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12-O-Tetradecanoylphorbol-13-Acetate (TPA)-Induced Gene Sequences in Human Primary Diploid Fibroblasts and Their Expression in SV40-Transformed Fibroblasts

Peter Angel, Hans Jobst Rahmsdorf, Annette Pöting, Christine Lücke-Huhle, and Peter Herrlich

Kernforschungszentrum Karlsruhe, Institut für Genetik und Toxikologie, Postfach 3640, D-7500 Karlsruhe 1, Federal Republic of Germany

We have isolated cDNA sequences from TPA-treated primary human fibroblasts, which indicate RNA species that are coordinately regulated after treatment of these cells with either ultraviolet light, mitomycin C, the UV-induced factor EPIF, or TPA. The levels of RNA are elevated in Bloom syndrome (cells of two out of three patients). After transformation with SV40 one of the sequences is overexpressed while another one is reduced. Both genes maintain their inducibility by the agents mentioned.

Key words: human genetic stress response, Bloom syndrome, metallothionein IIa, UV-induced extracellular protein-inducing factor, cDNA cloning

The complete transformation of primary fibroblasts into tumor cells has been dissected into several genetic steps. The first step—immortalization—can be imitated by the experimentally induced expression of any one of a number of genes [1–5] and can be induced by treatment with carcinogens [7]. Immortalized cells become tumorigenic if transfected by one member of a second group of genes [1–7] or if treated with a tumor promoter [8]. These observations suggest that the process of complete transformation requires at least two steps. The involvement of additional steps is likely, eg, the complete transformation of immortalized cells by carcinogens seems to require two events, a frequent and a rare one [9].

Although current views implicate constitutive expression of a number of genes in the transformed cell [10–14], it is conceivable that carcinogens and tumor pro-

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moters act by inducing transient functions, eg, a mutator function. This possibility may be supported by the spontaneously elevated mutation rate in the human cancer risk disease Bloom syndrome [15,16].

We have observed earlier that skin fibroblasts from patients with Bloom syndrome express a number of proteins at an elevated rate which normal cells express only when treated with a tumor promoter or with a carcinogen [17,18]. We have used molecular hybridization and cDNA cloning techniques to define RNA sequences that change in abundance after TPA (12-O-tetradecanoylphorbol-13-acetate) treatment. We show that these RNAs are coordinately induced by either carcinogen or TPA treatment and that their expression is elevated spontaneously in cells from two of three patients with Bloom syndrome. Upon transformation of human skin fibroblasts with SV40, the expression of one of the sequences remains elevated. This RNA codes for metallothionein and its elevated expression appears to confer better surviving ability after gamma-ray, X-ray or α-ray treatment. Another RNA species coding for a secreted protein is reduced in the transformed cell. Inducibility by TPA or by the UV-induced factor EPIF [18] is maintained.

MATERIALS AND METHODS

Cells and Culture Conditions

Most cells and their origin have been described [17,18]. SV40-transformed normal human fibroblasts (GM 637) and Xeroderma pigmentosum group A fibroblasts (XP12 Ro) were obtained from JE Cleaver (San Francisco, CA); XPGM 2994 were from the Human Genetic Mutant Cell Repository (Camden, NJ). The cells were grown in monolayers using Dulbecco's minimal essential medium supplemented with 15% fetal calf serum and antibiotics. The pH was maintained at 7.2 and the temperature at 37°C. To ensure optimal growth conditions, cells were seeded at 5×10^5/10-cm petri dish and the experiments performed 72 hr later.

