In Vitro transformation of LW13 rat liver epithelial cells[1]

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ABSTRACT

A rat liver epithelial cell line designated LW13 was established using a sequential sedimentation method. The cell line retained many normal properties of liver epithelial cells and showed some structural and functional features resembling those of liver parenchymal cells. LW13 cells became malignant after the introduction of exogenous transforming EJ Ha *ras* gene. Tumors produced by inoculation of the transformed cells into baby rats contained areas of poorly differentiated hepatocellular carcinoma. In *situ* hybridization analysis confirmed the random rather than specific integration of exogenous *ras* gene into host chromosomes. Furthermore, an at least tenfold increase in the expression of the endogenous c myc gene was detected among transformed cell lines, suggesting the involvement of the c myc proto oncogene in the in *vitro* transformation of rat liver epithelial cells by EJ Ha *ras* oneogene.

Key words: *LW13 cell line, in vitro transformation, EJ Ha ras integration, c myc expression.*

INTRODUCTION

In recent decades rat liver cell cultures have been utilized by many investigators for various purposes in liver cancer research. Although it was generally believed that in *vivo* most of the hepatomas were derived from hepatocytes, none of the hepatocyte cultures used for in *vitro* studies could survive longer than several weeks keeping their normal functional activities. On the other hand, rat liver epithelial cells (RLEC) could develop

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into long term cell lines or strains. While none of them showed all the hepatic functions, they maintained some of them, including hormone responses to synthesize tyrosine aminotransferase (TAT).

In vitro studies with RLEC have afforded useful information to hepatocarcinogenesis research. In some experiments, spontaneously transformed RLEC were capable of producing carcinoma like tumors [1], while chemically transformed RLEC resulted only in a small portion of hepatocellular carcinomas associated with a wide range of other tumor types [2]. The high heterogeneity of tumors formed by transformed RLEC may to some extent reflect the original relationship between hepatocytes and culturable RLEC. Therefore studies with RLEC may also be helpful in finding out the real progenitor(s) of hepatocellular carcinoma *in vivo*.

It has become apparent that oncogenes might be involved profoundly into liver cancer formation. Among these oncogenes, activated ras and/or myc genes were detected in chemically induced rat liver cancer [3] and particularly in human primary hepatoma [4]. With fibroblast system there is some evidence suggesting a cooperation of ras and myc genes during the malignant transformation [5]. So far, to this point, studies with rat liver cells gave some controversial results. Simultaneous activation of ras and myc has been reported for several systems. But for myc gene, while some reports indicated that c myc expression was essential for transformation either being associated with early [6] or late stage [7], other data argued that elevated myc expression might be related to immortalization of RLEC rather than ras induced transformation [8].

Several laboratories have reported transformation of RLEC by EJ Ha ras gene [9 −11], but the possibility of formation of hepatocellular carcinoma by these transformed cells through transplantation was not mentioned. And the molecular basis of this ras induced transformation remained to be elucidated. In addition, it would be interesting to investigate whether the integration of the exogenous ras gene into a specific host chromosome is a common phenomenon in the RLEC system,as has been reported previously [12].

In this paper we described the establishment of the rat liver epithelial cell line LW13. Subsequently, we transformed this cell line with the EJ Ha ras gene,aiming at analyzing the behavior of Ha ras and other oncogenes during the transformation process, mimicking *in vivo* hepatocarcinogenesis to some extent.

METHODS AND MATERIALS

A. *Establishment of LW13 cell line*

The method used for the establishment of the cell line was basically similar to that described by G. M. Williams [23]. Briefly, 10 d old male Wistar rats were cervically dislocated, the extirpated livers were washed, minced and digested by D Hanks' buffered 0.3% trypsin in flask with magnetic bar stirring at 37 C for 10 min. After decantation the trypsinization process was repeated twice. Combined cell supernatants were centrifuged, resuspended in HamF12 10%BS medium,and dispersed into culture flasks. After 20 min incubation at 37 C, the medium containing unattached cells was transferred to a second set of flasks for another 20 min incubation. Thereafter, still unattached cells in the supernatants were transferred to a third set of flasks and incubated again as described. The final supernatants were discarded and the flasks containing attached cells were covered with HamF12 10%BS medium, supplemented with insulin, glutamine, glucose and antibiotics. Subcultures were maintained in DMEM HamF12 10%BS (or FCS) medi-

urn. Cells were passaged when they were grown just to confluency. The medium was changed twice per week.

B. Characterization of the LW13 cell line

Slides for chromosome studies were prepared by standard techniques using hypotonic treatment of colcimid ar−rested cultures [13] and were analyzed after Giemsa trypsin banding. Determination of TAT activity was performed after the method of Granner [14]. Examination of ultrastructures was carried out with glutaraldehyde and OsO_4 fixed cells as described [15]. Sections were examined with JEOL JEM100B transmission electron microscope (TEM), Measurement of growth curve was done by daily counting duplicate flasks passaged with an initial density of 2×10^5 cells/flask.

C. Plasmid DNA

For transformation of the LW13 cells, a plasmid consisting of pSV2 neo sequences [16] and a 6. 6 kb EJ Ha ras transforming oncogene [17] incorporated into the BamHI site of pSV2 neo was employed:a 460 bp fragment of v Ha ras in plasmid BS9 [33] was used as probe for the detection of transfected EJ Ha ras sequences; a 2. 2 kb SstI BamHI fragment generated from 6.6 kb EJ Ha ras, which could not be hybridized with endogenous rat cellular ras sequences, was used as a probe for in situ hybridization of chromosomes; the probe used for the detection of the ex−pression of endogenous c myc gene was a 420bp pstI pstI fragment of mouse c myc exon 2.

