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12-*O*-Tetradecanoylphorbol 13-acetate-inducible proteins are synthesized at an increased rate in Bloom syndrome fibroblasts

(Fanconi anemia/ataxia–telangiectasia/ultraviolet light/sister chromatid exchange/two-dimensional gel electrophoresis)

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ABSTRACT **A set of proteins, which in normal fibroblasts were barely, if at all, detectable, were synthesized at an increased rate in fibroblasts from patients with Bloom syndrome (BS). The same set of proteins was induced in normal human fibroblasts by treatment with 12-*O*-tetradecanoylphorbol 13-acetate (TPA). In BS cells, TPA caused a further 2-fold increase in the rate of synthesis. Production of these proteins was inhibited by the addition of fluocinolone acetonide to the culture medium. One of the proteins (XHF_1) present at high levels in BS fibroblasts and in TPA-treated cells was also induced by irradiation with ultraviolet light. This protein was secreted into the culture medium. Most other TPA-inducible proteins were cytoplasmic. Among other human mutants prone to chromosome aberrations we found one of three tested cases of Fanconi anemia and one case of ataxia–telangiectasia that showed increased spontaneous rates of synthesis of the TPA-inducible proteins. In these cases, however, the induction by TPA was like that seen in healthy fibroblasts.**

Bloom syndrome (BS) is a human high-cancer-incidence condition that is transmitted as an autosomal recessive mutation (1). The only consistent cellular feature is an increased frequency of spontaneous symmetric quadriradial chromatid interchanges and of sister chromatid exchanges (SCEs) (2–4). Somatic cell hybrids between lymphocytes from a BS patient and normal lymphocytes have a normal level of SCEs (5, 6). Slow DNA chain growth seems to be characteristic of BS cells (7, 8). BS cells also display an increased sensitivity to UV light or ethyl methanesulfonate (8–10), repair deficiencies (11–14), and a spontaneous mutation rate 10 times higher than that of normal cells (15).

We have examined BS cells in conjunction with cells from patients with other genetic diseases in order to try to detect the altered gene product. Mutations causing a change of charge, a deletion, or a frame shift are detectable at the protein level by two-dimensional polyacrylamide gel electrophoresis, at least for the more abundant proteins (16–20). In such an experiment we found in BS fibroblasts several new proteins that normal human fibroblasts did not synthesize. This suggested that the mutation might be pleiotropic and prompted us to examine normal fibroblasts treated with agents producing effects similar to one or more facets of the BS phenotype—e.g., high frequency of SCEs, increased spontaneous mutation rate, and increased rate of cellular transformation (21–23). Among agents causing such conditions, ultraviolet light and the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA) induced a protein pattern in normal fibroblasts partly or completely identical to the pattern in fibroblasts from patients with BS.

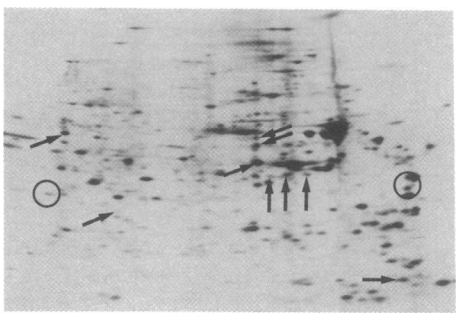

FIG. 1. Constitutive expression of proteins in BS 2189 fibroblasts from a patient with BS. The cells were in the 18th passage for this experiment. An autoradiogram of an IEF two-dimensional gel is shown. Arrows point to proteins XHF_1 and a–h (see Fig. 2). Proteins a and b seem to occur as double spots. Protein h is not resolved in all gels. The basic proteins are on the left. Fibroblasts from three other patients with BS were labeled and produced similar patterns (not shown).

