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Assessment and Establisment of a WKA Rat Fetus-Derived Cells for Oncogene Transfection and Analysis of the Transformation-Associated Antigens[1]

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SUMMARY

In our search for the tumor antigen expression that was strictly associated with the cell transformation, we first established a new recipient cell line with stable phenotypes as the non-transformants for activated oncogene transfection. An inbred Wister-King-Aptekman (WKA) rat fetus-derived fibroblast line, WFB, showed a strict contact inhibition and serum dependency on the cell monolayer culture, and a complete anchorage dependency of cell growth with a normal diploid phenotype of the chromosome. This cell also showed a complete loss of tumorigenicity in syngeneic WKA rats as well as nude mice even if more than 10^7 cells were injected subcutaneously. Although the transformation efficiency of WFB cells to the activated H-ras oncogenes was lower than that of NIH3T3, WFB indicated a more enhanced sensitivity for the cell transformation when compared to those of BALA3T3 mouse cells and NRK-49F rat cells. The transformed WFB cells developed tumors in the syngeneic rats as well as nude nice, and killed the host rapidly. The morphology of transformed WFB tumors demonstrated the histological architecture that is consistent with fibrosarcomas. There was no difference in this histological feature among the WFB tumors that were transformed by oncogenes, including polyoma middle T DNA. The data indicated that WFB is applicable for tumor DNA transfection experiments in detecting the transforming genes of cellular DNA. Furthermore, the more important point is that the strict phenotype of WFB cells as nontransformed cells could be very useful for the investigation of the expression of "tumor antigens" that is virtually associated with the cell transformation.

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INTRODUCTION

The transfection of tumor DNAs has extensively clarified the cell transformation process by use of the activated cellular oncogenes(1, 2, 3). This also may provide us with a new opportunity to analyze the transformation-associated antigens on the cell surface, because it becomes possible to compare the antigens between parental non-transformed cells and their transformant clones, and to identify "true" tumor antigens.

The transfection experiments usually employ NIH3T3 cells as the recipient for DNA transfection, since NIH3T3 cells have the highest sensitivity for transformation assay using activated oncogenes among several nontransformed rodent lines such as BALB3T3 and Swiss3T3 mouse cells and 3Y1 and NRK-49F rat cells(4). However, NIH3T3 cells frequently indicated spontaneous piling-up foci on the monolayer culture, and sometime small colonies in the soft agar were observed, although these background activities were usually minimal if an adequate clone of this line was employed. Furthermore, NIH3T3 is not adeguate for analysis of the antigen expression, since this line was derived from Swiss mouse that was not completely inbred for the genetic background. This aspect of NIH3T3 means a disadvantage for experiments in which transformation associated antigens on the cell surface were analysed.

The crucial issue of our research for these "true tumor antigens" is to establish the most adequate reciqient cells that maintain their strict phenotypes as the non-transformants. We have investigated such recipient cells for tumor DNA transfection experiments that have not only strict characteristics as nontransformed cells but relatively high sensitivity for the cell transformation by the oncogenes.

In this paper we showed that the WFB cell which was derived from inbred WKA rat fetus could be one such candidate of the recipient cells, because this line showed a more enhanced sensitivity for transformation than BALA3T3 mouse cells and rat kidney-derived NRK-49F cells while maintaining strict characteristics as the nontransformant.

MATERIALS AND METHODS

Animals

BALB/c male nude mice and inbred Wister-King-Aptekman (WKA) rats were obtained from CLEA Japan Inc., Shizuoka, Japan. In the experiment, 6 to 8 week-old male nude mice and 8 to 10 week-old male rats were employed.

Cells

The whole tissue of WKA rat fetus of 14-15th day of gestation was minced with scissors in 10 m*l* phosphate buffered saline (PBS) containing 0.05% trypsin, and was treated for 5 minutes at 37 C. Ten m*l* of the complete medium (MEM containing 5% FCS and 292 μg/m*l* of L-glutamine) were added to the mixture.

It was centrifuged at 1000 rpm for 10 min, and the pellet was washed twice with ice-cold PBS. Then, the pellet was cultured in tissue culture fiasks (Costar Models 3275 and 3150, Costar, Cambridge, MA.) with 5% CO_2 at 37 C. After 2-3 weeks of cultivation, the confluent monolayer consisting mainly of spindle-formed fibroblasts in the culture flasks was observed.