Small and large scale preparations of plasmid DNA were carried out with alkali SDS methods similar to those de−scribed by Maniatis [18,19]. A Bio Gel A 150m column was used in some cases to separate plasmid from RNA. Raw DNA was further purified by repeated CsCl (Merck) centrifugation after Maniatis [2()] to obtain circular plasmid DNA for transfection.

D. Transfection of LW13 cells

The transfection of EJ Ha ras gene into LW13 cells was carried out using standard calcium phosphate precipi−tation technique. Briefly, 5 μg pSV2 neo Ha ras or pSV2 neo plasmid DNA and 10−20 μg high molecular weight salmon testis DNA were mixed, and incubated with cells (passage 25−30) grown to 50−80% confluency in 60 mm culture dish after precipitation with $CaCl_2$ and HEPES buffered saline. Transfectants were selected by addition of the antibiotic G418.

E. Preparation of radiolabeled probes

Probes used for nucleic acid hybridization were labeled by incorporation of [α−^{32}P] dCTP(NEN) with a nick translation kit (Amersham N5500), probes used for in situ hybridization were labeled by ^{3}H dATP or^{3}H dCTP (Amersham) with the same kit or with the Amersham Multiprimer labelling Kit RPN1600Y. Radiolabeled DNA was isolated by Sephadex G50 chromatography.

F, In situ hybridization

The method was based on that of Chandler [21]. Normally, 15 μl of hybridization solution containing l. 5×10^4 cpm of the ^{3}H labeled 2.2 kb Ha ras DNA probe was applied to each chromosome spread for overnight hybridiza−tion. Banded chromosomes (Wright stain) were compared to standard karyotype of the rat [22] and Levan's nomen clature for banded chromosomes was adopted [13]. A slight modification of the method was the use of 95% ethanol containing O. 04% HCl for destaining to prevent loss of silver grains.

G. Extraction and hybridization of nucleic acids

The method of extraction of genomic DNA was based on that of Herrman [24] and that of total RNA preparation on the guanidine hydrochloride procedure of Strohman et al [25]. Identification of transfected Ha ras was achieved by Southern blot technique [26]. Ten or more μg genomic DNA, digested by restriction enzymes, was hybridized with the probe after eleetrophoresis and transferred to Hybond N membranes (Amersham). Expression of RNA was ana−lyzed by Northern blot technique [27]. Denaturation and electrophoresis of RNAs were carried out in the presence of formaldehyde. Glyoxal was used in RNA dot blot experiments.

H. Soft agar assay and tumorigenicity test

Soft agar assay was done in 60 mm culture dishes containing 0.5% agar as the lower layer and 0.33% agarose as

the top layer in DMEM−F12−10%FCS medium. Cells were seeded at a density of 2×10^4 per dish and dishes were monitored for 3 wk. Tumorigenicity tests were performed as follows: confluent cells were harvested and resuspended in Hanks' solution at a concentration of 2×10^7 cells/ml, and 0.1 ml such suspension was inoculated subcutaneously into the back of each of the ld−old Wistar baby rats. Animals were inspected for 5 m.

RESULTS

A. Establishment and characterization of the LW13 hepatic epithelial cell line

The sequential sedimentation and passage of trypsinized cell suspensions from livers of 10 d-old rats gave rise to 3 sets of flasks with different cell populations. While an epithelial cell population developed in the third set of flasks and was further subcultured, the cells from the first and second sets of flasks were discarded due to their dominance of fibroblast−like cells. Subcultured epithelial cells were of an adherent, polygonal type, growing to flat monolayer (Fig 1). Generally the cells had one nucleus with 1−3 round or oval nucleoli. This epithelial -like growth pattern remained unchanged during long term propagation. The developed cell line was designated as LW13.
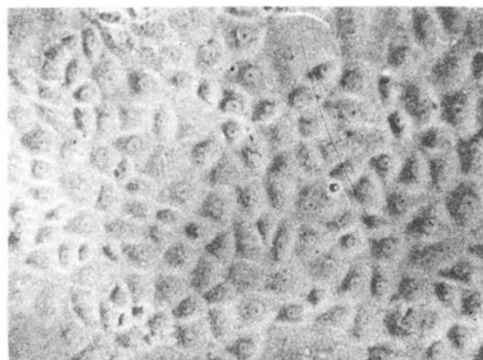

Fig 1. Morphology of LWI3 cells at passage 6 with polygonal shaped cells grown just to confluen− cy. Phase contrast. ×240
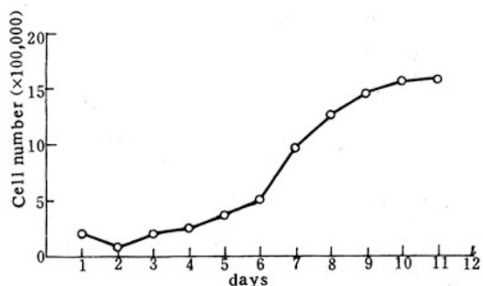

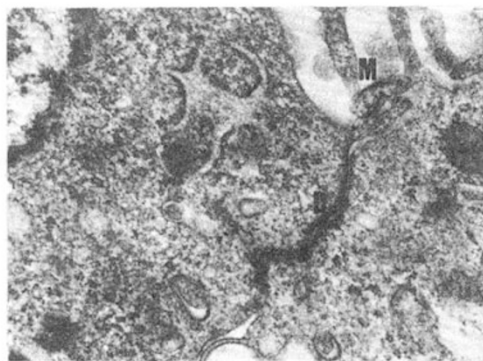**Fig 2.** Growth curve of LWl3 cells in 10% BS complete medium.