RNA Purification

Cells were lysed in 7 M urea, 2% SDS, 0.35 M NaCl, 1 mM EDTA, 10 mM Tris HCl, pH 8.0, and the nucleic acids were extracted several times with phenol-chloroform. The RNA was obtained as pellet by centrifugation through CsCl [19]. The RNA pellet was taken up in 10 mM Tris HCl, pH 7.5, and precipitated by ethanol. Poly A^+ RNA was purified on oligo-dT-celulose [20].

cDNA Library and Screening

Double-stranded cDNA [21] was synthesized from 10 μg of poly A^+ RNA isolated from normal human fibroblasts (Berlin-2) which had been treated with 20 ng/ml TPA for 8 hr. The presence of induced RNAs was tested by in vitro translation in a reticulocyte system. The double-stranded cDNAs were tailed with oligo-dC and inserted into the dG-tailed Pst I site of pBR 322. After annealing, the constructs were transformed into competent Escherichia coli C 600. About 1,200 tetracycline-resistant, ampicillin-sensitive colonies were obtained from 30 ng recombinant plasmid. Individual colonies were grown on L-broth agar plates containing tetracycline at 15 μg/ml and transferred to nitrocellulose by toothpicks. Triplicate filters were prepared. The transferred bacterial clones were permitted to grow to colonies of 2 mm diameter. One filter was stored at 4°C in a sealed bag. The cells on the other two filters were

lysed with 10% SDS and the DNA denatured and adsorbed to the filters using 0.5 M NaOH, 1.5M NaCl [21]. The filters were transferred consecutively to 1.5M NaCl, 0.5 M Tris, pH 8.0, and to 0.18 M NaCl, 10 mM NaH_2PO_4, pH 7.4, 1 mM EDTA. The filters were then dried, baked at 80°C for 2 hr, and washed at 65°C in 50 mM Tris HCl, pH 8.0, 1 M NaCl, 1 mM EDTA, 0.1% SDS for 2 hr to remove cell debris. Prehybridization was carried out at 42°C for 6 hr. The mixture contained 50% formamide, 0.9 M NaCl, 50 mM NaH_2PO_4, pH 7.4, 5 mM EDTA, 0.1% SDS, bovine serum albumin, ficoll, and polyvinyl pyrrolidone each, 100 μg/ml small, denatured salmon sperm DNA, 1 μg/ml poly A. One filter was hybridized with radioactive, single-stranded cDNA prepared from poly A^+ RNA of nontreated growing cells, the other with cDNA to RNA from TPA-treated cells. The hybridization mixture contained 2×10^6 cpm ^{32}P-cDNA in 1.5 ml of prehybridization buffer. Incubation was at 42°C for 14 hr. The filters were washed three times at room temperature with 2× SSC, 0.1% SDS and two times at 68°C with 1× SSC, 0.1% SDS, dried, and put on film (Kodak X-Omat AR) using an intensifier screen. For the screening see Results and Figure 1.

Dot Blot RNA Hybridizations

Dot blot hybridizations were performed essentially according to Kafatos et al [22]. The required amount of total or poly A^+ RNA was dried in a vacuum centrifuge, taken up in 20 μl 50% formamide, 6% formaldehyde, 1× TBE (90 mM Tris, 90 mM boric acid, 2.5 mM EDTA pH 8.0), heated at 37°C for 15 min and then at 65°C for 3 min, diluted with 180 μl 10× SSC, and spotted onto nitrocellulose filters. After baking at 80°C, the filters were prewashed twice at 65°C in 4× SSC, 0.02% each of bovine serum albumin, ficoll, and polyvinyl pyrrolidone, 0.1% SDS, and 3.3% PiPPi (100% PiPPi is 200 ml 1 M NaH_2PO_4 + 300 ml 1 M Na_2HPO_4 + 200 ml 7.5% $Na_4P_2O_7$ + 300 ml H_2O). To the second wash 20 μg/ml salmon sperm DNA were added. Hybridization was performed at 65°C in 4× SSC, 10 mM EDTA, 0.1% SDS, 20 μg/ml salmon sperm DNA, and 50 ng/ml nick-translated probe DNA (specific activity 3×10^7–10^8 cpm/μg DNA) for 18 hr. The filters were washed at 65°C consecutively in 2× SSC, 1× SSC, and 0.5× SSC containing 0.1% SDS, 3.3% PiPPi, and were dried and put on film using an intensifier screen.

