Effect of tumor promoters, protease inhibitors, and repair processes on x-ray-induced sister chromatid exchanges in mouse cells

(12-*O*-tetradecanoylphorbol 13-acetate/malignant transformation/DNA repair/recombination/carcinogenesis)

HATSUMI NAGASAWA AND JOHN B. LITTLE

Department of Physiology, Harvard University School of Public Health, 665 Huntington Avenue, Boston, Massachusetts 02115

ABSTRACT   The induction of sister chromatid exchanges (SCE) in the second postirradiation mitosis was studied in mouse 10T½ cells irradiated with 400 rads (4 grays) and maintained in stationary growth for several hours after x-ray exposure (similar to liquid holding recovery experiments in bacterial cells). X-irradiation with no recovery period induced few SCE. With short recovery intervals, however, the SCE frequency rose in parallel with the increase in survival, reaching a maximum increase of 2-fold after 4 hr; SCE declined with longer recovery intervals. The influence of postirradiation incubation with the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and with the protease inhibitors antipain and leupeptin was studied on spontaneous, x-ray-induced (no recovery), and recovery-induced (4 hr) SCE. TPA (0.1 μg/ml and 1.0 μg/ml) increased the frequency of both spontaneous and direct x-ray-induced SCE, but not of recovery-induced SCE. Incubation with the protease inhibitors suppressed both TPA- and recovery-induced SCE, but had no effect on direct x-ray-induced SCE. These results are discussed in relation to the hypothesis that promotional events in carcinogenesis may involve the expression of mutational damage in cells by mitotic segregation.

Early experiments with mouse skin carcinogenesis demonstrated that repeated application of certain noncarcinogenic agents such as croton oil after treatment with a low dose of a known carcinogen could greatly enhance the frequency of induced·tumors (1–3). These results led to the hypothesis that carcinogenesis is a two-stage process involving both initiating and promoting factors. Classical promoting agents are neither carcinogenic nor mutagenic by themselves (3, 4), but they appear to complete a process begun by the initiating agent (3). The phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) has been shown to be one of the active components in croton oil. TPA has since been found to have a broad spectrum of biologic effects. These include effects on cell membrane function (5–7), the inhibition of cell differentiation (8–11), the enhancement of DNA, RNA, and protein synthesis (12, 13), and the induction of ornithine decarboxylase (14) and protease activity (15, 16).

Recently, Kinsella and Radman (17) presented the hypothesis that the tumor-promotion phenomenon is related to the induction of an aberrant mitotic segregation event allowing the expression by segregation of specific recessive genetic or epigenetic chromosomal changes present in initiated cells. Tumor promoters would act by inducing enzymes necessary for the genetic recombination that would lead to such segregational events. In support of this hypothesis, Kinsella and Radman (17) presented experimental evidence that the tumor promoter TPA induced sister chromatid exchanges (SCE) in cultured mammalian cells, whereas a nonpromoting TPA derivative did not. They assumed that the frequency of SCE could be considered

as a cytological indication of cellular recombinational activity (18, 19).

We have previously studied the induction of SCE in mammalian cells by x-rays (20). We found that while x-irradiation induced many chromosome aberrations, it was very inefficient by itself in inducing SCE. However, when the cells were placed under conditions favoring the recovery from potentially lethal damage (similar to liquid holding recovery in bacterial cells) for several hours after irradiation, a significant enhancement in SCE was observed (20). In the present investigation, we have examined the effect of incubation with TPA and two protease inhibitors on spontaneous, x-ray-induced and "recovery-induced" SCE.

