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Isolation and Characterization of *ras*-Transfected BALB/3T3 Clone Showing Morphological Transformation by 12-O-Tetradecanoyl-phorbol-13-acetate

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The transformation frequency of mouse BALB/3T3 cells was significantly enhanced after transfection with an activated *ras* oncogene (v-Ha-*ras*) followed by treatment with a tumor promoter, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), suggesting that the *ras* oncogene acted as an initiator in two-stage carcinogenesis. A cell clone (Bhas42) containing the *ras* oncogene was isolated from the *ras*-transfected BALB/3T3 cells. Bhas42 cells were flat and showed contact inhibition, but the addition of TPA to quiescent Bhas42 cultures resulted in a dramatic change of cell morphology to spindle shape, doubling of the cell population, and increased DNA synthesis.

Key words: Transformation — *ras* oncogene — TPA — BALB/3T3

Multi-stage mechanisms are widely accepted in theories explaining the development of tumors in animals and humans, and at least two stages, initiation and promotion, have been demonstrated in many studies.[1–5] The initiation process is thought to include one or several mutational events which may occur in certain oncogenes.[6] Initiation is not supposed to lead to cancer by itself, but a subsequent treatment of the initiated cells with a tumor promoter, such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA), can efficiently produce cancer cells.[7] This process has been reproduced in *in vitro* transformation assay systems of cultured rodent cell lines, such as BALB/3T3 and C3H10T1/2.[2,8] Although the cell transformation assays are appropriate for the analysis of *in vitro* tumorigenicity, we can only see the end product of the multi-stage process. As a consequence of this feature of the *in vitro* systems, it has been difficult to separate each stage in the sequential course of events. It was demonstrated recently that C3H10T1/2 cells[9] and primary cultured cells of Chinese hamster[10] are transformed when transfected with an activated *ras* gene and subsequently treated with a tumor promoter. These results strongly suggest that a cell clone which has a *ras* oncogene but normal morphological characteristics can be isolated and should be readily transformed by TPA treatment. In this study we successfully isolated such clones, Bhas42 being one of them. This clone, established from mouse BALB/3T3 cells by transfection with an activated *ras* oncogene, showed normal cell morphology and contact inhibition, and was morphologically transformed by TPA treatment.

MATERIALS AND METHODS

Cell Lines and Cell Culture Mouse BALB/3T3 cells, A31-1-1 clone isolated by Kakunaga and Crow,[11] were obtained through the Japanese Cancer Research Resources Bank. The cells were cultured in Eagle's minimum essential medium (Nissui, Tokyo), supplemented with 10% FCS, in a humidified incubator at 37° in an atmosphere of 5% CO_2 in air.

Plasmid DNA A plasmid pBR322 DNA containing Ha-MuSV-DNA, clone H1 (v-Ha-*ras*, 11.5 kb), originally isolated by Ellis *et al.*,[12] was obtained from Dr. T. Hirakawa (Ajinomoto Co., Tokyo). A new *Bam*HI recognition site was introduced at the end of the inserted *ras* sequence (2.5 kb). pSV2-*neo*, constructed by Dr. E. Southern and Dr. P. Berg, was obtained through Dr. T. Hirakawa. The plasmid DNAs were prepared from *E. coli* HB101 by a modification of the cleared lysate method.[13,14]

Reagents Fetal calf serum was purchased from Filtron Pty. (Altoka, Victoria, Australia) and was prescreened for the absence of spontaneously transformed foci. TPA was purchased from Con-

solidated Midland Co., Brewster, NY., and was dissolved in acetone. 1α,25-Dihydroxycholecalciferol (1α,25(OH)$_2$D$_3$; biologically active vitamin D$_3$) was a product of Teijin Co. (Tokyo) and was dissolved in ethanol. Insulin, purchased from Sigma Chemical Co. (St. Louis, MO), was dissolved in 0.1*N* acetic acid. [^{3}H]Thymidine (specific activity 1 mCi/mmol) was obtained from Amersham International (Bucks, UK).