The cells were treated with 0.05% trypsin +0.02% EDTA for 3 min at room temperature, and the single cell suspension was used for cell cloning in 60 mm petri dishes (# 3002, Falcon Plastics, Oxnard, Ca.). Approximately 200 single cells were seeded on the petri dishes, and were cultured with complete medium. Three to four weeks later, microscopic cell colonies were obtained, and picked up with Pasteur pippetes. The same procedure was repeated for one selected colony, and thus WFB was obtained. Currently, WFB has been continuously cultured more than 200 passage generations. We studied several of the growth characteristics of cells *in vitro,* such as the doubling time, morphological observation, serum dependency and chromosomal pattern. The doubling time was determined as follows. One hundred cells/well were seeded into microtiter plates (# 3040, Falcon Plastics), and the number of cells adherent to these cells was established 24 h later. Nonadherent cells were removed, the medium was replenished, and the amount of time necessary to increase the number of adherent cells by 2-fold was determined.

The chromosome was analysed by the conventional method. NIH3T3 and BALA3T3 mouse cells and NRK-49F rat cells were also employed to assess the transformation sensitivity as controls for that of WFB cells.

Tumorigenicity Assay in vitro and in vivo

The anchorage-independent cell growth in 0.3% soft agar culture was determined for inocula, ranging from 10^2 to 10^3 cells, as described previously(5, 6). The cell growth was scored microscopically at the 3rd week after plating, and the colony formation was expressed as % of plating officience=(No. of clusters-No. of original cell aggregates)$\times$100/No. of viable nucleated cells plated.

C-C36 and C-C26 cells were used for soft agar culture controls. The cells have been previously shown to be highly tumorigenic *in vitro* and *in vivo*(7). In order to characterize the tumorigenicity in vivo of cells, these cells were resuspended in PBS and inoculated subcutaneously into the backs of BALB/c nude mice or WKA rats with various concentrations of cells. At weekly intervals, the tumor development

was assessed. In a separate experiment, the histological features of tumors developed in vivo were also investigated.

DNA Transfection and Transformation Assay

Transformation of WFB cells with 6.6 kb EJras(8) separated from genomic DNA digested by BamHI or polyoma middle T DNA 5229 bp fragments inserted into the BamHI site of pBR322 plasmid DNA (pPyMT) was carried out by the calcium phosphate technique described elsewhere(5). Briefly, approximately 100 ng of 6.6 kb EJras DNA with 7 to 10 μg of calf thymus DNA (Pharmacia, Uppsala, Sweden) or 2 to 5 μg of EcoRI-digested pPyMT DNA was dissolved in 1 m*l* of 250 mM $CaCl_2$ buffered with 25 mM Hepes (pH 7.12). This solution was added to 1 m*l* of 280 mM NaCl-1.5 mM Na_2HPO_4-25 mM Hepes (pH 7.12). After 30 min at room temperature, 0.5 m*l* was added to each 60 mm petri dish (# 3002, Falcon Plastics, Oxnard, Ca.) containing 5 m*l* of complete medium and approximately 5×10^5 WFB cells. The cultures were incubated under CO_2 at 37 C for 6 h with a subsequent shock for 5 min in 10% dimethylsulfoxide. After incubation for 18 h, the cells were trypsinized, plated again in 60 mm petri dishes (# 3002), and fed every 2-3 days. After 21 days of cultures, piling-up foci on the monolayer of cells were counted for each dish. Some dishes in which WFB was transfected with EJras DNA were used for obtaining the transformed cells. After 18 h of transfection, $1-5\times10^4$ of transfected WFB cells was transferred into 60 mm petri dishes containing 0.3% soft agar (Bactoagar, Difco Laboratories, Detroit, MI.) on a baselayer of 0.6% agar. Colonies of transformed cells were picked up from the agar after 10 days of cultivation, and transformed WFB clones were obtained. Single cell cloning was also performed for these transformed cells. One to two hundred single cells were seeded on petri dishes (# 3002, Falcon Plastics), and cultured with complete medium. After two weeks, piling-up foci of cell colonies with a high saturation density were picked up. Thus several transformed cells were obtained. In this paper, we described representatively the characteristics of transformed W14 and W31 clone.

