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### Ⅲ. 研究成果の刊行物・別刷



## Detection of initiating as well as promoting activity of chemicals by a novel cell transformation assay using v-Ha-ras-transfected BALB/c 3T3 cells (Bhas 42 cells)

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### Abstract

Cell transformation assay using BALB/c 3T3 cells, C3H10T1/2 cells and others, can simulate the two-stage carcinogenesis utilized for formation of transformed foci. A sensitive cell transformation assay for tumor initiators as well as promoters has been developed using a v-Ha-ras-transfected BALB/c 3T3 cell line, Bhas 42; these cells are regarded as initiated in the two-stage paradigm of carcinogenesis. To distinguish between initiation and promotion, the initiation assay involves a 2-day treatment of low-density cells, obtained one day after plating, with a test chemical, and the promotion assay involves treatment of near-confluent cells with a test chemical for a period of 12 days (Day 3–14). When Bhas 42 cells were treated with tumor initiators, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine and 3-methylcholanthrene, transformed foci were induced in the initiation assay but not in the promotion assay. In contrast, tumor promoters, 12-*O*-tetradecanoylphorbol-13-acetate, lithocholic acid and okadaic acid, gave negative responses in the initiation assay but positive responses in the promotion assay. The results were reproducible with various treatment protocols. Sixteen polycyclic aromatic hydrocarbons were examined using both assays. Benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene induced focus formation only in the initiation assay. Increase of focus formation was observed in the promotion assay with benzo[*e*]pyrene, benzo[*ghi*]perylene, 1-nitropyrene and pyrene. Benz[*a*]anthracene, benz[*b*]anthracene, chrysene and perylene showed positive responses in both initiation and promotion assays. Results of initiation and promotion assays of acenaphthylene, anthracene, coronene, 9,10-diphenylanthracene, naphthalene and phenanthrene were negative or equivocal. The present Bhas assays for the detection of either/both initiating and promoting activities of chemicals are sensitive and of high performance compared with other cell transformation assays.

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**Keywords:** Transformation; Bhas 42 cells; Complete carcinogen; Initiation and promotion

### 1. Introduction

Chemical carcinogens can be divided into two categories, i.e., initiators and promoters, based on the two-stage model of carcinogenesis [1,2]. Most initiators can be detected by various genotoxicity tests, the results

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of which are used for carcinogenicity prediction and for regulatory purposes by authorities [3,4]. In the case of tumor promoters, several methods have been proposed [5], but none of them have been routinely used for regulatory purposes. Therefore, to develop a method for detection of non-genotoxic carcinogens with various mechanisms of action is a major challenge for the safety evaluation of chemicals [5,6]. The utilization of additional screening tests covering a wide range of carcinogenic processes has advantage before contemplating *in vivo* long-term carcinogenicity experiments for chemical safety assessment.

The cell transformation assays using BALB/c 3T3 cells [7,8] and C3H10T1/2 cells [9,10] can simulate the process of two-stage animal carcinogenesis [11,12]. Formation of transformed foci is the consequence of the complex process of transforming cells to a malignant state. Since these assays can detect both initiating and promoting activities [13,14], their inclusion as screening tools is anticipated to be useful for detection of not only tumor initiators but also tumor promoters such as non-genotoxic carcinogens. In spite of this expectation, cell transformation assays have not been accepted as a routine screening method, because of the laborious and time-consuming procedure compared with the routine genotoxicity assays [15].

We have developed a sensitive cell transformation assay for detecting tumor promoters using Bhas 42 cells [16] that was established by Sasaki et al. [17]. The cells, v-Ha-*ras*-transfected BALB/c 3T3 cells, are considered as initiated in the two-stage transformation paradigm [18]. The assay method has many advantages, e.g., high sensitivity, short experimental period, use of smaller amounts of materials, and simplicity of the procedure.

After establishing the promotion assay using Bhas cells, various chemicals including tumor initiators were examined for their potency of tumor-promoting activity. Some initiators are known to have tumor promotion capacity in animal experiments (complete carcinogens). However, *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and 3-methylcholanthrene (MCA), as examples of initiators, did not show clear positive response in our preliminary study. In the promotion assay, Bhas cells were treated with these chemicals when the cells were near confluence. Since initiators need several cell divisions for fixation of gene mutation [19–21], we designed a new treatment protocol in which cells were seeded at a lower density and allowed to divide several times in culture dishes after treatment with test chemicals.

In the present work, tumor initiators and promoters were examined in the newly developed protocol with various treatment schedules in order to define the most

effective protocol for detection of initiators and promoters. The effect of repeated treatment with MCA at initiating and/or promoting period with several time Schedules was compared with that of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as a model promoter. In addition, several polycyclic aromatic hydrocarbons (PAHs) were tested with the novel protocol to validate the assay.

## 2. Materials and methods

### 2.1. Cell cultures

Minimum essential medium (MEM) was obtained from Nissui Pharmaceutical, Tokyo, Japan. Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12) was purchased from GIBCO Laboratories, Grand Island, NY. Fetal bovine serum (FBS) was obtained from Moregate, Bulimba, Australia.

Bhas 42 cells were routinely cultured in MEM supplemented with 10% FBS (M10F) in a humidified 5% CO<sub>2</sub> incubator at 37 °C. The cells were subcultured using 0.25% trypsin (GIBCO) before reaching confluence. For transformation assays, Bhas 42 cells were cultured in DMEM/F12 supplemented with 5% FBS (DF5F).

### 2.2. Chemicals

Acenaphthylene, benzo[*a*]pyrene (B[*a*]P), benzo[*ghi*]perylene (B[*ghi*]P), coronene, 7,12-dimethylbenz[*a*]anthracene (DMBA), lithocholic acid (LCA), MCA, naphthalene, okadaic acid, perylene, phenanthrene and pyrene were obtained from Wako Pure Chemical Industries (Osaka, Japan). Anthracene, benz[*a*]anthracene (B[*a*]A), benz[*b*]anthracene (B[*b*]A) and MNNG were obtained from Kanto Chemical (Tokyo, Japan). Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), benzo[*e*]pyrene (B[*e*]P) and TPA were obtained from Sigma Chemical Co. (St. Louis, MO). Chrysene was obtained from Avocado Research Chemicals (Lancashire, UK), 1-nitropyrene (1-NP) from Aldrich (St. Louis, MO), and 9,10-diphenylanthracene (9,10-DPhA) from Merck (Rahway, NJ). These chemicals except for coronene were dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries); coronene was suspended in 0.5% carboxymethylcellulose sodium salt solution (CMC-Na; Wako Pure Chemical Industries). Final concentrations of these solvents in experimental medium were adjusted to less than 0.1% in the case of DMSO or less than 1% in the case of CMC-Na.

