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Enhancement of colony forming ability in the lung by transfer of the v-fos oncogene into a ras-transformed rat 3Y1 cell line

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Transfer of the v-fos oncogene into a rat 3Y1 cell line transformed by v-H-ras, which is tumorigenic but non-metastatic, enhanced lung metastasis, depending on the amount of fos-related transcripts. Enhancement of the metastatic potential was associated with increases in tumor growth rate i.m. of inoculated cells but not the rate of in vitro cell growth, irrespective of the addition of tissue (e.g. lung) extract to the regular medium. These results suggest that the v-fos oncogene increased the malignancy by altering biological factors of the recipient cells responsible for cell growth and/or survival rate in vivo.

Fos oncogene; Lung metastasis; DNA transfer; Growth; Ras oncogene; Cell line, 3Y1

1. INTRODUCTION

Amplification, structural change or the expression of several oncogenes seems to be related to the metastatic potential of certain tumor cells [1–6]. Transfer of the active c-H-ras modifies the metastatic potential [7–9]. It was also noted that expression of the fos oncogene which encodes nuclear protein [10–12], was increased in highly metastatic cell lines of rat spontaneous mammary carcinoma [13]. We reported that transfer of the v-fos gene into a rat cell line transformed by v-src enhanced the metastatic potential to the lung [14,15]. We have now obtained evidence that transfer of the v-fos oncogene into a rat cell line transformed by v-H-ras also enhanced metastasis in the lung and the growth rate in vivo, with no change in the potential for growth in vitro.

2. MATERIALS AND METHODS

2.1. *Cells and DNA transfection*

A transformed rat fibroblast cell line, HR-3Y1-3, was established by transfection of the cloned v-Ha-ras gene, Ha-MuSV L clone [16], into a normal rat fibroblast cell line, 3Y1-B clone 1-6 [17]. The cloned v-fos gene, pFBJ-2 (10 μg/dish) [18], was co-transfected with pSV2-neo (1 μg/dish) [19] into HR-3Y1-3 (1–3 $\times$ 10^5 cells/dish) by calcium phosphate precipitation, as described [14,15]. Independent colonies resistant to 400 μg/ml Geneticin (G418, Gibco), one colony from one dish, were isolated and designated as fos-HR-3Y1-301 to 309. Fos-HR-3Y1-308a and 308b were isolated from one dish. As the control, pBR322 and pSV2-neo were cotransfected into HR-3Y1-3 cells. Mixed populations of the combined colonies (about 200 colonies) resistant to G418 were designated as fos-HR-3Y1-300 or neo-HR-3Y1-300, respectively. All the cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

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2.2. *Southern blot analysis and Norther blot analysis*

Southern blot analysis was performed, as described [14,15]. The blot was autoradiographed using Kodak XAR-5 film at −80°C for 48 h. Northern blot analysis was performed, as described [14,15]. RNA prepared by the guanidinium/cesium method was electrophoresed through 1.2% agarose/1.8 M formaldehyde gel and blotted to nitrocellulose.

2.3. *Experimental lung metastasis in rats*

To assess the extent of experimental metastasis, 1 $\times$ 10^6 of viable cells were injected into the tail vein of syngeneic female F344 rats and these rats were killed when moribund (20–35 days after inoculation). The lungs were removed, fixed with Bouin's solution and examined for the presence of metastases. Several metastatic lung nodules were explanted in vitro and cultured in DMEM medium containing 10% FBS and G418 (400 μg/ml). These cell lines were designated by adding -F1 to the parent cell lines.

2.4. *Tumor development*

The size of the tumor formed by the i.m. inoculated cells (1 $\times$ 10^6) into the thigh of F344 rats was monitored until the rats died.

2.5. *In vitro growth rate*

Cells were seeded at 1 $\times$ 10^5 in a ∅ 60-mm dish and cultured in DMEM with 10% FBS, then were harvested with trypsin and counted every 24 h for 5 days.

