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	100.0
	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	100.0
	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 2×10^4 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 2×10^3 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 2×10^4 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_1, 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_1 together with S9 mix which contained 5% rat S9 (Kikkoman, Chiba, Japan), 2 mM HEPES (Sigma Chemical Co.), 5 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphatase (Sigma Chemical Co.) and 4 mM β-$NADP^+$ (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 (2×10^3 cells/mL) than in the promotion assay (2×10^4 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).

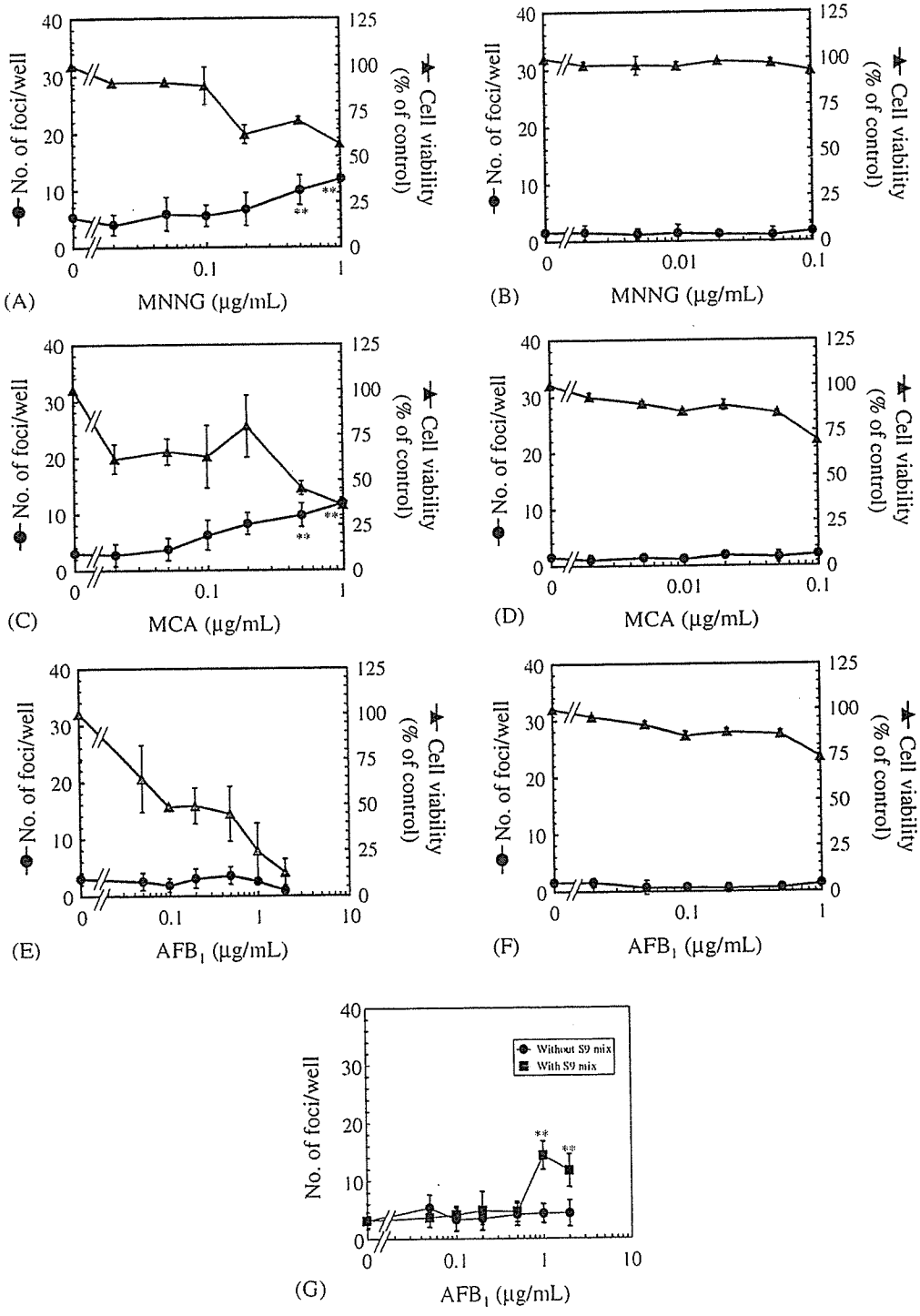

Fig. 1. Results of transformation assays of initiators. A, C and E show results of initiation assay and B, D and F show results of promotion assay. G shows result of metabolic activation in AFB_1 initiation assay. $^{*}p < 0.05$, compared with solvent control. $^{**}p < 0.01$, compared with solvent control.

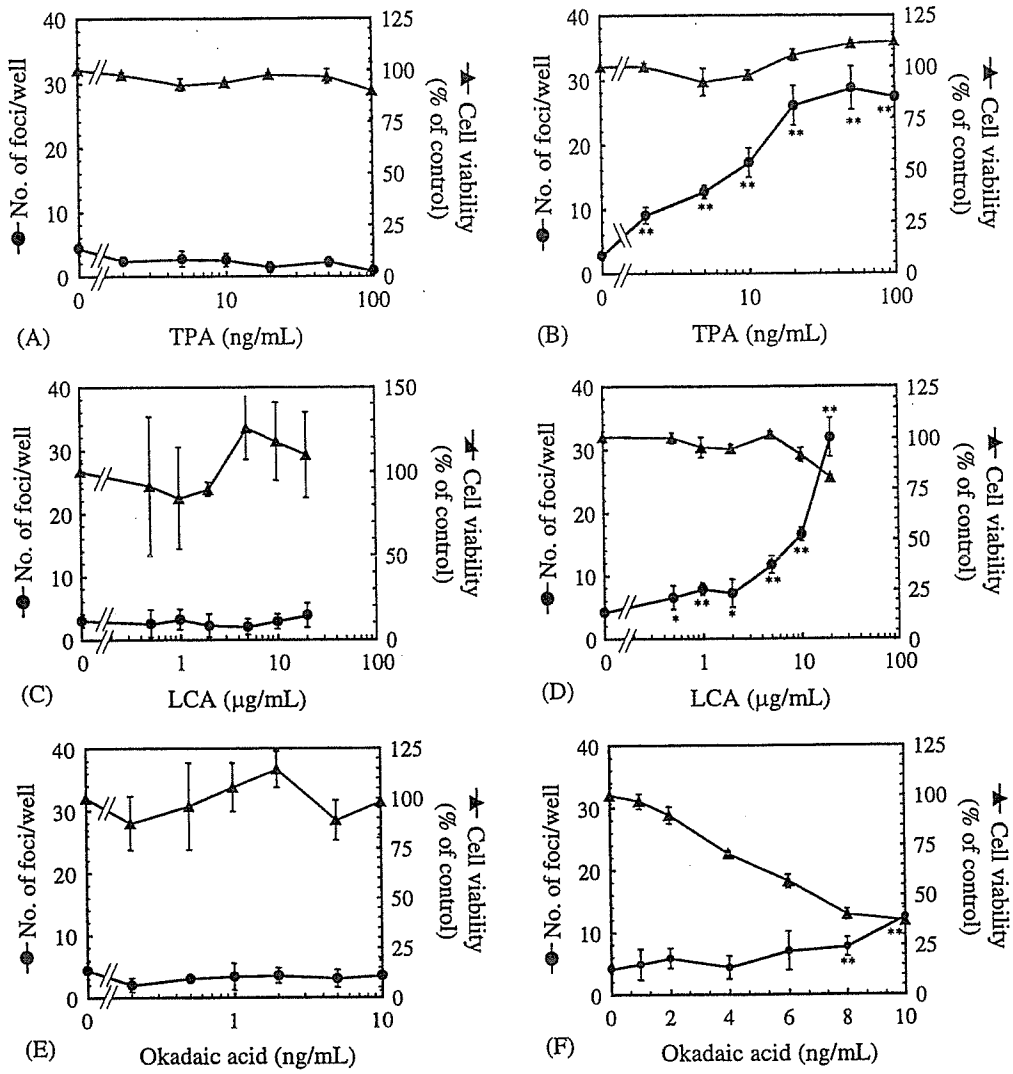

Fig. 2. Results of transformation assays of promoters. A, C and E show results of initiation assay and B, D and F show results of promotion assay. $^{*}p < 0.05$, compared with solvent control. $^{**}p < 0.01$, compared with solvent control.