DNA Transfection and Detection of Transformed Foci DNA transfection was performed by the modified procedure reported by Wigler *et al.*[15] BALB/3T3 cells were seeded at a density of 10^6 cells per plate (diameter 100 mm) and cultured for 20 hr. The medium was replaced with fresh medium 4 hr before transfection. DNA solution (40 μg/ml in 240m*M* $CaCl_2$) containing equal amounts of *ras* DNA and calf thymus DNA was added drop by drop to an equal volume of HBS-PO$_4$ buffer (42m*M* HEPES, 274m*M* NaCl, 1.4m*M* NaH_2PO_4, 1.4m*M* Na_2HPO_4; pH 7.1) with gentle mixing by air bubbling. The mixture was kept at room temperature for 30 min. DNA (20 μg in 1 ml) was added to each dish and the cells were incubated with the DNA for 6 hr at 37°. The cells were then treated with MEM containing DMSO (30%) for 30 sec[16] and cultured for another 18 hr. The cells were treated with tumor promoters for 2 weeks and after that incubated without the promoters for another 3 weeks. At the end of the culture, six weeks after the transfection, the cells were fixed and stained with Giemsa and transformed foci were identified.[8]

Co-transfection[17] of v-Ha-*ras* DNA with pSV2-*neo* was carried out with 21 μg of DNA mixture containing 1 μg of pSV2-*neo* DNA (*ras*:*neo*= 10:1) as described above. Antibiotic G-418 was added to the cultures at 18 hr after the transfection and culture was continued for two weeks with medium changes twice a week. Among many G-418-resistant colonies, those which had flat shape were selectively cloned at the end of the culture.

DNA-DNA Hybridization The methods for DNA preparation and dot blot hybridization have been described previously.[18] The DNA was dissolved in water and was heated for 10 min at 95°, quickly chilled in an ice-water bath and incubated with an equal volume of 1*N* NaOH at room temperature for 20 min. After neutralization of the DNA sample, it was applied to a nitrocellulose filter. The filter was dried and baked for 2 hr at 80°.

The denatured ^{32}P-labeled probe DNA (10^6 cpm) labeled by nick translation methods[19] was applied to the blotted nitrocellulose filter in Denhardt's solution. The filter was incubated for 18 hr at 65° and was washed under a stringent condition. The filter was then exposed to X-ray film (Kodak X-OMAT RP film).

Southern blot hybridization experiment was done following the protocol reported by Southern.[20] High-molecular-weight DNA (10 μg each) was digested by restriction endonuclease *Bam*HI (50 units). The DNA sample was then loaded on agarose gel (1% agarose in 0.04*M* Tris-HCl, 0.002 *M* EDTA-2Na, 0.019*M* acetic acid). Lambda phage DNA digested with *Hin*dIII and ϕX174 phage DNA digested with *Hae*III were used as size markers. After the electrophoresis, the gel was soaked in 0.5*M* NaOH and 1.5*M* NaCl for 30 min followed by neutralization in 0.5*M* Tris-HCl and 1.5*M* NaCl for 30 min. The DNAs were then transferred to a nitrocellulose filter (Schleicher & Schuell type BA85) by the blotting technique. Hybridization was done as described above.

Measurement of DNA Synthesis in Quiescent Cultures The extent of DNA synthesis was measured in terms of the incorporation of [^{3}H]thymidine by pulse labeling for 12 hr.[8] Cells were plated at 10^5 cells/dish (diameter 35 mm) and cultured for 8 days to obtain quiescent cultures. Then the medium was changed to depleted medium which was prepared as reported before.[8] [^{3}H]Thymidine (0.2 μCi/dish) was added to the culture dishes every 12 hr for 7 days. At the end of the labeling, cells were lysed with 1*N* NaOH and the lysates were precipitated by 10% trichloroacetic acid. The precipitates were collected on glass fiber filters (Whatman GF/C). The filters were washed and dried, and the radioactivity was measured. In parallel with the measurement of DNA synthesis, cell growth was evaluated by counting cell numbers under a microscope.

RESULTS

Enhancement of Transformed Foci by Transfection with v-*ras*^{H} DNA Followed by TPA Treatment The transfection of BALB/3T3 cells with *ras* oncogene increased the number of transformed foci from 0 foci per dish, when only pBR322 DNA was transfected, to 4.8, as shown in Table I. Further increment of the number of transformed foci to 15.2 per dish was observed when the *ras* oncogene-transfected BALB/3T3 was treated with TPA (300 ng/ml). In addition, the size of the foci was much larger with TPA treatment than without TPA (Fig. 1). These results clearly indicate that TPA enhances the transformation of BALB/3T3 cells previously transfected with v-Ha-*ras* DNA.