### 2.3. Cell growth assay

Cell growth assays, using the standard crystal violet absorption method [22], were applied to dose range finding for initiation and promotion assays. Additionally, the cell growth assay was performed concurrently with every transformation assay. In the case of initiation assay, cell numbers were adjusted to  $2 \times 10^3$  cells/mL in M10F and the cell suspension was seeded onto 24-well microplates at 0.5 mL per well (Day 0). Three wells were prepared for each test concentration. After a 24 h

Table 1  
Results of initiation and promotion assays for initiators and promoters

Chemical	Concentration	S9 mix	Initiation assay		Promotion assay	
			Number of foci/well	Cell viability	Number of foci/well	Cell viability
MNNG	0 µg/mL	–	5.3 ± 1.8	100.0	1.5 ± 0.5	100.0
	0.002 µg/mL	–	N.E.		1.5 ± 1.2	96.4
	0.005 µg/mL	–	N.E.		1.2 ± 1.0	96.1
	0.01 µg/mL	–	N.E.		1.3 ± 1.5	95.8
	0.02 µg/mL	–	4.0 ± 1.8	90.6	1.2 ± 0.4	98.4
	0.05 µg/mL	–	5.8 ± 2.9	90.8	1.0 ± 1.3	97.4
	0.1 µg/mL	–	5.5 ± 1.9	88.7	1.7 ± 1.2	93.1
	0.2 µg/mL	–	6.7 ± 2.9	62.1		tox.
	0.5 µg/mL	–	10.0 ± 2.6	69.6		tox.
	1 µg/mL	–	12.0 ± 1.5	57.0		tox.
MCA	0 µg/mL	–	3.0 ± 1.1	100.0	1.5 ± 0.5	100.0
	0.002 µg/mL	–	N.E.		1.0 ± 0.9	93.6
	0.005 µg/mL	–	N.E.		1.3 ± 0.5	89.7
	0.01 µg/mL	–	N.E.		1.2 ± 0.4	85.5
	0.02 µg/mL	–	2.7 ± 2.0	61.6	1.8 ± 0.8	88.7
	0.05 µg/mL	–	3.7 ± 2.0	65.6	1.5 ± 1.0	84.7
	0.1 µg/mL	–	6.2 ± 2.6	62.7	2.0 ± 1.3	69.2
	0.2 µg/mL	–	8.2 ± 1.9	79.6		tox.
	0.5 µg/mL	–	9.7 ± 2.1	45.2		tox.
	1 µg/mL	–	11.8 ± 4.0	35.8		tox.
AFB1	0 µg/mL	–	3.0 ± 1.1	100.0	1.5 ± 0.5	100.0
	0.02 µg/mL	–	N.E.		1.5 ± 0.8	95.9
	0.05 µg/mL	–	2.5 ± 1.5	64.2	0.7 ± 1.2	91.5
	0.1 µg/mL	–	1.8 ± 1.2	48.8	0.7 ± 0.5	85.2
	0.2 µg/mL	–	3.0 ± 1.7	49.3	0.5 ± 0.8	87.4
	0.5 µg/mL	–	3.5 ± 1.5	44.7	0.7 ± 0.5	86.0
	1 µg/mL	–	2.5 ± 0.5	24.5	1.3 ± 0.8	73.4
	2 µg/mL	–	0.8 ± 1.0	12.5		tox.
AFB1	0 µg/mL	+	3.2 ± 1.5	N.E.	N.E.	
	0.05 µg/mL	+	3.7 ± 1.6	N.E.	N.E.	
	0.1 µg/mL	+	4.2 ± 1.5	N.E.	N.E.	
	0.2 µg/mL	+	4.8 ± 3.3	N.E.	N.E.	
	0.5 µg/mL	+	4.7 ± 1.8	N.E.	N.E.	
	1 µg/mL	+	14.3 ± 2.4	N.E.	N.E.	
	2 µg/mL	+	11.7 ± 2.8	N.E.	N.E.	
TPA	0 ng/mL	–	4.3 ± 1.0	100.0	2.8 ± 0.4	100.0
	2 ng/mL	–	2.3 ± 0.5	97.5	9.0 ± 1.3	100.0
	5 ng/mL	–	2.7 ± 1.2	92.8	12.7 ± 1.0	92.7
	10 ng/mL	–	2.5 ± 1.0	93.8	17.2 ± 2.2	95.9
	20 ng/mL	–	1.3 ± 0.8	97.6	26.0 ± 3.0	105.5
	50 ng/mL	–	2.2 ± 0.8	96.8	28.7 ± 3.3	111.1
	100 ng/mL	–	0.8 ± 1.3	90.0	27.3 ± 2.9	112.2
	LCA	0 µg/mL	–	3.0 ± 1.1	100.0	4.2 ± 0.8
0.5 µg/mL		–	2.5 ± 2.2	91.2	6.5 ± 1.9	99.3
1 µg/mL		–	3.2 ± 1.6	84.1	7.8 ± 1.0	94.7
2 µg/mL		–	2.2 ± 1.8	89.4	7.2 ± 2.2	94.0
5 µg/mL		–	2.0 ± 1.3	125.7	11.7 ± 1.4	101.1
10 µg/mL		–	2.8 ± 1.2	117.7	16.5 ± 1.0	91.0
20 µg/mL		–	3.8 ± 1.9	109.7	31.8 ± 3.1	79.7
Okadaic acid		0 ng/mL	–	4.3 ± 1.4	100.0	4.2 ± 0.8
	0.2 ng/mL	–	2.0 ± 1.1	87.5	N.E.	
	0.5 ng/mL	–	3.0 ± 0.6	96.0	N.E.	
	1 ng/mL	–	3.3 ± 2.2	105.5	4.8 ± 2.5	97.1

Table 1 (Continued)

Chemical	Concentration	S9 mix	Initiation assay		Promotion assay	
			Number of foci/well	Cell viability	Number of foci/well	Cell viability
	2 ng/mL	–	3.5 ± 1.2	114.5	5.8 ± 1.6	90.1
	4 ng/mL	–	N.E.		4.3 ± 1.9	70.7
	5 ng/mL	–	3.0 ± 1.4	89.0	N.E.	
	6 ng/mL	–	N.E.		7.0 ± 3.1	56.8
	8 ng/mL	–	N.E.		7.7 ± 1.5	40.3
	10 ng/mL	–	3.5 ± 2.4	98.0	12.5 ± 1.4	37.0

N.E., not experimented; tox.: toxic.

cultivation, cells were treated with a fresh medium containing a test chemical. The cells were fixed with a 10% formalin on Day 3 and stained with a 0.1% crystal violet (CV) solution. CV was extracted from stained cells in each well with 0.5 mL of a solution containing 0.02 mol/L hydrochloric acid in 50% ethanol. Optical density of CV extracted from stained cells was measured at 540 nm, and the results were expressed as percentage of absorbance compared to the solvent control culture.

In the growth testing for the promotion assay, cell numbers were adjusted to  $2 \times 10^4$  cells/mL in DF5F and seeded onto 24-well microplates at 0.5 mL per well. After a 3-day cultivation, medium was replaced with the one containing a test chemical. Cells were then fixed and stained on Day 7, and optical density of CV extracted from stained cells was measured as above.

#### 2.4. Transformation assay for initiating activity (Bhas initiation assay)

The procedure for the initiation assay using BALB/c 3T3 cells [23] was adopted for the present Bhas initiation assay. Cell numbers were adjusted to  $2 \times 10^3$  cells/mL in M10F and the cells were seeded onto each well of six-well microplates in 2 mL amounts (Day 0). Six wells were prepared for each test concentration. After a 24 h cultivation, cells were treated with a fresh medium containing a test chemical. Culture medium was replaced with DF5F without test chemical on Day 3; thereafter, culture medium was changed twice a week with fresh DF5F without test chemical. On Day 24, the cells were fixed with methanol and stained with a 5% Giemsa solution. Transformed foci were judged from morphological characteristics: deep basophilicity, dense multilayering of cells, and random orientation of cells at the edge of foci.