2.6. *Effect of lung extract on in vitro growth*

Preparation of lung extract and assay of the growth stimulating activity were done according to Yamori et al. [20]. Briefly, cells were suspended in DMEM containing 1% fetal bovine serum and plated on a ∅ 60-mm dish at a concentration of 5 $\times$ 10^4 cells. After incubation for 24 h at 37°C, the attached cells were counted (time 0 count) and fed DMEM containing 10 mM Hepes buffer and 100 μg of pro-

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tein of the lung extract per ml prepared from fresh lungs of normal F344 rats. Growth index was defined by dividing the cell counts at each time point by time 0 counts.

3. RESULTS

3.1. *Southern blot analysis*

Fig.1 shows Southern blot analysis of the genomic DNA from the transfectants, digested with *Sma*I. All the cell lines had a common 13 kb DNA fragment which contained the cellular fos (c-fos) gene. All of the v-fos transfected cell lines had an additional 4.0 kb fragment containing the entire coding region of the v-fos originated from the transfected pFBJ-2 DNA.

In some transfectants, various additional hybridizing fragments were evident, showing random integration of the v-fos DNA upon transfection. Second generations of fos-transferred cells established from lung colonies (fig.1, below) retained the exogenous fos DNA.

3.2. *Northern blot analysis*

Fig.2 shows Northern blot analysis of the RNA hybridized with the fos-specific probe. To compare the amount of fos-related transcripts, the filters were washed and rehybridized with an actin probe [21] which detects a 2.1-kb actin-specific transcript, confirmed in advance not to differ in expression among the cell lines. Two sizes of the fos-related transcripts (3.4 kb and 5.6 kb) were seen in some of the transfectants (fos-HR-3Y1-301, 307, 308a, 308b). Other fos-transfected cell lines and control cell lines (neo-HR-3Y1-300, HR-3Y1-3, 3Y1-1-6) expressed little fos-related transcripts. Second generations, such as fos-

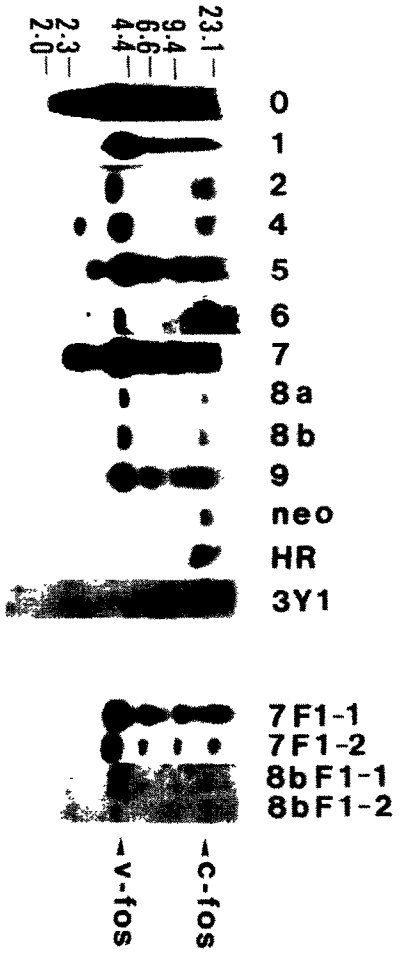

Fig.1. Southern blot analysis of the presence of the v-fos DNA sequences in *Sma*I-digested DNA (20 μg) from pFBJ-2-transfected HR-3Y1-3 cells. Hybridization was performed with the ^{32}P-labeled fos-specific *Pst*I DNA fragment (1 kb) of p-fos-1 [18]. Lanes 0–9, fos-HR-3Y1-300 to fos-HR-3Y1-309; lane neo, neo-HR-3Y1-300; lane HR, HR-3Y1-3; lane 3Y1, 3Y1-B clone 1–6; lanes 7F1-1 to 8bF1-2, fos-HR-3Y1-307F1-1 to fos-HR-3Y1-308bF1-2. Arrows show 13 kb c-fos and 4.0 kb v-fos DNA fragments.