Control experiments were performed by using NIH3T3 cells transfected with pBR322 and v-Ha-*ras* DNAs. When the pBR322 DNA

Table I. Transformation of BALB/3T3 and NIH3T3 Cells by v-Ha-*ras* DNA Followed by Treatment with Tumor Promoters

Recipient cells	DNA (μg/dish)	Promoters	Total No. of foci/ total No. of dishes	Foci/dish
NIH3T3 (JCRB0615)	Calf thymus (10) +pBR322 (10)	—	0/2	0
	Calf thymus (10) +v-Ha-*ras* (10)	—	38/2	14
BALB/3T3 (JCRB0601)	Calf thymus (10) +pBR322 (10)	Ethanol[a)]	0/5	0
		TPA	1/5	0.2
		1α,25(OH)$_2$D$_3$	0/5	0
		Insulin	0/3	0
	Calf thymus (10) +v-Ha-*ras* (10)	Ethanol[a)]	24/5	4.8
		TPA	76/5	15.2
		1α,25(OH)$_2$D$_3$	47/5	9.4
		Insulin	15/3	5.0

Concentration of chemicals added: ethanol, 0.5%; TPA, 300 ng/ml; 1α,25(OH)$_2$D$_3$, 5 μg/ml; insulin, 5 mg/ml.

a) Ethanol: Results for acetone and acetic acid are not shown, but were similar.

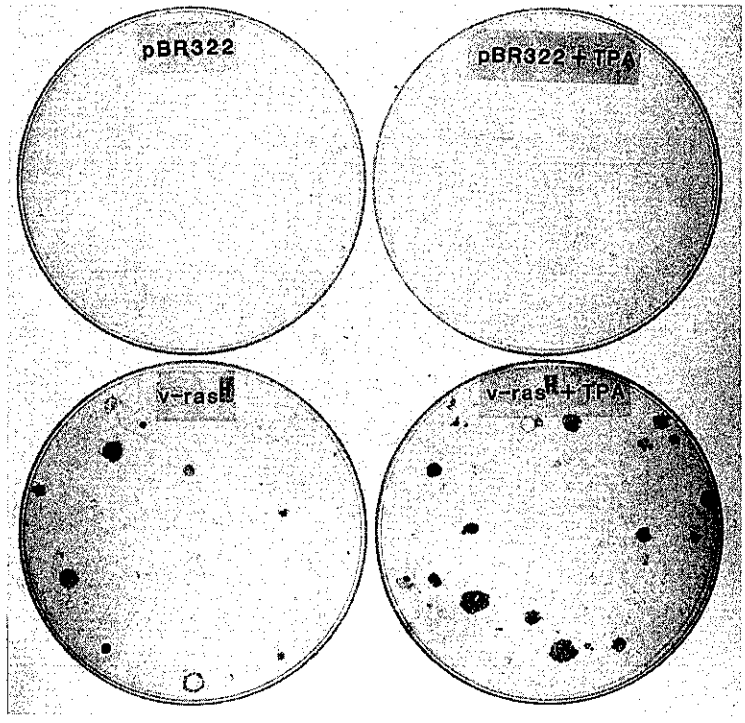

Fig. 1. Transformation assay with v-Ha-*ras* (v-*ras*^{H}) oncogene DNA in mouse BALB/3T3 cells. Either pBR322 or v-Ha-*ras* DNAs were transfected to BALB/3T3 cells 20 hr after seeding of 10^6 cells per 100 mm dish. Either *ras* or pBR322 DNAs (10 μg) were added to each dish with 10 μg of carrier DNA (calf thymus) in the presence of $CaPO_4$ and the dish was incubated for 6 hr. The cells were then treated with DMSO followed by treatment with or without TPA as described in "Materials and Methods." At 6 weeks after the addition of DNA, cells were fixed and stained.

was introduced into NIH3T3 cells, no significant increase in the number of transformed foci was observed (Table I). On the other hand, transformation of the NIH3T3 cells was clearly enhanced, without treatment by TPA, when v-Ha-*ras* DNA was used for the transfection.