#### 2.5. Transformation assay for promoting activity (Bhas promotion assay)

Bhas promotion assay was conducted according to the procedure reported by Ohmori et al. [16]. Cell numbers were adjusted to  $2 \times 10^4$  cells/mL in DF5F and seeded onto each well of six-well microplates in 2 mL amounts (six wells

per test concentration) (Day 0). Medium was replaced with fresh medium containing a test chemical on Day 3, Day 7 and Day 10, and then with fresh medium without the test chemical on Day 14. On Day 21, the cells were fixed with methanol and stained with a 5% Giemsa solution for focus counting.

#### 2.6. Metabolic activation in initiation assay

In the case of metabolic activation of AFB<sub>1</sub>, cultures of Bhas 42 cells were started under the same conditions as those of the initiation assay. Three wells were prepared for each test concentration. After a 24 h incubation, cells were treated with AFB<sub>1</sub> together with S9 mix which contained 5% rat S9 (Kikkoman, Chiba, Japan), 2 mM HEPES (Sigma Chemical Co.), 5 mM MgCl<sub>2</sub>, 33 mM KCl, 5 mM glucose-6-phosphatase (Sigma Chemical Co.) and 4 mM β-NADP<sup>+</sup> (Oriental Yeast, Osaka, Japan). After a 3 h treatment, all plates were washed with phosphate-buffered saline and provided fresh M10F. The culturing was continued until Day 24 and the cells were fixed and stained for focus counting.

#### 2.7. Transformation assay with various schedule of treatments

Cells were seeded onto six-well microplates as with the initiation assay. MCA (100 ng/mL) or TPA (50 ng/mL) was added at various time schedules during cell-growth phase (initiation stage) and/or stationary phase (promotion stage). Three wells were prepared for each set of conditions. Cells were cultivated until Day 24, and then fixed and stained for focus counting.

#### 2.8. Statistical analysis and criteria of judgment

Results of initiation and promotion assays were evaluated as follows. After *t*-test analysis, chemicals showing significant increase ( $p < 0.05$ ) of focus number at more than two consecutive concentrations were judged positive. Chemicals showing statistically significant effect at only one concentration were considered equivocal. Negatives were those which induced no statistically significant increase of transformed foci at any concentrations tested.

### 3. Results

#### 3.1. Establishment of initiation assay

Preliminary examination with MNNG and MCA in the Bhas promotion assay revealed little response in the formation of transformed foci. Then, an experimental

procedure was designed in which Bhas 42 cells were seeded at one-tenth lower cell density ( $2 \times 10^3$  cells/mL) than in the promotion assay ( $2 \times 10^4$  cells/mL), a procedure that will permit cells to divide several times after the treatment with test chemicals. With the protocol described in Section 2.4, MNNG and MCA induced a significant number of foci (Fig. 1A and C).

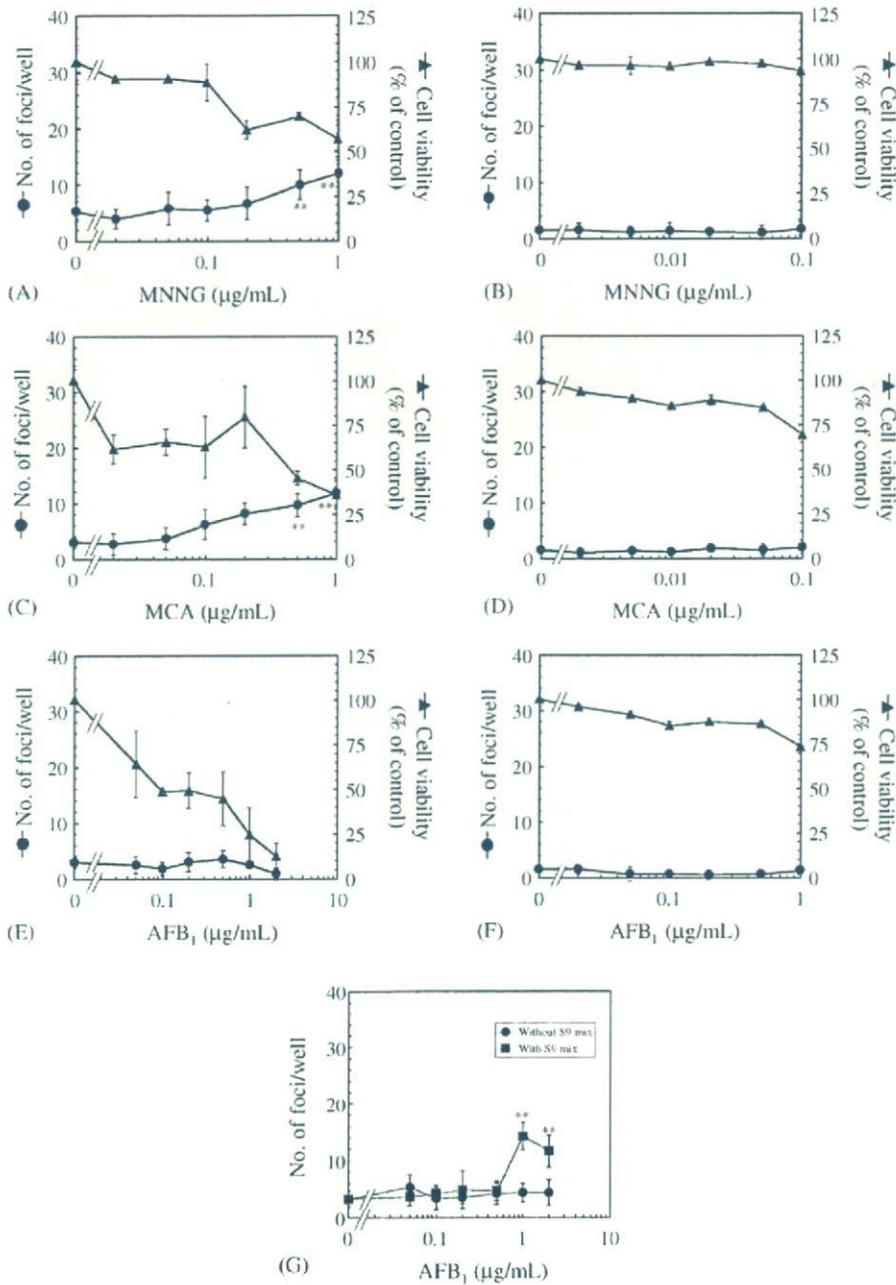


Fig. 1. Results of transformation assays of initiators. A, C and E show results of initiation assay and B, D and F show results of promotion assay. G shows result of metabolic activation in AFB<sub>1</sub> initiation assay. \* $p < 0.05$ , compared with solvent control. \*\* $p < 0.01$ , compared with solvent control.