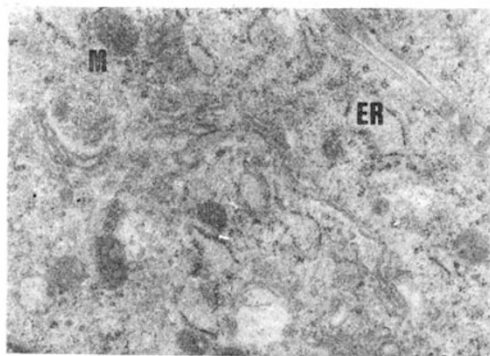**Fig 3.** Ultrastructure of LWl3 cells showing the pres- ence of desmosomes (D) and microvilli(M). × 17600.

Fig 4. Ultrastructure of cytoplasm of LW13 cells show- ing the presence of free mitochondria(M) and di- lated rough endoplasmic reticulum(ER). × 11500.

A growth curve of LW13 cells at passage 28, fed with 10%BS complete medium, is shown in Fig 2. The average doubling time was 39 h. For LW13 ceils at passage 12 the number of confluent cells 15 d after passaging was 6.03×10^4 cells/cm^2, revealing the property of density inhibition of the cells.

Chromosome analysis at passage 15 showed that from 50 metaphases, 90% were diploid while the rest of the cells had chromosome numbers of 42 ± 1 or 40. The dominant diploid karyotype was stable for more than 50 passages.

LW13 cells maintained the hormonal responsiveness of synthesizing TAT which might be regarded as a kind of hepatocyte specific function. Tab 1 shows the statistically significant increment of TAT activity of LW13 cells after dexamethasone treatment.

Tab 1. Activity of Tyrosine Aminotransferase in LW13 cells

Samples	TAT dexamethasone[a]	
	untreated	treated
LW13[b] (lysates)	(mU/min/mg protein)[d]	
Exp 1	0.672	0.88
Exp 2	0.71	0.87
Exp 3		0.85
newborn rat liver (homogenate)	2.52[c]	

Notes :

(a) 10^{-5}M, 24h pretreated; (b) at passage 16; (c) mean value of duplicate experiment; (d) T value given by t test is 36.8 which corresponds to p <0.01, indicating the increase of specific activity of TAT after dexamethasone induction is statistically significant.

TEM not only showed the existence of desmosomes which confirmed the epithelial nature of LW13 cells (Fig 3) but also abundant mitochondria, dilated endoplasmic reticulum (Fig 4) and in particular the intercellular structures resembling bile canaliculi with microvilli limited by junctional complexes (Fig 5). These characteristics are similar to those described for adult rat hepatocyte [28]. No virus particles were found.

Fig 5. Ultrastructure of LW13 cells showing the presence of intercellular structure resembling bile canaliculi (C). ×6700.
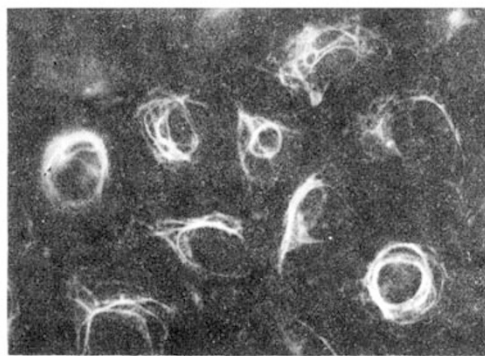

Fig 6. The presence of keratin filaments in LW 13 celjls . ×600

Finally, LW13 cells were shown to be immunostained positively for anti keratin antibodies (Fig. 6). Even at passage 50 the cells were still anchorage dependent and nontumorigenic.

Based on the above characterization it appears obvious that LWl3 is a normal liver epithelial cell line possessing at the same time some structural and functional properties of rat liver parenchymal cells.

B. Transformation of LW13 cells by exogenous Ha ras gene

After transfection, G418 resistant colonies were visible 7 12 d later in selection medium. An average of approximately 20 colonies appeared among 10^6 transfected cells. Morphologies of colonies of LW13 cells transfected with vector alone and of many colonies transfected with pSV2 neo Ha ras were similar to those of LW13 cells, but some pSV2 neo Ha ras transfected colonies showed completely different morphology. Both kinds of colonies were then picked up with clone ring and propagated into cell lines. 3 pSV2 neo alone transfected (N3−N5), 4 pSV2 neo Ha ras transfected hut morphologically unchanged (H1−H4), and 9 pSV2 neo Ha ras transformed (HA HI) colonies were analyzed. Fig 7 shows a representative transformed cell line HD. The transformed cells grew in multilayer, proliferated rapidly, and formed foci in soft agar (Fig 8). Typically, cell density of clone HD could reach up to $4.4 \times 10^5/cm^2$ which was 7.3 fold as high as normal untransformed LW13 cells in complete medium. These transformed cells could give rise to tumors when inoculated into 1 d old rats, while LW13 cells, pSV2 neo transfected LW13 cells as well as many pSV2 neo Ha ras transfected but morphologically unchanged LW13 cells all failed to produce tumors up to 5 months in vivo. Most of the tumorigenic cells were also capable of producing metastases as shown in Tab 2.
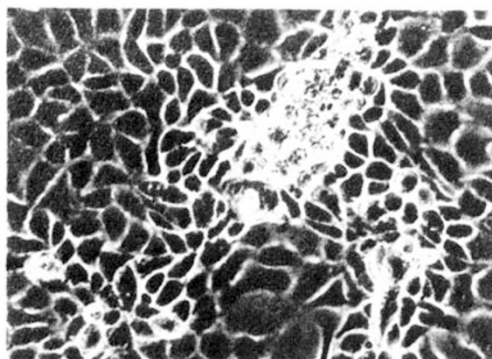