We also studied the effects of two other chemicals, $1\alpha,25(OH)_2D_3$ and insulin, on the *ras*-oncogene transfected BALB/3T3 cells. Both compounds are known to be active as tumor promoters *in vitro*.[8, 21, 22] The number of foci induced by $1\alpha,25(OH)_2D_3$ was twice as high as in the control experiment. Transformation was not increased, in comparison with the control, when insulin was added to the medium.

We randomly picked up several foci induced by the v-Ha-*ras* DNA alone and by the combination of v-Ha-*ras* transfection and TPA treatment to confirm that the *ras* DNA is present in these BALB/3T3 cells. All cultures of these colonies retained their transformed morphology and grew to much higher densities than control BALB/3T3 cells. Total DNAs of these clones were then analyzed by the dot blot hybridization method with a probe for *ras*-oncogene, and they all showed acquisition of the probe sequence (Table II, clone numbers 5–7).

Isolation of BALB/3T3 Clone Containing v-Ha-*ras* DNA v-Ha-*ras* and pSV2-*neo* plasmid DNAs were mixed together in the ratio of 10:1 and transfected to BALB/3T3 cells by the calcium-phosphate method as described in "Materials and Methods." We repeated the transfection several times and obtained sixty clones which were resistant to G-418 and found that five clones had acquired the *ras* DNA sequence. Among these clones, three

Table II. Analysis of *ras* Sequence by Dot Blot Hybridization in Various Cell Clones

Clone number[a]	Cells	Treatment	Amount of DNA[b] 5	10	20 (μg)
1	BALB3T3	-			
2	BALB3T3	pSV2-neo			
3	BALB3T3(TF)	ras			
4	BALB3T3(TF)	ras			
5	BALB3T3(TF)	ras+TPA			
6	BALB3T3(TF)	ras+TPA			
7	BALB3T3(TF)	ras+TPA			
8	Bhas42	ras+pSV2-neo			
9	Bhas30	ras+pSV2-neo			

a) 1) Parental BALB/3T3 without exogenous DNA. 2) BALB/3T3 with pSV2-*neo* plasmid. 3, 4) Two independent BALB/3T3 clones transformed by v-Ha-*ras* oncogene. 5–7) Three independent BALB/3T3 clones transformed by v-Ha-*ras* oncogene with TPA treatment. 8) Clone Bhas42 established after co-transfection with v-Ha-*ras* and pSV2-*neo* DNAs. 9) Clone Bhas30 established after co-transfection with v-Ha-*ras* and pSV2-*neo* DNAs.

b) Dot blot hybridization was performed as described in the text.

clones showed non transformed characteristics and two clones showed transformed morphology. Typical clones having each characteristic were named Bhas42 and Bhas30, respectively. Both Bhas42 and Bhas30 cells were subcloned and used for the following experiments.

The doubling time of Bhas42 cells was prolonged to about 24 hr from the 15 hr of BALB/3T3 cells (Fig. 2). Bhas42 clone as well as control BALB/3T3 ceased their growth when they reached confluency several days after starting the cultures, showing flat-shaped non-transformed morphology. The morphology of these cells in the quiescent state is shown in Fig. 3. However, the doubling time of Bhas30 cells was equivalent to that of BALB/3T3 cells (Fig. 2), and this morphologically altered clone kept on growing up to sixteen days, although the growth rate slowed down once a confluent state was reached, indicating that Bhas30 is a transformed clone.

When TPA was added to either parental BALB/3T3 cells or BALB/3T3 cells transfected with pSV2-*neo* plasmid DNA, the cell morphology did not change, as shown in Fig. 3. We examined thirty BALB/3T3 clones resistant to G-418 and none of them showed morphological changes when treated with TPA.