MATERIALS AND METHODS

Cells. BS 2189 fibroblasts were obtained from K. Sperling (Berlin) (listed as entry 74 in ref. 24). The BS patient was a 6-year-old Turkish boy. Fibroblasts were obtained from three other patients with BS. Normal fibroblasts were freshly obtained from the skins of four young healthy individuals. We name the cells after the city from which the individuals came. The origins of other cells with or prone to chromosomal abnormalities are given in the legend of Table 1.

Patterns of Pulse-Labeled Proteins. Cells were grown in monolayer in standard Petri dishes in Dulbecco's minimal essential medium supplemented with 20% fetal calf serum. About 24 hr prior to confluence, the medium was discarded and replaced by 2 ml of Dulbecco's minimal essential medium containing 3% fetal calf serum, 10 μM methionine, and [^{35}S]methionine at 100 μCi/ml (1 Ci = 3.7×10^{10} becquerels). Labeling was for 2 hr at 37°C. The cells were scraped from the Petri dish in 8 ml of 10 mM sodium phosphate buffer (pH 7.5) containing 150 mM NaCl, 3 mM KCl, and 0.5 mM EDTA. The cells were sedimented and disrupted in 50 μl of 1% sodium dodecyl sulfate containing 5% (vol/vol) 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride. In order to detect new protein spots reliably, efforts were made to protect proteins

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Abbreviations: TPA, 12-O-tetradecanoylphorbol 13-acetate; SCE, sister chromatid exchange; BS, Bloom syndrome; IEF, isoelectric focusing.

after cell lysis and to resolve the whole spectrum of acidic, neutral, and basic proteins. Isoelectric focusing (IEF) and nonequilibrium pH gradient electrophoresis were each used in combination with sodium dodecyl sulfate/acrylamide electrophoresis in the second dimension (16, 25).

RESULTS AND DISCUSSION

Fibroblasts from Several Patients with BS Express Characteristic Proteins. The pattern of pulse-labeled proteins from the fibroblasts of four BS patients contained a set of 9 spots not synthesized in normal fibroblasts (Figs. 1 and 2). They range between approximately 20 and 70 kilodaltons in mass and from acidic to basic in charge. The increase in synthesis was observed in all four BS cultures, but the degree of increase varied with the donor patient. It was most noticeable for XHF_1, which was markedly increased in all BS cells tested (see quantitation in Table 1). In all normal fibroblasts, the basal levels of most of the proteins under consideration were barely, if at all, detectable (Fig. 2 *Left*; see also XHF_1 synthesis in Table 1). Note however, that the rate of synthesis of protein d in two out of four normal fibroblast preparations reached that seen in BS cells (compare Fig. 1 with the first two panels in Fig. 2). The syn-

