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Transforming Capacity of the Plasmid Containing SV40 Promoter in NIH3T3 Fibroblast Cells

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SV 40 Promoter 를 갖는 Plasmid 에 의한 NIH3T3 섬유아세포의 형질전환

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ABSTRACT: The plasmid pKOneo, containing SV40 transcriptional promoter, has been used in the mouse tumorigenicity assay for oncogene studies. This assay employs a cotransfection of NIH3T3 fibroblast cells with the desired DNA and the plasmid pKOneo. This oncogene assay, however, has been speculated due to the SV40 transcriptional promoter in the plasmid pKOneo. This research was designed to investigate if the plasmid pKOneo alone is capable of transforming NIH3T3 fibroblast cells. The NIH3T3 subclones were established after the NIH3T3 cells were transfected with the plasmid pKOneo alone. The estabilished NIH3T3 subclones, containing the exogeneous plasmid pKOneo in their chromosomes, were examined for their expression of transformation-associated parameters. The results indicate that this plasmid pKOneo alone has positive effects on transformation of NIH3T3 cells after integration into cellular chromosomes.

KEY WORDS□Oncogene, Tumorigenicity, SV40 Promoter, Cell Transformation.

The transfer of cellular genomic DNA into NIH3T3 fibroblast cells has been employed to study oncogenes that present in some tumors and capable of causing foci of morphologically transformed NIH3T3 fibroblast cells. These NIH3T3 cells were adapted to grow immortally in monolayer culture and passaged extensively *in vitro*, even though they still have growth control (Murray *et al.*, 1981; Perucho *et al.*, 1981; Pulciani, 1979). In this transfection foci assay, NIH3T3 mouse fibroblast cells were treated with a calcium phosphate precipitate of the DNA of interest, and then monitored for development of dense clumps of morphologically altered cells, called foci, against a background monolayer of confluenet fibroblasts.But,only about 20 percent of the tumor cell lines tested have yielded active oncogenes in this NIH3T3 cell transfection foci assay (Dubersstein, 1984).

Wigler and his colleagues have developed the alternative mouse tumorigenicity assay to detect oncogenes in the majority of tumors and tumor cell lines that have not been amenable to study with the NIH3T3 tumorgenicity assay (Fasano *et al.*, 1984). This mouse turmogenicity assay is based on the synthesis of a cotransfection pro-

cedure, which bypasses the search for foci, and which directly tests the ability of an entire population of cotransfected NIH3T3 cells to form a tumor in a nude mouse after injection of cells. This is based on the fact that cotransfection of NIH3T3 cells with a calcium phosphate precipitate of high molecular weight cellular DNA and the plasmid pKOneo renders mammalian cells resistant to the drug G418. The selected NIH3T3 cells, expected to represent the genomic characteristic of the tumor, in the medium containing G418 after transfection was, then, injected into a nude mouse to see if it develops a tumor (Fasano *et al.*, 1984).

However, it has been speculated that the tumorgenicity assay has a bias for cell transformation due to cotransfected plasmid pKOneo containing the SV40 transcriptional promoter. This speculation is based on the reports that analysis of some tumors caused by slow transforming retroviruses led to the identification of intergration sites of proviruses with specific tumors (Rather *et al.*, 1985). Therefore, the present work was undertaken to examine if the plasmid pKOneo alone is capable of transforming NIH3T3 fibroblast cells. The NIH3T3 subclones containing plasmid pKOneo in their chromosomes have been studied for their expression of transformation-associated parameters.

MATERIALS AND METHODS

The plasmid pKOneo was generously provided by Dr. D. Pintell (University of Missouri-Columbia, Columbia, MO., USA). This plasmid was introduced into *Escherichia coli* for amplication and isolated according to a scaled up version of the procedure of Birnboim and Doly (1979).

For estabilishing NIH3T3 subclones with the plasmid pKOneo, 300 nano-grams of pKOneo were added as a calcium phosphate precipitate to NIH3T3 cell culture in an 100 mm culture dish according to the procedure of Rhee (1987). These transfected cells were then incubated in the culture medium containing G418 (0.4 gram per liter) from Gibco (Grand Island, NY., USA) for about two weeks. The NIH3T3 subclones resistant to G418 were cloned using cloning cylinders and propagated in the culture medium under 5% carbon dioxide at 37 °C.

Two million cells of each NIH3T3 subclone were injected subcutaneously into the interscapular region of each nude mouse (3 to 4 week old male) of Aphymice Nude-nu purchased from Harlan Spriague Dawely, Inc. (Indianapolis, Indiana, USA) at once. Tumor growth was monitored every 7 days. Prior to death from tumor growth, the mice were sacrificed and the tumor tissues from each animal were saved to obtain DNA for later use.