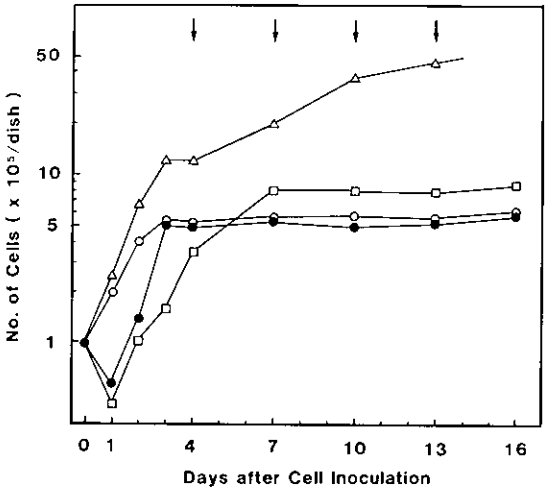

Fig. 2. Comparison of cell growth of various BALB/3T3 cell lines transfected with exogenous DNAs. Cells were seeded at a density of 10^5 cells/dish at day 0. (●) Parental BALB/3T3; (□) BALB/3T3 transfected with pSV2-*neo*; (○) Bhas42, BALB/3T3 transfected with v-Ha-*ras* and pSV2-*neo* (not transformed); (△) Bhas30, BALB/3T3 transfected with v-Ha-*ras* and pSV2-*neo* (transformed). Arrows indicate the timing of medium changes.

When TPA was applied to the quiescent Bhas42 cells, a dramatic change of morphology was observed as seen in Fig. 3F and Fig. 3H. Within 36 hr after the addition of TPA, all the quiescent cells which had been flat became spindle-shaped and no longer showed contact inhibition. The morphology of Bhas42 cells after TPA treatment appeared identical to that of cells obtained from foci of chemically transformed BALB/3T3. The Bhas42 maintained its sensitivity to contact inhibition and predisposition for morphological transformation by TPA after continuous subculturing without TPA treatment. Another characteristic of Bhas42 was its high spontaneous transformation frequency which was 100 times that of BALB/3T3.

We further examined the presence of integrated *ras* oncogene in both Bhas42 and Bhas30 by Southern blot hybridization. High-molecular-weight DNAs from these cells were digested by restriction endonuclease *Bam*HI and subjected to 1% agarose gel electrophoresis. DNAs were then transferred to nitrocellulose membrane filters and hybridization was carried out by using [^{32}P]thymidine-labeled *ras* oncogene as a probe. As shown in Fig. 4, 2.5 kb bands were seen in both Bhas42 and Bhas30, and the intensity of this band was much higher in Bhas42 than in Bhas30. In addition, multiple discrete bands other than 1.4 kb, 2.5 kb and 3.2 kb bands were observed only in Bhas42, indicating that the exogenous *ras* DNA was integrated in various sites of chromosomal DNA in Bhas42. No change in the banding pattern of Bhas42 was observed after the addition of TPA. Two distinct bands (designated as 3.2 kb and 1.4 kb in the figure), commonly observed in Bhas42, Bhas30 and control BALB/3T3 cells, were possibly derived from the intrinsic c-*ras* gene of mouse BALB/3T3 cells.

Enhancement of Cell Proliferation and DNA Synthesis of Bhas42 Clone by TPA Treatment

We further examined the TPA-induced transformation of Bhas42 cells by measuring the number of cells and DNA synthesis.