Southern Blot Hybridization of Cellular DNA

In order to confirm the insertion of transfected DNA into WFB, EJras-transformed W14 and W31 cell DNA was assessed by the Southern blot hybridization technique. Cell DNAs were prepared from cultured cells as described elsewhere(5). After cleavage of cellular DNA (20 μg) and 6.6 kb EJras fragments by SacI, DNAs were run by 0.9% agarose gel electrophoresis, denatured in situ, and transferred onto a nitrocellulose membrane filter (Bio-Rad Laboratories, Richmond, Ca.) with a modification of the technique of Southern(10). DNA fragments immobilized on the membrane filter were hybridized with ^{32}P-labeled EJras DNA and detected by

autoradiography.

RESULTS

Growth Characterisrics of WFB

The morphological feature of WFB at approximatily 200 passage generations is shown in Fig. 1, demonstrationg the fibroblastic nature with spindle-form cells. The growth of WFB on the confluent monolayer of cell culture was strictly inhibited by the cell-cell contact. Fig. 2 indicates the chromosomal analysis of WFB. It demonstrates 2n+XY of the karyotype that means a normal characteristic of WFB chromosome. We analyzed several characteristics of the cell growth (Table 1). The doubling time of WFB was almost twice of that of C-C36 and C-C26 which are highly tumorigenic mouse colon tumor lines(7). WFB also showed an overt serum dependency for the cell proliferation in contrast to colon tumor lines, and eventually the cell growth was completely inhibited when cultured with 1% FCS. Table 1 also indicates the tumorigenic potentials *in vitro* and *in vivo*. WFB could not form colonies in soft agar as well as syngeneic WKA rats and nude mice, even when more than 10^7 cells were inoculated. These data indicate the strict nature of WFB line as non-transformed cells.

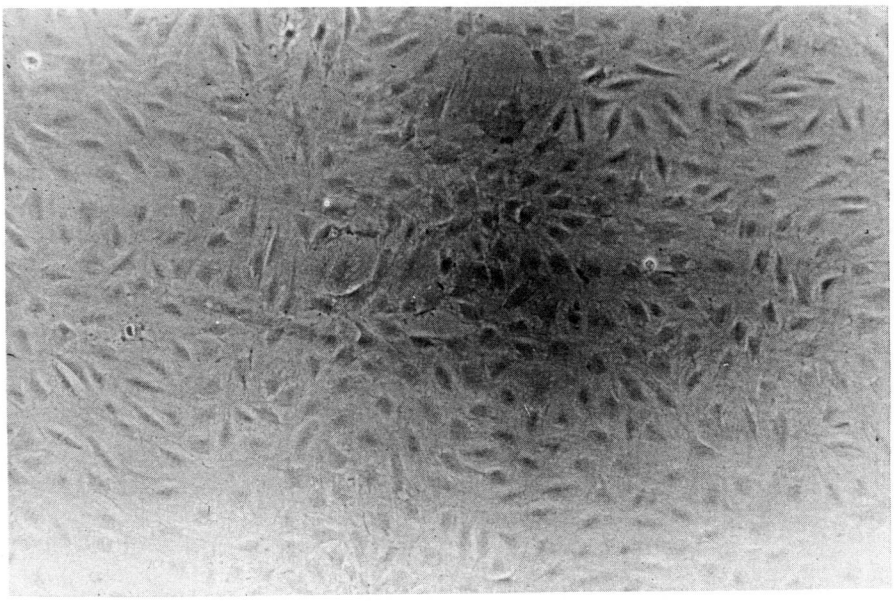

Fig. 1 A phase contrast microscopic feature of WFB at approximately 200th passage generations, demonstrating the fibroblastic nature of cells in morphology. 100×.