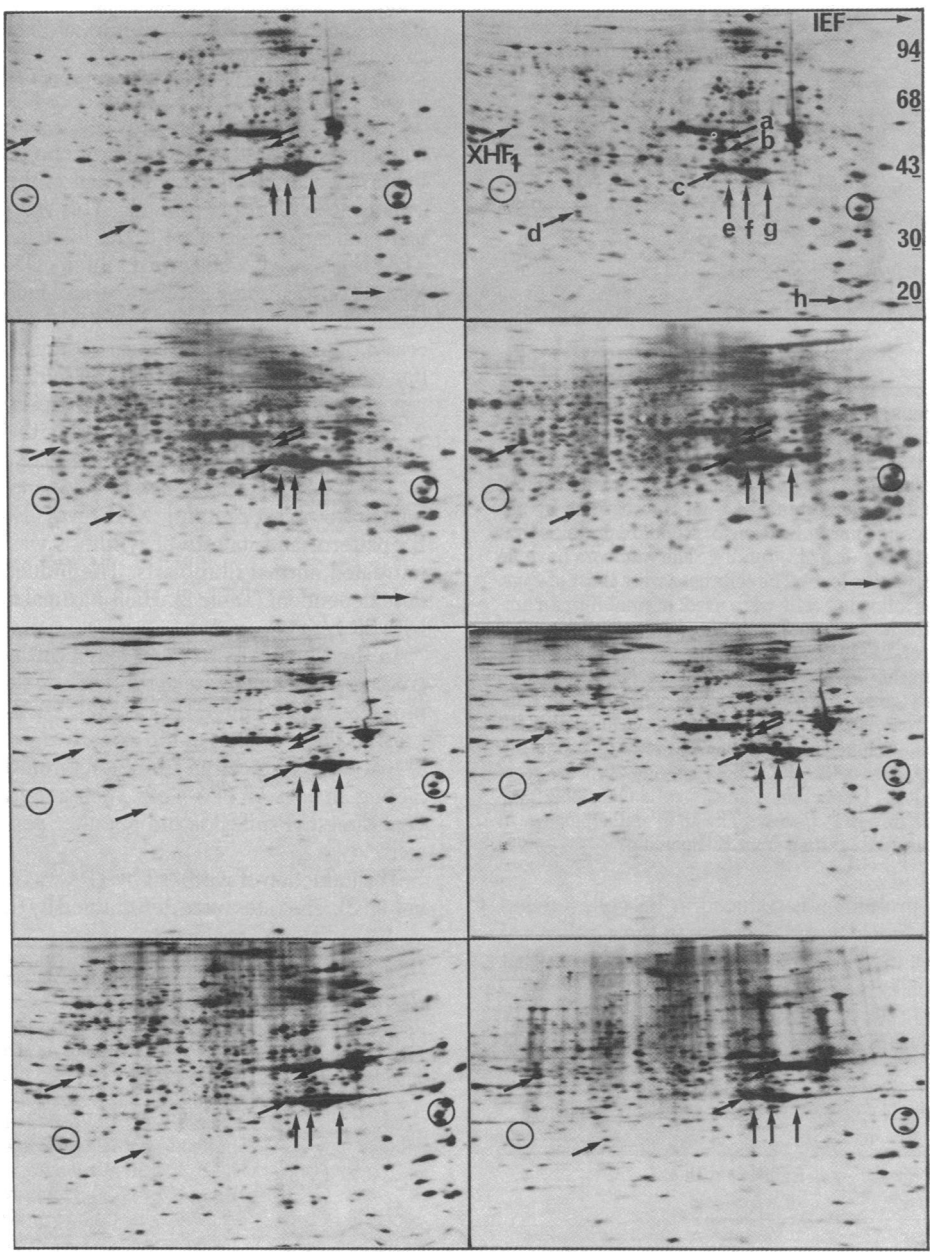

FIG. 2. Induction by TPA of protein synthesis in human fibroblasts. Proteins were separated by IEF in the first dimension. The second dimension was electrophoresis in the presence of sodium dodecyl sulfate; the calibration in *Top Right* is in kilodaltons. Four different lines of normal fibroblasts were labeled prior to the addition of TPA (*Left*) and 8 hr after TPA (*Right*). From top to bottom: forearm skin fibroblasts Berlin 1 (passage 16), foreskin fibroblasts Freiburg (passage 4), forearm skin fibroblasts Berlin 2 (passage 8), and forearm skin fibroblasts München (passage 16). The TPA concentration was 20 ng/ml. With respect to protein induction there was little variance between 5 and 200 ng/ml. Also, exponential and confluent cultures gave similar induction rates. The pictures selected here demonstrate the variation due to two-dimensional resolution and due to differences from one cell line to the other. In two cases we followed the cell cycle distribution through the experiment. According to flow cytometry, 55% of Berlin 1 and 69% of BS 2189 were in G1 during exponential growth, 15% of both were in G2. At 10 hr after TPA addition, a larger proportion of both cell types (32%) were in G2.