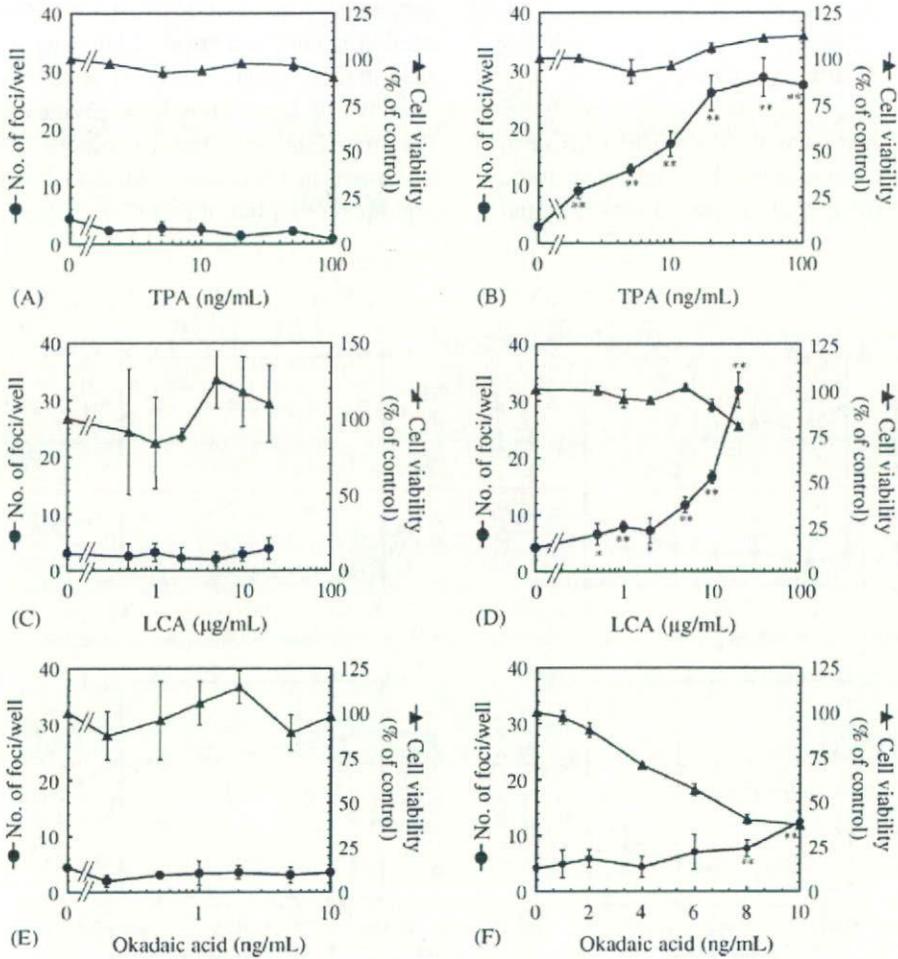


Fig. 2. Results of transformation assays of promoters. A, C and E show results of initiation assay and B, D and F show results of promotion assay. \**p* < 0.05, compared with solvent control. \*\**p* < 0.01, compared with solvent control.

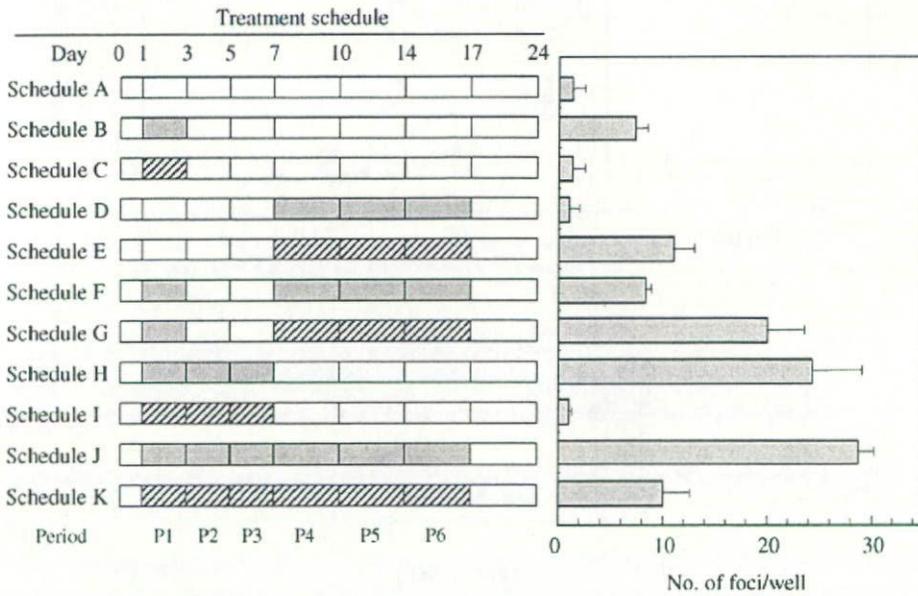


Fig. 3. Result of transformation assay under various time schedules. In treatment schedule, solid blocks represent treatment with 100 ng/mL MCA and diagonal blocks represent treatment with 50 ng/mL TPA.

### 3.2. Transformation assays of initiators

The results are summarized in Table 1. Fig. 1A and B shows the results of Bhas initiation and promotion assays of MNNG, respectively. Significant increase of focus formation was observed in the initiation assay at the concentrations of 0.5  $\mu\text{g}/\text{mL}$  and above. As mentioned above, MNNG did not induce transformed foci in the promotion assay up to 0.1  $\mu\text{g}/\text{mL}$ . With concentrations over 0.2  $\mu\text{g}/\text{mL}$  in the promotion assay, cell flattening with enlarged cell size and without increased cell number was observed after triple dosing with concentrations over 0.2  $\mu\text{g}/\text{mL}$  in the promotion assay.

Results of the initiation and promotion assays for MCA are shown in Fig. 1C and D. MCA showed a response similar to that with MNNG. MCA induced transformed foci in the initiation assay at concentrations of 0.5  $\mu\text{g}/\text{mL}$  and higher. In the case of the promotion assay, no focus formation was observed at concentrations up to 0.1  $\mu\text{g}/\text{mL}$  and severe cytotoxicity was observed at concentrations over 0.2  $\mu\text{g}/\text{mL}$ .

AFB<sub>1</sub> showed negative results in both initiation and promotion assays (Fig. 1E and F). In the case of promotion assay, 1  $\mu\text{g}/\text{mL}$  of AFB<sub>1</sub> was the maximum concentration tested because of its cytotoxicity. Metabolic activation of AFB<sub>1</sub> by cytochrome P450 (CYP) 2A6, 3A4 and 1A2 is necessary to exert mutagenic and carcinogenic activities [24,25]. Fig. 1G shows results of the initiation assay of AFB<sub>1</sub> metabolically activated by S9

mix, and there was significant foci induction at 1 and 2  $\mu\text{g}/\text{mL}$ .

### 3.3. Transformation assays of tumor promoters

Initiation and promotion activities of three tumor promoters were also examined and the results are summarized in Table 1. Fig. 2A and B shows results on TPA. No increase of focus number was observed in the initiation assay up to 100 ng/mL (Fig. 2A). In contrast, significant increase of focus formation was observed in the promotion assay at all concentrations tested up to 100 ng/mL (Fig. 2B).

Fig. 2C and D shows results on LCA. As in the case of TPA, transformed foci were not induced in the initiation assay at all concentrations tested (Fig. 2C), whereas transformed foci were observed in the promotion assay at all concentrations tested (Fig. 2D).

Okadaic acid, up to 10 ng/mL, did not induce transformed foci (Fig. 2E) in the initiation assay. A significant increase of focus formation was observed in the promotion assay at 8 and 10 ng/mL at which high cytotoxicity was shown (Fig. 2F).