MATERIALS AND METHODS

Cell Culture. C3H 10T½ clone 8 mouse embryo-derived fibroblasts (21, 22) were maintained at 37°C in a humidified 95% air/5% CO_2 atmosphere in Eagle's basal medium (BME, GIBCO catalogue no. F-15) supplemented with 10% fetal calf serum inactivated for 30 min at 56°C, penicillin (50 units/ml) and streptomycin (50 μg/ml). Cells used were from passages 8–13. For experiments, 10^5 cells were seeded in T-30 Falcon plastic flasks and returned to the incubator until they reached confluency (about 10^6 cells per flask). The culture medium was then changed at daily intervals, and the experiment was begun on the third day. By this time, the cells were in stationary growth. Only 0–3% of the cell population was in the DNA synthesis phase as determined by pulse-labeling with [³H]thymidine, and the addition of fresh medium no longer acted as a stimulus to DNA synthesis or cell division.

X-Irradiation. The cells were irradiated aerobically while in confluent (stationary) growth with a General Electric Maximar x-ray generator operating at 220 kV and 15 mA with 1 mm-Al added filtration, yielding an absorbed dose rate of 80 rads/min (1 rad = 0.01 gray). Immediately after irradiation either the cultures were trypsinized and the cells were distributed in fresh medium at low density into four T-30 Falcon flasks or the medium was renewed and the cultures were returned to the incubator for 4-hr repair incubation prior to trypsinization.

SCE. After trypsinization the cells were distributed into four T-30 Falcon flasks at a density of about 3×10^5 cells per flask and incubated with complete medium to which the thymidine analogue 5-bromodeoxyuridine (BrdUrd) had been added to a final concentration of 10 μM. BrdUrd is incorporated into the newly synthesized daughter strand of DNA. The cells were incubated with BrdUrd for two rounds of cell replication (40–48 hr), allowing BrdUrd substitution in both DNA strands in one chromatid, but only in one strand of its sister chromatid. Colchicine at a final concentration of 2 μM was added to each

Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; SCE, sister chromatid exchanges; BrdUrd, 5-bromodeoxyuridine.

culture 4–5 hr before fixation in order to arrest the cells in metaphase. Mitotic cells were fixed by the hypotonic method and the chromosomes were spread by air-drying (23).

The chromosomes were stained by the fluorescence plus Giemsa technique (24) for the differential staining of sister chromatids. They were initially stained in the fluorochrome Hoechst 33258 (final concentration 5 μg/ml in double-distilled water) for 20 min at 24°C, rinsed in water, then mounted with phosphate buffer at pH 6.8. The slides were then exposed to black light from a bank of General Electric 15T8/BL bulbs in order to allow the photochemical reaction to occur whereby the chromatids fluoresce differentially. Hoechst 33258 fluoresces more efficiently when bound to poly(dA-dT) than when bound to poly(dA-BrdUrd). Finally, the slides were stained for 10 min in 3% Giemsa solution, allowing recognition of SCE among the differentially stained sister chromatids. The stained mitotic preparations were photographed through a light microscope, and SCE were scored directly from the photographic negatives. In each experiment, the number of SCE in 20–30 mitotic cells were scored for each data point. Because the 10T½ cells are aneuploid (near tetraploid), the results were expressed as the mean frequency of SCE per chromosome.

TPA and Protease Inhibitors. TPA (lot 007) was obtained from Consolidated Midland Co. (Brewster, NY). A stock solution containing 5 mg/ml was made with spectranalyzed grade acetone (Aldrich) and kept in amber bottles at −20°C until used in experiments. Its potency was checked by measuring its ability to enhance transformation induced in 10T½ cells by 100 rads of x-rays (25). The protease inhibitors antipain and leupeptin were kindly provided by T. Matsushima, Institute of Medical Sciences, University of Tokyo. Stock solutions containing either agent at 10 mg/ml were prepared in double-distilled water and kept at −20°C prior to use. TPA and protease inhibitors were further diluted in complete medium; the cells were incubated in their presence beginning immediately after the postirradiation subculture until fixation 44–48 hr later.