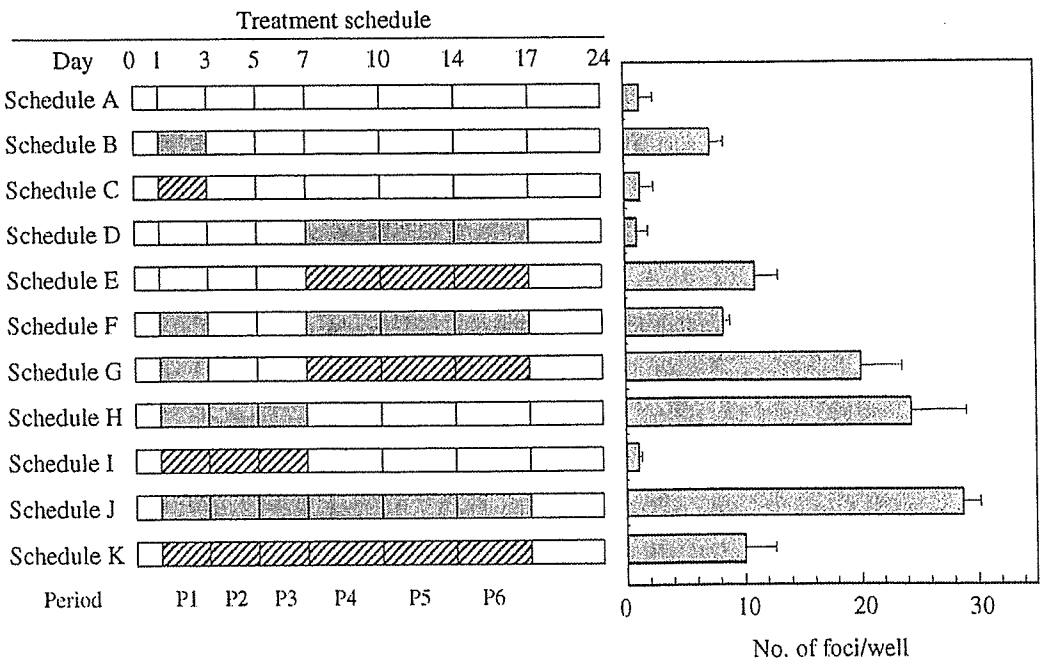

Fig. 3. Result of transformation assay under various time schedules. In treatment schedule, solid blocks represent treatment with 100 ng/mL MCA and diagonal blocks represent treatment with 50 ng/mL TPA.

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 μg/mL and above. As mentioned above, MNNG did not induce transformed foci in the promotion assay up to 0.1 μg/mL. With concentrations over 0.2 μg/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 μg/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 μg/mL and higher. In the case of the promotion assay, no focus formation was observed at concentrations up to 0.1 μg/mL and severe cytotoxicity was observed at concentrations over 0.2 μg/mL.

AFB_1 showed negative results in both initiation and promotion assays (Fig. 1E and F). In the case of promotion assay, 1 μg/mL of AFB_1 was the maximum concentration tested because of its cytotoxicity. Metabolic activation of AFB_1 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_1 metabolically activated by S9 mix, and there was significant foci induction at 1 and 2 μg/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-

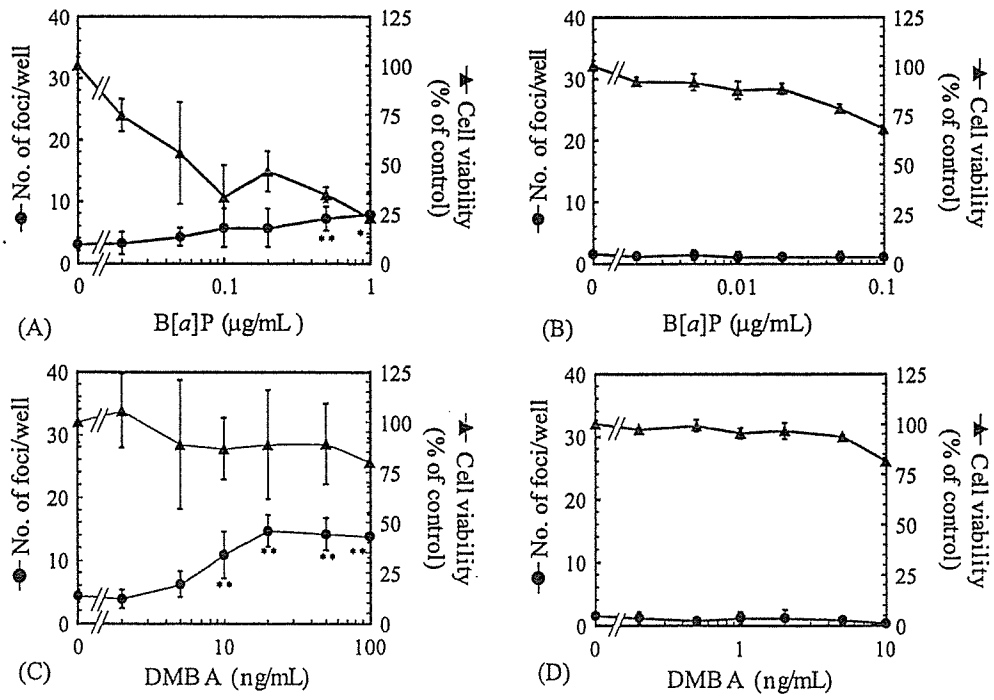

Fig. 4. Results of initiation and promotion assays of B[*a*]P and DMBA (Group I). A and C show results of initiation assay and B and D show results of promotion assay. $^{*}p < 0.05$, compared with solvent control. $^{**}p < 0.01$, compared with solvent control.

ment schedules. When Bhas 42 cells were seeded at 2×10^3 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