### 3.4. Transformation assays with various schedules of treatment

Fig. 3 shows results of treatment with MCA (100 ng/mL) and/or TPA (50 ng/mL) under various treat-

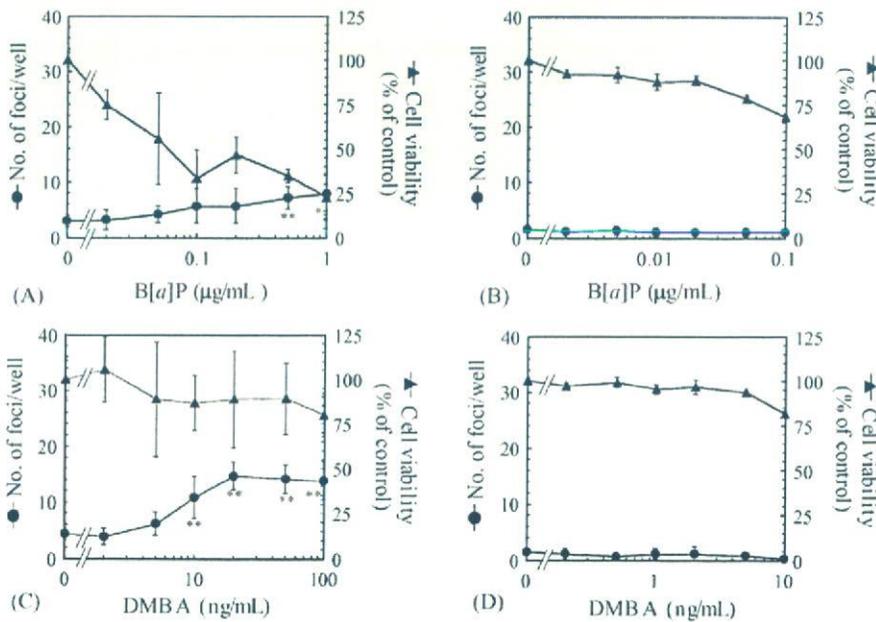


Fig. 4. Results of initiation and promotion assays of B[a]P and DMBA (Group I). A and C show results of initiation assay and B and D show results of promotion assay. \* $p < 0.05$ , compared with solvent control. \*\* $p < 0.01$ , compared with solvent control.

ment schedules. When Bhas 42 cells were seeded at  $2 \times 10^3$  cells/mL, cells were in growth phase almost until Day 7. In order to treat cells repeatedly during the period of growth phase, change of medium containing MCA or TPA was performed every 2 days (periods 1–3 in Fig. 3). After Day 7, when cells became confluent, the change of MCA- or TPA-containing medium was carried out twice a week during the subsequent period (periods 4–6 in Fig. 3).

Schedules B–E were performed as confirmation test of initiation and promotion assays for MCA and TPA. The number of transformed foci in Schedule F did not increase from that in Schedule B, indicating that the additional treatment with MCA during stationary phase had little effect. In contrast, MCA-treatment during cell-growth phase followed by TPA-treatment during stationary phase clearly enhanced the formation of transformed foci (Schedule G). Repeated treatment with MCA during

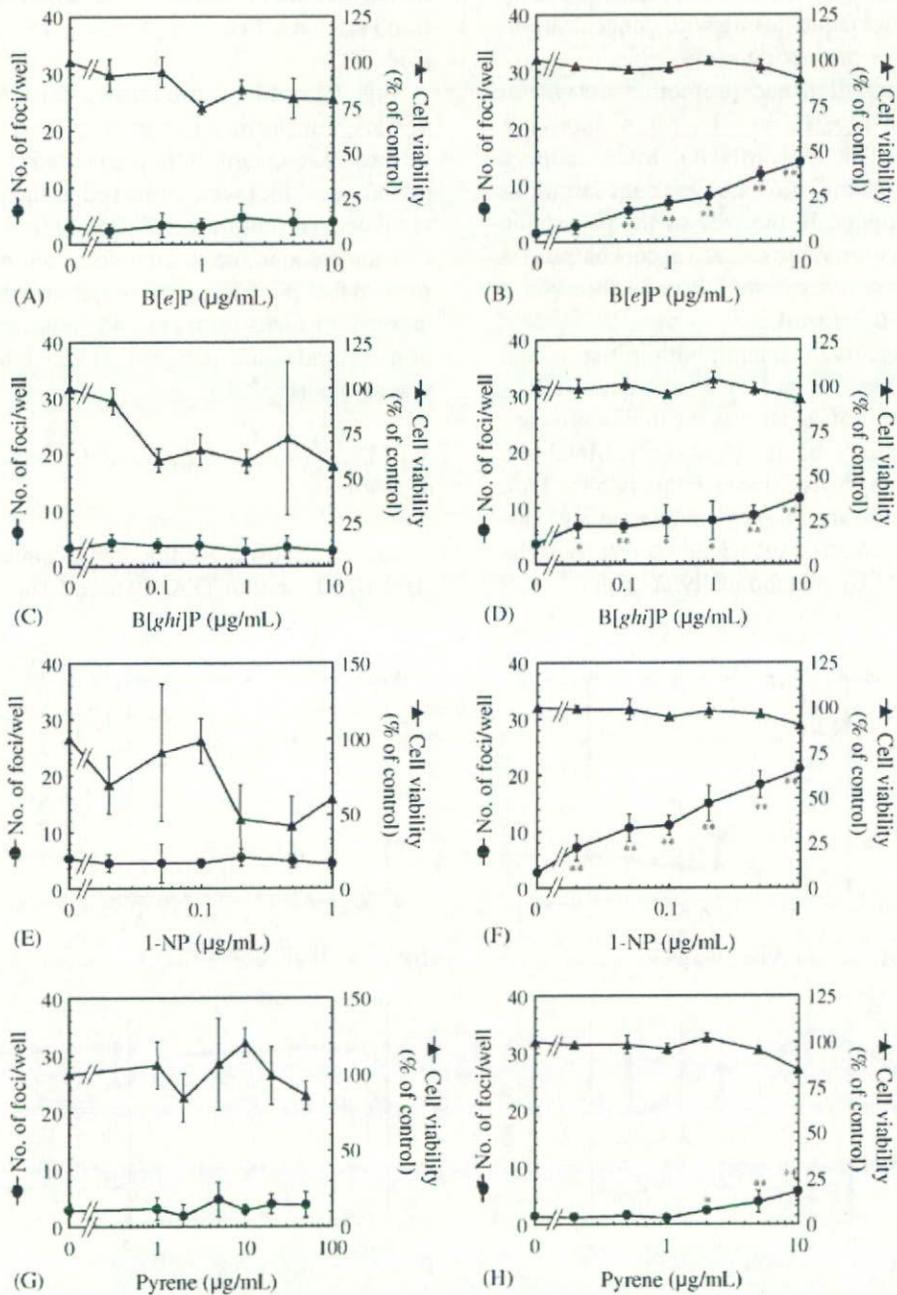


Fig. 5. Results of initiation and promotion assays of B[e]P, B[ghi]P, 1-NP and pyrene (Group II). A, C, E and G show results of initiation assay and B, D, F and H show results of promotion assay. \* $p < 0.05$ , compared with solvent control. \*\* $p < 0.01$ , compared with solvent control.

the cell-growth phase (Schedule H) and the whole period (Schedule J) increased the number of transformed foci. It is remarkable that cell growth was retarded and cells did not reach subconfluence on Day 7 because of the cytotoxicity of MCA. Repeated TPA treatment during growth phase (Schedule I) was ineffective for inducing transformed foci. The number of transformed foci in Schedule K (TPA treatment during cell-growth phase and stationary phase) did not increase from that in Schedule E.

