 24 h later by the use of 5 ml of 41.7%

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(w/v) PEG (MW 1000, Baker Chemical Co., Phillipsburg, NJ) containing 15% dimethyl sulfoxide followed by extensive washing with serum-free DMEM.⁸⁾ The cells were grown for 24 h and refed with selective medium containing 800 µg/ml of G418 plus 10⁻⁵ M ouabain. Hybrid clones formed colonies at a frequency about 10⁻⁴ during a 4- to 6-week cultivation period. Fifty independent hybrid clones were isolated. Since micronuclei were efficiently induced in these hybrids by colcemid, microcell transfer of neo-integrated human chromosomes in the hybrids to mouse A9 cells was performed. The cells were seeded in a 25 cm² flask (Costar No. 3025, Cambridge, MA) 2 days before micronucleation. Micronuclei were induced by colcemid (0.05 µg/ml) in DMEM containing 20% FCS for 48 h. The flasks were replaced by prewarmed serum-free DMEM containing 10 µg/ml of cytochalasin B (Sigma Chemical Co., St. Louis, MO) and placed in acrylic inserts filled with warmed water.⁹⁾ The flasks in the inserts were centrifuged in a fixed-angle rotor at 10,000g for 1 h at 34°C. The microcell pellets were resuspended in 10 ml of serum-free DMEM and filtered in series through 8 µm, 5 µm and 3 µm polycarbonate filters (Nucleopore, Pleasanton, CA). The purified microcells were centrifuged and resuspended in 2 ml of serum-free DMEM containing 100 µg/ml of phytohemagglutinin (PHA). The microcells were attached to the A9 cell monolayers by incubating them for 15 min at 37°C. The cells were fused by the use of 47% PEG (w/v) (MW 1540, Wako Pure Chemical Industries, Ltd., Osaka) solution (3 ml) for 1 min followed by extensive washing in serum-free DMEM. After 24 h, the cells were trypsinized and split

into 3 plates (100 mm) with selective medium containing 800 µg/ml G418. The resulting microcell hybrids were cloned at 10 to 14 days after selection. Microcell hybrid colonies were formed at a frequency of about 10⁻⁵.

Four colonies were isolated from each parental hybrid clone and expanded for karyotyping. Two hundred microcell clones were isolated and analyzed for the presence of intact human chromosome by Q-banding plus Hoechst 33258 staining.¹⁰⁾ Table I shows that thirteen A9 cell clones were found to contain one copy or two copies of an intact human chromosome 1, 2, 5, 6, 7, 8, 10, 11, 12, 15, 18, 19 or 20 (Table I and Fig. 1). The other microcell hybrids contained no intact human chromosome but had additional human chromosome fragments or rearranged chromosomes. The frequency of transfer of intact human chromosomes marked with exogenously added pSV2-neo gene (number of microcell clones which contain intact human chromosome/number of microcell clones isolated = 21/200) is apparently lower than that of an endogenously marked chromosome (X/autosome translocation which contains HGPRT gene) (31/44) described before.¹⁾ These results suggested that the exogenously added gene may induce chromosomal instability which results in the rearrangements or deletions during the transfer process.

We next examined whether human chromosomes in the A9 microcell clones were tagged with pSV2-neo gene. High-molecular-weight DNAs were isolated from 13 A9 microcell clones containing a single intact human chromosome and digested with *Hind* III (Bethesda Research Laboratories, Gaithersburg, MD) which cuts at a single site within the pSV2-neo gene. The DNAs were also

Table I. Transfer of a Normal Human Chromosome with the pSV2-neo Gene to Mouse A9 Cells via Microcell Fusion