UV and TPA treatment, in vitro translation, two-dimensional (2D) gel electrophoresis, the preparation of EPIF, and the technique of surviving colony count have been described elsewhere [17,18,23].

RESULTS

The Isolation of cDNA Clones Complementary to TPA-Induced mRNA Species

The TPA-induced mRNAs were quite abundant and could be detected by in vitro translation without difficulty. The translation products have been resolved by two-dimensional gel electrophoresis (Fig 3. in Schorpp et al [18]. An example of the actual RNA preparation used for cloning is shown in Figure 1. We prepared cDNA clones starting from poly A^+ RNA of TPA-treated human fibroblasts and isolated those that hybridized to RNA species that showed an altered abundance after TPA treatment. This was achieved by differentially hybridizing to radioactive total cDNA that had been prepared using either poly A^+ RNA from TPA-treated or from control

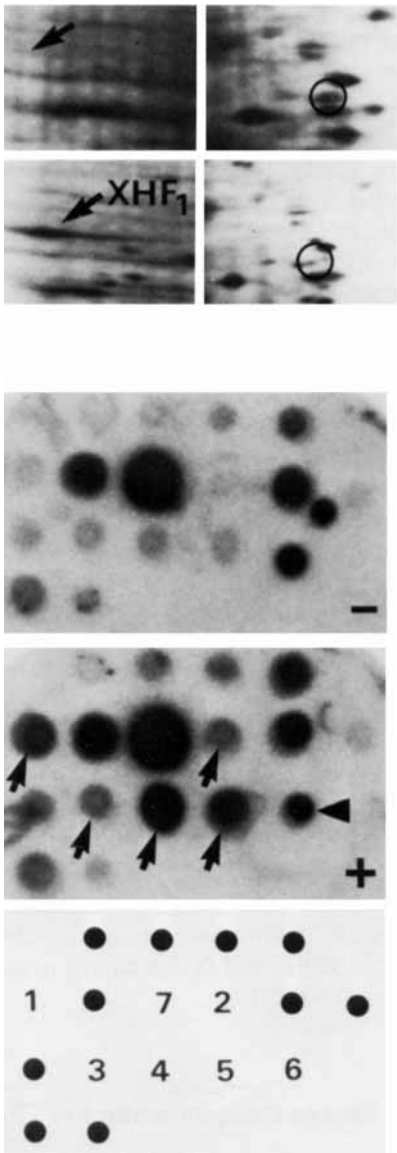

Fig. 1. Screening of the cDNA library. Poly A^+ RNA was prepared from logarithmically growing human skin fibroblasts (Berlin-2) and from a parallel culture that had been treated with 20 ng/ml TPA for 8 hr. a) Poly A^+ RNA (0.2 μg) was added to 10 μl of a reticulocyte lysate and incubated in the presence of ^{35} S-methionine (4 mCi/ml final conc.) at 30°C for 1 hr. Total protein was resolved by 2-D PAGE [17]. Two regions from the autoradiograms were selected: the area resolving protein XHF1 and an area from the acidic region showing a spot that is reduced. The upper panels were from control cells, the lower panels from TPA-treated cells. The RNA coded also for the other inducible proteins (not

cells. The abundant sequences that have been investigated further have been numbered 1 to 6 (Fig. 1). Clone 7 hybridized equally well to both probes and has been used as a standard clone. Clones 1, 2, 4, and 5 hybridized strongly to cDNA prepared from TPA-treated cells, while clone 3 was labeled only slightly more intensively than to control cDNA. Clone 6 showed a reduced hybridization signal with cDNA made from TPA-treated cells.

