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Presence of Fibroblast-transforming Genes in Normal DNA of Several Mouse and Rat Strains*

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Abstract—*Cellular DNA of the inbred mouse strains BALB/c, C3Hf, C57BL, CBA, DBAf, GRS and ND2 and the inbred rat strains BN, SD and WAG was shown to oncogenically transform murine fibroblasts of the continuous cell line BALB/3T3. Subcutaneous inoculation of transformed cells into BALB/c mice led to the rapid development of sarcomas. For transformation the DNA had to be fragmented to a size smaller than 23 kilobase pairs (kbp), with a lower limit of 6.5 kbp. No transforming virus could be rescued from the transformed lines by infection with a murine leukemia virus.*

INTRODUCTION

NORMAL cellular DNA of every vertebrate species tested contains nucleotide sequences that are homologous to the oncogenes of acute transforming retroviruses [1]. These cellular homologues, designated as c-*onc*, are thought to play an important role in embryogenesis [2]. A high rate of expression of some c-*onc* genes has been found in several tumours, including human neoplasms. This is thought to be brought about by, among others, chromosomal translocation, leading to the insertion of a c-*onc* gene close to an active cellular promoter [3]. Another mechanism is the positioning of promoter/transcription-enhancing elements of viral origin in the vicinity of a c-*onc* gene [4]. Obviously, such cellular oncogenes play an important role in some forms of carcinogenesis, although not an exclusive one.

Exposure of fibroblasts from continuous cell lines such as NIH/3T3 to a DNA precipitate from tumours may lead to oncogenic transformation [5, 6]. This so-called transfection method is a powerful tool for the detection of activated oncogenes in various tumours, including human ones. Such activated fibroblast-transforming genes have repeatedly proved to be homologous to retroviral oncogenes [7]. Nucleotide sequence analysis indicated that point mutations resulting in a single amino acid substitution may be responsible for oncogene activation [8].

High-molecular-weight DNA isolated from normal tissues does not transform NIH/3T3 cells. However, Cooper *et al.* [9] and Krump-Konvalinkova and Van den Berg in our institute [10] have found that fragmentation leads to normal cellular DNA becoming transforming. The frequency of transformation by normal cellular DNA fragments is greatly increased by preinfection of the recipient cells with a murine leukemia virus [10]. This is presumably due to the action of the proviral long terminal repeat with its promoter and transcription-enhancing sequences.

The question of whether the transfectable normal oncogene (*tno*) is present not only in the DNA of BALB/c mice but also in that of other mouse strains and rats was investigated.

MATERIALS AND METHODS

Animals

Cellular DNA was isolated from spleens of male mice of the following strains: BALB/c, CBA, C3Hf, C57BL, DBAf, GRS and ND2. Rats of the strains WAG, BN and SD were also used as donors.

Transformed BALB/3T3 cells obtained in transfection experiments were injected subcutaneously into 2-month-old female BALB/c mice, 10^6 cells per animal. The animals were examined twice a week for palpable tumours and kept until 6 months after the inoculation.

Cell cultures

BALB/3T3 cells were purchased from Flow Laboratories, Rockville, MD, U.S.A. They were

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grown according to the 3T3 scheme [11] in Dulbecco's modification of minimal essential medium containing 10% foetal calf serum and antibiotics. One dish seeded with BALB/3T3 cells was infected with a cell-free preparation of Rauscher murine leukaemia virus (R-MuLV), cultured for 10 passages according to the 3T3 scheme and then stored, frozen in liquid nitrogen. Productive infection was checked by reverse transcriptase assay and immunofluorescence using an antiserum to the p30 antigen of R-MuLV. For transfection studies an ampule of cells was thawed, the cells cultured and then passaged once.

DNA isolation

Spleens were removed aseptically and a monocellular suspension was prepared in phosphate-buffered saline (PBS). The cells were washed × 3 in PBS and then transferred to TEN buffer (20 mM Tris, 10 mM EDTA, 100 mM NaCl, pH 8) at a maximum of 2×10^8/ml. An equal volume of TEN containing 0.4 mg proteinase K per ml and 4% sarkosyl was then added under continuous stirring. This mixture was incubated overnight at 37°C. Afterwards an equal volume of PCI (250 g phenol, 150 ml chloroform, 30 ml isoamyl alcohol) was added. The suspension was shaken for 1 hr and then transferred to a Corex centrifuge tube and spun for 20 min at 9000 rev/min at room temperature. The supernatant was collected and again subjected to extraction with PCI. Following this, it was extracted with chloroform only. The upper layer was very cautiously withdrawn and 10 mM Tris + 1 M NaCl, pH 7.4, was added, resulting in a 0.2-M NaCl concentration. DNA was then precipitated with 2 vols of absolute ethanol at -20°C. The DNA was removed with a Pasteur pipette and air-dried. It was then added to 1 mM Tris + 0.01 M EDTA, pH 7.9; the DNA concentration was about 1 mg/ml. The tube containing the DNA was turned continuously at 4°C until the substance was dissolved. The DNA was incubated for 2 hr at 37°C with RNase A (0.1 mg/ml), extracted once with PCI and then with chloroform, and subsequently precipitated with cold ethanol. Isolated DNA was fragmented by repeatedly pressing it through a syringe with a needle of 0.5 or 0.6 mm diameter or by sonication. The molecular weight was then determined by electrophoresis overnight in 0.8% agarose gel, using HindIII digested lambda phage DNA fragments as markers.