Fig 7. Morphology of the pSV2 neo Ha ras transformed malignant cell line (HD). ×240

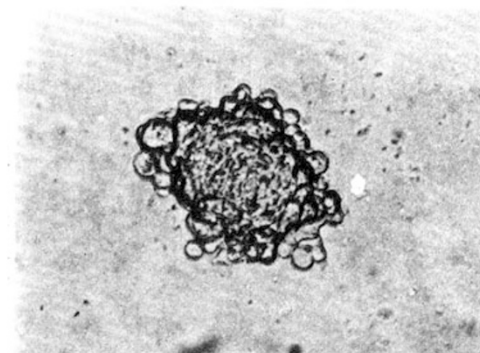**Fig 8.** A representative focus produced in soft agar by the tumorigenic cell line HA. ×115

Tab 2 summarizes the results obtained from the transfection experiments.

Southern blot analysis confirmed that only pSV2 neo Ha ras transfected cell lines had integrated exogenous ras gene, and that individual clones might have different amounts of copies of the gene (Fig 9). RNA dot blot analysis indicated that only tumorigenic cell lines expressed high levels of Ha ras gene (Fig 10), suggesting that

Tab 2. Summary of transfection experiments

Transfected plasmid	Cell type	Morphology	Growth in soft agar	Tumorigenicity*	Metastasis*	Average weight of tumors (g)
	LW 13	N	-	--	--	--
pSV2-neo	N3	N	-	--	--	--
	N4	N	-	--	--	--
	N5	N	-	--	--	--
pSV2-neo -EJ-ras	H1	N	-	--	--	--
	H2	N	-	--	--	--
	H3	N	-	--	--	--
	H4	N	-	--	--	--
pSV2-neo -EJ-ras	HA	GC	+++	10/10	2/10	2.8(3wk)
	HB	C	+	4/4	0/4	21.4(11wk)
	HC	GC	+	4/4	1/4	12.7(6wk)
	HD	C	+	5/5	4/5	18.4(10wk)
	HE	C	+	5/5	2/5	16.5(6wk)
	HF	GC	ND	4/4	0/4	16.9(6wk)
	HG	C	ND	5/5	0/5	11.9(6wk)
	HH	C	ND	5/5	4/5	14.7(6wk)
	H1	C	+	5/5	3/5	14.3(6wk)

Notes: N normal   - no foci   - negative up to 5 months
GC greatly changed   +++   big foci   * number of rats appeared positive/ of rats tested
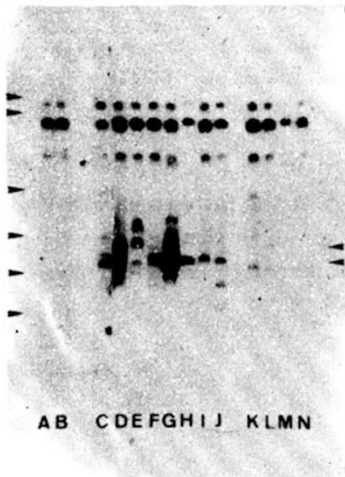C changed   +   small foci
ND not done

Fig 9. Detection of exogenous EJ−Ha−*ras* gene among trans-fetted cell lines by Southern blot. 15 μg genomic DNA extracted from each cell line was analyzed as described in procedure. laneA, LW13; laneB, N3; laneC, HA; lanesD−J,HC−HI seven cell lines; lanesK−N,H1−H4 four cell lines. Arrowheads on the left site indicate λ −Hind III DNA marker, from top to bottom: 2.0, 2.3, 4.4, 6.6, 9.4, 23. 1 kb respectively; two arrowheads on the right side indicate the exogenous Ha−*ras*. Serial number of all cell lines are listed in Table 2.

Fig 10. Experssion of the Ha−ras gene as detected by dot blot, Graded amounts of samples were 10, 2.5, 0. 625 μg total RNA. lane A,LW13; lane B,N3; lane C,N5; dane D, H1; lanesE−I, HG, HI, HF, HD and HA respectively.

overexpressed Ha -ras was a causal factor of transformation.

It was interesting to note that the tumors grown from Ha -ras gene transformed cells (e. g. line HD) contained areas of poorly - differentiated hepatocellular carcinomas which consisted of nonuniform cells with nuclei of different sizes and darkly stained nuclear membrane (Fig 11). This indicated that LW13 cells, as a liver epithelial cell line, could contribute to the formation of hepatocellular carcinomas, when transformed by the activated oncogene, Undifferentiated anaplastic carcinoma cells, however, were also found in tumors derived from the same line(Fig 12).
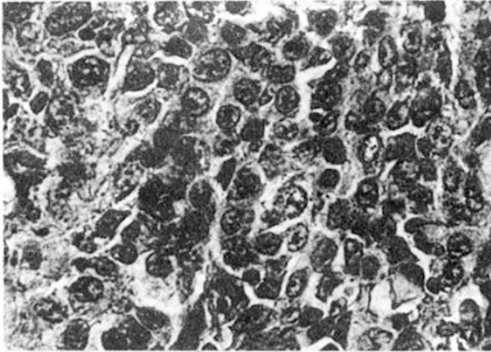