The cDNA clones were characterized by hybrid-promoted translation and sequencing experiments to be described elsewhere [24]. Clones 1, 4, and 5 select RNA that codes for the secreted protein XHF 1 [17,18] (Fig. 1). Clone 3 represents part of the metallothionein IIa gene sequence [25].

Coordinate Regulation by TPA, UV, MMC, and EPIF

The cDNA clones indicate RNA species that are rapidly induced by TPA in primary fibroblasts except for clone 6 RNA, which is reduced. The increased levels of RNA at 8 hr after TPA were determined by dot blot hybridizations (Fig. 2). The increases were already detectable at 2 hr after TPA (not shown). As anticipated by the data on the protein level [18], ultraviolet light (UV), mitomycin C (MMC), or the induced extracellular protein-inducing factor EPIF induced similar changes in RNA abundance as did TPA. Examples are shown in Figures 2 and 4. The RNA complementary to cDNA clone 7 did not respond to any of the agents, nor did actin mRNA. The coordinate regulation by carcinogenic and cocarcinogenic agents is an astonishing observation because it suggests that both groups of agents share a mechanism of action although it does not imply that this action is related to the assumed roles in carcinogenesis.

Elevated RNA Levels in Cells From Patients With Bloom Syndrome

Cells from patients with the autosomal recessive genetic disease Bloom syndrome are characterized by chromosomal instability. The instability is detected as a high spontaneous rate of sister chromatid exchanges [26] or mutations [15,16]. Presumably as a consequence of chromosomal instability the patients run an increased risk of developing cancer [26]. Skin fibroblasts from Bloom patients were examined for their content in RNA sequences complimentary to the cDNA clones isolated. Cells from two of three patients had extraordinarily high levels of the RNA species without any treatment (Fig. 3). The fact that one Bloom patient differed in this respect suggests that the phenotypic entity may fall into different complementation groups. The obvious speculation that the spontaneous chromosome instability and the elevated expression of TPA-inducible RNAs are part of an endogenous state of "tumor promotion" cannot be further substantiated at this time, and we realize that alternative interpretations are possible (see notes of caution in Mallick et al [17]). Since all

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shown) [17,18]. b) Double-stranded cDNA was synthesized from 10 μg of poly A^+ RNA from TPA-treated cells. The cDNAs were tailed with oligo-dC and inserted into the dG-tailed Pst I-site of pBR 322. Examples are shown of the 1,200 tetracycline-resistant, ampicilline-sensitive E coli C 600 colonies that had been obtained by transformation with cDNA plasmids. After lysis of the cells and binding of the DNA, the nitrocellulose filters were hybridized to ^{32}P-cDNA synthesized from the cytoplasmic poly A^+ RNA present in either nontreated cells (−) or TPA-treated cells (+; 20 ng/ml for 8 hr). Clones 1, 4, and 5 were more abundant in the TPA-cDNA probe, clones 2 and 3 were slightly more abundant, while clone 6 was reduced in the TPA probe. Clone 7 mRNA was present in equal amounts in both probes. Dots mark other colonies that have not been examined further.