### 3.5. Initiation and promotion assays of PAHs

Results described above showed that MCA induced foci in the initiation assay without metabolic activation by S9 mix. This means that Bhas 42 cells have the capacity to metabolize PAHs, like the parental cell line BALB/c 3T3. Sixteen PAHs were evaluated for their initiating and promoting activities using the present methods. Response to PAHs in both methods could be

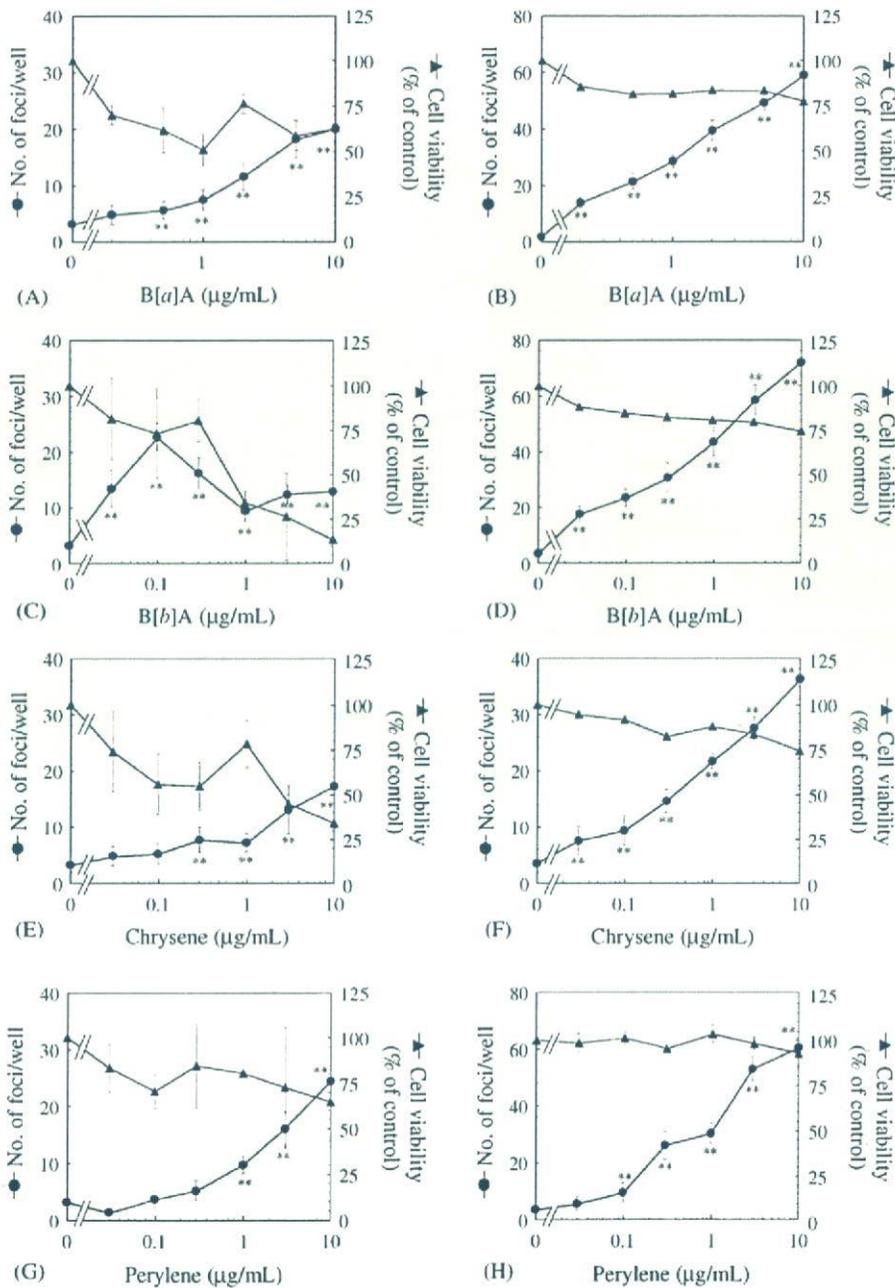


Fig. 6. Results of initiation and promotion assays of B[a]A, B[b]A, chrysene and perylene (Group III). A, C, E and G show results of initiation assay and B, D, F and H show results of promotion assay. \* $p < 0.05$ , compared with solvent control. \*\* $p < 0.01$ , compared with solvent control.

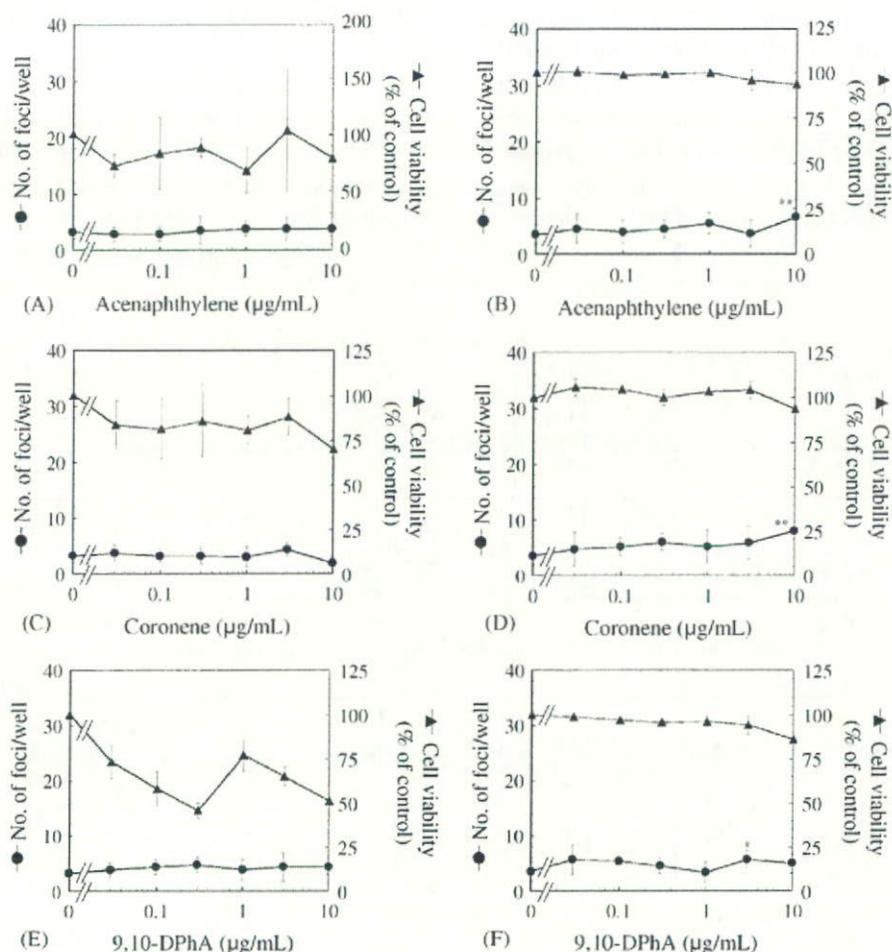


Fig. 7. Results of initiation and promotion assays of acenaphthylene, coronene and 9,10-DPhA (Group IV). A, C and E show results of initiation assay and B, D and F show results of promotion assay. \* $p < 0.05$ , compared with solvent control. \*\* $p < 0.01$ , compared with solvent control.

categorized into four groups: showing only initiation activity (Group I), only promotion activity (Group II), both initiation and promotion activities (Group III), and equivocal or negative response in both methods (Group IV).