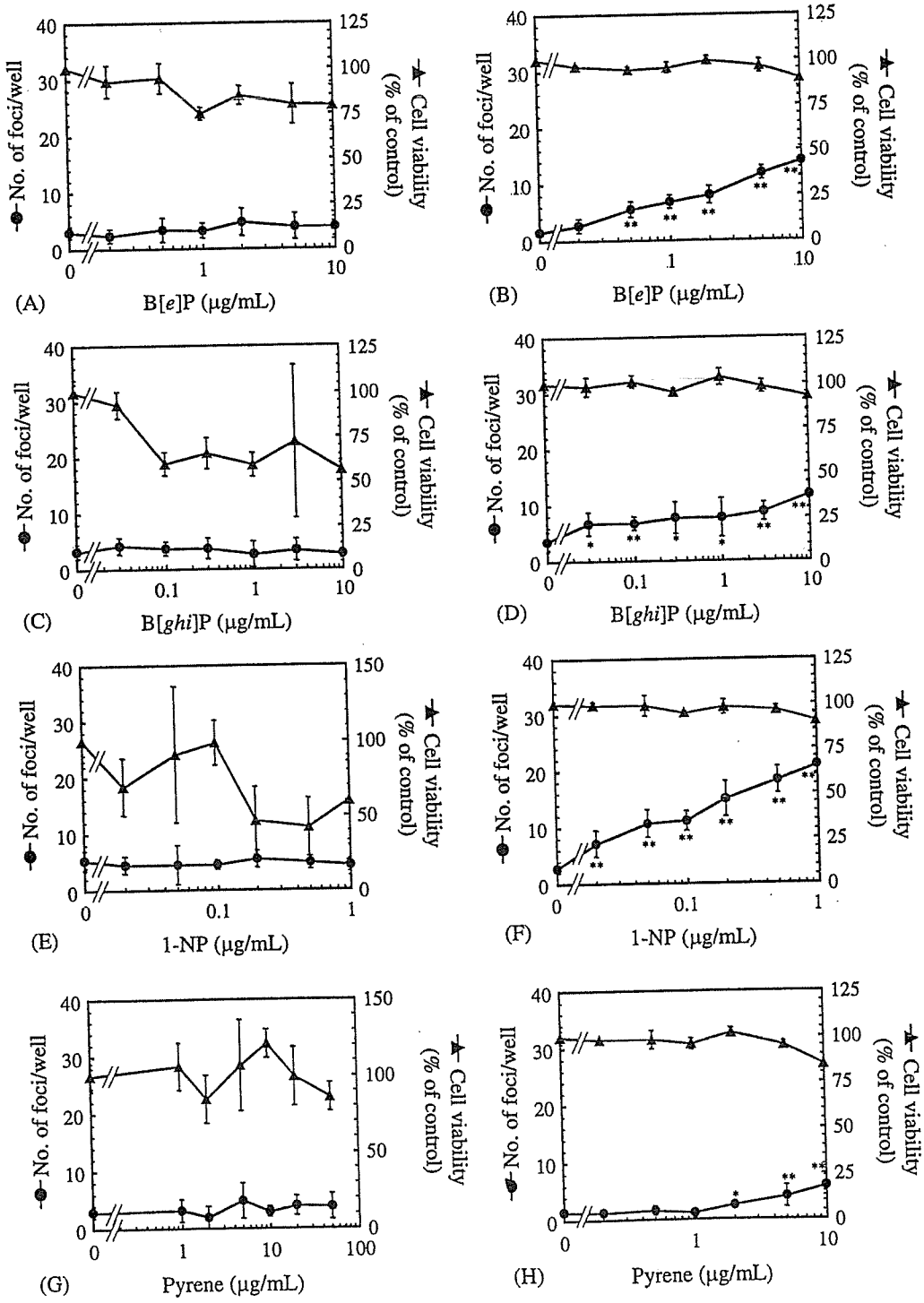

Fig. 5. Results of initiation and promotion assays of B[*e*]P, B[*ghi*]P, 1-NP and pyrene (Group II). A, C, E and G show results of initiation assay and B, D, F and H show results of promotion assay. $^{*}p < 0.05$, compared with solvent control. $^{**}p < 0.01$, compared with solvent control.

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 categorized into four groups: showing only initiation

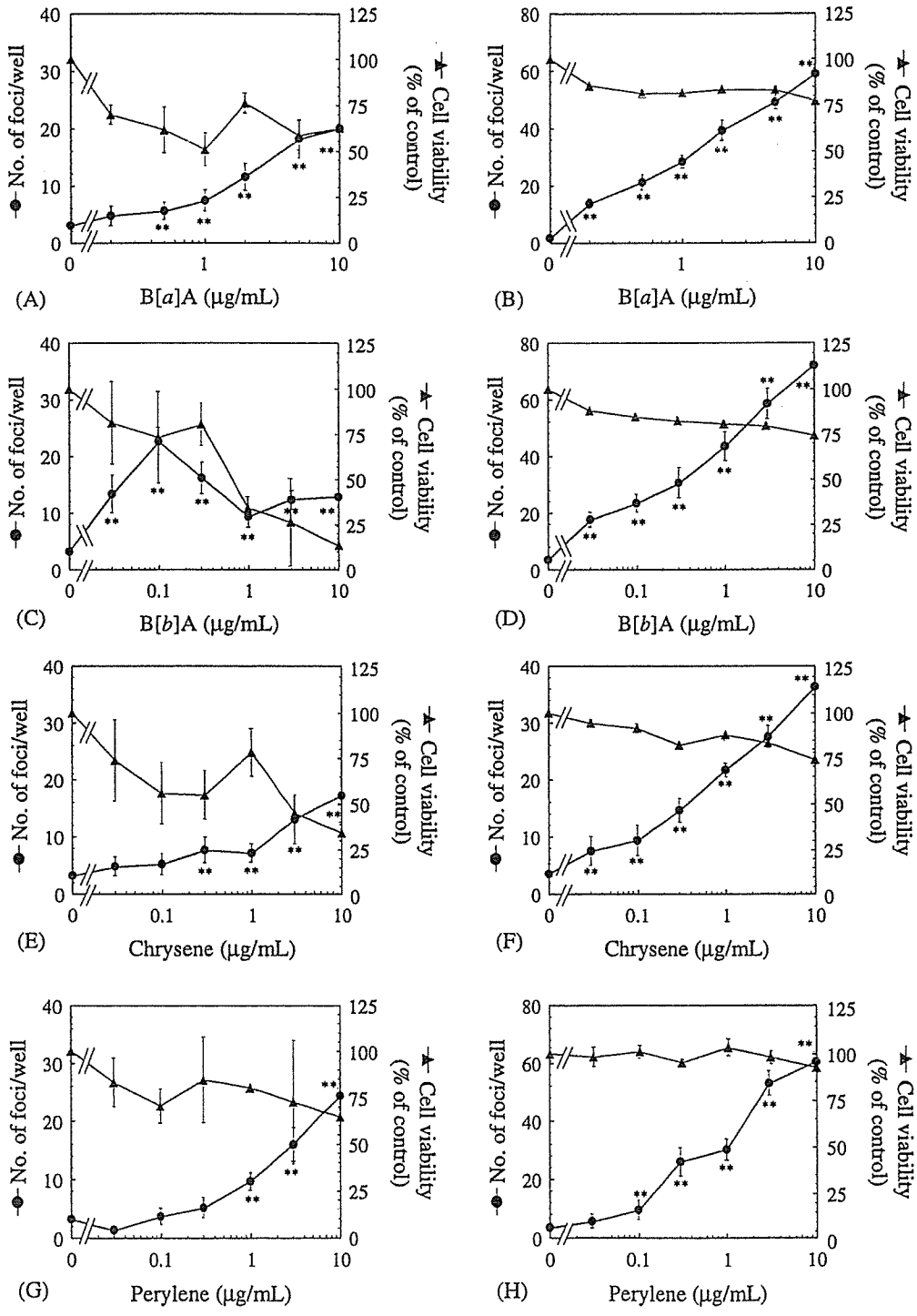

Fig. 6. Results of initiation and promotion assays of B[*a*]A, B[*b*]A, chrysene and perylene (Group III). A, C, E and G show results of initiation assay and B, D, F and H show results of promotion assay. $^{*}p<0.05$, compared with solvent control. $^{**}p<0.01$, compared with solvent control.