Microcell hybrid clones	Human chromosome found in microcell hybrids	Presence of pSV2-neo (No. of <i>Hind</i> III fragment, No. of <i>Kpn</i> I fragment)	Integration site of pSV2-neo
A9(Neo-1)-4	#1	+ (2, 1)	1p34-p36
A9(Neo-2)-1	#2	+ (2, 1)	ND
A9(Neo-5)-4	#5	+ (2, 1)	ND
A9(Neo-6)-3	#6	+ (3, 1)	6q25-q26
A9(Neo-7)-2	#7	+ (4, ND)	ND
A9(Neo-8)-1	#8	+ (2, 1)	ND
A9(Neo-10)-3	#10	+ (5, ND)	ND
A9(Neo-11)-1	#11	+ (2, 1)	11p11
A9(Neo-12)-4	#12	+ (2, 1)	12p12
A9(Neo-15)-2	#15	+ (3, 2)	ND
A9(Neo-18)-5	#18	+ (2, 1)	ND
A9(Neo-19)-1	#19	+ (2, 1)	19p13
A9(Neo-20)-3	#20	+ (2, 1)	ND

ND: Not determined.

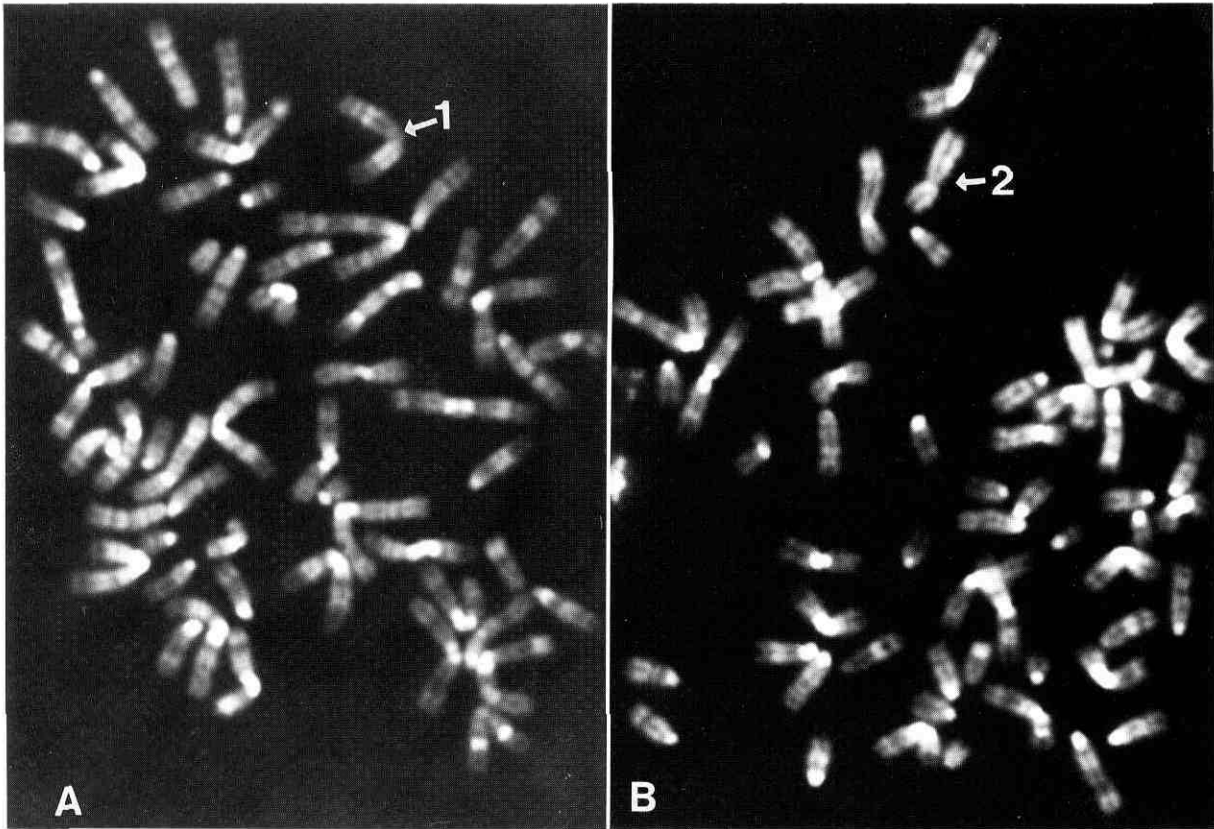


Fig. 1. Quinacrine plus Hoechst 33258 stained metaphase plates of mouse A9 cell clones containing a single human chromosome. An arrow indicates the human chromosome. A) A9(Neo-1)-4 containing human chromosome 1. B) A9(Neo-2)-1 containing human chromosome 2.