RESULTS

Spontaneous and X-Ray-Induced SCE. Fig. 1 presents the results of an experiment in which replicate stationary cultures of 10T½ cells were irradiated with either 0 to 400 rads, then subcultured at various intervals from 0 to 24 hr later at low density into BrdUrd-containing medium in which they were maintained for 48 hr before the SCE frequency was measured. As can be seen in Fig. 1, the spontaneous SCE frequency was about 0.1 per chromosome; this result is typical for many experiments performed with 10T½ cells. X-irradiation with 400 rads (no recovery) enhanced SCE about 20–30% over base line (spontaneous) levels. When the cells were allowed several hours of repair incubation, however, the SCE frequency increased; a maximal enhancement of about 2-fold was seen with a recovery interval of 4 hr. The frequency of SCE declined with longer recovery times, reaching baseline levels by 12–24 hr. A recovery interval of 4 hr or longer prior to subculture of cells exposed to 400 rads led to about a 2.5-fold enhancement in cell survival in these experiments.

Effect of TPA. The influence of incubation with two concentrations of TPA on spontaneous, direct x-ray-induced (0 hr) and recovery-induced (4 hr) SCE is shown in Table 1. Exposure to TPA at either concentration for 48 hr had no toxic effect on the cells as measured by colony-forming ability. Previous results (25) showed that incubation with 0.1 μg of TPA per ml for 2 weeks had no effect on the viability of 10T½ cells. TPA did appear to stimulate cell proliferation in these experiments. Therefore, in order to obtain the optimal number of mitotic cells that had gone through two generations in the presence of
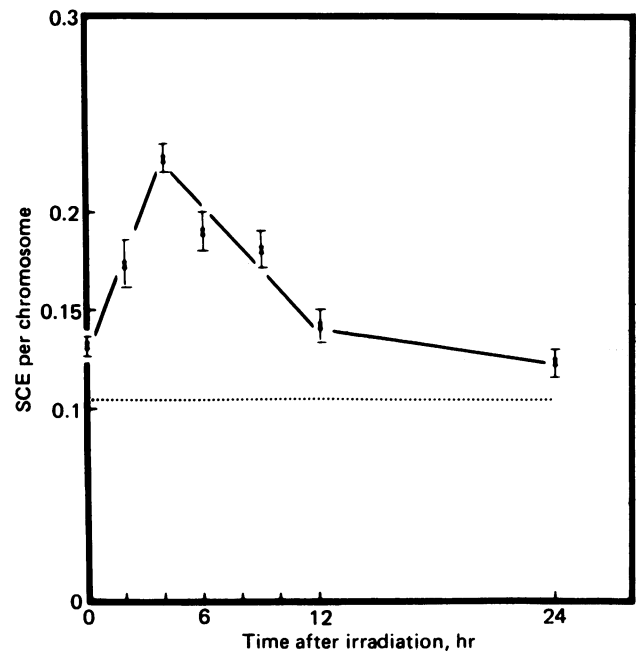

FIG. 1.   Induction of SCE in mouse 10T½ cells irradiated with 400 rads while in stationary growth and allowed recovery intervals of 0–24 hr before subculture at low density. The cells were harvested and SCE were measured after two rounds of replication. The horizontal dotted line represents the spontaneous SCE frequency.

BrdUrd, replicate cultures incubated with TPA were fixed at several intervals between 36 and 48 hr after subculture at low density into BrdUrd- and TPA-containing medium. The optimal incubation time was 48 hr for control cultures and 44 hr for TPA-treated cultures. Three experimental points in Fig. 1 were studied: spontaneous (0 rad) exchanges; SCE induced by 400 rads with no recovery (immediate subculture); and SCE induced by 400 rads followed by a 4-hr recovery interval prior to subculture.