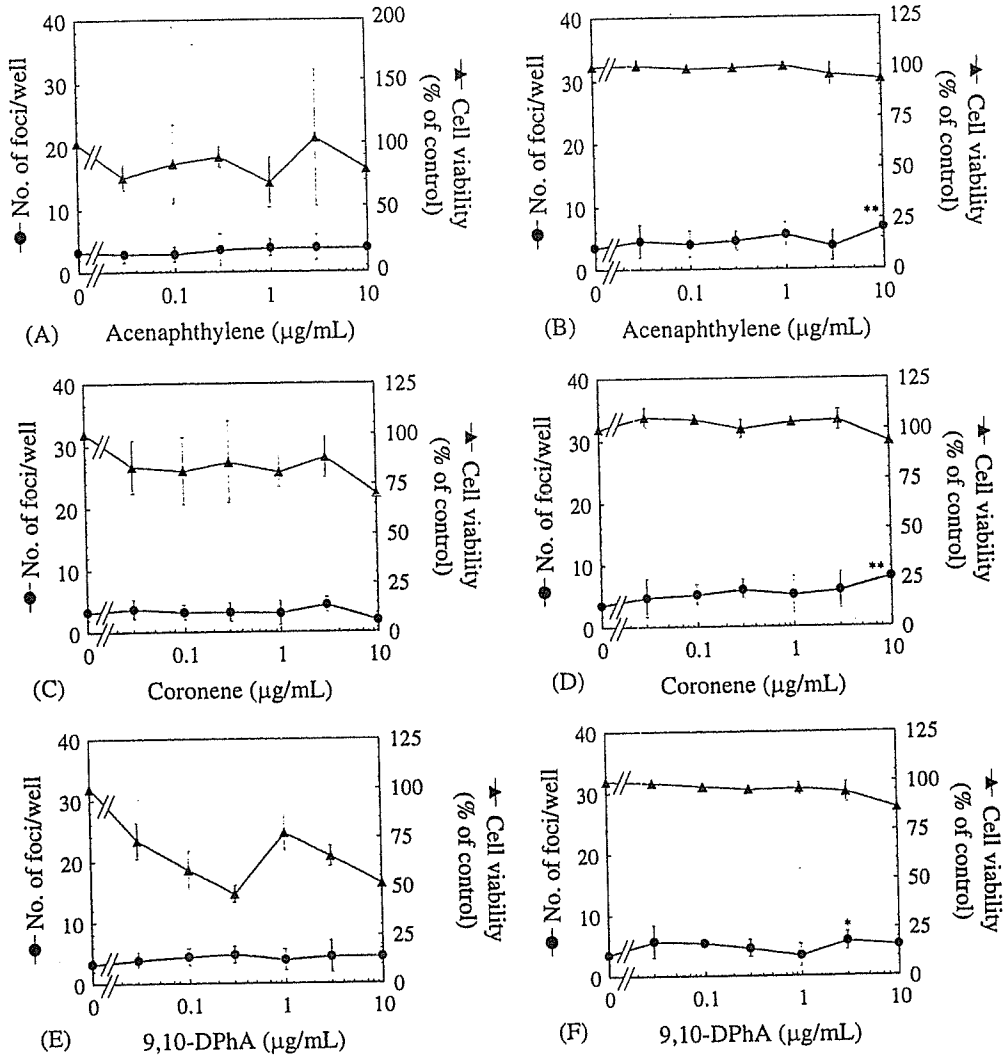

Fig. 7. Results of initiation and promotion assays of acenaphthylene, coronene and 9,10-DPhA (Group IV). A, C and E show results of initiation assay and B, D and F show results of promotion assay. $^{*}p < 0.05$, compared with solvent control. $^{**}p < 0.01$, compared with solvent control.

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 μg/mL (Fig. 4A) and DMBA at concentrations of 10 ng/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 μg/mL and above (Fig. 6A), chrysene at concentrations of 0.2 μg/mL and above (Fig. 6E) and perylene at concentrations of 1 μg/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 μg/mL, but it decreased at concentrations over 0.2 μg/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 μg/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-

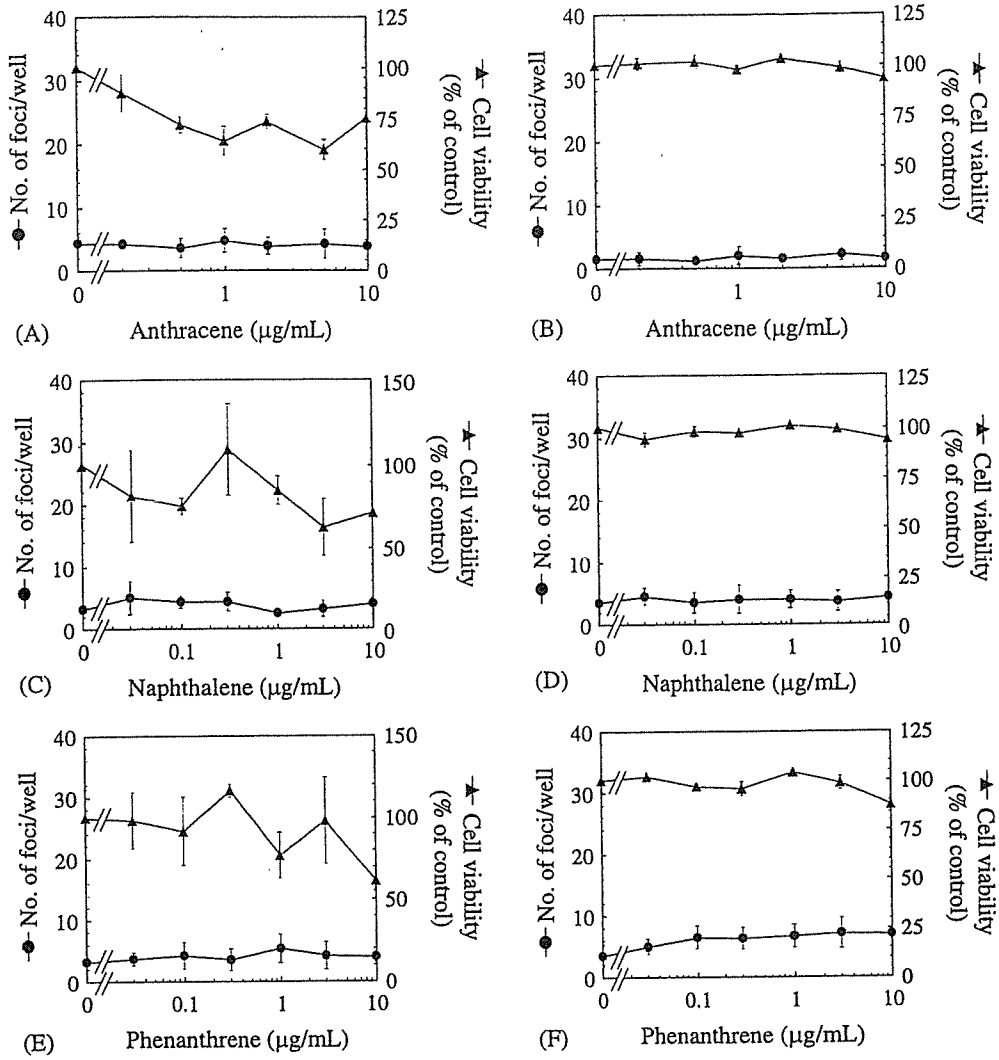

Fig. 8. Results of initiation and promotion assays of anthracene, naphthalene and phenanthrene (Group IV). A, C and E show results of initiation assay and B, D and F show results of promotion assay. $^{*}p < 0.05$, compared with solvent control. $^{**}p < 0.01$, compared with solvent control.