digested with *Kpn* I (Toyobo Co., Ltd., Osaka) which has no restriction site within the pSV2-neo plasmid DNA. Cleaved DNAs were analyzed by electrophoresis in 1% agarose gels and transferred to nitrocellulose filters.¹¹⁾ The filters were baked, prehybridized and hybridized in 50% formamide-0.75 M sodium chloride-0.075 M sodium citrate at 42°C to ³²P-labeled pSV2-neo plasmid DNA. The *Hind* III-digested DNA from the clones which contain either human chromosome 1, 2, 5, 8, 11, 12, 18, 19 or 20 gave only 2 bands (Fig. 2) and *Kpn* I digest of these DNA gave one band (Table I), suggesting that one copy of pSV2-neo gene is integrated in these cellular DNAs. The other 4 A9 clones containing chromosome 6, 7, 10 or 15 showed 3 or more bands in the *Hind* III-digested DNA's, one of which was identical to the unit length 5.7 kb of pSV2-neo plasmid (Fig. 2). This suggests tandem integrations in a head-to-tail form. The integrations of pSV2-neo on the human chromosomes were directly confirmed by chromosomal *in situ* hybridization analysis. The methods used were those described

by Harper *et al.*¹²⁾ The pSV2-neo plasmid DNA was labeled by nick translation using tritium-labeled dATP, dGTP, and dTTP. A specific activity of 4×10^7 cpm/ μ g of DNA was obtained. The hybridized slides were washed with SSC, coated with NTB2 nuclear track emulsion (Kodak) and exposed for 2 weeks at 4°C. Exposed slides were developed and stained with quinacrine and Hoechst 33258. The results show that human chromosomes 1, 6, 11, 12, and 19 were tagged with pSV2-neo and the integration sites of the plasmid were determined (Table I). Figure 3A shows a partial chromosomal spread of A9(Neo-11)-1 containing one copy of human chromosome 11 and an identical spread with a grain on 11p11 (Fig. 3B).

The stability of the human chromosome in these A9 cell microcell hybrids was examined. The karyotypic analyses of A9(Neo-6)-3, A9(Neo-2)-1, A9(Neo-11)-1 and A9(Neo-12)-4 were performed at passage 12 to 14 after the cells had been grown in the growth medium containing G418. The results showed that about 95% of