As can be seen in the first column of Table 1, TPA at a final concentration in the medium of 0.1 or 1.0 μg/ml increased the frequency of spontaneous SCE. TPA also appeared to enhance direct x-ray-induced SCE (column 2, Table 1) but to have no significant effect on the frequency of recovery-induced exchanges (column 3). In order to determine whether these changes in SCE frequencies might involve only a fraction of the cell population, we examined the effect of TPA (1 μg/ml) on the distribution of SCE among cells in the three experimental groups. The results are presented in Fig. 2. Both TPA and irradiation appear to have led to an increase in the exchange

Table 1. Effect of TPA on spontaneous and x-ray-induced SCE*

| TPA, | | 400 rads[†] | |
μg/ml	0 rads	0 hr	4 hr
0	0.105 ± 0.005 (1%)	0.135 ± 0.007 (2.2%)	0.194 ± 0.01 (0%)
0.1	0.168 ± 0.007 (0%)	0.175 ± 0.007 (3.8%)	0.181 ± 0.006 (3.4%)
1	0.199 ± 0.007 (3.8%)	0.172 ± 0.005 (10.5%)	0.192 ± 0.009 (7.8%)

* Results expressed as mean number of SCE per chromosome ± 1 SEM of pooled data from four separate experiments, and represent a total of 75–100 cells for each point. Figures in parentheses are the percentage of cells containing chromosomes with 3 or more SCE.
† Zero hr, no recovery interval (immediate subculture); 4 hr, 4-hr recovery interval (delayed subculture).
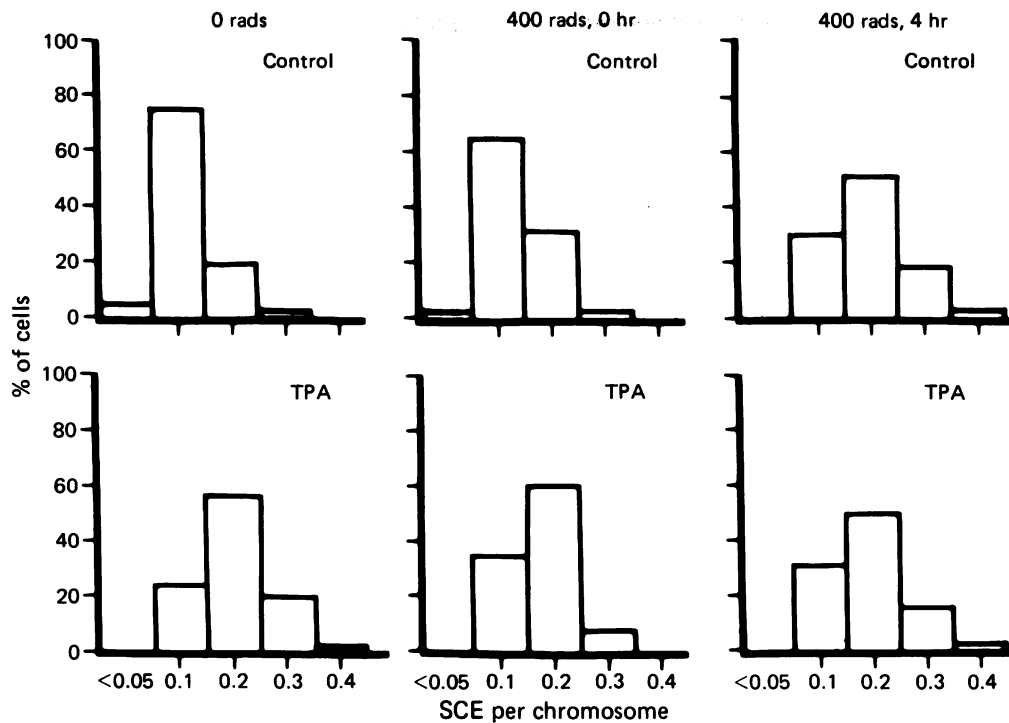
FIG. 2. Cellular distribution of spontaneous, x-ray-induced, and recovery-induced (4 hr) SCE in control and TPA-treated (1.0 μg/ml) cultures. Results are pooled from four separate experiments and expressed as the mean number of SCE per chromosome; 75–100 cells were scored for each group.

frequency among all cells, though the distribution appears slightly broader in the irradiated groups allowed to recover for 4 hr. A subpopulation of cells with a great many SCE, such as was reported by Kinsella and Radman (17) in TPA-treated cultures, was not observed in these experiments.