Table 1. TPA-induced synthesis of protein XHF_1 in various human fibroblasts

Cells	Relative rate of protein synthesis: Spontaneous	Time after TPA: 4 hr	8 hr	24 hr	SCEs/cell
Normal fibroblasts					
Berlin 1	<0.01	0.3	0.5	0.9	6.6
Freiburg	<0.01		0.9	1.1	
A 1829	0.04		0.5	0.7	
BS					
BS 2189	1.0	1.0	1.9		54.6
BS Kiel	0.9		2.0	2.7	49
GM 1492	0.4	1.5	2.0	2.0	73.6
Other cells with or prone to chromosomal abnormalities					
Fanconi anemia					
1424	<0.01	0.5	0.6	1.1	8.8
CRL 1196	0.07	0.5	0.7	1.5	7.6
GM 2362	0.4	0.7	1.2	1.2	
Ataxia–telangiectasia	0.1			1.2	
Xeroderma pigmentosum	0.01				
18 P^-	0.3		0.9	1.4	20

Fibroblasts from various sources were pulse-labeled and the proteins were resolved by two-dimensional electrophoresis as in Fig. 1. Where indicated the cells had been treated with TPA at 20 ng/ml at various times prior to pulse-labeling. SCEs were determined in nontreated cells as in ref. 26. Numbers were obtained from fluorograms by microdensitometer readings and normalization for standard proteins that did not change synthesis rate (see also ref. 27). The numbers have no dimension; 0.01 is the detection level. The cells used were those of Figs. 1 and 2. In addition, the following cells were used: normal human amniotic fibroblasts A 1829 in passage 10 (obtained from T. Schroeder, Heidelberg); BS Kiel (ref. 28) in passage 20; GM 1492, obtained from the Human Genetic Mutant Cell Repository (Camden, NY) through P. Cerutti, passage 28. 1424 are cells from an 11-year-old boy with Fanconi anemia (obtained from K. Sperling), passage 8. Fanconi cells CRL 1196 (passage 20), ataxia–telangiectasia cells CRL 1343 (passage 18), and xeroderma pigmentosum cells CRL 1223 (passage 20) were from the American Type Culture Collection. Fanconi cells GM 2362 (passage 30) were obtained through P. Cerutti. The 18 P^- cells (passage 5) lack part of chromosome 18 (obtained from K. Sperling).

thesis of at least two proteins was reduced in BS cells (circled in Figs. 1 and 2). The increase and decrease in the synthesis of several gene products in BS cells support the contention that the causative mutation is pleiotropic.

Induction of Proteins in Normal Human Fibroblasts by UV Light and by TPA. To find out whether the BS protein patterns could be correlated with the BS phenotype, we tried to mimic in normal cells some manifestations of the phenotype and then examine the resulting proteins. This was done by treating normal human fibroblasts with UV light or the tumor promoter TPA. We chose UV because it inhibits DNA replication (29) and leads to chromosome aberrations and increased SCEs (30). We chose TPA because tumor promoters have been reported to increase SCEs (21, 31) and because they enhance tumorigenesis (for review see ref. 32). Both treatments have recently been reported to cause changes in gene expression (27, 33–43).

Table 2. Induction by UV of protein XHF_1 synthesis in normal human fibroblasts

UV dose, J/m²	Relative rate of synthesis
0	<0.01
10	0.2
20	0.6
30	0.8
40	1.1

Normal fibroblasts (Berlin 1) were UV irradiated with a germicidal lamp at a dose rate of 0.1 J/m² per sec (incident dose at surface of cells). Prior to UV irradiation, the medium was decanted and the cells were washed twice with warm phosphate-buffered saline. The cells were then incubated with normal medium for 37 hr before pulse-labeling.