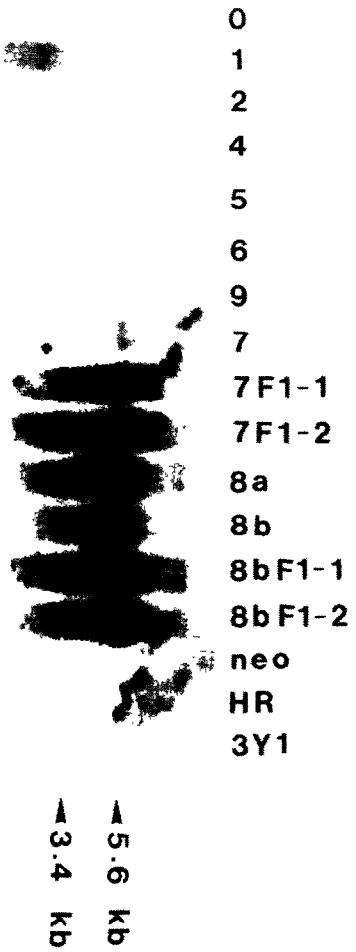

Fig.2. Northern blot analysis showing the fos-related transcript in v-fos-transferred HR-3Y1-3 cells. RNA on the filters was hybridized with the same probe used for Southern blot analysis and after that with an actin probe showing 2.1 kb actin [21] as an internal quantitative control for the amounts of RNA on the filters. The relative level of fos-related transcripts was calculated by dividing the density of fos bands by that of actin bands on the autoradiographed X-ray films. The values are listed in table 1. The density was measured using a Video Densitometer (Bio-Rad).

Table 1

Presence and expression of the v-fos gene, and experimental lung metastasis of fos-transfected HR-3Y1-3 cells

Cell line	Experiment[a]	4.0 kb of fos DNA[b]	Relative level of fos expression[c] (RNA)	Lung metastasis	
				No. of lung nodules[d]	Incidence[e]
fos-HR-3Y1-307	A	+	3.5	153.2 ± 54.8	6/6
fos-HR-3Y1-308b	A	+	6.6	182.7 ± 11.1	7/7
fos-HR-3Y1-309	A	+	0	0	0/6
neo-HR-3Y1-300	A	−	0	0	0/6
HR-3Y1-3	A	−	0	0	0/6
fos-HR-3Y1-301	B	+	8.8	269.0 ± 30.5	6/6
fos-HR-3Y1-302	B	+	0	0.5 ± 0.3	1/6
fos-HR-3Y1-305	B	+	0	4.4 ± 1.9	5/5
fos-HR-3Y1-308a	B	+	10.3	214.9 ± 45.7	6/6
fos-HR-3Y1-304	C	+	0	0.8 ± 0.2	1/6
fos-HR-3Y1-306	C	+	0	0.2 ± 0.2	1/6
fos-HR-3Y1-307F1-1	C	+	21.9	334.2 ± 40.8	6/6
fos-HR-3Y1-307F1-2	C	+	16.0	136.2 ± 10.4	5/6
fos-HR-3Y1-308bF1-1	D	+	6.4	361.0 ± 30.3	6/6
fos-HR-3Y1-308bF1-2	D	+	6.4	233.4 ± 40.3	6/6

[a] Rats were examined 33 days in experiment A, 35 days in experiment B, 22 days in experiment C, and 20 days in experiment D after i.v. inoculation
[b] All the cell lines have a common 13 kb of c-fos DNA
[c] The level was determined by dividing the density of fos transcripts by that of the actin band
[d] Mean ± SD
[e] Numbers of rats bearing lung metastasis/total rats

HR-3Y1-307F1-1 and 308bF1-2, also expressed fos-related transcripts.