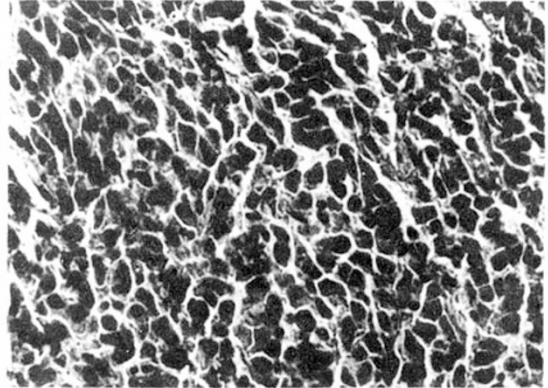**Fig 11.** Poorly- differentiated hepatocellular carcinoma produced by HD cell line. Hematoxylin stain. ×200

Fig 12. Anaplastic carcinoma existed in some parts of tumors produced by HD cell line. Hematoxylin stain. ×150.

C. Incorporation of exogenous Ha- ras gene

To investigate the relationship between the position of the integrated Ha -*ras* gene and transformation, a radiolabeled 2.2kb probe which could not be hybridized with rat cellular sequences (Fig 13) was used to localize the human EJ-Ha -*ras* gene on rat chromosomes by in situ hybridization technique.
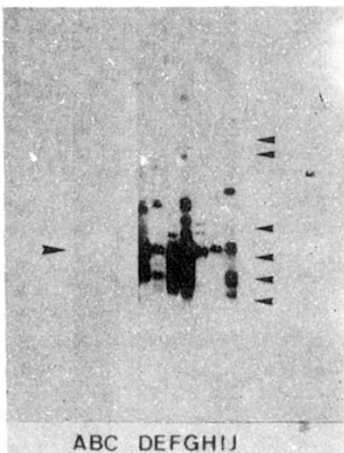
Fig 13. Identification of homology between the 2.2kb probe, rat cellular Ha - *ras* and exogenous EJ - Ha - *ras*. 15μg BamHI digested genomic DNA of each cell line was hybridized with the 2.2kb probe, lane A, LW13; lane B, N3; lane C, N5; lanes D−J, HC, HD,......HI; arrowheads on the right side indicate λ − Hind III DNA marker, from top to bottom: 2.0, 2.3, 4.4, 6.6, 9.4, 23.1 kb respectively; the left arrowhead indicates 6. 3 kb band.

The incorporated sites of EJ -Ha -*ras* were determined by chromosome banding

through emulsion. Karyotype analysis showed that the cell lines HD, HE, HF and HI investigated were aneuploid. But except for the abnormal chromosome No. 13 found in HD cell line and chromosome No. 2 found in HA cell line, there was no other apparent chromosome translocation or band deletion.

In the present work, at least 50 metaphases of each cell line were examined microscopically and the positions of hybridized spots were recorded. Furthermore, according to the altered karyotype of tumorigenic cells, chromosome photographs were pooled and classified into 8 categories, based on their main characteristics (chromosome length and arm ratio). In Tab 3, the chromosomes of 25 metaphases from each of 4 transformed cell lines, HD, HE, HF and HI, were photographed and grouped into 8 categories. For each category, the average length of chromosomes, the number of chromosomes actually counted (in 25 metaphases)etc,were listed. Above all, the percentages of total chromosome length of each category in the total chromosome length of all categories (Tab 3,5) were calculated. In Tab 4, the total number of silver grains found in each 25 *in situ*

Tab 3. Classification and data analysis of chromosomes from in situ hybridized metaphases

	Cell Lines	chromosome category							
		I	II	III	IV	V	VI	VII	VIII
Chromosomes included (No.) (1)		1	2	3-5	6-8, X	9-10	11-13	14-16	17-20, Y
average length of each chromosome* (2)		10.62	9.46	6.83	5.43	4.63	3.57	3.84	3.02
numbers of	HD	111	82	213	305	276	136	168	448
chromosomes	HE	64	62	173	177	121	102	135	208
actually counted	HF	69	65	144	234	183	123	147	293
(3)	HI	77	72	182	260	251	125	159	379
total length	HD	1179	776	1455	1656	1278	486	645	1353
of chromosomes	HE	680	587	1181	961	560	364	518	628
actually counted* *	HF	733	615	984	1271	847	439	564	885
(4)	HI	818	681	1243	1412	1162	446	611	1144
summed total length	HD	8828							
of chromosomes	HE	5479							
of all categories	HF	6338							
(5)	HI	7518							
(%)calculated percentages	HD	13.4	8.8	16.5	18.8	14.5	5.5	7.3	15.3
of chromosome total length	HE	12.4	10.7	21.5	17.5	10.2	6.6	9.4	11.5
of each category in that of	HF	11.6	9.7	15.5	20.0	13.4	6.9	8.9	14.0
all categories * * *	HI	10.9	9.1	16.5	18.8	15.5	5.9	8.1	15.2

Notes: * refer to standard karyotype of the Norway rat [22];
          * * from (1)×(2);
          * * * from (3)/(4);

hybridized metaphases of 4 different cell lines and the observed number of silver grains in different categories were given. For comparison, the expected number of silver graihs which would represent a random distribution of silver grains were also listed in Tab, 4. A X^2 test was adopted to check up whether the silver grains actually observed in photographs are signals (statistically significant) or only a general background (statistically insignificant comparing to the expected values). The results demonstrated that among the different cell lines HD, HE, HF and HI, most of the hybridized spots accumulate in different categories. However, within one cell line, most of the hybridized spots are confined to one definite category. The X^2 test indicated that an abundance of spots in the definite category reflects a significant signal rather than a general badkground. Apparently, these results demonstrated that for an individual cell line the integration sites are on the same chromosome and this can be explained by its clonal origin.When different cell lines are compared, however, EJ−Ha−ras is found to be incorporated into rat chromosomes in a random pattern.