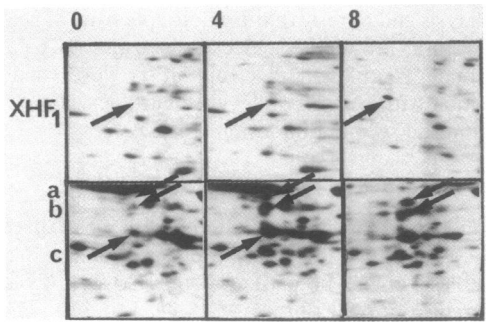

FIG. 3. Time course of induction by TPA. At various times (hr) after the addition of TPA to the culture medium at 20 ng/ml, cells were pulse-labeled and resolved as in Fig. 1. Only areas of interest are shown (nomenclature and location within the two-dimensional gels as in Fig. 2 *Top Right*).

Treatment of human fibroblasts with UV light reliably induced one protein, which we called XHF_1 ["X" by analogy with protein X of *Escherichia coli* (44, 45) and "HF" to designate a human fibroblast protein]. XHF_1 was also characteristic of the BS pattern, and its rate of synthesis was barely detectable in untreated normal fibroblasts. The induction of XHF_1 was UV dose dependent (Table 2). Half-maximal induction was reached with 20 J/m².

In the fibroblasts from all four healthy individuals TPA increased the *de novo* rate of synthesis of the complete set of "BS proteins," including XHF_1 (Fig. 2). Furthermore, TPA also repressed the synthesis of the same two proteins (circled in Fig. 2) that were reduced in fibroblasts from BS patients. Nonpromoting analogs of TPA—e.g., 4-*O*-methyl TPA—and the solvent dimethyl sulfoxide did not alter gene expression in these cells.

The induction of synthesis by TPA was rapid (examples shown in Fig. 3). The rates were determined by the microdensitometry

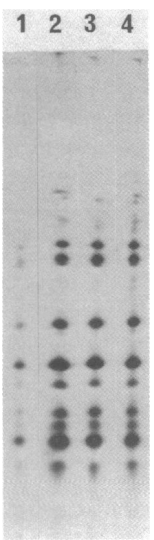

FIG. 4. Peptide pattern of protein XHF_1 after limited proteolysis. In order to test the identity of protein spots from different two-dimensional gels, spots were cut out from the dried gel (avoiding fluorography) and placed in slots of a sodium dodecyl sulfate/15% polyacrylamide gel together with 0.1 μg of V8 protease in 100 μl of sample buffer (see ref. 46). Electrophoresis was started after 30 min at room temperature and was for 15 hr at 40 mA. A fluorogram of the dried gel is shown. The following XHF_1 probes were compared: Lane 1, XHF_1 from IEF of noninduced normal fibroblasts (München). Because of the low level of synthesis, samples from several gels had to be combined. Lane 2, XHF_1 from nonequilibrium pH gradient electrophoresis of BS 2189 cells; lane 3, XHF_1 from IEF of the same BS cells. Lane 4, XHF_1 from IEF of TPA-induced normal fibroblasts (München). Nine fragments are identical in all four samples. Other fragments of minor quantity are probably derived from contaminating protein species.

of autoradiograms such as the one shown in Fig. 1 and were plotted versus time (not shown). The lag period was only 2 hr. For XHF_1 the rate of synthesis ultimately achieved by TPA or UV treatment was the same as the constitutive rate in BS cells (0.4–1.1; see Tables 1 and 2). The high spontaneous rate of expression in BS cells could be approximately doubled by further treatment with TPA (Table 1).

To determine whether the BS proteins were identical to the newly expressed proteins in TPA- and UV-treated normal cells we subjected proteins of identical migration behavior in two-dimensional gels to limited proteolysis (46) and compared their peptide patterns. An example is shown in Fig. 4. Protein XHF_1 isolated from several sources always yielded the same nine peptide fragments upon digestion with staphylococcal V8 protease in sodium dodecyl sulfate. Several other proteins (a, b, and d) were examined by the same technique, with equivalent results.

Properties and Possible Functions. Most induced proteins were cytoplasmic. Protein XHF_1 was secreted. By chasing with nonradioactive methionine after a pulse, we found that it moved into the culture medium within 2 hr (Fig. 5). The proteins a and b were probably glycosylated, as suggested by a change in migration after treatment with tunicamycin (not shown).