thalene, and phenanthrene are shown in Fig. 8. These chemicals were entirely negative in both initiation and promotion assays at concentrations up to 10 μg/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[a]		Ames' test				*E. coli*		Metabolic cooperation		MLA		References
			Initiation assay	Promotion assay	Carcinogenicity	Promotion	Frameshift mutation[b]		Missense mutation[c]		S9 (−)	S9 (+)	S9 (−)	S9 (+)	S9 (−)	S9 (+)	
							S9 (−)	S9 (+)	S9 (−)	S9 (+)							
Group I																	
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		+	−	+		+	+	+	I	−	−	−	+	−	+	[41,42,44,48,55,60]
AFB1	1162-65-8	1	+[d]	−	+[e]		I			+			−	+			[39,42,55]
Group II																	
TPA	16561-29-8		−	+		+		−		−			+				[1,42,62]
LCA	434-13-9		−	+	−		−	−	−	−	−	−	+		−	−	[41,44,47,60,62]
Okadaic acid	78111-17-8		−	+		+		−		−				I			[30,54,56]
B[e]P	192-97-2	3	−	+	−	+	−	+	−	−	−	−	−				[38,41–44]
B[ghi]P	191-24-2	3	−	+	−	−	−	+									[38,49,50]
1-NP	5522-43-0	2B	−	+	+		I	I									[39,49,50,58]
Pyrene	129-00-0	3	−	+	−	I	−	+	−	I	−	−					[38,41,42,44]
Group III																	
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]
Group IV																	
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			−	±													
Naphthalene	91-20-3	2B	−	−	−		−	−	−	−	−	−			−	+	[40–42]
Phenanthrane	85-01-8	3	−	−	−	−	−	−	−	−	−	−					[38,41,42,44]

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

[a] Carcinogenicity was tested by skin application to mice and promoting activity was assessed by mouse-skin initiation-promotion assay.

[b] Frameshift mutation was detected with TA97, TA98, TA1537 and TA1538.

[c] Missense mutation was detected with TA100 and TA1535.

[d] Initiating activity of AFB1 required metabolic activation with S9.

[e] 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_1, 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 chemicals 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_1, 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_1 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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An Inter-laboratory Collaborative Study by the Non-Genotoxic Carcinogen Study Group in Japan, on a Cell Transformation Assay for Tumour Promoters Using Bhas 42 cells

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Summary — The Bhas promotion assay is a cell culture transformation assay designed as a sensitive and economical method for detecting the tumour-promoting activities of chemicals. In order to validate the transferability and applicability of this assay, an inter-laboratory collaborative study was conducted with the participation of 14 laboratories. After confirmation that these laboratories could obtain positive results with two tumour promoters, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and lithocholic acid (LCA), 12 coded chemicals were assayed. Each chemical was tested in four laboratories. For eight chemicals, all four laboratories obtained consistent results, and for two of the other four chemicals, only one of the four laboratories showed inconsistent results. Thus, the rate of consistency was high. During the study, several issues were raised, each of which were analysed step-by-step, leading to revision of the protocol of the original assay. Among these issues were the importance of careful maintenance of mother cultures and the adoption of test concentrations for toxic chemicals. In addition, it is suggested that three different types of chemicals show positive promoting activity in the assay. Those designated as T-type induced extreme growth enhancement, and included TPA, mezerein, PDD and insulin. LCA and okadaic acid belonged to the L-type category, in which transformed foci were induced at concentrations showing growth-inhibition. In contrast, M-type chemicals, progesterone, catechol and sodium saccharin, induced foci at concentrations with little or slight growth inhibition. The fact that different types of chemicals similarly induce transformed foci in the Bhas promotion assay may provide clues for elucidating mechanisms of tumour promotion.

Key words: *Bhas 42 cells, cell transformation assay, inter-laboratory collaborative study, tumour promoter.*

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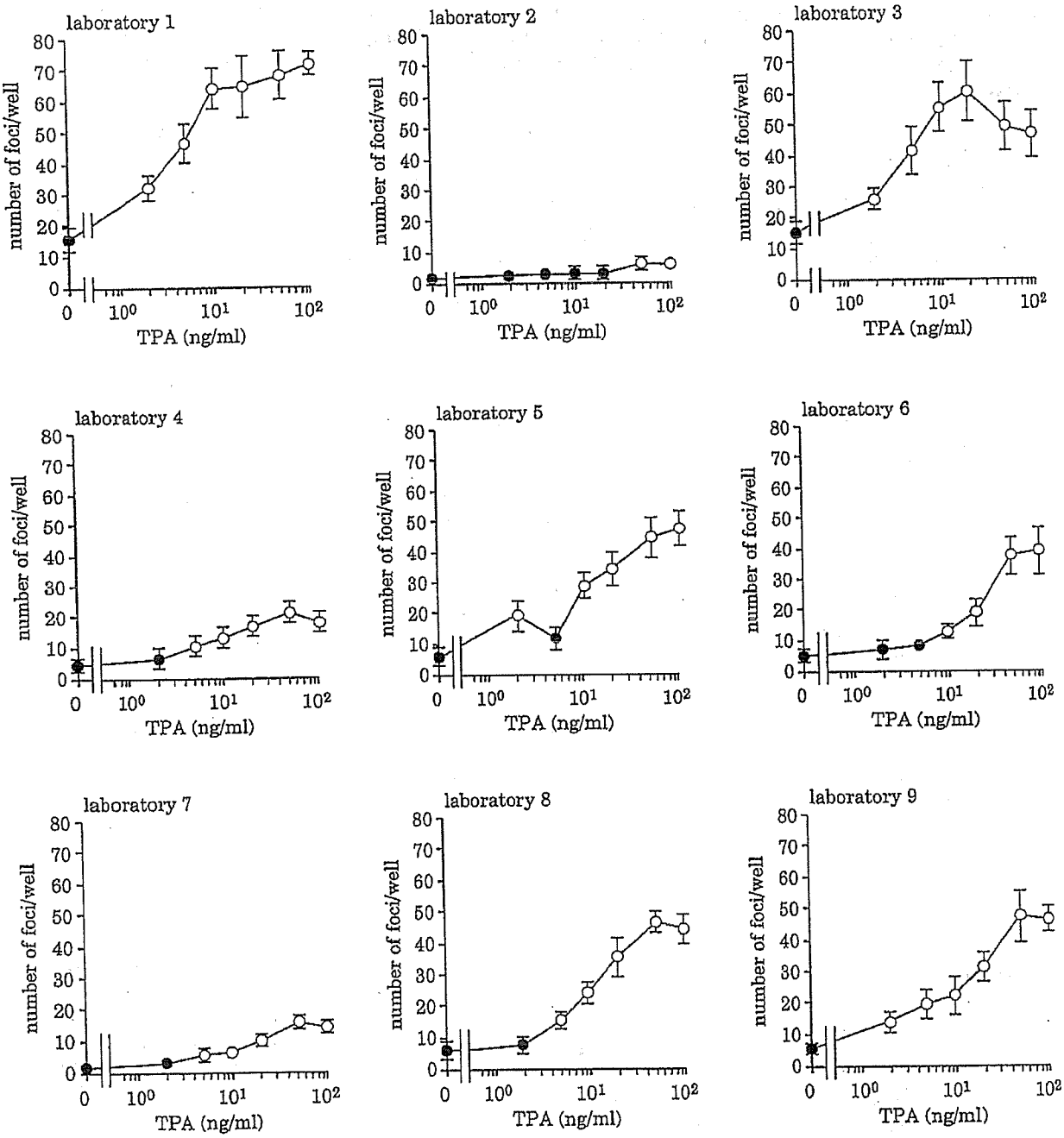
Introduction