3.3. *Experimental lung metastasis*

As shown in table 1, no colony was seen in the lung of the rats inoculated with HR-3Y1-3 or neo-HR-3Y1-300, in which the fos-related transcript was not evident. Lung colonies were few in fos-transferred cell lines not expressing detectable fos transcripts. These cell lines produced no pulmonary metastases, either macroscopically or microscopically, even 60 days after i.v. inoculation. On the other hand, the cell lines (fos-HR-3Y1-307, 308b, 301, 308a) expressing fos-related transcripts, produced a number of lung colonies. The cell lines of second generation (fos-HR-3Y1-307F1-1 through 308bF1-2) also expressed a large amount of fos-related transcripts and the metastatic potential to the lung was high.

3.4. *Tumor development*

Growth rate of the tumors produced by cell lines with relatively high levels of fos expression (fos-HR-3Y1-307 in fig.3A,B and 308a, 307F1-1 in fig.3B), was larger than that of cell lines with little expression of fos-related transcript (fos-HR-3Y1-309 in fig.3A and fos-HR-3Y1-306 in fig.3B), or of the control cell line (neo-HR-3Y1-300 in fig.3A).

3.5. *In vitro growth*

As shown in fig.4A, in vitro growth rate and saturation density of the cells were much the same among the cell lines examined and did not correlate with the relative level of fos expression.

Fig.4B shows the effect of lung extract on in vitro cell growth. The growth rate of the highly metastatic cell line, fos-HR-3Y1-308a did not surpass that of low metastatic cell lines, fos-HR-3Y1-309, or neo-HR-3Y1-300.

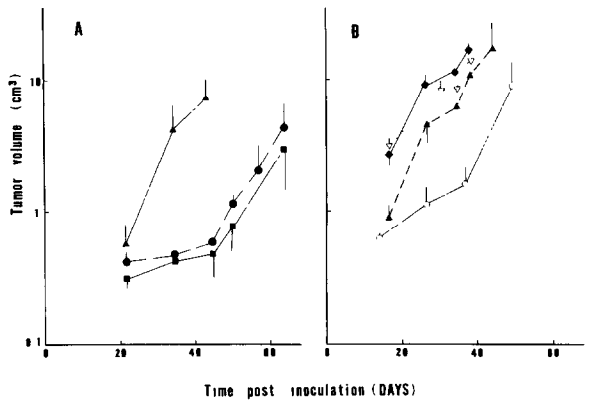

Fig.3. Change in size of tumors formed by inoculation of v-fos-transferred HR-3Y1-3 cells. Two experiments are shown (A,B). Tumor size was measured using a caliper after i.m. inoculation into the thigh of five F344 rats. The growth curve represents the mean of tumor volume calculated by $ab^2/2$ (a, long axis; b, short axis). Bars show SD. (▲) fos-HR-3Y1-307; (●) fos-HR-3Y1-309; (■) neo-HR-3Y1-300; (◆) fos-HR-3Y1-308a; (▽) fos-HR-3Y1-307F1-1; (○) fos-HR-3Y1-306.

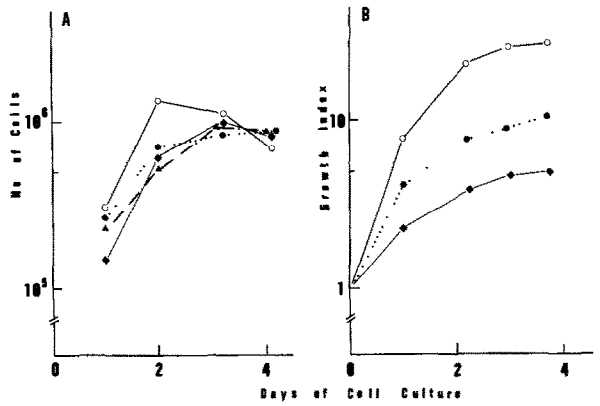

Fig.4. (A) In vitro growth rate of the v-fos and/or neo-transferred HR-3Y1-3 cells. 1×10^5 cells were seeded in triplicate on 60 mm culture dishes and cultured in DMEM containing 10% FBS. Numbers of cells were counted every 24 h for 5 days. (▲) fos-HR-3Y1-307; (◆) fos-HR-3Y1-308a; (●) fos-HR-3Y1-309; (○) neo-HR-3Y1-300. (B) Effect of rat lung extract on the growth of fos-transferred cells. Growth medium was replaced at time 0 with serum-free DMEM containing 100 μg protein of lung extract per ml. Growth index was determined as described in section 2. The symbols are the same as those in (A).