Some of the properties of XHF_1 are reminiscent of the plasminogen activator (37, 47, 48). These properties include its size, inducibility by TPA, and the fact that it is secreted. The following points however, argue against an identity between the two proteins: (*i*) plasminogen activator is not inducible by UV in fibroblasts from normal adult individuals (47); (*ii*) XHF_1 is an abundant protein detectable by staining the gels; (*iii*) we have not yet been able to detect a protease activity of XHF_1, or ascribe a [^{3}H]diisopropyl fluorophosphate binding activity to it (not shown).

One could argue that the induced proteins are (*i*) a response to a major attack such as a heat shock (49, 50), (*ii*) typical of particular phases of the cell cycle, or (*iii*) due to slowed growth. These suggestions can probably be ruled out as follows. (*i*) After heat shocking normal cells, we observed new proteins that were unrelated to those seen after TPA treatment (not shown). (*ii*) The proteins are not characteristic of any phase of the cell cycle, because cultures of untreated cells had a similar cell cycle distribution with large G1 peaks. TPA-treated cells were shifted to G2 (see legend to Fig. 2 and ref. 51). Finally, we failed to detect cell cycle specific proteins in synchronized cultures (not shown; see also ref. 52). (*iii*) Arresting cellular proliferation by confluent growth, serum deprivation, or treatment with hydroxyurea or mitomycin C did not induce the BS proteins. Con-