Fig. 4 shows results of two chemicals showing positive responses only in the initiation assay (Group I). B[a]P at concentrations of 0.5 and 1  $\mu\text{g}/\text{mL}$  (Fig. 4A) and DMBA at concentrations of 10  $\text{ng}/\text{mL}$  and above (Fig. 4C) induced transformed foci in the initiation assay only. In the case of the promotion assay, no transformed foci were induced at any concentrations tested for B[a]P (Fig. 4B) or DMBA (Fig. 4D).

Chemicals classified in Group II consisted of B[e]P, B[ghi]P, 1-NP, and pyrene, which showed positive responses only in the promotion assay (Fig. 5). No increase of foci was observed in the initiation assay with these chemicals (Fig. 5A, C, E and G). In contrast, they induced significant increase of foci in the promotion assay (Fig. 5B, D, F and H).

Results for Group III are shown in Fig. 6. B[a]A at concentrations of 0.5  $\mu\text{g}/\text{mL}$  and above (Fig. 6A), chrysene at concentrations of 0.2  $\mu\text{g}/\text{mL}$  and above (Fig. 6E) and perylene at concentrations of 1  $\mu\text{g}/\text{mL}$  and above (Fig. 6G) induced foci in the initiation assay. In the initiation assay of B[b]A, the number of foci increased on increasing the dose up to 0.1  $\mu\text{g}/\text{mL}$ , but it decreased at concentrations over 0.2  $\mu\text{g}/\text{mL}$  (Fig. 6C). In the promotion assay, a marked increase in focus formation was observed with these chemicals at a non-cytotoxic concentration range from 0.1 to 10  $\mu\text{g}/\text{mL}$  (Fig. 6B, D, F and H).

Fig. 7 shows results of initiation and promotion assays of acenaphthylene, coronene, and 9,10-DPhA. No increase of foci was observed in the initiation assay at all concentrations tested for acenaphthylene (Fig. 7A), coronene (Fig. 7C) and 9,10-DPhA (Fig. 7E). These chemicals weakly induced foci in the promotion assay at the highest concentration (Fig. 7B, D and F). Results of initiation and promotion assays for anthracene, naph-

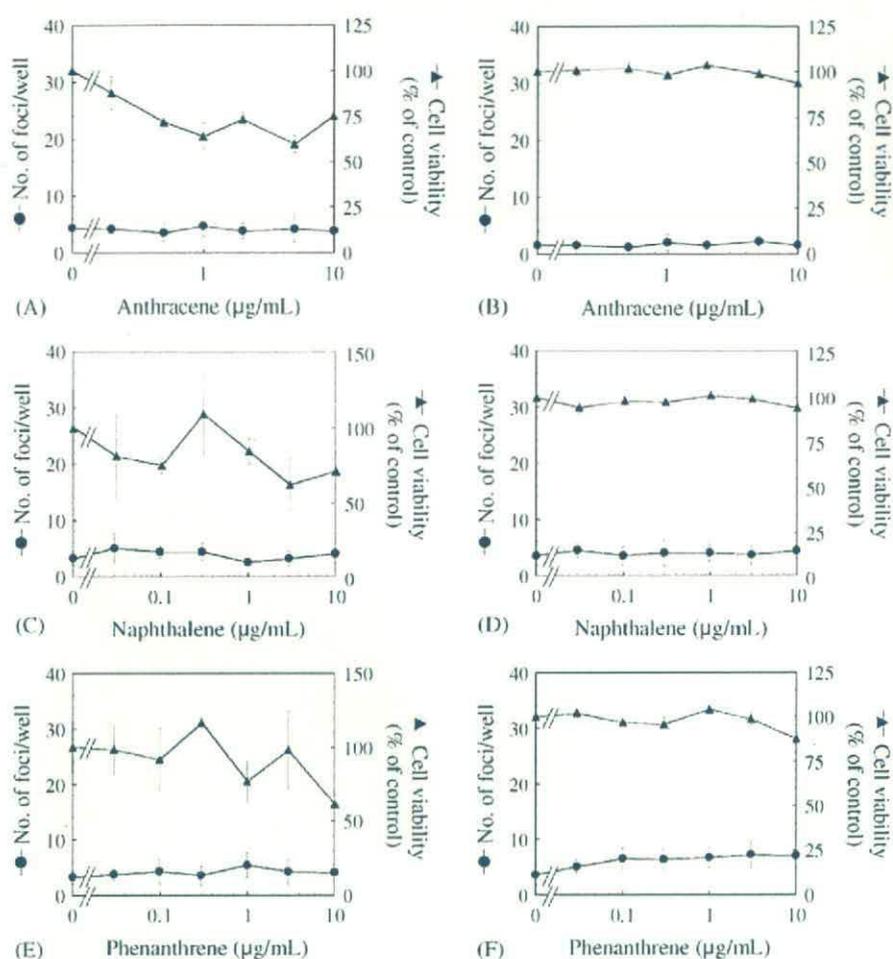


Fig. 8. Results of initiation and promotion assays of anthracene, naphthalene and phenanthrene (Group IV). A, C and E show results of initiation assay and B, D and F show results of promotion assay. \* $p < 0.05$ , compared with solvent control. \*\* $p < 0.01$ , compared with solvent control.

thalene, and phenanthrene are shown in Fig. 8. These chemicals were entirely negative in both initiation and promotion assays at concentrations up to  $10 \mu\text{g/mL}$ .

#### 4. Discussion

In addition to the Bhas promotion assay, we have developed a method for evaluation of initiating activity of chemicals using Bhas 42 cells. In the promotion assay, cells are treated with test chemicals at subconfluent condition, in which further cell division is limited, whereas the initiation assay was designed for cells at a lower cell density to be able to divide several times after treatment with test chemicals. The fact that tumor initiators could transform Bhas 42 cells only under cell proliferating conditions was important in aiding understanding of the two-stage model of chemical carcinogenesis. MNNG and MCA, both tumor initiators, were positive in the initiation assay but negative in the promotion assay. In contrast, TPA, LCA, and okadaic acid, tumor promoters

[26–31], were positive in the promotion assay but were negative in the initiation assay.

Some tumor initiators are considered to have tumor-promoting activity. This recognition came from the experimental observation that repeated application at subtumorigenic doses can induce tumors in mouse skin without any post-treatment with a promoter [1]. In order to probe why repeated treatment with MNNG or MCA could not induce transformed foci in the Bhas promotion assay, an experiment with various schedules of treatment was conducted (Fig. 3). This demonstrated that MCA was effective only when cells were in a dividing phase, consistent to the current notion for the mechanism of initiator action, i.e., the fixation of genetic damage resulting in genetic alteration, namely mutation, after several cell divisions. On the contrary, the promoter, TPA, induced transformed foci when cell division was limited but it was not effective during the cell-dividing phase. It is well known that tumor promoters inhibit the interaction with neighboring cells (metabolic cooperation) [32–35].