4. DISCUSSION

Transfer of the v-fos oncogene into a ras-transformed rat cell line (HR-3Y1-3) enhanced colony-forming ability in the lung (table 1), depending on the amount of fos-related transcripts. Although other investigators suggested that transfer of the active c-H-ras modified the metastatic potential [7–9], our cell line transformed by v-H-ras (HR-3Y1-3) produced no metastatic nodules in the lung. Furthermore, no remarkable alteration in the expression of v- or c-H-ras was seen among the fos-transferred cell lines and the control cell line (data not shown).

Highly metastatic transfectants with a high level of fos expression showed a higher growth rate after i.m. inoculation than did cell lines with little fos expression (fig.3), but not correlating with the growth rate in vitro. Difference in metastatic potential of certain cell lines has been explained by response of the cells to tissue-originated growth factors. Yamori et al. [20] reported that the addition of lung extract to culture medium selectively stimulated the growth of a highly metastatic clone of mouse colon tumor cells. This was, however, not the case in our experiments, as growth rate of the highly metastatic cell line (fos-HR-3Y1-308a) did not surpass that of the low metastatic cell line (fos-HR-3Y1-309) or the control cell line (neo-HR-3Y1-300) when lung extract was added to the culture medium (fig.4B). Similar results were obtained by adding muscle extract, prepared from thighs of syngeneic F344 rats (unpublished data). In preliminary experiments, fos-transferred highly metastatic cell lines tended to be more resistant to splenic effectors than did the controls. Thus, increases in lung metastasis and growth rate i.m. of the H-ras-transformed 3Y1 cell line by fos transfer may be attributed to the augmentation of cellular resistance to host defence systems. Alternatively, fos transfer might have promoted expression of angiogenesis factors, advantageous for cellular growth in vivo. Determination of biological factors responsible for fos-transferred highly metastatic cell lines are under way.

Fos protein is a nuclear compound and binds with a high affinity to DNA cellulose, in vitro [10,11]. We observed that the nuclei of fos-HR-3Y1-308a, expressing a high level of fos transcripts, were immunostained with antibodies specific for the v-fos protein, while those of neo-HR-3Y1-300 did not (data not shown). Recent evidence suggests that the nucleoprotein complex containing the fos product binds to the AP-1 recognition site of the fat specific element 2 (FSE 2) located upstream of the adipocyte P2 (aP2) gene [12,22]. AP-1, also a nuclear oncoprotein encoded by c-jun [23] and identical to the fos-associated protein p39 [24], is considered to form DNA-binding protein complexes with fos protein that functions as a transcriptional regulator [22]. How fos-associating proteins such as AP-1 are related to the function of the v-fos in our cell lines remains to be clarified.

We reported [14,15] that the transfer of the v-fos oncogene into a rat cell line transformed by v-src (SR-3Y1-2) increased metastasis to the lungs. These cell lines showed both experimental and spontaneous metastatic potentials, while fos-transferred HR-3Y1-3 cell lines produced few spontaneous metastases in the lung. Furthermore, the metastatic potential of the fos-transferred SR-3Y1-2 cell lines correlated with the degree of invasiveness. Therefore, genes responsible for metastatic potential, the expression of which is affected by the v-fos products, appear to depend on the type of recipient cells. Identification of genes regulated by the v-fos product in our cell systems, is the subject of ongoing study.