Chemicals active in carcinogenesis according to the two-stage model can be divided into two categories, initiators and promoters (1). Most initiators can be detected by using various genotoxicity tests, some of which are used for carcinogenicity evaluation of chemicals by regulatory authorities (2). In the case of tumour promoters, several detection methods have been reported (3–13), but none of them have been accepted for regulatory purposes. Meanwhile, the existence of non-genotoxic carcinogens has presented a major challenge for the toxicity screening of chemicals. Since tumour promoters are considered to be one kind of non-genotoxic carcinogen, the development of reliable and applicable screening methods for the detection of tumour promoters is an important goal.

In vitro cell transformation tests, involving BALB/c 3T3 cells or C3H10T1/2 cells, can simulate the process of two-stage animal carcinogenesis, and can

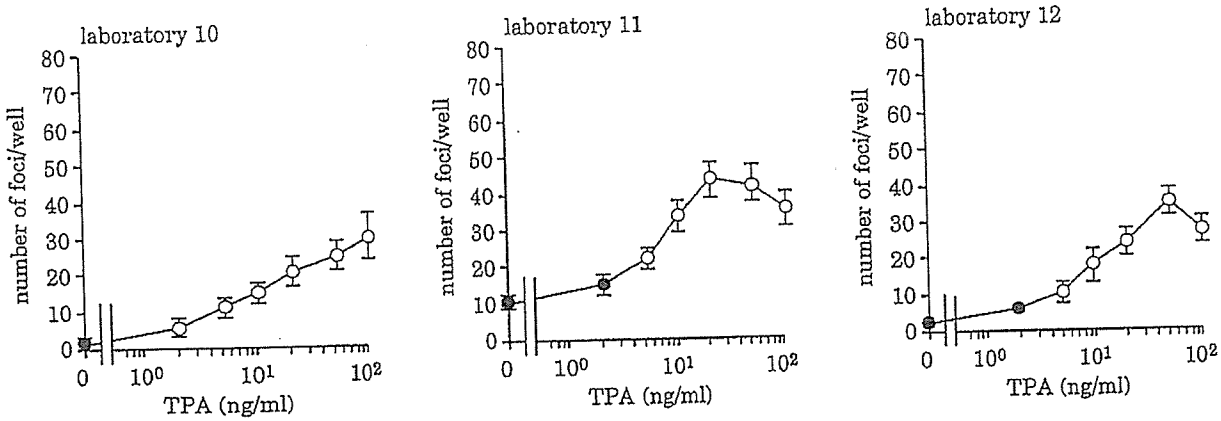
detect both the initiating and the promoting activities of chemicals. Recently, Ohmori *et al.* have developed an *in vitro* cell transformation assay for tumour promoters, by using Bhas 42 cells, a v-*Ha*-ras-transfected BALB/c 3T3 cell line, which are considered to be initiated cells in the two-stage transformation model of carcinogenesis (14). This cell transformation assay has many advantages: a) treatment with an initiating agent and subsequent cultivation for an expression period can be omitted; b) the experimental period is

Figure 2: Transformation frequencies with TPA in the Bhas promotion assay



○ = *significant point compared to control.*

Figure 2: continued



○ = *significant point compared to control.*

shortened from 4–6 weeks to 2.5–3 weeks after cell inoculation; and c) the transformation frequency is high, so only three to six wells in 6-well plates are required for each dose, instead of 10–20 6cm dishes. In order to gain wider awareness of this method, it was necessary to confirm its applicability and transferability between laboratories.

The aim of this report is to describe an inter-laboratory collaborative study on the Bhas promotion assay. Fourteen laboratories, belonging to the Non-Genotoxic Carcinogen Study (NGCS) Group in the Environmental Mutagen Society of Japan, participated in the project. Here, we describe four consecutive studies, during which the method originally reported by Ohmori *et al.* (14) was examined and improved after the analysis of each stage of the project.

Materials and Methods

Media, cells and culture conditions

Minimal Essential Medium (MEM) was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan),

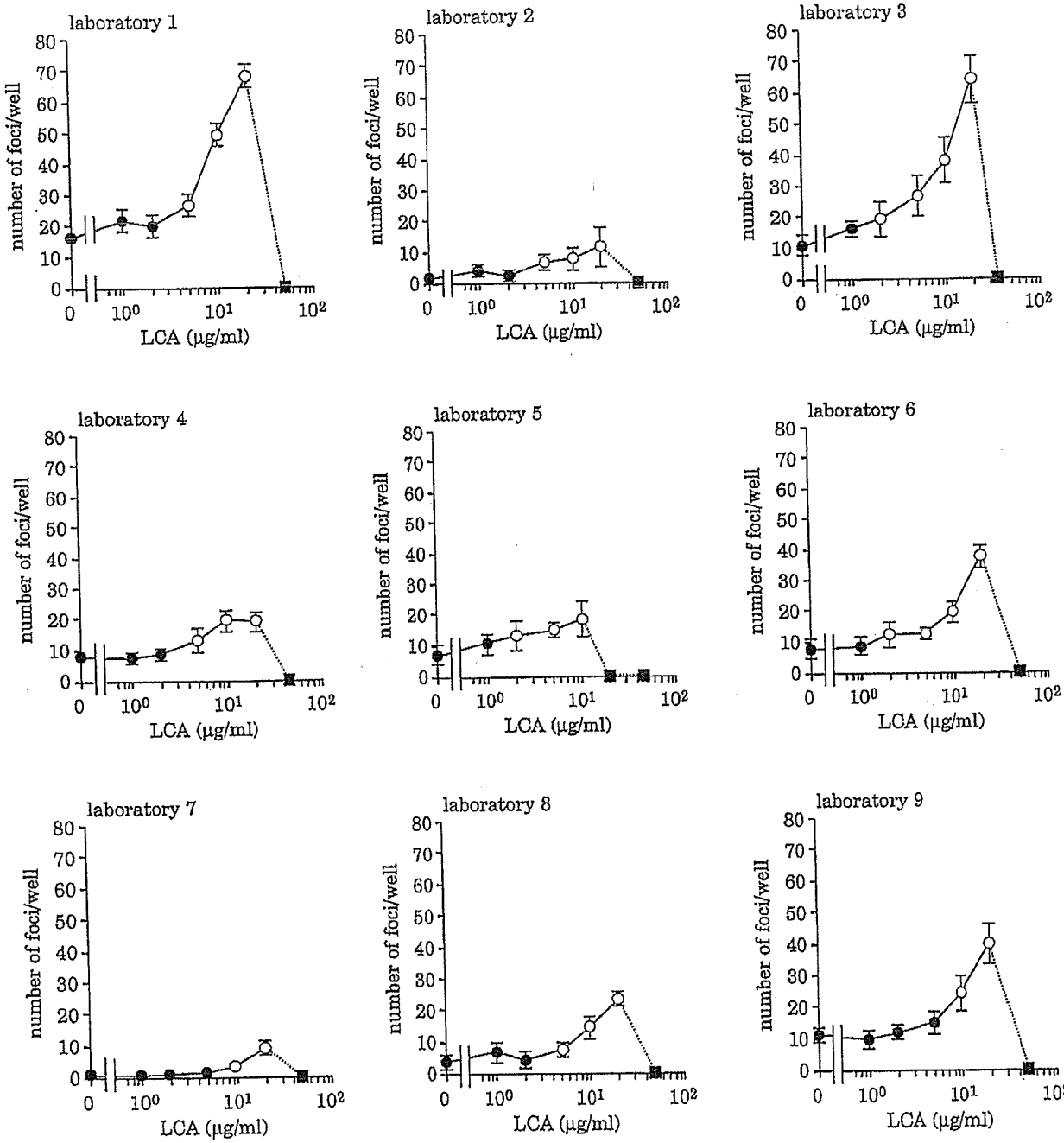
Table 1: Evaluation of the promotion assay for TPA, based on four criteria for transformation (Study II)