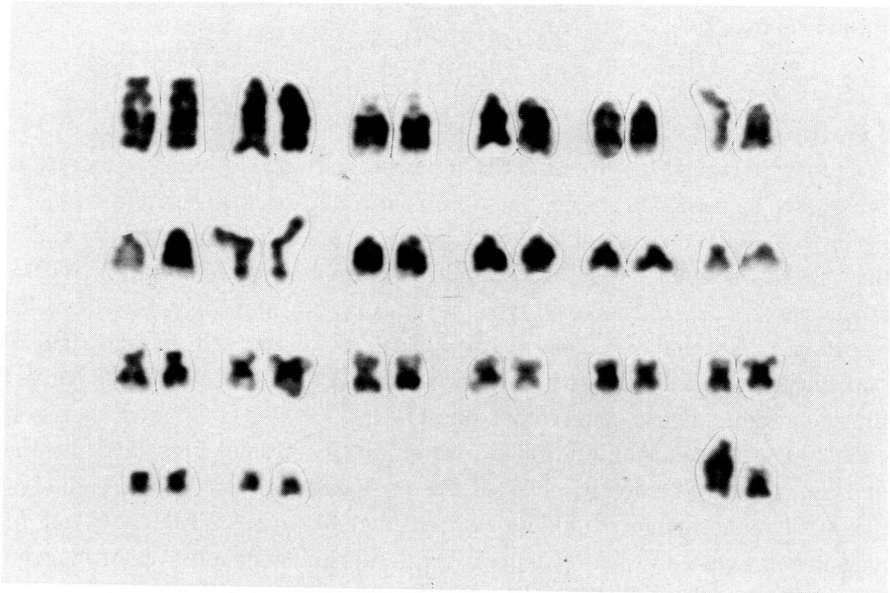

Fig. 2 A chromosomal analysis of WFB cells, demonstrating 2n+XY of the karyotype, and that there is no overt translocation among the chromosomes.

Table 1 *Several characteristics of WFB cells in vitro and in vivo.*

	WFB	C-C26	C-C36
doubling time (hr)	37.0	16.3	16.5
saturation density ($\times 10^4 cm^2$)			
5% FCS	13.8±1.1	N. D.	61.5±13.4
2.5%	7.0±1.4	N. D.	58.0± 2.8
1%	2.0±1.7	N. D.	52.0± 4.2
colony formation in 0.3% agar (% plating efficiency)			
10^2 seeded/60 mm dish	0.	70.4±3.9	81.2± 5.8
10^3	0.	59.6±6.8	68.2± 6.4
10^4	0.	N. D.	N. D.
tumorigenicity *in vivo* (% palpable tumor developed at 3–5 weeks after injection)			
10^7 injection to nude mice	0.	N. D.	N. D.
10^7 injection to WKA rats	0.	/	/

Transformation of WFB by Oncogenes

In order to assess the sensitivity of WFB cells of WKA rat origin for transformation by the oncogenes, the cells were transfected by the calcium phosphate precipitation technique with 6.6 kb EJras that was separated by BamHI cleavage of the genomic activated-H-ras(8), and polyoma middle T DNA that was inserted into

plasmid pBR322(9).

As shown in Table 2, WFB was more sensitive to EJras oncogene than BALB3T3 mouse cells and NRK-49F rat cells. NIH3T3 cells had the highest sensitivity, as previously reported by several investigators(4), among the four lines employed in the experiments.

However, NIH3T3 cells transfected with calf thymus DNA alone or without DNA showed the spontaneous transformed-like foci on the cell monolayer, although it was usually small in size (data not shown). The number of transformed foci in NRK-49F cells showed approximately one third of WFB when transfected by EJras. NRK-49F cells indicated an increased sensitivity in the experiments using pPyMT. However, this line usually showed a relatively high rate of cell growth, so that it was frequently very difficult to maintain the cells in a good condition even if the culture medium was exchanged daily.

In contrast, WFB has not demonstrated any spontaneous piling-up foci, and was very easy to maintain *in vitro* because it kept a complete contact inhibition on the monolayer culture for three to four weeks of the transfection experiments.