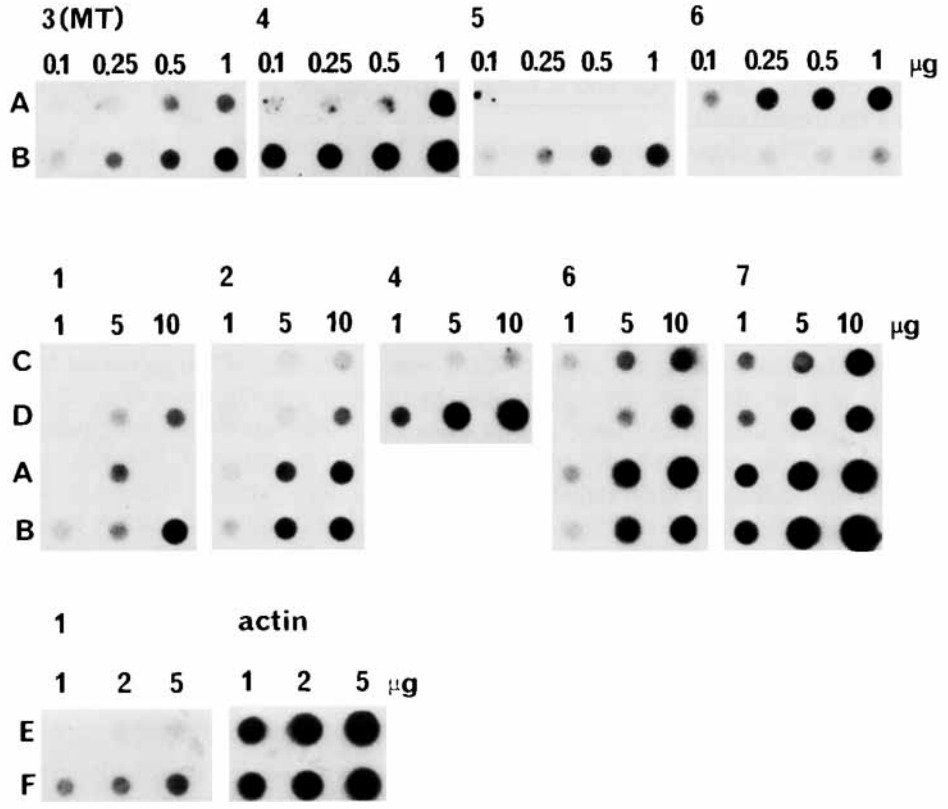

Fig. 2. TPA, UV, and MMC regulate mRNA abundance. Normal human fibroblasts (Berlin-2) were treated with 20 ng TPA/ml for 8 hr (B) or harvested in the logarithmic growth phase (A). Fibroblasts from a patient with Cockayne's syndrome (GM 1856) were UV-irradiated (9 J/m^2) (D) or nonirradiated (C) and harvested 48 hr later. SV40-transformed normal human fibroblasts (GM 637) were treated with 1 µg/ml mitomycin C (F) for 8 hr or nontreated (E). The amounts of total RNA indicated (second and third rows) or poly A$^+$ RNA (first row) were dotted onto nitrocellulose and probed with nick-translated gene probes. The cDNA clones appear as numbers above each set. A pipetting error occurred at the "5 µg spot" of 1A. This signal should be added to the 5 µg spot of 1B. For all experiments, clone 7 and actin served as control hybridizations. Examples are shown: clone 7 in row 2 and actin in row 3. Responses following MMC treatment were also observed for the cDNA clones not shown here. UV inductions done with normal human fibroblasts required larger dose but resulted in similar increases of RNA.

biopsy specimens were, to our knowledge, from peripheral skin, differences owing to site of biopsy are not likely [27]. We hope of course that the cloned sequences may help to clarify these points.

Expression of TPA-Inducible Sequences in Transformed Cells

Although the gene products mediating the cancerogenesis-promoting effect of TPA may not be among the cloned sequences, we were interested to investigate whether transformation influenced the expression of these genes. We chose SV40