Transfection

Eighteen hours before transfection 3×10^5 BALB/3T3 cells were seeded into plastic Petri dishes (60 mm diameter) and incubated at 37°C in an atmosphere containing 5% CO_2. At 1 hr before transfection cultures were refed with growth medium. Also at 1 hr before transfection $CaCl_2$ (2.5 M in distilled water and sterilized by filtration) was slowly added to the DNA sample. Thirty minutes later the contents of the tube containing DNA were pipetted very slowly into a tube with an equal volume of autoclaved buffer (250 mM HEPES, pH 7.1, and 1.5 mM Na_2HPO_4/NaH_2PO_4). In the meantime nitrogen was bubbled continuously through the tube. A precipitate formed within 30 min at room temperature; this was then gently deposited on the Petri dishes containing BALB/3T3 cells. The amount of DNA added was 50–75 μg per dish. The cultures were incubated for 7 hr at 37°C under 5% CO_2. The medium was then removed but a small layer of DNA was left behind on top of the cells. Three millilitres of fresh growth medium were added. Eighteen hours later the cells were trypsinized and transferred to ten dishes. As soon as the cultures containing infected BALB/3T3 cells reached confluency, medium with 5% foetal calf serum was used. Foci of transformed cells could be scored at 2 weeks after transfection. Cultures containing uninfected BALB/3T3 cells were overlaid with 0.6% agarose in growth medium after a week. Once a week 0.5 ml medium was added to the cultures. Colonies of transformed cells were scored at 5 weeks after transfection.

Foci or colonies of transformed cells were detached from the dish by means of a Pasteur pipette and deposited in wells of a microtitre plate. If growth occurred, the contents of the wells were then transferred to a tissue culture flask. Transformed sublines obtained from uninfected BALB/3T3 cells were injected into BALB/c mice. Transformed lines obtained from infected BALB/3T3 cells were screened for the release of a transforming virus by a standard focus assay on BALB/3T3 cells [12].

RESULTS

The transformation rate of uninfected BALB/3T3 cells exposed to different batches of mouse and rat DNA is presented in Table 1. Spontaneous transformation is negligible in this assay: one colony per ten dishes. The addition of unfragmented rodent DNA to the cultures had no significant effect. However, shearing of the DNA resulted in a large number of colonies of transformed cells. The data are based on two separate experiments per DNA sample. It was not technically feasible to simultaneously compare the samples from all mouse and rat strains. With one batch of DNA a two-fold variation in the number of colonies per dish was noted. DNA from

all mouse and rat strains tested produced colonies of cells with anchorage independence. The average number of colonies was approximately the same for all DNA batches. The transforming activity amounted to 1–2 colony-forming units/ μg DNA.

Transformed cells obtained in various transfection experiments were inoculated into BALB/c mice. Tumour incidences are presented in Table 2. Although control BALB/3T3 cells also produced some tumours, the frequency was considerably higher and the latency period was considerably shorter when transformed cells were inoculated. Ten tumours arising in this experiment were examined histopathologically and proved to be sarcomas.

Preinfection of BALB/3T3 cells with R-MuLV promotes the transforming activity of fragmented mouse or rat DNA. Foci of transformed cells can be detected within 2 weeks. The transforming activity amounts to 2–4 focus-forming units/μg DNA. Also in this series of experiments, all DNA samples proved to have transforming activity after fragmentation (Table 3). The rate of spontaneous transformation was somewhat higher than in the uninfected BALB/3T3 cell cultures: 0.5 foci of transformed cells per dish. In each experiment at least two foci were picked and transferred to a flask. After one passage the supernatants were tested for transforming activity on BALB/3T3 cells. None of the lines released a transforming virus. Three sublines of uninfected BALB/3T3 cells transformed by BALB/c DNA were infected with R-MuLV by cocultivation with the irradiated BALB/3T3 cells producing this virus. Reverse transcriptase assay revealed that the three transformed lines had been infected. However, they did not release a focus-forming virus.

BALB/c DNA was sheared by either repeated pressing through a syringe or by sonication. The results of transfection with DNA samples of varying modal molecular weight as given in Table 4 indicate that fragments larger on the average than 23 kbp or smaller than 6.5 kbp have no transforming activity.