Lab No.	1: No. of concentrations with significant effect	2: Significant at consecutive concentrations	3: No. of concentrations showing a 3-fold increase	4: No. of concentrations showing a 2-fold increase	Judgement based on 1 + 2 + 3	Judgement based on 1 + 4
1	6	yes	4	6	+	+
2	2	yes	2	5	+	+
3	6	yes	4	5	+	+
4	5	yes	3	5	+	+
5	5	yes	4	6	+	+
6	4	yes	3	5	+	+
7	5	yes	5	6	+	+
8	5	yes	4	5	+	+
9	6	yes	5	6	+	+
10	5	yes	6	6	+	+
11	5	yes	4	5	+	+
12	5	yes	5	6	+	+

and Dulbecco's Modified Eagle's Medium/Ham's F12 Medium (1:1; DMEM/F12) was the product of GIBCO Laboratories (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Moregate (Bulimba, Queensland, Australia).

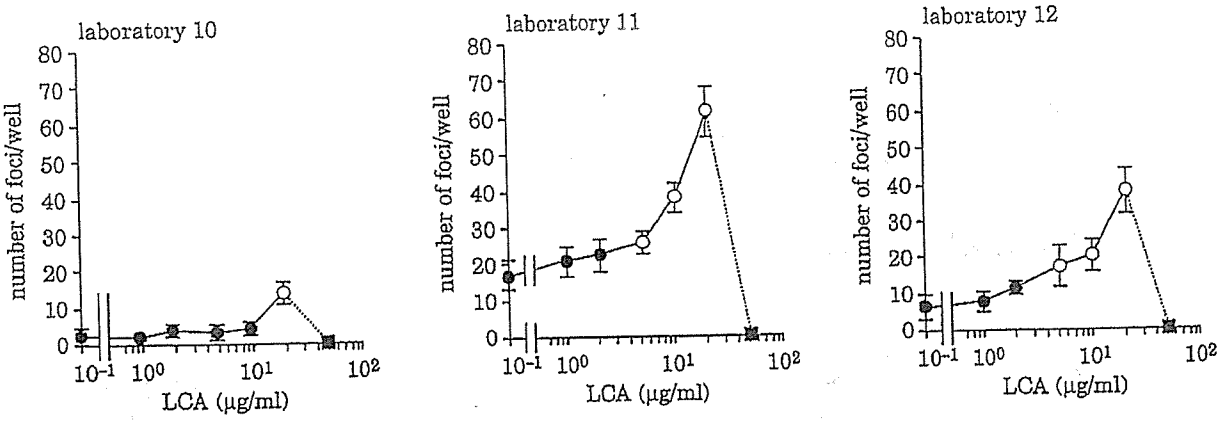
Bhas 42 cells were routinely cultured in a medium consisting of MEM supplemented with 10% FBS (M10F) at 37°C, in an atmosphere of 95% air and 5% CO_2. The cells were subcultured by using 0.25% trypsin (Wako Pure Chemical

Figure 3: Transformation frequencies with LCA in the Bhas promotion assay



○ = *significant point compared to control;* ■ = *point of cell death because of toxicity.*

Figure 3: continued



○ = *significant point compared to control;* ■ = *point of cell death because of toxicity.*

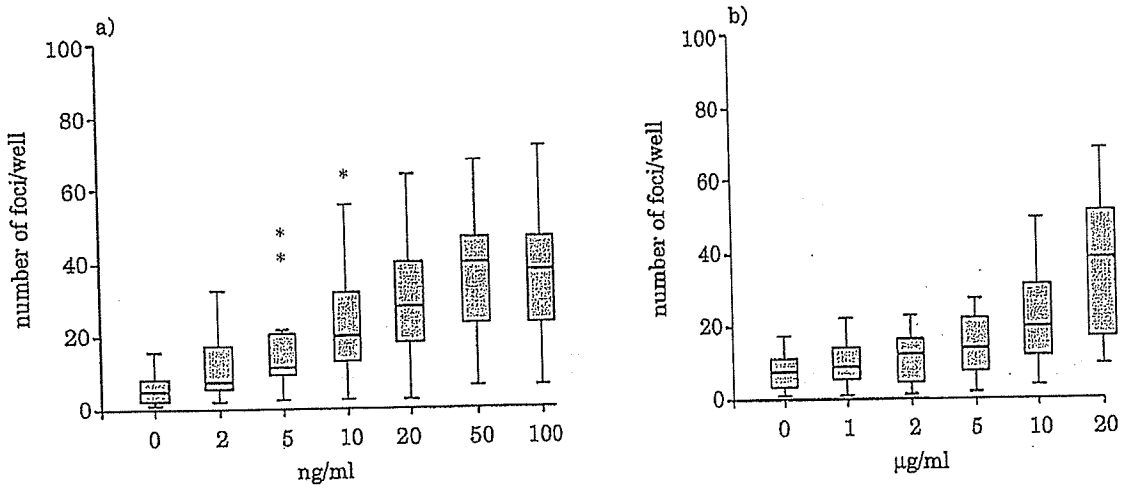
Industries, Osaka, Japan). It is necessary to keep the cell density at no more than 60–70% confluence. For the collaborative study, Bhas 42 cells were distributed to each laboratory by the management team. After the expansion of the number of cells in each laboratory, aliquots of the cells were kept frozen at -80°C. Each experiment was performed by using an aliquot of these stock cells.

Plastic culture dishes and plates were obtained from Sumitomo Bakelite (Tokyo, Japan), Corning Inc. (New York, NY, USA), Nalge Nunc International (Denmark) and Iwaki (Tokyo, Japan).