While the distribution of SCE among cells appeared to increase randomly, their distribution among individual chromosomes did not. Data are presented in Table 1 that indicate the fraction of cells in each experimental group that contained chromosomes with three or more exchanges on them. This fraction ranged as high as 10.5% in TPA-treated cultures. If SCE production were a random event, the probability by Poisson statistics that a single chromosome would contain 3 or more SCE is below 1% for a mean frequency of 0.1–0.2 SCE per chromosome.

Effect of Protease Inhibitors. The effects of incubation with the protease inhibitors antipain and leupeptin on spontaneous, TPA-, and x-ray-induced SCE are tabulated in Tables 2 and 3.

Incubation with these inhibitors for 48 hr at a concentration of 50 μg/ml had no influence on cell viability in any of these experimental groups. The experimental design was similar to that in Table 1; the data were pooled from three separate experiments, each of which showed the same results. The effects of antipain and leupeptin were similar. Incubation with these inhibitors for the 44- to 48-hr interval after subculture completely suppressed the enhancement in SCE induced by TPA in the spontaneous and 0-hr x-ray groups. A comparison of the 0- and 4-hr recovery groups (Tables 2 and 3) indicates that they also suppressed the enhancement in the SCE frequency that occurred in x-irradiated cells allowed 4 hr repair-incubation before subculture (recovery-induced SCE) (Fig. 1 and Table 1). On the other hand, they do not appear to have suppressed the direct x-ray-induced exchanges (0-hr group); this is particularly evident in the antipain experiments. The significance of the small but consistent increase in spontaneous SCE in the cells incubated with the protease inhibitors is not clear.

The effect of incubation with antipain on the distribution of

Table 2. Effect of incubation with the protease inhibitor antipain on x-ray- and TPA-induced SCE*

TPA, μg/ml	0 rads	400 rads	
		0 hr	4 hr
0	0.122 ± 0.006 (1.1%)	0.148 ± 0.001 (0%)	0.140 ± 0.007 (1.9%)
0.1	0.111 ± 0.005 (1.3%)	0.146 ± 0.007 (0%)	0.138 ± 0.006 (3.5%)
1	0.121 ± 0.007 (0%)	0.131 ± 0.007 (1.9%)	0.131 ± 0.006 (1.5%)

* Experimental plan and results expressed as in Table 1. Pooled data from three separate experiments representing a total of 65–95 cells for each group. All groups were incubated with antipain (50 μg/ml) after subculture.

Table 3. Effect of protease inhibitor leupeptin on x-ray- and TPA-induced SCE*

TPA, μg/ml	0 rads	400 rads	
		0 hr	4 hr
0	0.127 ± 0.006 (0%)	0.139 ± 0.007 (1.1%)	0.121 ± 0.007 (0%)
0.1	0.125 ± 0.006 (0%)	0.138 ± 0.007 (0%)	0.135 ± 0.007 (1.6%)
1	0.119 ± 0.005 (1.2%)	0.122 ± 0.006 (1.5%)	0.131 ± 0.005 (0%)