Tab 4. Analysis of the distribution of hybridized silver grains in chromosomes of individual category in 4 transformed cell lines

Cell	total number of silver		grain distribution in different categories							
Line	grains counted		I	II	III	IV	V	VI	VII	VIII
HD	305	expected*	40.9	26.8	50.3	57.3	44.2	16.8	22.3	46.7
		observed	236	14	3	8	6	8	5	25
		X^2	930.6	6.1	44.4	42.4	33.0	4.6	13.4	10.0
		P	$\ll 10^{-4}$	–	–	–	–	–	–	–
HE	158	expected	19.6	16.9	34	27.7	16.1	10.4	14.9	18.2
		observed	6	7	103	19	8	5	5	5
		X^2	9.4	5.8	140.0	2.7	4.0	2.8	6.6	9.6
		P	–	–	$\ll 10^{-4}$	–	–	–	–	–
HF	232	expected	26.9	22.5	36	46.4	31.1	16	20.6	32.5
		observed	8	16	18	137	14	10	8	21
		X^2	13.2	1.9	9.0	176.9	9.4	2.2	4.4	4.0
		P	–	–	–	$\ll 10^{-4}$	–	–	–	–
HI	189	expected	20.6	17.2	31.2	35.5	29.3	11.2	15.3	28.7
		observed	11	10	11	11	10	5	114	17
		X^2	4.5	3.0	13.0	16.9	12.7	3.4	636.7	4.8
		P	–	–	–	–	–	–	$\ll 10^{-4}$	–

Note: * value from multiplying total number of silver grains and value (5) in Tab 3

Further investigation into the relatively well banded chromosome spreads has testified the incorporation of the EJ -Ha -ras gene in HD at position lq22. in HE at 4q24,in HF at 7q13. and in HI at 15q25. Fig. 14 and 15 are representatives for hybridized metaphases of HD and HI, respectively. Fig. 16 shows the hybridized spots matched to special region of chromosomes in HD, HF and HI respectively.
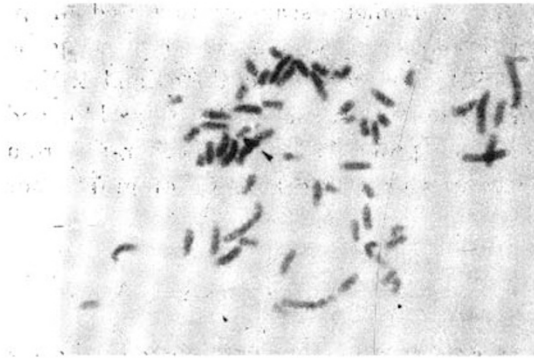
Fig 14. A typical hybridized metaphase of HD cell, arrowhead indicates the hybridized spots on chromosome No. 1.

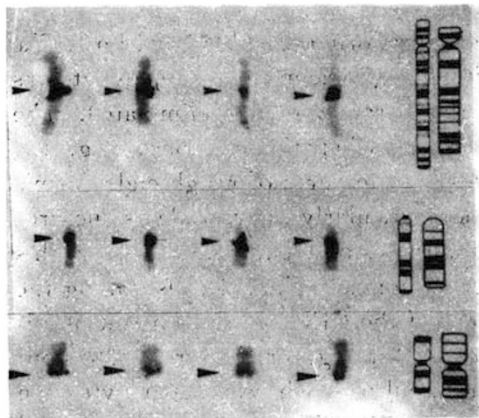**Fig 15.** A typical hybridized metaphase of a HI cell; arrowhead indicates the hybridized spots on chromosome No. 15.

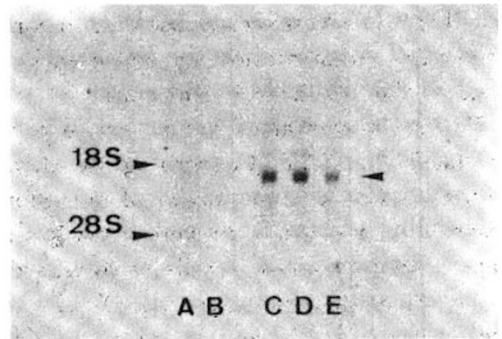**Fig 16.** Hybridized chromosomes of cell lines HD, HE and HI.

Fig 17. Overexpression of c *myc* among Ha *ras* transformed cell lines. 15 μg total RNA from each cell line was analyzed by Northern blot with a 420bp probe (see methods section), laneA, LW13; laneB, N3; laneC, HC; laneD,HD; laneE,HE; arrowhead on the right side indicates 2.5kb c *myc* band.