DISCUSSION

Fragmented cellular DNA from every mouse and rat strain tested can transform BALB/3T3 fibroblasts. As discussed by Hilkens *et al.* [13], the inbred mouse strains used in this investigation are quite genetically distinct. The same holds for the three rat strains maintained in our institute. The

Table 1. Transformation of BALB/3T3 cells after exposure to cellular DNA from mice or rats

	DNA sample from animal strain	Average No. of colonies of transformed cells per dish	
		Unfragmented DNA	Fragmented DNA
Mouse	BALB/c	0	12.3
	C3Hf	0.2	8.7
	C57BL	0	6.5
	CBA	0.1	14.2
	DBAf	0.1	10.1
	GRS	0	7.9
	ND2	0	7.3
Rat	BN	0.1	14.2
	SD	0	10.5
	WAG	0	6.2

Table 3. Transformation of BALB/3T3 cells infected with Rauscher murine leukaemia virus after exposure to DNA from mice or rats

	DNA sample from animal strain	Average No. of foci of transformed cells per dish	
		Unfragmented DNA	Fragmented DNA
Mouse	BALB/c	0.7	22.4
	C3Hf	1.1	18.9
	C57BL	0.4	25.0
	CBA	0.0	14.6
	DBAf	0.9	19.2
	GRS	0.2	21.3
	ND2	0.2	15.1
Rat	BN	0.5	12.2
	SD	1.0	19.2
	WAG	0.2	21.4

Table 2. Tumour production in BALB/c mice after subcutaneous inoculation of BALB/3T3 cells transfected with rodent DNA

Treatment of BALB/3T3 cells	No. of clones tested	No. of clones producing tumours	No. of tumours/ No. of inoculated mice	Average latency period (weeks)
None	2	1	2/10	22
Spontaneously transformed	1	1	4/5	10
Transfected with:				
BALB/c DNA	3	3	12/12	5
C3Hf DNA	3	3	9/10	7
CBA DNA	2	2	8/8	8
DBAf DNA	3	3	12/12	7
SD DNA	2	2	5/6	6

Table 4. Influence of the size of BALB/c mouse DNA fragments on transformation of infected BALB/3T3 cells

Treatment of DNA samples	Modal size of fragments in kilobase pairs	Average No. of foci of transformed cells per dish
None	24	1.2
5× shearing through a 0.6-mm needle	23.5	0.6
10× shearing through a 0.6-mm needle	16	16.7
5× shearing through a 0.5-mm needle	16	7.9
20× shearing through a 0.6-mm needle	13	18.6
10× shearing through a 0.5-mm needle	12	19.3
25× shearing through a 0.5-mm needle	10	21.0
5-sec sonication 17 u	9.5	14.9
5-sec sonication 10 u	6.5	8.8
5.sec sonication 7 u	4	0.2

reported transforming activity of fragmented cellular DNA is therefore not a peculiarity of the BALB/c mouse strains and its relatives. The oncogene *tno* seems to be ubiquitous in mice and rats.

Attempts to achieve transformation of either BALB/3T3 or NIH/3T3 cells with fragmented DNA from man, rhesus monkey, cat and dog have failed. Sheared calf thymus DNA, however, can transform these murine cells [Krump, unpublished results].

Some of the BALB/3T3 sublines transformed by DNA from the mouse strains BALB/c, C3H, CBA or DBA have been tested for amplification of known c-*onc* genes. No difference has been found in Southern blot patterns of the DNA from these sublines as compared with the parental BALB/3T3 line when the cloned retroviral oncogenes *alb, erb, fes, mos, myc, myb, Ha-ras, Ki-ras, sis* and *src* were used as probes [14]. The cellular oncogene *tno* detectable by transfection of 3T3 cells by fragmented normal mouse DNA does not seem to be homologous to any of these known oncogenes. The repeated failure to obtain a transforming virus from cells transfected with normal DNA and infected with R-MuLV suggests that *tno* is not a proviral oncogene that has been inserted into the germline by a retrovirus.

Some polymorphism of *tno* among the different mouse strains and particularly between rats and mice was anticipated. BALB/c mice were therefore hyperimmunized with irradiated syngeneic BALB/3T3 cells transformed by DNA from other mouse strains or from rats. It was hoped that genetic polymorphisms might be reflected by differences in the amino acid sequence of the *tno* gene product that would lead to antibody production by immunized BALB/c mice. Up to now, no antigen has been immunoprecipitated from radioactively labelled tumour cells by sera from immunized mice. This approach failed to detect the *tno* gene product. Its detection would have facilitated the isolation and characterization of the *tno* oncogene.

Fragmentation to pieces smaller than 23 kbp is required for normal mouse or rat DNA to exhibit transforming activity. This may be necessary to allow the recipient cell to process the incoming DNA to make it oncogenic. Processing may involve mutation at specific points in the *tno* oncogene, further fragmentation or demethylation. It is also possible that DNA fragments larger than 23 kbp cannot become integrated close to an active cellular promotor. The most likely hypothesis, however, is that the oncogene *tno* must be separated from a neighbouring *cis*-acting inhibitory sequence.

As fragments with a modal size smaller than 6.5 kbp cannot transform NIH/3T3 cells, it may be assumed that the size of *tno* is at least 4 kbp. This assumption accords with data obtained by Dr V. Krump in our institute with mouse DNA fractionated by ultracentrifugation on a continuous sucrose gradient after EcoRI digestion. The size of approximately 4–6.5 kbp is not particularly large. If *tno* should contain some introns, this oncogene would code for a small polypeptide such as some known growth factors. It is presently being attempted in our institute to clone the *tno* oncogene by means of the recombinant DNA technique for its further characterization.

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