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REFERENCES

[1] Vousden, K. and Marshal, C.J. (1984) EMBO J. 3, 913–917.
[2] Radinsky, R., Kraemer, P.M., Raines, M.A., Kung, H.J. and Culp, L.A. (1987) Proc. Natl. Acad. Sci. USA 84, 5143–5147.
[3] Schwab, M., Ellison, J., Busch, M., Rosenau, W., Varmus, H.E. and Bishop, J.M. (1984) Proc. Natl. Acad. Sci. USA 81, 4940–4944.
[4] Nau, M.M., Brooks, B.J., Carny, D.N., Gazdar, A.F., Battey, J.F., Sausville, E.A. and Minna, J.D. (1986) Proc. Natl. Acad. Sci. USA 83, 1092–1096.
[5] Little, C.D., Nau, M.M., Carny, D.N., Gazdar, A.F. and Minna, J.D. (1983) Nature 306, 194–196.

[6] Tatosyan, A.G., Galetzki, S.A., Kisseljova, N.P., Asanova, A.A., Zborovskaya, I.B., Spitkovsky, D.D., Revasova, E.S., Martin, P. and Kisseljov, F.L. (1985) Int. J. Cancer 35, 731–736.
[7] Collard, J.G., Schijven, J.F. and Roos, E. (1987) Cancer Res. 47, 754–759.
[8] Garbisa, S., Pozzatti, R., Muschel, R.J., Saffiotti, U., Ballin, M., Goldfarb, R.H., Khoury, G. and Liotta, L.A. (1987) Cancer Res. 47, 1523–1528.
[9] Pozzatti, R., Muschel, R., Williams, J., Padmanabhan, R., Howard, B., Liotta, L. and Khoury, G. (1986) Science 232, 223–227.
[10] Sambucetti, L.C. and Curran, T. (1986) Science 34, 1417–1419.
[11] Renz, M., Verrier, B., Kurz, C. and Muller, R. (1987) Nucleic Acids Res. 15, 277–292.
[12] Distel, R.J., Ro, H.S., Rosen, B.S., Groves, D.L. and Spiegelman, B.M. (1987) Cell 49, 835–844.
[13] Yukhi, N., Hamada, J., Kuzumaki, N., Takeichi, N. and Kobayashi, H. (1986) Jpn. J. Cancer Res. 77, 9–12.
[14] Taniguchi, S., Kawano, T., Mitsudomi, T., Kimura, G. and Baba, T. (1986) Jpn. J. Cancer Res. 77, 1193–1197.
[15] Kawano, T., Taniguchi, S., Nakamatsu, K., Sadano, H. and Baba, T. (1987) Biochem. Biophys. Res. Commun. 77, 173–179.
[16] Zaitsu, H., Tanaka, H., Mitsudomi, T., Matsuzaki, A., Ohtsu, M. and Kimura, G. (1988) Biomed. Res. 9, 181–197.
[17] Kimura, G., Itagaki, A. and Summers, J. (1975) Int. J. Cancer 15, 694–706.
[18] Curran, T., Peters, G., Beveren, C.V., Teich, N.M. and Verma, I.M. (1982) J. Virol. 44, 674–682.
[19] Southern, P.J. and Berg, P. (1982) Mol. Appl. Genet. 1, 327–341.
[20] Yamori, T., Iida, H., Tsukagoshi, S. and Tsuruo, T. (1988) Clin. Expl. Metastasis 6, 131–139.
[21] Nakajima-Iijima, S., Hamada, H., Reddy, P. and Kakunaga, T. (1985) Proc. Natl. Acad. Sci. USA 82, 6133–6137.
[22] Rauscher, F.J., iii, Sambucetti, L.C., Curran, T., Distel, R.J. and Spiegelman, B.M. (1988) Cell 52, 471–480.
[23] Varmus, H.E. (1987) Science 238, 1337–1339.
[24] Rauscher, F.J., iii, Cohen, D.R., Curran, T., Bos, T.J., Vogt, P.K., Bohmann, D., Tjian, R. and Franza, B.R., jr (1988) Science 240, 1010–1016.