D. Overexpression of endogenous c myc genes coupled with ras-induced transformation

The demonstration of the expression of *c-myc* gene with a radiolabeled 420bp mouse c *myc* exon 2 fragment by Northern blot analysis showed an overexpression Of this
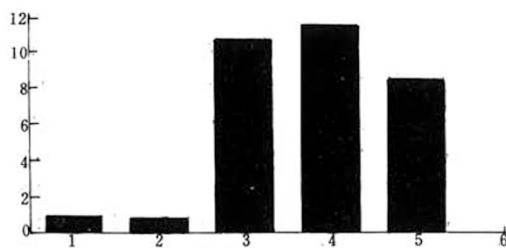

Fig 18. Expression of c *myc* gene. Signal intensity of Northern blot *x* ray film was measured by densitometric method with Shimadza dual wavelength thin-layer chromoto-scanner model CS 930. 15μg total RNA for each cell line was analyzed by Northern blot with 420bp probe, column1, LW13; column 2, N3; column 3, HC; column 4, HD; column 5, HE; column 6, H1.

gene in *ras* transformed LW13 cells (Fig 17). Densitometer scanning indicated an approximately 10 fold increase of RNA expression of the c *myc* gene in the tumorigenic cell lines HC, HD, HE, but not in LW 13. pSV2 *neo* transfected alone, and pSV2 neo Ha *ras* transfected nontumorigenic cells (Fig 18). Data from Northern blot and dot blot experiments with a radiolabeled 4.8kb c-myc probe (a fragment generated from pSVc myc 1[5]) were consistent with the results presented here (photographs not shown).

DISCUSSION

This paper describes a series of experiments, including the establishment of a rat liver epithelial cell line LW13, *in vitro* transformatin of LW13 cells by the EJ Ha *ras* gene,the detection of integrated exogenous ras gene and of expression of endogenous c *myc* gene. The results obtained provided additional information concerning *in vitro* transformation and hepatocarcinogenesis in the rat.

First of all, it seems essential to confirm the hepatic origin of LW13 cell line . The sequential sedimentation method used here permitted the attachment of hepatocytes as well as the nonparenchymal epithelial cells, but the fibroblasts were eliminated. The LW13 cells maintained the inducible potential to express hepatic functions, e.g. the production of TAT. Concerning the ultrastructures, the features of rough endoplasmic reticulum (RER), abundance of mitochondria, and particularly intercellular structures resembling bile canaliculi were similar to the characteristics of hepatocytes [28]. In this paper, however, LW13 cells were regarded rather as nonparenchymal cells,taking the following aspects into account: 1) neither the extent of the RER *nor* the amount of mitochondria of LW13 cells is as high as in hepatocytes; 2) it is generally agreed that nonparenchymal epithelial cells are easier to develop into cultures whereas hepatocytes can survive not longer than several weeks in culture; 3) they are smaller than parenchymal cells . We, therefore, considered LW13 cells as rat liver nonparenchymal epithelial cells having some characteristics of parenchymal cells.

Until recently it had widely been believed that hepatocytes are the origin of hepatoma cells. However, Fausto et al [29,30] pointed out that a kind of nonparenchymal epithelial cells, so called"oval cells", could be transformed, yielding moderately to well differentiated hepatocellular carcinomas after transfection by EJ Ha *ras*. Interestingly, these tumor cells could even express antigens of both hepatocytes as well as oval cells [31]. Tsao et al [32] described a diploid epithelial cell line from rat liver bearing phenotypic properties of oval cells. Although the exact relationship between hepatocytes, nonparenchymal oval cells and culturable epithelial cells such as LW13 remains to be further elucidated, the fact that LWl3 cells can be transformed *in vitro*, resulting in cells capable of producing tumors with areas of poorly differentiated hepatocellular carcinomas *in vivo* would support the hypothesis that nonparenchymal epithelial cells may contribute to the genesis of hepatocellular carcinomas *in vivo* under certain conditions.

With respect to the specific integration of exogenous ras gene in rat chromosomes as claimed by some authors [12], our data of *in situ* hybridization demonstrated only a random incorporation of exogenous EJ Ha ras. The existence of specific sequences which

may have the potential to facilitate the integration of transfected *ras* gene can not be excluded, although we have never found the incorporation of a human *ras* gene into rat chromosome No. 3.

One of the consequences of the *in vitro* transformation of LW13 cells is the overexpression of endogenous c *myc* gene. It is quite possible that c *myc* plays an important role during the *ras* induced oncogenesis. To investigate the functional relationship between *ras* and *myc* genes, c *myc* antisense RNA work has been carried out in further experiments by the present authors (unpublished data). A fragment of the mouse c *myc* gene was ligated into pSV2 plasmid in opposite orientation and was introduced into the *ras* transformed LW13 cells. Southern blot experiments confirmed the integration of this antisense c *myc* DNA sequences. Delayed growth initiation in low serum medium of antisense RNA expressing cells has been observed, indicating the influence of c *myc* gene expression on the malignant phenotype of LW13 cells transformed by the *ras* gene. A modification of the present system has been developed for further investigation of the c *myc* gene function.

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REFERENCES

[1] Diamond L, McFall R, Tashiro Y,Sabatini D. The WIRL 3 rat liver cell lines and their transformed derivatives. Cancer Res 1973; 33:2627 36.

[2] Tsao MS. Grisham JW. Hepatocarcinomas, cholangiocarcinomas, and hepatoblastomas produced by chemically transformed cultured rat liver epithelial cells. Am J Pathol 1987; 127:168 81.

[3] Makino R, Hayashi K, Sato S, Sugimura T. Expressions of c Ha *ras* and c *myc* genes in rat liver tumors. Biochem Biophys Res Comm 1984; 119(3):1096 102.

[4] Gu JR, Tian PK, Wang X, Huang LH, Pan ZM, Li XZ et al. N *ras gene*−a common oncogene presents in human primary hepatocellular carcinoma, a cell line 7404 derived from human hepatoma and human chronic myelogenous leukemia cell line(K562). Sci. Sin. 1985; 5(B):452 57.