Test chemicals

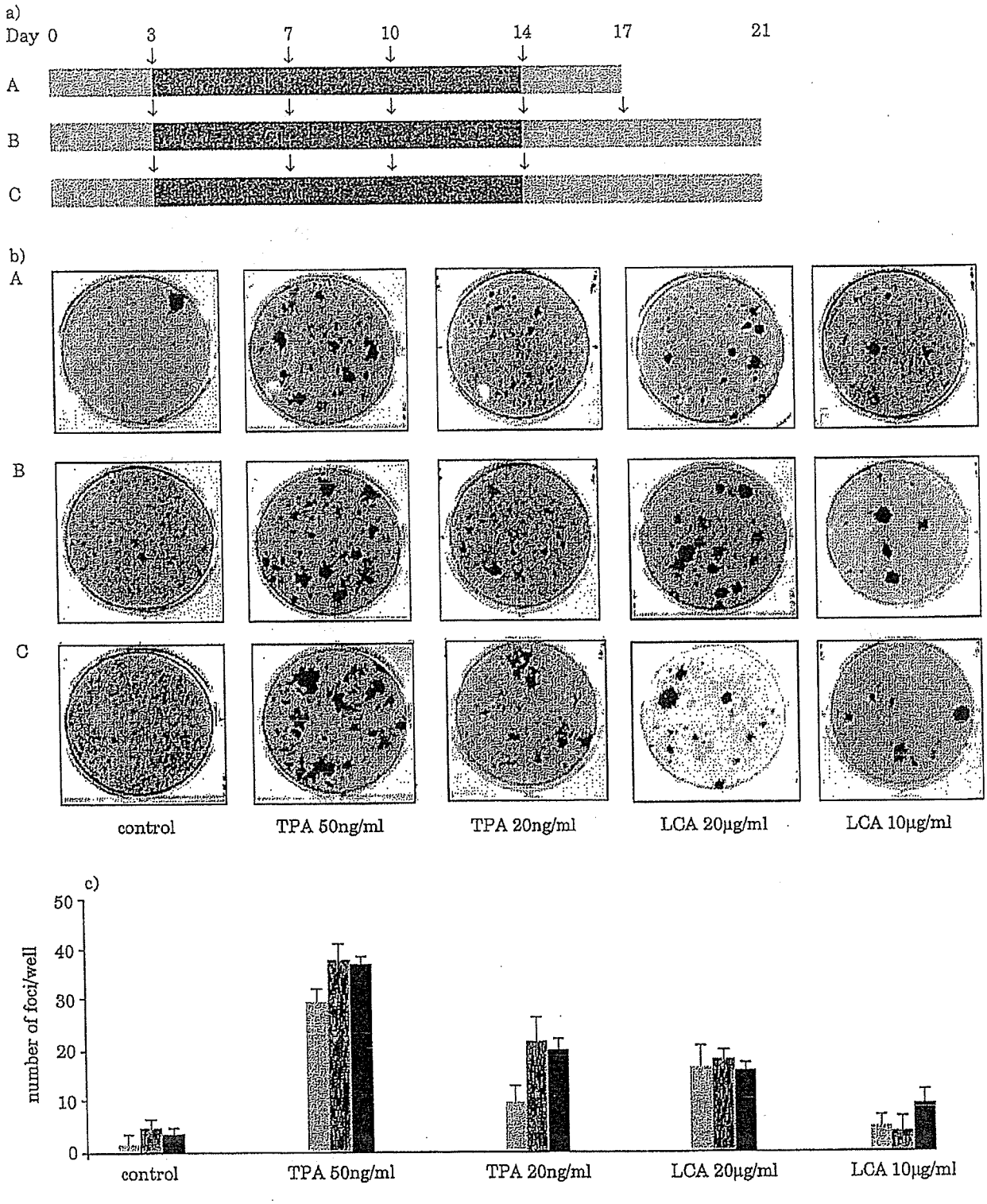
Catechol, dexamethasone, diethylstilboestrol (DES), lithocholic acid (LCA), okadaic acid, sodium saccharin and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 17β-oestradiol, insulin,

Figure 4: Box-whisker plots of transformation frequencies



From assays with a) TPA and b) LCA, conducted by 12 laboratories.

Figure 5: Effects of culture period on transformation frequencies with TPA and LCA



a) time schedules of Bhas promotion assay (dark grey boxes denote period of culture with medium containing TPA or LCA: arrows denote medium changes); b) Giemsa-stained wells of 6-well plates showing transformed foci; c) transformation frequencies induced with various time schedules.
= schedule A; = schedule B; = schedule C.

mezerein, phorbol 12,13-didecanoate (PDD), 4α-phorbol and progesterone were obtained from Sigma-Aldrich (St. Louis, MO, USA), and anthralin from Tokyo Kasei Kogyo (Tokyo, Japan). The purity level of 10 of the test chemicals was greater than 97%, that of LCA and TPA was greater than 95%, that of okadaic acid was greater than 85%, and that of insulin was 27USP units/mg.

The test chemicals, except insulin and sodium saccharin, were dissolved in dimethyl sulphoxide (DMSO, greater than 99.9%). Insulin was dissolved in 0.1mol/l hydrochloric acid, which, after more than 500 times dilution in the medium, had little effect on the cells. Sodium saccharin was dissolved directly in the medium. Chemical solutions were prepared by the management team and divided into aliquots, then distributed to the respective laboratories.

Cell growth assay for the determination of test concentrations

The crystal violet (CV) method was employed, as described previously (14). On Day 0, a cell suspension (0.5ml) of Bhas 42 cells in DMEM/F12 + 5% FBS (DF5F) at 2×10^4 cells/ml, was distributed into 24-well plates at 1×10^4 cells/well. On Day 3, the medium in each well was replaced with the medium containing the test chemicals. Three wells were used for each dose group. On Day 7, the cells were fixed with 10% formalin for 30 minutes, then washed with water, and stained with 0.1% CV solution for 30 minutes. After a thorough rinsing with water, the plates were dried. The stained CV in each well was extracted with 0.5ml of a solution consisting of 0.02mol/l HCl-50% ethanol. The optical density of each extract was measured at 540–570nm.

The chemical concentrations for transformation experiments were determined by using the results of the cell growth assays. Chemicals were classified into three types, according to their effects on cell growth. The first type of chemical (designated as T-type) exhibited marked enhancement of cell growth; the second type (L-type) showed cytotoxicity without growth enhancement; and the third type (M-type) promoted focus formation at concentrations having little effect on cell growth. TPA was included in the first group, and test concentrations for these chemicals were selected to cover from little effect on cell growth to growth enhancement. The L-type chemicals included LCA, and the M-type group comprised miscellaneous chemicals. With the latter two types, test concentrations were selected ranging from those exhibiting below 70% growth level to those having little effect on cell growth.

In the inter-laboratory collaborative study, the management team conducted trial cell-growth assays in advance for the test chemicals at tentative concentrations, set up according to the above principles. From these results, the test concentrations to be used by the participating laboratories were specified by the management team.

Bhas promotion assay

The original Bhas promotion assay protocol (14) was employed, with several modifications, as follows. Although M10F medium was used for routine cell culture, DF5F medium was used, not only for transformation experiments, but also for the mother cultures. In addition, it was critically important to use mother culture cells at around 60–70% confluence. A cell suspension of 2×10^4 cells/ml was prepared from a

Table 2: Evaluation of the promotion assay for LCA, based on four criteria for transformation (Study II)

Lab No.	1: No. of concentrations with significant effect	2: Significant at consecutive concentrations	3: No. of concentrations showing a 3-fold increase	4: No. of concentrations showing a 2-fold increase	Judgement based on	
					1 + 2 + 3	1 + 4
1	3	yes	1	2	+	+
2	3	yes	3	3	+	+
3	4	yes	2	3	+	+
4	3	yes	0	2	±	+
5	3	yes	0	3	±	+
6	4	yes	1	2	+	+
7	2	yes	2	2	+	+
8	3	yes	2	3	+	+
9	2	yes	1	2	+	+
10	1	no	1	1	±	+
11	3	yes	1	2	+	+
12	3	yes	2	3	+	+