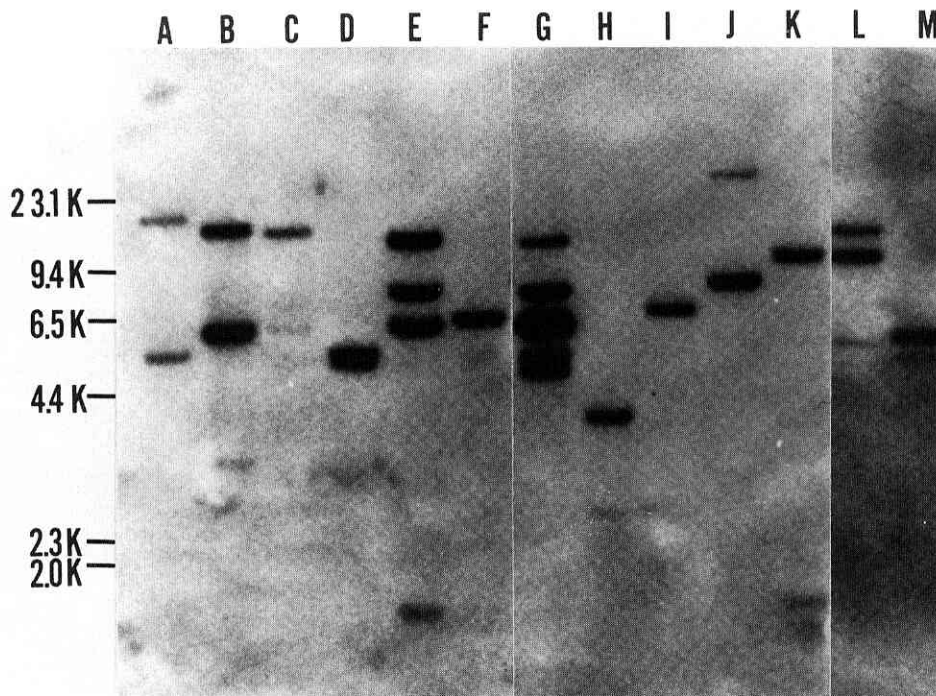


Fig. 2. Southern blot hybridization of *Hind* III restriction enzyme-digested cellular DNAs extracted from 13 A9 microcell hybrid clones containing human chromosome 1, 2, 5, 6, 7, 8, 10, 11, 12, 15, 18, 19, or 20 showing integration of the pSV2-neo plasmid DNA. Ten micrograms of DNA was digested with *Hind* III restriction endonuclease. Completely digested DNA was separated by 1% agarose gel electrophoresis and the blots were hybridized with ³²P-labeled pSV2-neo DNA. The fragment size was determined by comparison with DNA *Hind* III fragments stained with ethidium bromide. Lanes: A = A9(Neo-1)-4; B = A9(Neo-6)-3; C = A9(Neo-2)-1; D = A9(Neo-5)-4; E = A9(Neo-7)-2; F = A9(Neo-8)-1; G = A9(Neo-10)-3; H = A9(Neo-11)-1; I = A9(Neo-12)-4; J = A9(Neo-19)-1; K = A9(Neo-20)-3; L = A9(Neo-15)-2; and M = A9(Neo-18)-5.

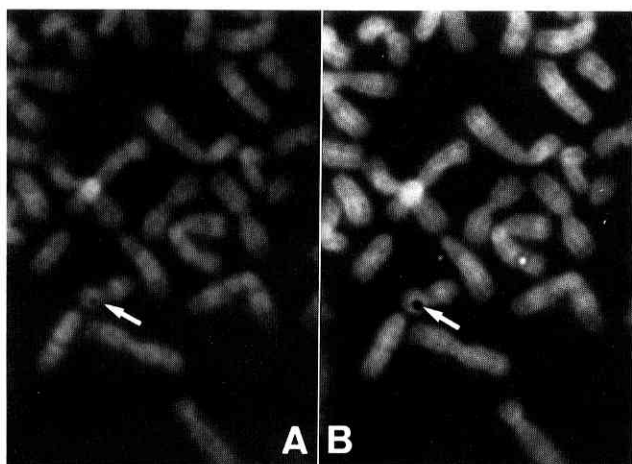


Fig. 3. Q-banded metaphase plates following *in situ* hybridization of A9(Neo-11)-1 indicating a single human chromosome 11 tagged with pSV2-neo gene. (Arrows indicate chromosome 11.) A) A Q-banded metaphase plate. B) The identical metaphase plate with an autoradiographic grain on 11p11.

the cell population of A9(Neo-2)-1 and A9(Neo-11)-1 stably retained the intact human chromosomes 2 and 11 respectively. On the other hand, 65% of the cell population of A9(Neo-6)-3 (95% of the cell population at passage 2 retained human chromosome 2) and A9(Neo-12)-4 did not contain the intact human chromosomes 2 and 12, respectively. Since these cells were still resistant to G418, chromosomal segments containing the pSV2-neo plasmid(s) are suggested to be present in the cells. Thus, the stability of neo-tagged human chromosomes in the A9 microcell hybrids grown in selective medium is variable among the hybrids. These results suggested that the microcells should be examined by karyotypic analysis to establish whether a large portion of the cells retained an intact human chromosome before performing experiments.