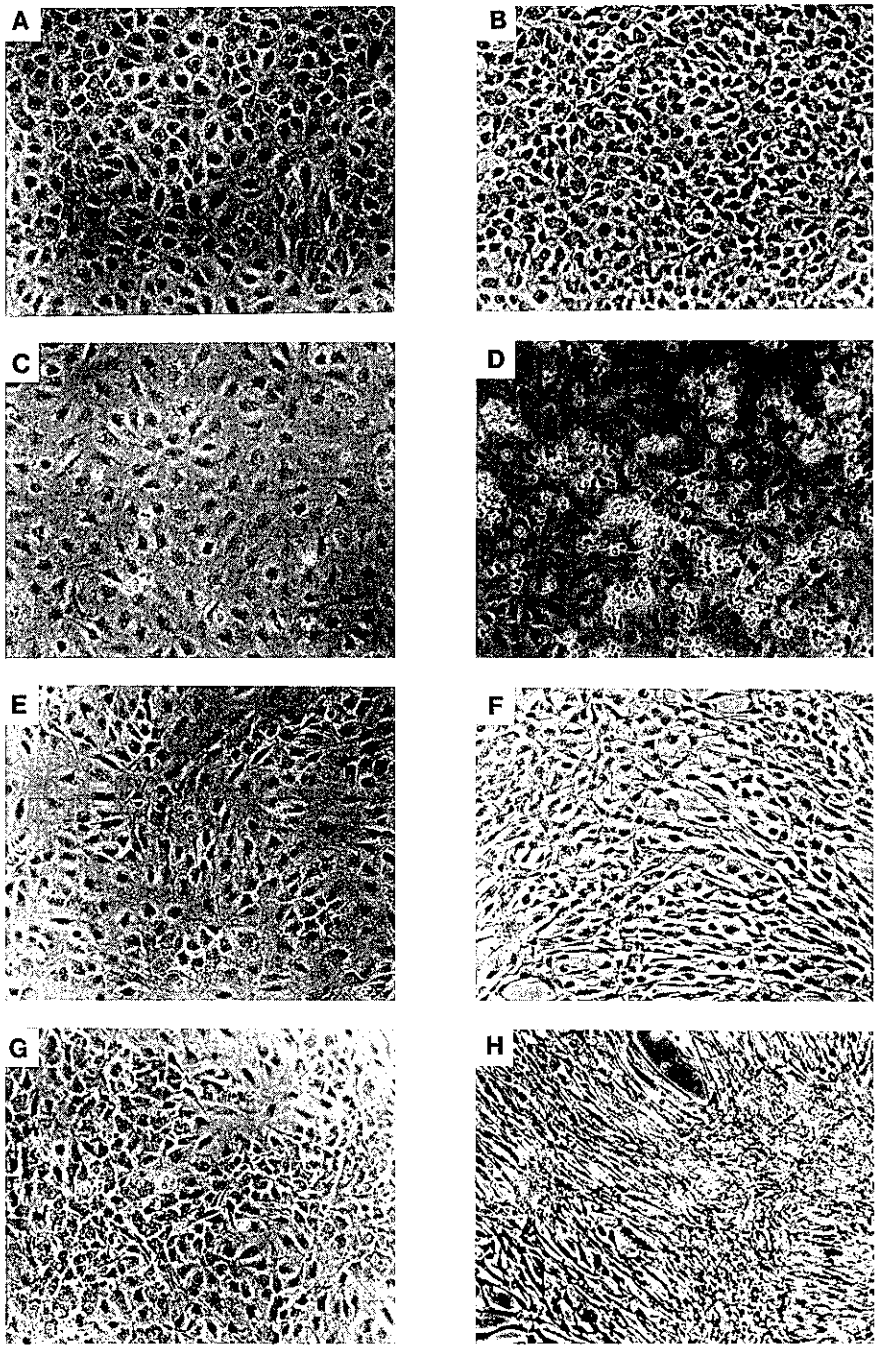

Fig. 3. Morphological changes of quiescent cells after the addition of TPA: (A) BALB/3T3 control at two weeks without TPA; (B) BALB/3T3 control at two weeks with TPA treatment; (C) BALB/3T3 with pSV2-*neo* at two weeks without TPA treatment; (D) BALB/3T3 with pSV2-*neo* at two weeks with TPA treatment; (E) Bhas42 control at 36 hr without TPA treatment; (F) Bhas42 at 36 hr with TPA treatment; (G) Bhas42 control at two weeks without TPA treatment; (H) Bhas42 at two weeks with TPA treatment.

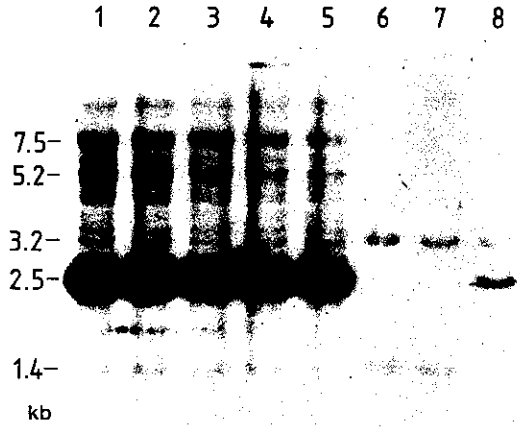

Fig. 4. Southern blot hybridization of Bhas42 and Bhas30 DNAs after digestion with restriction endonuclease *Bam*HI with *ras* sequence as a probe. All cells indicated in this experiment were prepared after they had reached the confluent state. 1 through 5, Bhas42. 1, day 0 (control). 2, One week without TPA. 3, Two weeks without TPA. 4, One week with TPA. 5, Two weeks with TPA. 6, BALB /3T3. 7, BALB/3T3 transfected with pSV2-*neo*. 8, Bhas30 prepared after confluent state. Lambda phage DNA digested with *Hin*dIII and ϕX174 phage DNA digested with *Hae*III were used as size markers.