[5] Land H, Parada LF, Weinberg RA. Tumorigenic conversion of primary embyro fibroblasts reqmres at least two cooperating oncogenes. Nature 1983; 304: 596 602.

[6] Huber BE, Heilman CA, Thorgeirsson SS. Gene expression in the progressive development of hepatocellular carcinoma in the rat. Proc Am Assoc Cancer Res 1986:27:7.

[7] Beer D, Coloma J, Schwarz M, Sawada N,Pitot H. Protooncogene expression in isolated gamma glutamyl transpeptidase positive hepatocytes. Proc Am Assoc Cancer Res 1986;27:79.

[8] Siaha S, Neal GE, Legg RF, Watson JV, Pearson C. The expression of c *myc* related to the proliferation and transformatiop of rat liver derived epithelial cells. Br J Cancer 1989;59:674 6.

[9] Sinha S, Marshall CJ, Neal GE. Ɣ Glutamyl transpeptidase and the *ras* induced transformation of a rat liver cell line. Cancer Res 1986; 46:1440 5.

[10] Houck KA, Michalopoulos GK, Strom SC. Introduction of a Ha *ras* oncogene into rat liver epithelial cells and parenchymal hepatocytes confers resistence to the growth inhibitory effects of TGF β, Oncogene 1989 ; 4 : 19 25.

[11] Huber BE, Cordingley MG. Expression and phenotypic alterations caused by an inducible transforming *ras* oncogene introduced into rat liver epithelial cells. Oncogene 1988; 3:245 56.

[12] Mckenna WG, Nakahara K, Musehel RJ. Site specific integration of Ha *ras* in transformed rat embryo cell-

s. Science 1988;241:1325 28.

[13] Levan G. Nomenclature of G bands in rat chromosomes. Hereditas 1974;77:37 52.

[14] Granner DK, Tomkins GM. 80. Tyrosine Aminotransferase (Rat Liver). In :Methods in Enzymology 1970; 17 (A): 633 37.

[15] McMahon JB, Richards WL, AA. del Campo, Song MH, Thorgeirsson SS. Differential effects of transforming growth factor β on proliferation of normal and malignant rat liver epithelial cells in culture. Cancer Res 1986; 46:4665 71.

[16] Southern PJ Berg P. Transformation of mammalian cells to antibiotics resistence with a bacterial gene under control of the SV40 early region promoter. J Mol Appl Genetics 1982; 1: 327 41.

[17] Shih C, Weinberg RA. Isolation of a transforming sequence from a human bladder carcinoma cell line. Cell 1982; 29:161 9..

[18] Sambrook J, Fritsch EF, Maniatis T. Small scale preparation of plasmid DNA. In:Molecular Cloning V. 1 : 1.25.

[19] Sambrook J, Fritsch EF, Maniatis T. Large scale preparation of plasmid DNA. In:Molecular Cloning V. 1 : 1.38.

[20] Sambrook J, Fritsch EF, Maniatis T. Purification of closed circular DNA by equilibrium centrifugation in CsCl Ethidium bromide gradients. In:Molecular Cloning V. 1 : 1.42.

[21] Chandler ME, Yunis JJ. A high resolution in situ hybridization technique for the direct visualization of labeled G banded early metaphase and prophase chromosomes. Cytogenet Cell Genet 1978; 22:352 56.

[22] Committee for a standardized karyotype of Rattus norvegicus. Standard karyotype of the Norway rat, Rattus norvegicus. Cytogenet Cell Genet 1973;12:199 205

[23] Williams GM. Weisburger EK, Weisburger JH. Isolation and long term cell culture of epithelial like cells from rat liver. ExpCellRes 1971;69:106 12.

[24] Herrmann BG, Frischauf A M. Isolation of genomic DNA. In:Methods in Enzymology 1987; V152.15:180 83.

[25] Strohman RC, Moss PS, Micou Eastwood J, Spector D. Messenger RNA for myosin polypeptidases: Isolation from single myogenic cell cultures. Cell 1977;10:265 73.

[26] Sambrook J, Fritsch EF, Maniatis T. Analysis of Genomic DNA by Southern hybridization. In: Molecular Cloning. V. 2:9.31.

[27] Sambrook J, Fritsch EF, Maniatis T. Analysis of RNA. In:Molecular Cloning. V. 1:7.37.

[28] Bruni C, Porter KR. The fine structure of the parenchymal cell of the normal rat liver. Am J Pathol 1965; 46: 691 756.

[29] Braun L, Goyette M, Yeswen P, Thompson NL, Fausto N. Growth in culture and tumorigenicity after transfection with the *ras* oncogene of liver epithelial cells from carcinogen treated rats. Cancer Res 1987; 47:4116 24.

[30] Braun L, Mikumo R, Fausto N. Production of hepatocellular carcinoma by oval cells: cell cycle expression of c *myc* and *p*53 at different stages of oval cell transformation. Cancer Res 1989;49:1554 61.

[31] Goyette M, Faris R, Braun L, Hixson D, Fausto N. Expression of hepatocyte and oval cell antigens in hepatocellular carcinoma produced by oncogene transfected liver epithelial cells. Cancer Res 1990;50:4809 17.

[32] Tsao MS, Smith JD, Nelson KG, Grisham JW. A diploid epithelial cell line from normal adult rat liver with phenotypic properties of 'oval' cells. Exp Cell Res 1984; 154:38 52.

[33] Ellis RW, Defeo D, Maryak JM, Young HA, Shih TY, Chang EH et al. Dual evolutionary origin for the rat genetic sequences of Harvey Murine Sarcoma Virus. J Virol 1980;36:408 20.