The presence or absence of human isozymes listed in Table II was examined in these A9 microcell hybrids.

Table II. Expression of Human Isozymes and Chromosome Identification in A9 Microcell Hybrids

Microcell hybrids	Human chromosomes														Human chromosome present
	1	2	5	6	7	8	10	11	12	14	15	18	19	20	
	Isozymes														
	PEPC	MDH1	HEXB	ME1	GUSB	GSR	GOT1	LDHA	LDHB	NP	MP1	PEPA	GPI	ADA	
A9(Neo-1)-4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	1
A9(Neo-2)-1	-	+	-	-	-	-	-	-	-	-	-	-	-	-	2
A9(Neo-5)-4	-	-	+	-	-	-	-	-	-	-	-	-	-	-	5
A9(Neo-6)-3	-	-	-	+	-	-	-	-	-	-	-	-	-	-	6
A9(Neo-7)-2	-	-	-	-	+	-	-	-	-	-	-	-	-	-	7
A9(Neo-8)-1	-	-	-	-	-	+	-	-	-	-	-	-	-	-	8
A9(Neo-10)-3	-	-	-	-	-	-	+	-	-	-	-	-	-	-	10
A9(Neo-11)-1	-	-	-	-	-	-	-	+	-	-	-	-	-	-	11
A9(Neo-12)-4	-	-	-	-	-	-	-	-	+	-	-	-	-	-	12
A9(Neo-15)-2	-	-	-	-	-	-	-	-	-	-	+	-	-	-	15
A9(Neo-18)-5	-	-	-	-	-	-	-	-	-	-	-	+	-	-	18
A9(Neo-19)-1	-	-	-	-	-	-	-	-	-	-	-	-	+	-	19
A9(Neo-20)-3	-	-	-	-	-	-	-	-	-	-	-	-	-	+	20

+: Human and mouse isozymes were detected. -: Only mouse isozymes were detected.

PEPC=peptidase C; MDH1=malate dehydrogenase; HEXB=hexosaminidase B; ME1=malic enzyme; GUSB= β -glucuronidase; GSR=glutathione reductase; GOT1=glutamateoxaloacetate transaminase; LDHA=lactate dehydrogenase A; LDHB=lactate dehydrogenase B; NP=purine nucleoside phosphorylase; MP1=mannose phosphate isomerase; PEPA=peptidase A; GPI=glucose phosphate isomerase; ADA=adenosine deaminase.

The cells (10^7) were washed twice with phosphate-buffered saline. Pelleted cells were resuspended in 90 μ l of cell extract buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 20 mM KCl, 0.1 mM dithiothreitol and 10% glycerol. Ten microliters of 5% Triton X-100 was added to the cell suspension and the cells were kept in ice for 20 min. The cell lysate was centrifuged at 13,500g for 30 min at 4°C and the supernatants were collected. The cell extract (2 μ l) was applied to cellulose acetate plates (Helena Laboratories, Beaumont, TX) and separated for 20 min to 60 min at 4°C. After electrophoresis, plates were overlaid with 1% melted agar containing enzyme substrate and a soluble dye which forms colored precipitates upon reaction with the enzyme's product. As shown in Table II, the presence of a specific human chromosome identified by karyotypic analysis in each of the A9 microcell hybrids was confirmed by the presence of human

isozyme whose gene is assigned to the specific human chromosome.

Currently, the library of microcell hybrids described here has been successfully used for chromosome transfer experiments via microcell fusion, confirming the library to be useful as a donor cell source for transfer of an individual chromosome to other cells.¹³⁾

The authors wish to thank Prof. M. Umeda, Yokohama City University, for his continuous encouragement and the Japanese Cancer Resources Bank for providing NTI-4 and A9 cells. This study was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan, a Grant-in-Aid for the Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare of Japan and a Grant from the Science and Technology Agency, Japan.

(Received January 11, 1989/Accepted March 25, 1989)

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