Consequently, transformed foci of WFB could be easily recognized. Some dishes in which WFB was transfected with EJras DNA were used for obtaining the transformed cells. At 18 h after oncogene transfection, $1-5\times10^4$ of the cells were brought into the soft agar culture contaning 0.3% agar. Approximately 10 days later, as shown in Fig. 3, colonies in the agar were detected. Then these colonies of transformed WFB cells were picked up from the agar, and several clones were obtained. In this paper we analyzed two clones, designated as W14 and W31, for several growth charateristics of the transformed phenotypes. As shown in Table 3, the tumorigenic potential of these cells was assessed *in vitro* and *in vivo*. Both of these clones demonstrated a very high capability to grow in the soft agar. Furthermore, these cells also showed a high tumorigenic nature and an extensive growth potential of cells in the syngeneic rats and nude mice. When 10^6 W31 were injected

Table 2 *Transformation efficiency of WFB by EJras and polyoma middle T DNA.*

cells	*No. of foci per μg DNA of*[1]	
	EJras	pPyMT
WFB	110.0	0.1
NIH3T3	3800.0	7.0
BALB3T3	30.0	N. D.
NRK-49F	35.0	0.6

[1] No. of foci was determined as described in Materials and Methods. Approximately 21 days later of DNA transfection, the piling-up foci in the petri dishes were counted.

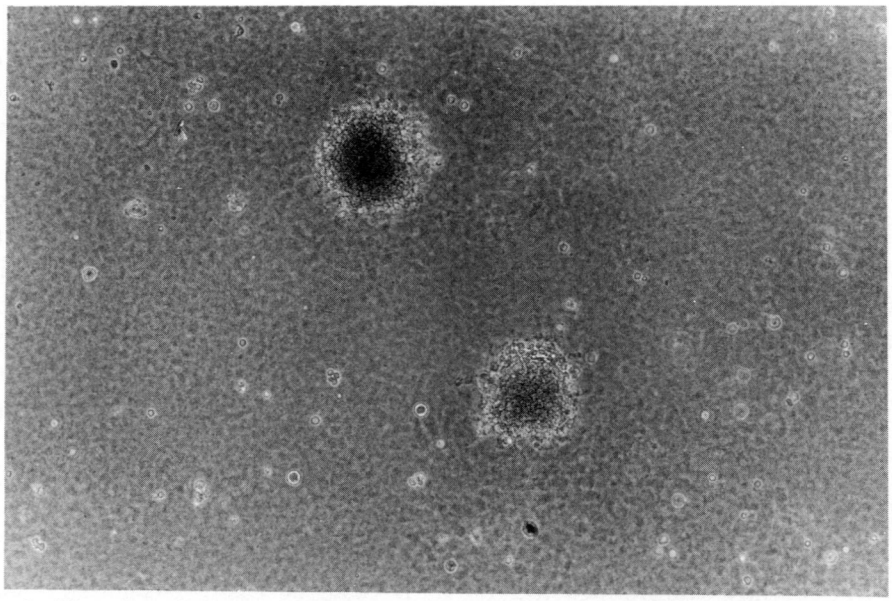

Fig. 3 The colony formation of transformed WFB cells in the soft agar. WFB was transfected with 6.6 kb EJras DNA by calcium phosphate precipitation, and at 18 hr later $1-5\times10^4$ cells were brought into 0.3% soft agar culture. A phase contrast microscopic feature of the colony was taken at 10 days later of the agar culture. 100×.

Table 3 *Tumorigenic potentials in vitro and in vivo of EJras-transformed WFB clones, W14 and W31.*

	WFB	W14	W31
colony formation in 0.3% agar (% plating efficiency)			
10^4 seeded/60 mm dish	0.	47.0±15.1	85.3±10.4
tumorigenicity in vivo[1] (10^6 inoculum/animal)			
nude mice	0.	100.0	100.0
WKA rats	0.	100.0	100.0

[1] Three to five animals for each group were injected with 10^6 cells subcutaneously, and were observed for the tumor development. The data is expressed as % tumor incidence.

subcutaneously into syngenic rats, W31 developed tumors as large as 10 mm in diameter even at one week after the injection.

Although the tumorigenic potential in vitro of W14 was somewhat reduced, the tumor development alone was almost similar to that of the W31 clone (data not shown).