Table 2  
Summary of Bhas initiation and promotion assays for 22 tested chemicals, and published carcinogenicity and mutagenicity assay summaries

Chemical	CAS no.	IARC	Bhas 42 assay		In vivo carcinogenicity <sup>a</sup>		Ames' test		Missense mutation <sup>c</sup>		E. coli		Metabolic cooperation		MLA		References
			Initiation assay	Promotion assay	Carcinogenicity	Promotion	Frameshift mutation <sup>b</sup>	Missense mutation <sup>c</sup>	S9 (-)	S9 (+)	S9 (-)	S9 (+)	S9 (-)	S9 (+)	S9 (-)	S9 (+)	
<b>Group I</b>																	
MNNG	70-25-7	2A	+	-	+	+	+	+	+	+	+	+	+	+	+	+	[42,44-46,48,50,55]
MCA	56-49-5		+	-	+	+	+	+	+	+	+	+	+	+	+	+	[42,44,45,48,55]
B[a]P	50-32-8	2A	+	-	+	+	+	+	+	+	+	+	+	+	+	+	[38,41,42,55,57,61]
DMBA	57-97-6		+	-	+	+	+	+	+	+	+	+	+	+	+	+	[41,42,44,48,55,60]
AFB1	1162-65-8	I	+	-	+	+	+	+	+	+	+	+	+	+	+	+	[39,42,55]
<b>Group II</b>																	
TPA	16561-29-8		-	+	-	-	+	-	-	-	-	+	+	-	-	-	[1,42,62]
LCA	434-13-9		-	+	-	-	+	-	-	-	-	+	+	-	-	-	[41,44,47,60,62]
Okadaic acid	78111-17-8		-	+	-	-	+	-	-	-	-	+	+	-	-	-	[30,54,56]
B[e]P	192-97-2	3	-	+	-	-	+	-	-	-	-	-	-	I	-	-	[38,41-44]
B[gh]P	191-24-2	3	-	+	-	-	+	-	-	-	-	-	-	-	-	-	[38,41,49,50]
I-NP	5522-43-0	2B	-	+	-	-	+	-	-	-	-	-	-	-	-	-	[39,49,50,58]
Pyrene	129-00-0	3	-	+	-	-	+	-	-	-	-	-	-	-	-	-	[38,41,42,44]
<b>Group III</b>																	
B[a]A	56-55-3	2A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	[38,42,53,55]
B[b]A	92-24-0		+	+	+	+	+	+	+	+	+	+	+	+	+	+	[51]
Chrysene	218-01-9	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	[38,41,42,59]
Perylene	198-55-0	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	[38,41,50,52,60]
<b>Group IV</b>																	
Acenaphthylene	208-96-8		-	±	-	-	-	-	-	-	-	-	-	-	-	-	
Anthracene	120-12-7	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	[38,41,42,44,60]
Coronene	191-07-1	3	-	±	-	-	-	-	-	-	-	-	-	-	-	-	[38,41,43]
9,10-DPhA	91-20-3	2B	-	±	-	-	-	-	-	-	-	-	-	-	-	-	[40-42]
Naphthalene	85-01-8	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	[38,41,42,44]

+, positive result; -, negative result; ±, equivocal result; I, inconclusive result.

<sup>a</sup> Carcinogenicity was tested by skin application to mice and promoting activity was assessed by mouse-skin initiation-promotion assay.

<sup>b</sup> Frameshift mutation was detected with TA97, TA98, TA1537 and TA1538.

<sup>c</sup> Missense mutation was detected with TA100 and TA1535.

<sup>d</sup> Initiating activity of AFB1 required metabolic activation with S9.

<sup>e</sup> Carcinogenicity of AFB1 was shown in liver.

Tsuchiya and Umeda [36] have demonstrated that a subconfluent phase of MNNG-initiated BALB/c 3T3 cells was the most sensitive stage for the induction of transformed foci by TPA.

Bhas 42 cells were considered as initiated cells by virtue of transfection of the *v-Ha-ras* gene. Here, however, we demonstrated that the additional treatment with initiating chemicals is effective for inducing transformed foci. Carcinogenesis is now considered to be a multi-step phenomenon. Sugimura stated in his review that several mutations could lead to tumor formation [37]. Thus, the results are interpreted as showing that initiator-induced mutations of oncogene(s) other than *ras* oncogene in Bhas 42 cells can result in the induction of cell transformation.

These discussions may pertain to a specific *in vitro* experimental assay, but the phenomena support the hypothetical mechanism for tumor formation in the mouse skin, where repeated treatment with tumor initiator causes genetic damage of several oncogenes in the skin cells. The basement cell layer of mouse skin comprises actively dividing stem cells, wherein genetic damage can be fixed and result in mutations. Therefore, we hypothesize that tumor formation from tumor initiator exposure does not involve promoting activity, such as disturbed interaction between neighboring cells, i.e., loss of metabolic cooperation, but involves fixation and accumulation of genetic damage after cell divisions.

In order to investigate the applicability of Bhas initiation and promotion assays to an *in vitro* screening for carcinogenesis, various PAHs and other initiators and promoters were evaluated by the assays; these could be classified into four groups (Table 2). Group I consists of chemicals showing positive results in the initiation assay but negative in the promotion assay. MNNG, MCA, AFB<sub>1</sub>, and carcinogenic PAHs were classified in this group. Group II chemicals are negative in the initiation assay but positive in the promotion assay. Tumor promoters, TPA, LCA, and okadaic acid, are Group II chemicals. These promoters are also negative in bacterial mutation assays. Group III consists of chemicals showing positive response in both initiation and promotion assays (B[a]A, B[b]A, chrysene and perylene). Group IV consists of chemicals showing equivocal or negative response in both assays. These results showing that there are various kinds of PAHs with initiating and/or promoting activities suggest carcinogenic risk in mixtures of PAHs such as soot from exhaust gas, and particulate matter in polluted air.

In the present study, a metabolic activation system was not used in the examination of PAHs. Some chemi-

cals negative in the Bhas initiation assay (Groups II and IV) are positive in the bacterial reverse mutation assay in the presence of an exogenous metabolic activation system (Table 2). It is, therefore, necessary to include a metabolic activation system in the Bhas initiation assay. To incorporate the metabolic activation system into the Bhas assay, we tried to modify the present Bhas initiation assay. AFB<sub>1</sub>, as a model initiator that requires metabolic activation by CYP enzymes such as 2A6, 3A4 and 1A2 [24,25], was used to validate the incorporation of the metabolic activation system. AFB<sub>1</sub> did not induce transformed foci in the initiation assay in the absence of the metabolic activation system but it induced transformed foci in its presence.

In summary, Bhas assays for the detection of both initiating and promoting activities of chemicals are more sensitive and economical than other cell transformation assays, and deserve consideration as a promising screening tool. However, the results obtained are still limited, and further studies are necessary to confirm the applicability of the Bhas assays especially in the presence of an exogenous metabolic activation system. In addition, the Bhas assays may be able to play an important role for understanding the mechanism of chemical carcinogenesis.

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