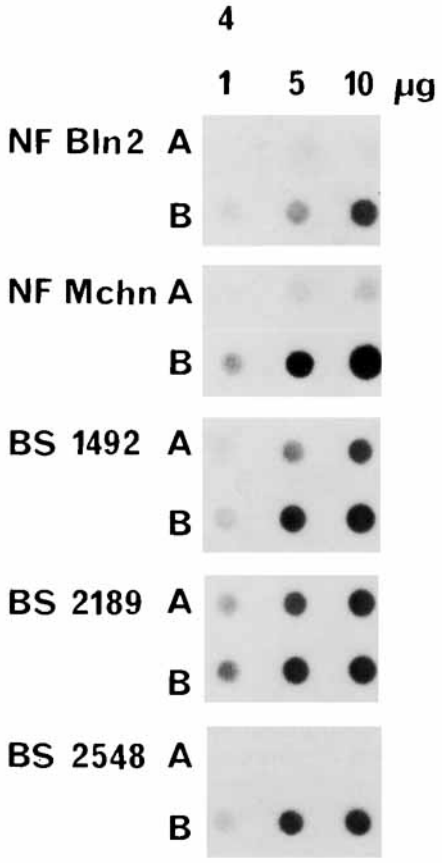

Fig. 3. Elevated expression of clone 4 (XHF1)-mRNA in two out of three Bloom cells. Cell lines of normal human fibroblasts and of Bloom syndrom fibroblasts were either untreated (A) or treated with 20 ng TPA/ml for 8 hr (B). The indicated amounts of total RNA were dotted onto nitrocellulose filters and probed with ^{32} P-labeled clone 4 cDNA. Normalization of the dots were as in Figure 2.

transformation of skin fibroblasts because we had obtained the aforementioned data using the normal counterpart of these cells. Both SV40-transformed normal fibroblasts and SV40-transformed fibroblasts from a patient with Xeroderma pigmentosum (group A) had threefold higher metallothionein RNA levels than the corresponding nontransformed primary fibroblasts. The noninduced abundance of XHF1-RNA, however, was fivefold lower in both transformed cells than in the primary fibroblasts (Fig. 4). Both transformed and nontransformed cells respond to TPA (not shown) or EPIF (Fig. 4) to the same degree. Thus, in these two pairs of cells transformation had a differential effect on the genes examined. We do not know whether the cells were isogenic, since they were not from the same individual. It cannot be determined at this point whether the sequences described are involved in transformation since normal and transformed cells differ in many respects. Our working hypothesis is that XHF1 represents an extracellular differentiation function that is lost by transformation and that increased metallothionein RNA levels cause growth advantage. Suggestive