One million cells of each NIH3T3 subclone were plated and incubated in an 100 mm tissue culture dish containing 10 m*l* of medium with 10% fetal bovine serum (FBS) for one and half days. The cells were trypsinized and treated according to the method of Kirshan (1975) for the analysis of the cell cycle distribution by flow cytometer from Coulter (Hieleah, Fla., USA). At same time, the medium in the remaining dishes was replaced with the same medium containing 0.1% FBS and the cells were allowed to grow for additional two days before analysis by flow cytometry. One million cells of each NIH3T3 subclone were also plated and incubated in an 100 mm culture dish containing 10 m*l* of medium with 10% FBS for 10 days to observe the density of cells. These subclones were trypsinized and the number of cells per each dish were counted using either a hemacytometer and microscope, or a particle counter from Particle Data, Inc. (Elmhust, Ill., USA) after phase-contrast photomicrographs were taken with a Zeiss inverted microscope.

For preparation of high molecular weight DNA with cells from tissue cultures and with tumor tissues from nude mice, the NIH3T3 subclones were trypsinized and lysed with sarcosyl and tumor tissues from nude mice were finely ground using a pestle in a mortar and lysed with sarcosyl. These cells and sarcosyl mixtures were centrifuged using cesium chlroride (CsCl) density gradient to extract DNA. The remaining CsCl was removed by dialysis and the extracted DNAs were further purified by phenol and chloroform extraction. DNA samples were digested with Hind III endonuclease, and sub-

jected to 0.7% agarose gel electrophoresis and nitrocellulose filter blot transfer by the method of Southern (1976). Filter blot DNAs were hybridized with the probe of pKOneo.

RESULTS AND DISCUSSION

To establish the subclones of NIH3T3 cells containing the SV40 transcriptional promoter, the NIH3T3 cells were transfected with the plasmid pKOneo alone. This plasmid contains neomycin resistant gene under control of SV40 promoter and renders mammalian cells resistant to G418 (Fasano *et al.*, 1984; Southern and Berg, 1982). Seven NIH3T3 subclones resistant to G418 were isolated and designated as NIHKO1, NIHKO2, NIHKO3, NIHKO4, NIHKO5, NIHKO6, and NIHKO7, respectively.

The tumorigenicity assay was performed with these seven NIH3T3 subclones as one of transformation-associated parameters. Two million cells of each NIH3T3 subclone were injected into one nude mouse at once and three nude mice were used to test each NIH3T3 subclone. Normal NIH3T3 cells were used as a negative control. The time of the first appearance of tumors and their subsequent growth were observed. The subclones NIHKO1, NIHKO2, NIHKO4, and NIHKO6 appeared to be tumorgenic. The subclones, NIHKO3, NIHKO5, and NIHKO7, however, did not respond in this tumorgenicity assay (Table 1). All tumors developed were designated as KO1, KO2, KO4, and KO6, respectively, according to the corresponding name of the subclones. The subclones NIHKO1 and NIHKO2 developed tumors in all mice in 3 weeks after injection. But, the subclone NIHKO6 caused tumors in all three mice in 5 weeks and the subclone NIHKO4 induced tumors in two mice in 5 weeks. The sizes of tumors of KO4 and KO6 were relatively smaller than those of KO1 and KO2. Apparently, the subclones NIHKO1 and NIHKO2 showed the higher tumor induction rate than the subclones NIHKO4 and NIHKO6.

High molecular weight DNAs extracted from NIH3T3 subclones and from tumor tissues were digested with Hind III endonucleases, fractionated on 0.7% agarose gel, blotted, and hybridized with the radioactively labelled probe of pKOneo (Fig. 1). Analyses of DNAs from seven subclones (Fig. 1 A lanes a to g) and from tumor tissues (Fig. 1 B lanes a to d) showed the Hind III to Hind III fragment, which is indicated by arrow, of neomycine resistant gene from exogenous pKOneo. Analysis of DNA from NIHKO7 (Fig. 1 A lane g) resulted in a smear band which indicates that multiple copies of plasmid were incorporated into the DNA of cell line. These DNA analyses also confirmed that all tumors were derived from the corresponding subclone cells injected since Southern blot pattern of tumor tissue DNA samples (Fig. 1 B lanes a, b, c, and d) displayed identical with that of the corresponding subclone DNA samples (Fig. 1A lanes a, b, d, and f). It was very interesting that the subclone NIHKO7 did not develop tumors in all three mice (Table 1) even with many number of gene copies incorporated into genome (Fig. 1 A lane g).