* Experimental plan and results expressed as in Table 2. Pooled data from three separate experiments. All groups were incubated with leupeptin (50 μg/ml) after subculture.
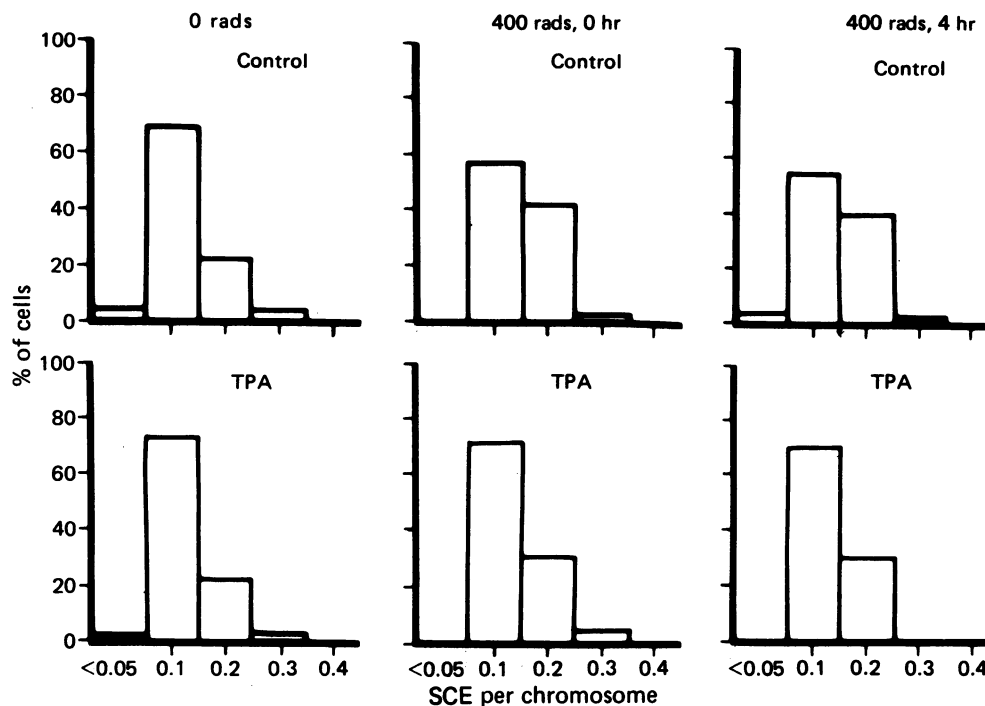
FIG. 3. Cellular distribution of spontaneous, x-ray-induced, and recovery-induced (4 hr) SCE in control and TPA-treated (1.0 μg/ml) cultures incubated in the presence of 50 μg of antipain per ml. Results are pooled from three separate experiments; 65–95 cells were scored for each group.

SCE among cells is shown in Fig. 3. The changes in the distribution of SCE as compared with Fig. 2 again appear to have involved the entire cell population.

DISCUSSION

When cultured mammalian cells are irradiated with x-rays or UV light while in the density-inhibited stationary phase of growth, and subculture to low density (a stimulus to cell proliferation) is delayed for several hours, an enhancement occurs in cell survival. This phenomenon has been termed recovery from potentially lethal damage (26, 27). It is associated with a decline in mutations after UV exposure (28, 29), and appears to reflect the activity of molecular DNA repair processes (30)—in particular the excision repair pathway for UV-induced damage (30, 31). This phenomenon is thus similar to liquid holding recovery in bacterial cells.

When such recovery experiments were carried out with malignant transformation as the end point, results almost identical to those in Fig. 1 were found (27); the transformation frequency induced by 400 rads of x-rays rose about 3-fold with recovery intervals of 3–4 hr, but declined with longer recovery periods. This observation suggests that a relationship exists between the induction of SCE and malignant transformation. On the other hand, the frequency of gross chromosomal aberrations declined steadily with increasing repair time, reaching a minimum at 4–6 hr; the decline in aberration frequency paralleled the enhancement in cell survival (20). On the basis of these results, the hypothesis has been proposed (32) that the changes in the transformation and SCE frequencies that occur with increasing recovery intervals result from the action of two distinct DNA repair processes that influence the fixation of the initial DNA damage: a rapid process acting primarily on lethal lesions such as strand breaks and a slower process acting primarily on mutational lesions such as base damage. The present results suggest an alternative hypothesis, which relates this phenomenon to promotional events.