The histology of these tumors in the syngenic rats is shown in Fig. 4. The spindle form cells with various nuclear sizes proliferated profoundly with frequent

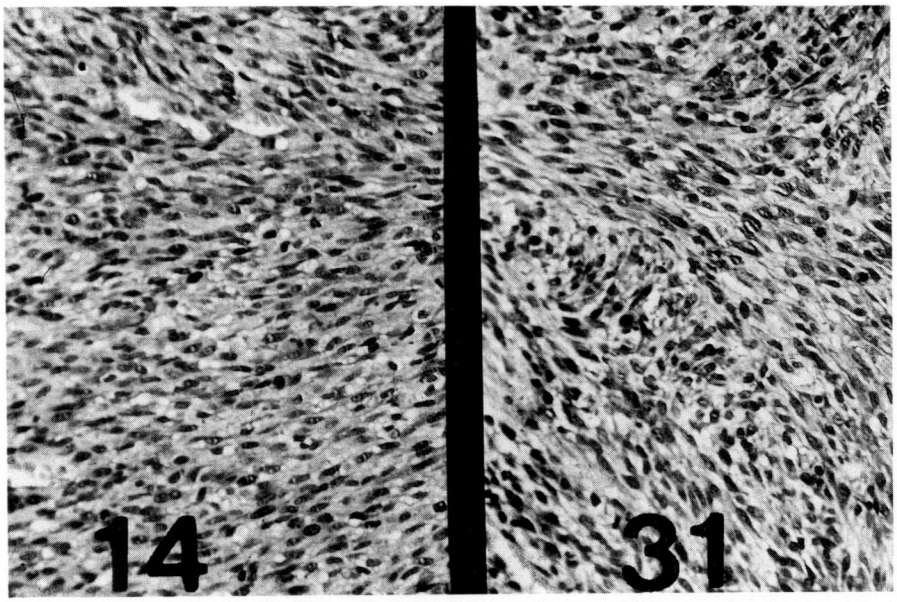

Fig. 4 The histology of the transformed WFB clones that developed in the syngeneic WKA rats. A picture in the left(14) is the histology of W14 tumor, and the right(31) is W31 tumor. Both of these tumors show that spindle form cells with highly atypical nuclei are proliferating with frequent mitoses, indicating fibrosarcomas. 200×, hematoxyline-eosin stain.

miteses. These histological features are the same as those tumors developed in nude mice, and are consistent with those of fibrosarcomas.

Southern Blot Analysis

In order to assess the insertion of EJras oncogene into the tumorigeic W14 and W31 cells, the DNA of these cells was transferred to a nitrocellulose membrane by Southern blotting, and was hybridized with ^{32}P-labeled EJras DNA probe. It is clearly demonstrated that when DNA was digested by the SacI restriction enzyme, a specific DNA sequence with 2.9 kb in size was detected in W14 and W31, but not in WFB (Fig. 5).

DISCUSSION

The tumor DNA transfection has detected the activated cellular oncogenes during the past several years, since cellular DNA from methylcholanthrene-induced mouse fibrosarcomas was demonstrated to be able to induce the transformation of NIH3T3 non-transformed cell by Weinberg *et al*(11).

Thereafter, these works provide us with a great understanding of not only the oncogenesis of the mammalian cells but also the biological significance of the