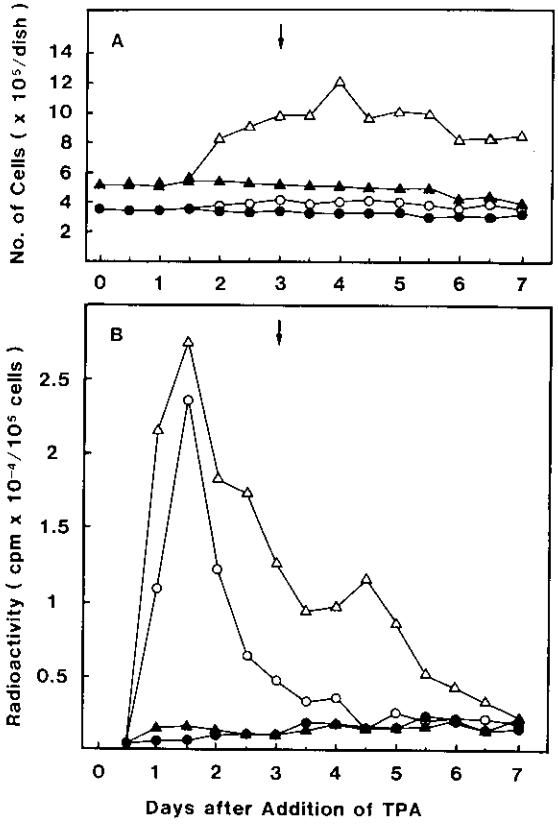

Fig. 5. Alterations in quiescent Bhas42 cells 0–7 days after TPA treatment. (A) Cell number, (B) DNA synthesis. (○) BALB/3T3 with TPA treatment; (●) BALB/3T3 without TPA treatment; (△) Bhas42 with TPA treatment; (▲) Bhas42 without TPA treatment. Arrows indicate medium changes.

Cultures of Bhas42 and BALB/3T3 cells were started at a density of 10^5 cells per 35 mm culture dish. When the cells had grown to reach contact inhibition at the density of about $3.5–5\times10^5$ cells/35 mm dish, 1 μg of TPA was added to both cultures. The number of cells and the amount of DNA synthesis were measured thereafter. The number of Bhas 42 cells was synchronously doubled within 36 hr after the addition of TPA, while cultures without TPA treatment showed no increase in cell number. As mentioned before, the TPA-transformed Bhas 42 cells were spindle-shaped and retained this morphology throughout the experiment (7 days after the addition of TPA). The number of BALB/3T3 cells did not change during one week with or without the addition of TPA (Fig. 5A) and no alteration was observed in their flat morphology.

DNA synthesis, measured at several occasions during the experiment by pulse labeling with [^{3}H]thymidine for 12 hr, was quickly increased after the addition of TPA in both Bhas42 and BALB/3T3 cells (Fig. 5B). The maximum incorporation of [^{3}H]thymidine was observed 36 hr after the addition of TPA in both cell clones.

Although the incorporation of thymidine in BALB/3T3 cells rapidly decreased within 4–5 days to the background level, Bhas42 cells retained their DNA synthesis at an elevated level until 7 days. No detectable incorporation of [^{3}H]thymidine was found in either Bhas42 or BALB/3T3 cells when TPA was not added to the cultures.

Discussion

It is clear from our results that the transformation of *ras*-transfected BALB/3T3 cells was enhanced by the addition of the tumor promoters, TPA and $1\alpha,25(OH)_2D_3$ (biologically active vitamin D_3). Similar results were reported by other authors using mouse C3H10T1/2 cells and primary cells of Chinese hamsters *in vitro*[9, 10] and a mouse skin system *in vivo*.[6] These findings indicate that *ras* oncogene can act as a tumor initiator both *in vitro* and *in vivo*. If this is the case, BALB/3T3 cells transfected by *ras* oncogene are expected to yield clones that show characteristics of initiated cells.