To provide an additional measure of the transformed phenotype, the cell density of each subclone was measured after it reached confluency. One million cells of each NIH3T3 subclone

Table 1. *Tumorigenicity test of normal NIH3T3 cells and of NIH3T3 subclones containing the plasmid pKOneo.*

Subclone name	number of mice with tumors per total mice on days after injection of cells						Tumor designation
	14	21	28	35	42	49	
NIHKO1	0/3	3/3	3/3	3/3	3/3	3/3	KO1
NIHKO2	0/3	3/3	3/3	3/3	3/3	3/3	KO2
NIHKO3	0/3	0/3	0/3	0/3	0/3	0/3	
NIHKO4	0/3	1/3	1/3	2/3	2/3	2/3	KO4
NIHKO5	0/3	0/3	0/3	0/3	0/3	0/3	
NIHKO6	0/3	2/3	2/3	3/3	3/3	3/3	KO6
NIHKO7	0/3	0/3	0/3	0/3	0/3	0/3	
NIH3T3 (negative control)	0/3	0/3	0/3	0/3	0/3	0/3	

This test was performed as described in the text. Each nude mouse was injected with two million cells of one of NIH3T3 subclones or of normal NIH3T3 cells. Three nude mice were used to test each NIH3T3 subclone. All tumor tissues were saved to obtain DNA for later use.

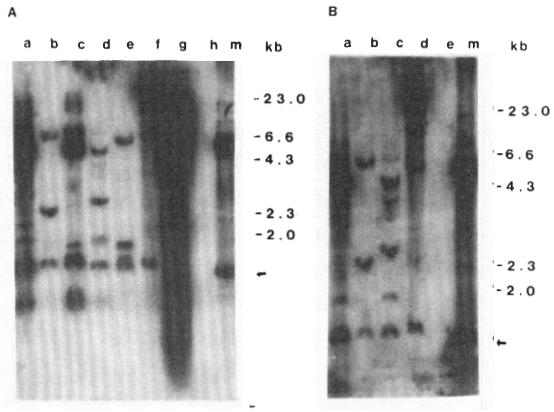

Fig. 1. *Southern blot analyses of the exogenous DNAs from NIH3T3-subclones and from tumor tissues.*
The NIH3T3 subclones were established by transfection with the plasmid pKOneo and all tumors were induced by injection of these subclones into nude mice. Arrow indicates the presence of Hind III to Hind III fragment of neomycin resistant gene from the plasmid pKOneo. All DNAs were digested with Hind III endonuclease, fractionated on 0.7% agarose, blotted, and hybridized with the probe of pKOneo.
(A) Identification of exogenous pKOneo in NIH3T3 subclones. High molecular weight DNAs were extracted from G418 resistant subclones of NIHKO1 (a), NIHKO2 (b), NIHKO3 (c), NIHKO4 (d), NIHKO5 (e), NIHKO6 (f), and NIHKO7 (g) after transfection of NIH3T3 cells with the plasmid pKOneo. The normal NIH3T3 cell DNA (h) and the plasmid pKOneo DNA (m) were digested with Hind III and loaded on the same gel as control markers.
(B) Identification of exogenous pKOneo in tumor tissues. High molecular weight DNAs were extracted from tumor tissues of KO1 (a), KO2 (b), KO4 (c), and KO6 (d) after injection of subclones into nude mice. The normal NIH3T3 cell DNA (e) and the plasmid pKOneo DNA (m) were digested with Hind III and loaded on the same gel as control markers.

were plated and incubated in an 100 mm culture dish containing 10 m*l* of medium with 10% FBS for 10 days. The medium was then replaced every 3 to 4 days. Phase-contrast photomicrographs (Fig. 2) were taken before trypsinization of cells to count total number of cells per dish (Table 2). The data in Table 2 indicate that all estabilished subclones containing the plasmid pKOneo did not seem to have contact inhibition. The relative growth rates of subclones were much higher than that of normal NIH3T3 cells. Phase-contrast

Table 2. *The number of cells per 100 mm culture dish under confluent condition.*

cell line	number of cells per 100 mm culture dish	
NIHKO1	25.0	million cells
NIHKO2	12.7	million cells
NIHKO3	15.7	million cells
NIHKO4	17.6	million cells
NIHKO5	22.4	million cells
NIHKO6	26.4	million cells
NIHKO7	11.0	million cells
NIH3T3(control)	8.0	million cells

One million cells were plated and incubated in an 100 mm dish containing 10 m*l* of medium with 10% fetal bovine serum for 10 days. The medium was replaced every 3 to 4 days. The cells were trypsinized and total number of cells were counted. Normal NIH3T3 cells were used as control.