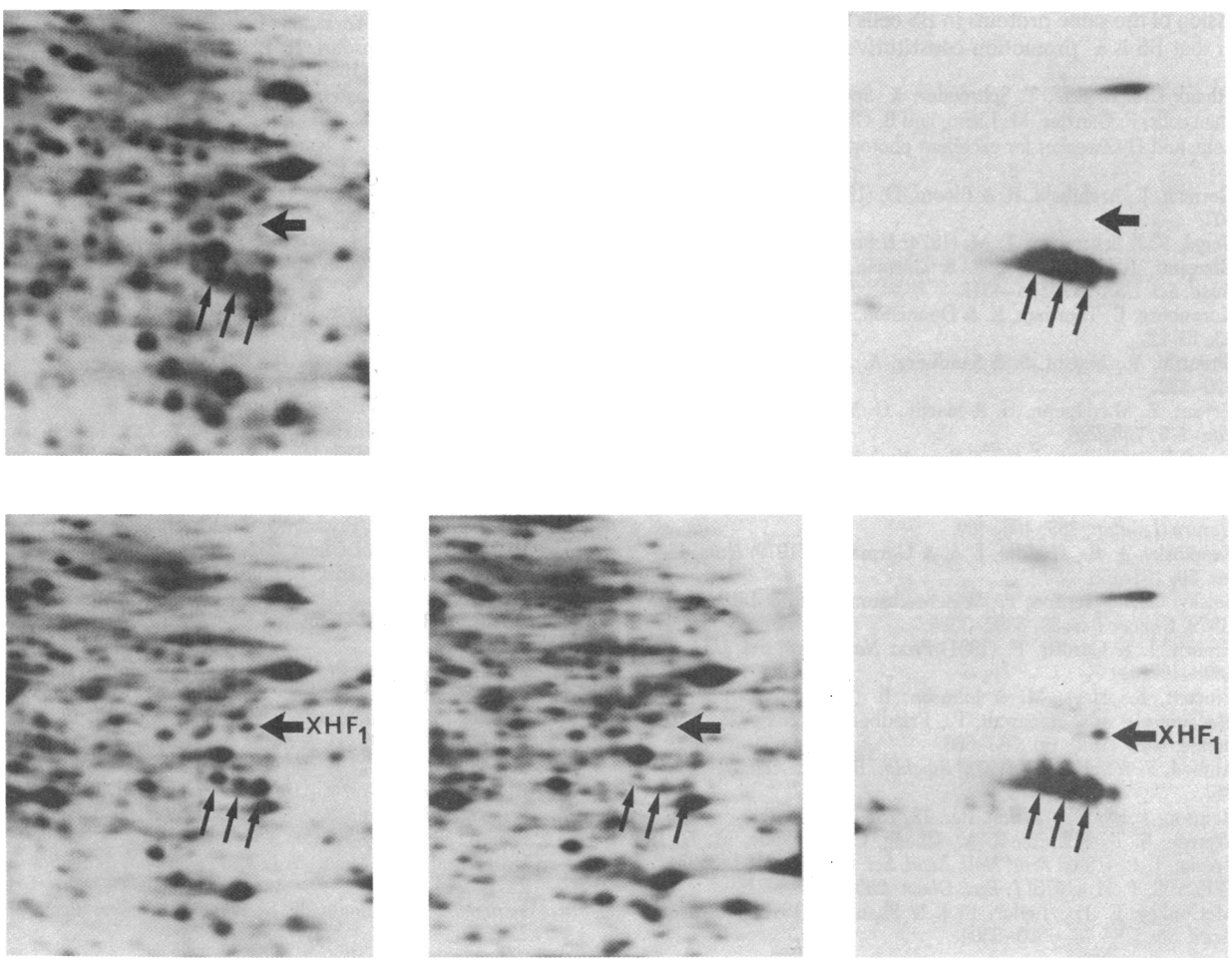

FIG. 5. Protein XHF_1 is secreted. Pulse-labeled proteins were separated by nonequilibrium pH gradient electrophoresis. (*Upper*) Untreated fibroblasts Berlin 1. (*Lower*) Same culture treated for 9 hr with TPA at 20 ng/ml. (*Left*) Radioactive pulse at 7–9 hr; total cellular proteins were resolved directly after the pulse. (*Center*) Cells were treated with chase medium (Dulbecco's minimal essential medium and 1 mM nonradioactive methionine) for 2 hr subsequent to the pulse, and the cellular proteins were then resolved. (*Right*) Media of these cultures were collected and centrifuged at 400 × *g* for 5 min after the addition of 20 μg of bovine serum albumin per ml. Trichloroacetic acid (final concentration 10%) was added to the supernatant and the precipitated proteins were collected by centrifugation at 28,000 × *g* for 30 min. The pellet was washed twice with ethanol and dissolved for nonequilibrium pH gradient electrophoresis. The thick arrow points at the position of XHF_1. Three other secreted proteins (not inducible by TPA) are marked by thin arrows. The basic proteins are to the right.

fluent and starved cells could, however, still be induced successfully by TPA (not shown). This also argues against a simple coupling of the protein pattern to the growth retardation of BS cells (6, 7).

A correlation between spontaneous XHF_1 expression and the frequency of SCEs is suggested by the data in Table 1 but requires further confirmation. XHF_1 should also be tested for a possible involvement in clastogenesis (11, 53). We note in this context an increased spontaneous XHF_1 synthesis occurring in some cells with other genetic deficiencies (Table 1).

As we have shown, there is a remarkable similarity in the protein synthesis patterns of TPA-treated normal cells and of BS cells. If the TPA-inducible (and BS) proteins were involved in bringing about some of the phenotypic characteristics of BS, known inhibitors of tumor promotion and of carcinogenesis may either block the synthesis of these proteins or act on them. Fluocinolone acetonide, a steroidal antiinflammatory agent, did indeed prevent the TPA-dependent induction (not shown). This substance is reported to be an inhibitor of the early effects of TPA (54). Early effects are more likely to occur in culture than late effects. An inhibitor of late stage of promotion, retinoic acid (54, 55), did not affect the protein synthesis pattern in any detectable way (not shown). These data indicate that the induced proteins may be involved in early TPA effects. The constitutive expression of the same proteins in BS cells further supports the notion that BS is a "promotion-constitutive mutant" (21).

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