First, these results confirm those of Kinsella and Radman (17) indicating that exposure to tumor promoters alone can induce

SCE, and that this induction is suppressed by protease inhibitors. We found a significant elevation in the SCE frequency after incubation with TPA alone at 0.1 μg/ml. This concentration was well below the level that affected the viability (cloning efficiency) of 10T½ cells. Kinsella and Radman (17) hypothesized that TPA acts by inducing the enzymes necessary for genetic recombination and that these same enzymes are involved in the production of SCE. We suggest that these enzymes may also be induced by x-irradiation, if the cells are placed under suitable recovery conditions. If this were true, an increase in SCE during recovery would be expected.

The hypothesis that the same underlying mechanism is responsible for both TPA-induced SCE and the enhancement in SCE induced during liquid holding recovery from x-ray damage is supported by two experimental observations reported herein. First, incubation with TPA did not further enhance the SCE frequency seen in irradiated cells allowed a 4-hr recovery period (Table 1). Second, the protease inhibitors suppressed both TPA-induced and recovery-induced SCE, but not direct x-ray-induced SCE (Tables 2 and 3).

The finding that the induction of SCE by TPA was suppressed by the protease inhibitors is of particular interest in light of the recent observation (33) that TPA-enhanced malignant transformation of x-irradiated 10T½ cells was inhibited by incubation with antipain or leupeptin during the expression period. Protease inhibitors have also been shown to suppress tumor promotion by TPA *in vivo* (34, 35). Protease enzymes appear to be closely associated with the development of malignant transformation. TPA has been shown to lead to the induction of plasminogen activator in tissue culture (16). The exact role of proteases in the carcinogenic process remains unclear, however, though it has been recently proposed that it may involve the derepression of a variety of genes via proteolytic cleavage of protein repressors (36). A particularly interesting example is the involvement of proteases in the induction of error-prone DNA repair in bacterial cells (36, 37). This repair process can be inhibited by incubation with the protease inhibitor antipain (37).

Genetics: Nagasawa and Little

Proc. Natl. Acad. Sci. USA 76 (1979) 1947

The enhancement in the frequency of SCE induced by TPA is relatively small compared with the levels induced by certain chemical agents such as mitomycin C. It might therefore be argued that it is due to some nonspecific toxic effect of the agent on the cells. We could not demonstrate any effect on the viability of 10T½ cells at the concentrations employed. Furthermore, incubation with TPA at 0.1 μg/ml has been shown to be nonmutagenic (38) and to induce neither chromosome aberrations (17) nor malignant transformation (25, 39) in mammalian cells. Finally, Kinsella and Radman (17) found that the nonpromoting TPA derivative 4-O-methyl-TPA, which was equally as toxic as TPA, did not induce SCE. Because of the nature of the technique, we can only measure the frequency of SCE induced during two rounds of replication. The promoting effect may be associated with the successive crossing-over that would occur during the cell proliferation accompanying the prolonged exposure to TPA that is required for tumor promotion *in vivo* (1–3) or *in vitro* (25, 39).

As can be seen in Table 1, the distribution of SCE among chromosomes was not random in TPA-treated cells. Unlike Kinsella and Radman (17), however, we found SCE to be randomly distributed among cells with no evidence for a subpopulation containing a very high frequency of SCE. This discrepancy may be attributed to differences between cell lines (they used Chinese hamster V-79 cells), or to the fact that their BrdUrd concentrations were 3- to 10-fold higher than ours.

The present results suggest that the hypothesis bears further investigation that one mechanism for the promotion of carcinogenesis induced by physical and chemical agents may involve the facilitation of expression of mutational damage in cells by mitotic segregation. Furthermore, the results shows how this mechanism may be involved in the induction of transformation by x-rays. To substantiate these hypotheses will require the demonstration that genetic recombination as well as such segregational events actually occur in mammalian cells. Another phenomenological approach would be to examine the extent to which other agents that induce SCE act as tumor promoters.

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