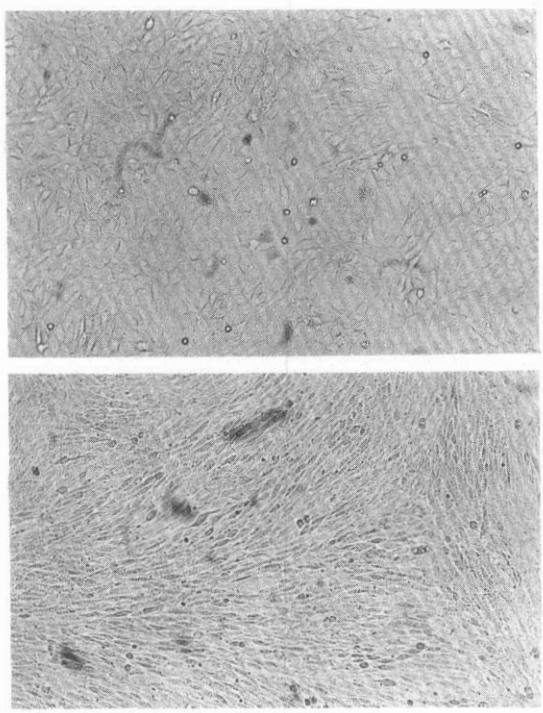

Fig. 2. *Phase-contrast photomicrographs of NIH3T3 cells (top) and NIHKO1 cells.*
The preparations of cells for the data in Table 2 were used to take phase-contrast photographs just before cell trypsinization.

photomicrographs (Fig. 2) of cells showed clear morphological transformation of cells as observed by other investigators (Demetriose *et al.*, 1984;

Table 3. *Percent (%) of cells in the cell cycle after flow cytometry.*

culture condition	cell line	% of cells in G0/G1 phases	% of cells in S,G2, and M phases
cells under sub-	NIHKO1	62.5	37.5
confluent and 10%	NIHKO2	63.8	36.2
FBS condition	NIHKO3	60.8	39.2
	NIHKO4	57.8	42.2
	NIHKO5	54.7	45.3
	NIHKO6	59.3	40.7
	NIHKO7	62.1	37.9
	NIH3T3	61.0	39.0
cells under serum-	NIHKO1	88.8	11.2
deprived and 0.1%	NIHKO2	90.8	9.2
FBS condition	NIHKO3	91.5	8.5
	NIHKO4	90.7	9.3
	NIHKO5	90.8	9.2
	NIHKO6	90.7	9.3
	NIHKO7	91.0	9.0
	NIH3T3	94.6	5.4

One million cells of each subclone were plated and incubated in an 100 mm tissue culture dish containing 10 m*l* of medium with 10% FBS for one and half days. These cells under subconfluent condition were trypsinized for the analysis of flow cytometry. At this time, the medium in the remaining dishes was replaced with the same medium containing 0.1% FBS and incubated for additional two days. These quiescent and growth-arrested cells were trypsinized for the analysis of flow cytometry. The data from flow cytometry were normalized. Normal NIH3T3 cells were used as control.

Land *et al.*, 1983).

To study the serum dependancy of the NIH3T3 subclones, the subclones were subjected to serum deprived and serum supplied conditions. These cells were then trypsinized for the analysis by flow cytometry as described in Table 3. Serum deprivation resulted in quiescent and growth-arrested cessation of cell growth. The flow cytometry data in Table 3 showed about 5% population of normal NIH3T3 cells stayed in S, M, and G2 phases under serum deprived condition, and about 40% population spent in S, M, and G2 phases under subconfluent and 10% serum condition. All estabilished NIH3T3 subclones by transfection showed that approximately 9 to 11% populations were in growing S, M, and G2 phases under serum deprived condition and that approximately 40% population spent in S, M, and G2 phases under subconfluent and 10% serum condition. All of these cell growth rate data under serum deprived and serum supplied conditions (Table 3) indicated that approximately same number of cells entered growing phases regardless of how many number of gene copies were introduced into genome (Fig. 1). It appeared to be higher rate of progression through cell-cycle by subclones than by NIH3T3 under serum deprived conditions. This mechanism causing higher rate was not very clear at this time, but it could be assumed that SV40 promoter in pKOneo plasmid for cotransfection caused higher growth rate of subclones in serum deprived condition since the growth rates of subclones were more or less very close one another except NIH3T3 cells.

These experiments clearly demonstrate that the plasmid containing the SV40 transcriptional promoter has a positive effect on cell transformation and reduces serum requirement after its integration into cellular chromosomes.

적 요

Mammalian cell 연구에 쓰기 위해 개발된 SV40 transcriptional promoter를 함유하는 pKOneo plasmid를 발암 유전인자 연구에 쓰이는 NIH3T3 쥐 세포에 stable transfection 시켜 7개의 subclones 얻었으며, 이 subclones이 갖는 세포 형질전환에 관한 여러가지 성질을 조사하였다. 실험결과에 따르면 stable transfection후 세포 염색체에 삽입된 pKOneo plasmid 자체만으로도 NIH3T3 세포의 형질전환을 크게 일으키는 것으로 사료되었다.

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