Although insulin is known to act as a tumor promoter, it had no enhancing effect on transformation, suggesting that the mechanism of action of this tumor promoter differs from that of the other two.

While the transformation of BALB/3T3 was clearly enhanced by TPA or $1\alpha,25(OH)_2D_3$ after the transfection with *ras* oncogene, the transformation was also induced by transfection of the *ras* oncogene alone (Table I). Since the transfection as such did not enhance the transformation frequency at all, *ras* oncogene is required in this process. It is difficult to specify the exact mechanisms by which *ras* oncogene may increase the spontaneous transformation of BALB/3T3 cells and in the Bhas42 clone.

The cell clone Bhas42 stopped its growth when the cells came in contact with each other (Fig. 2, Fig. 3 E and G), but was transformed morphologically by the addition of TPA (Fig. 3 F and H). Morphological alteration was also observed upon treatment of Bhas42 with $1\alpha,25(OH)_2D_3$ but not with insulin (unpublished data). These characteristics of Bhas42 cells were retained after subcloning. Thus, Bhas42 acted as a clone of initiated cells (in terms of morphology) in two-stage carcinogenesis. However, another cell clone, Bhas30, was permanently transformed right from the beginning, without TPA treatment. As the spontaneous transformation frequency of Bhas42 is much higher (approximately 0.01) than that of the parental BALB/3T3, the Bhas30 may have been generated from a cell clone similar to Bhas42 by spontaneous transformation, although the contents of *ras* sequence in the two clones were different.

At the moment, we cannot completely exclude the possibility that the Bhas42 clone may have appeared from a clonal variant with lower sensitivity to TPA or certain serum growth factors. However, no clone having characteristics like those of Bhas42 was obtained from BALB/3T3 transfected with *neo* alone. Thus we believe that *ras* oncogene in Bhas42 plays an important role in the transformation induced by TPA.

Southern blot hybridization showed many bands in Bhas42 (Fig. 5), indicating that the transfected *ras* sequences were integrated in more than one site of the chromosomes. One or several of these bands might be associated with the potential for transformation by TPA, although it is difficult to pinpoint the responsible band(s) among the many bands observed. The 2.5 kb band was observed in both Bhas30 and Bhas42, but the total DNA amount of this band was much higher in Bhas42. Since the 2.5 kb band is released from the intact plasmid DNA as indicated in "Materials and Methods," the 2.5 kb bands observed in both clones may have originated from plasmids integrated outside the two *Bam*HI recognition sites surrounding the *ras* sequence. The higher intensity of this band could be explained if the plasmid DNA frequently forms a concatemer after transfection, particularly when the DNA concentration is high.

The number of Bhas42 cells was increased two-fold at 36 hr after the addition of TPA (Fig. 5A) and cell division occurred synchronously. Morphological alterations were probably coupled with this cell division, since spindle-shaped cells also appeared at 36 hr after the addition of TPA. DNA synthesis was quickly stimulated by the addition of TPA in both Bhas42 and BALB/3T3 cells, indicating that the peak of DNA synthesis at 36 hr in Fig. 5A was not specific to Bhas42 cells. However, residual DNA synthesis was clearly maintained in Bhas42 until seven days after the addition of TPA. These results and the results of a preliminary study by flow cytometry (unpublished data) suggested that cell division was blocked near the end of the G_2 phase of the cell cycle in quiescent Bhas42, and the block was released synchronously by TPA. The cells appeared to proceed to the end

of another G_2 phase, where the cell division was blocked again. It seems that something else besides TPA is required for Bhas42 to exhibit continuous cell division.

As shown above, Bhas42 clone had characteristics of initiated cells, caused by the *ras* oncogene. Initiation associated with *ras* is probably only one pathway among many equivalent processes which may involve other oncogenes. Nevertheless, clone Bhas42 can now be used for substantial studies concerning the detailed mechanisms of the two-stage carcinogenesis initiated by *ras* oncogene.

Acknowledgments

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