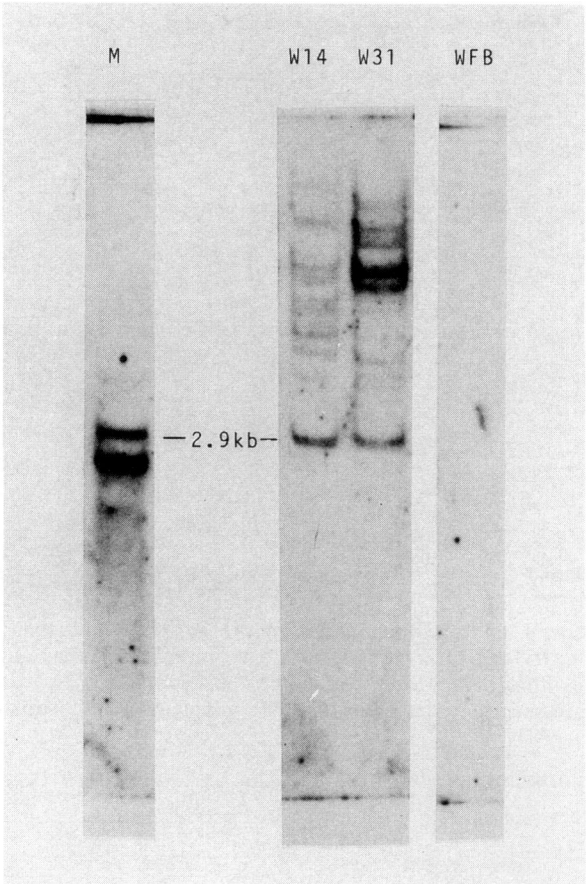

Fig. 5 Southern bot analysis for the integration of EJras oncogene to transformed WFB cells. 20 μg of 6.6 kb EJras and cell DNA that were digested by Sac I was run on a 0.9% agarose gel electrophoresis. DNAs of W14 and W31 demonstrate clearly the insertion of 2.9 kb specific DNA sequence of EJ ras.

functions of protooncogenes in the cells(1, 2). However, approximately 80% of tumor DNAs including human as well as experimental tumors could not transform NIH3T3 transfected with their DNAs. This means that NIH3T3 mouse cells are not sensitive to these cellular DNAs that may contain the activated oncogenes, or that activated or dominant oncogenes are not involved in their DNAs. Surtherland *et al* demonstrated recently that DNA could induce the transformation of foreskin fibroblasts but not NIH3T3 cells(12).

These results suggest that it is obviously important to select and establish the new recipient cells which are sensitive for the transformation assay using tumor DNAs, other than the already established cells such as NIH3T3, BALB3T3 and

NRK-49F.

In our search for cell surface antigen expression associated with the cell transformation, we have understood that the key point is to gain recipient cells for oncogene transfection experiments that maintain strictly phenotypes as the non-transformants, but still show sensitivity for the transformation assays by oncogenes. In this report, we introduced such a recipient line, designated as WFB, for the cell transformation, using tumor DNA trasnfection. This cell was derived from WKA rat fetus, and was continuously passaged *in vitro* more than 200 times. When transfected with EJras oncogene, WFB showed an easily recognized transformed foci and a higher sensitivity for cell transformation than that of BALB3T3 and NRK -49F cells, although it showed a lower sensitivity when compared to NIH3T3 cells. The EJras-transformed WFB clones, W14 and W31, could form rapidly growing colonies in the soft agar, and develop tumors in nude mice and syngeneic WKA rats. The histology of these tumors was consistent with that of fibrosarcomas.

It has already been shown that the primary rat fibroblast was not transformed with the activated oncogene alone such as H-ras, but transformed with additional oncogenes like c-myc(2, 13). This fact may support at least the two steps for oncogenesis, that is, one for cell immortalization and the other for promotion of the malignant transformation of cells(14). From this point of view, WFB may already be immortalized during *in vitro* cultivation of more than 200 times passage generations. It is also known that NIH3T3 cells frequently show relatively high spontaneous foci on the monolayer cultures, even though the transfection was performed using the NIH3T3 clones that showed initially the strict contact inhibition on the monolayer culture. This phenomenon sometimes causes difficulties for judging between the transformed and non-transformed foci of NIH3T3 cells, although the sensitivity to the transformation of cells is usually higher than other recipient cells.

On the other hand, WFB cells have continously shown their strict phenotyes as non-transformed cells in addition to the relatively high sensitivity of oncogene transformation assays. It shows a complete contact inhibition, serum dependency, long doubling time and complete anchorage dependency of cell growth. These characteristics are obviously very important for investigating the expression of the transformation-associated surface antigen on the cells(15, 16, 17). For this possibility, our preliminary studies using monoclonal antibodies showed that several newly expressed molecules were detected on the WFB cells transformed by EJras genes but not on non-transformed WFB cells. Furthermore, the transformed WFB cells were also sensitive to the cytotoxicity of NK cells or lymphokine-activated killer (LAK) cells, and there is a possibility that the molecules detected in the above experiments are those of candidates for the target structures involvinvg in the host immune surveillance against the tumors.

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