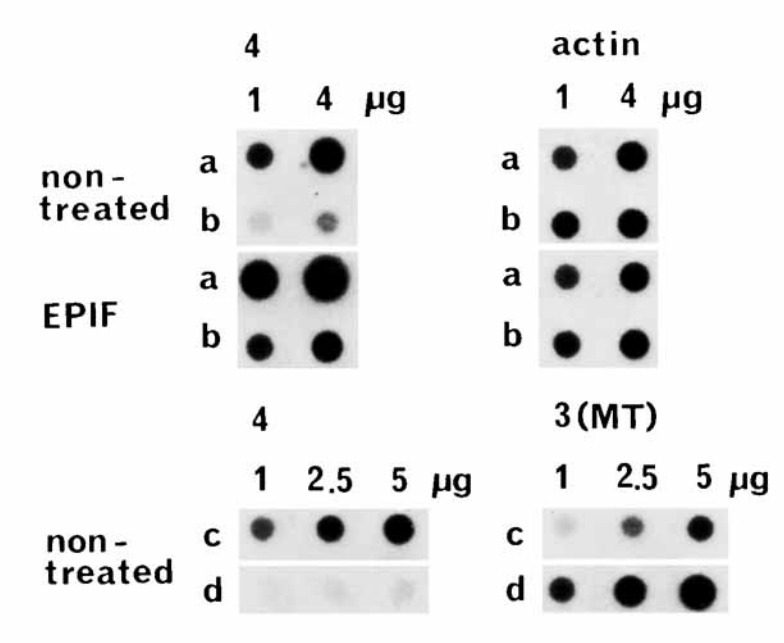

Fig. 4. Expression and inducibility of XHF 1 and metallothionein mRNA in primary and SV40-transformed human fibroblasts. Upper panel: Primary Xeroderma pigmentosum cells (group A, XPGM 2994, a) or SV40-transformed XP cells (XP12 Ro, b) were either untreated or treated with EPIF (from UV-irradiated XPGM 2994 cells 48 hr after irradiation) for 8 hr. The indicated amounts of RNA were dotted on nitrocellulose and probed with ^{32} P-labelled clone 4 cDNA or mouse α-actin cDNA, respectively. Lower panel: RNA was prepared from both normal human fibroblasts (NF Mchn, c) and SV40-transformed normal human fibroblasts (GM 637, d). The indicated amounts of RNA were probed with ^{32} P-labeled clone 4 cDNA and clone 3 cDNA (metallothionein cDNA). Note that exposure periods were tenfold longer than in Figure 3 to visualize expression of clone 4 mRNA in transformed cells.

support for this hypothesis comes from survival curves: Transformation of normal and Xeroderma fibroblasts led to increased resistance toward ionizing or α-radiation (Fig. 5).

DISCUSSION

It would not be surprising if transformed cells expressed other genes than normal cells. The transforming factor acts in a pleiotropic manner. This is reflected on the transcript level and has been used to characterize transformation-specific sequences [28–31]. One set of such transcripts is activated by transformation with SV40 [30] as is metallothionein RNA described in this paper. It thus appears that SV40 transformation and a tumor promoter regulate the same gene. Similarly, the protein MEP is elevated by either transformation or TPA [31]. Coordinate regulation by carcinogenic and cocarcinogenic agents does not, however, necessarily extend to SV40 transformation since the TPA-inducible gene for XHF1 responded in an opposite direction.

The TPA-inducible genetic program resembles vaguely the SOS response in E coli. We have called the program a stress response. The SOS response is essentially

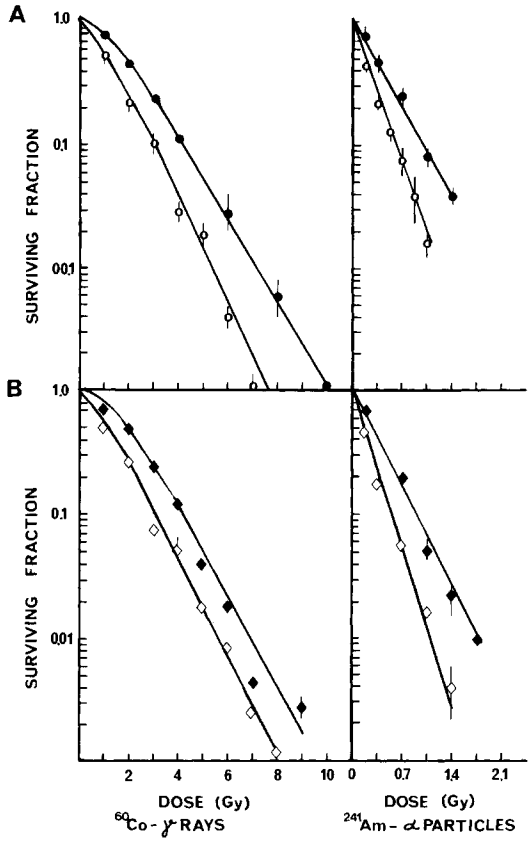

Fig. 5. Survival curves of primary human skin fibroblasts (open symbols) or SV40-transformed human fibroblasts (closed symbols) after exposure to either ^{60} Cobalt-gamma rays or ^{241} Americium-alpha particles. A) Cells derived from a healthy individual. B) Cells derived from a patient suffering from Xeroderma pigmentosum (group A). The data represent mean values of two to three independent experiments each ± standard error. A third pair of nontransformed and transformed skin fibroblasts gave similar results (not shown).

transient in nature but may lead to permanent genetic changes, eg, mutations [32]. The same dichotomy must be envisaged for the action of TPA. We have given evidence elsewhere that the stress response includes a gene amplifying function [33]. This exemplifies the transient nature of the stress response. Maintained expression of a carcinogen or tumor promoter-induced gene in transformed cells is a new facet of the response.

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