MOLECULAR AND CELLULAR BIOLOGY, May 1986, p. 1760–1766
0270-7306/86/051760-07$02.00/0

Vol. 6, No. 5

Induction of Metallothionein and Other mRNA Species by Carcinogens and Tumor Promoters in Primary Human Skin Fibroblasts

PETER ANGEL, ANNETTE PÖTING, UDO MALLICK,† HANS JOBST RAHMSDORF, MARINA SCHORPP, AND PETER HERRLICH*

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

Received 10 June 1985/Accepted 16 January 1986

We used nucleic acid hybridization and cDNA cloning techniques to isolate human sequences that respond to the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). These clones were used as probes to examine changes of gene expression that occurred after the proliferation of exponentially growing primary human fibroblasts was arrested. Transcript levels detected by these probes were increased coordinately by treatment of the cells with UV light, mitomycin C, TPA, or the UV light-induced extracellular protein synthesis-inducing factor EPIF (M. Schorpp, U. Mallick, H. J. Rahmsdorf, and P. Herrlich, Cell 37:861–868, 1984). Proteins coded for by these transcripts were characterized by hybrid-promoted translation and by cDNA sequencing. One of the cDNA clones was homologous to the metallothionein IIa gene, and one set of related clones selected RNA for the secreted TPA-inducible protein XHF1 (U. Mallick, H. J. Rahmsdorf, N. Yamamoto, H. Ponta, R.-D. Wegner, and P. Herrlich, Proc. Natl. Acad. Sci. USA 79:7886–7890, 1982).

We introduce an approach which may help to detect genetic elements linked both to proliferation control and to pathways that are triggered by growth arrest. We disturbed the proliferation of primary diploid human skin fibroblasts in culture and determined which genetic sequences responded. The arresting of proliferation may elicit SOS-type functions (18) directed toward eliminating the proliferation block or its consequences. It may also affect genes whose expression is linked to proliferation or which depend on negative or positive signals from cell cycle phases prior or subsequent to the block.

To detect genes that respond to growth interference we used molecular hybridization and cDNA cloning techniques to define RNA sequences that change in abundance in cells immediately after a given treatment. We report here on an analysis of six cDNA clones that were isolated. Using these cDNA clones as probes, we have begun to study the regulation and function of the induced sequences.

MATERIALS AND METHODS

Cells and culture conditions. Normal human fibroblasts (Munich and Berlin 2) were freshly obtained from the forearm skin of young healthy individuals (kindly provided by R. Eife, Munich, Federal Republic of Germany, and K. Sperling, Berlin, Federal Republic of Germany) and used for experiments between passages 10 and 16 (20, 26). Xeroderma pigmentosum fibroblasts GM2994 (group A) were from Human Genetic Mutant Cell Repository, Camden, N.J. The cells were grown in monolayers with Dulbecco minimal essential medium supplemented with 15% fetal calf serum–penicillin (100 U/ml)–streptomycin (100 μg/ml). 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 per 10-cm petri dish, and the experiments were performed 72 h later.

Essential materials. Oligo(dT)-cellulose and oligo(dT)-12-18 primer were from Collaborative Research, Inc., Waltham, Mass. Vanadyl-ribonucleoside complex was from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Avian myeloblastosis virus reverse transcriptase was from Life Sciences, Inc., St. Petersburg, Fla. *Escherichia coli* polymerase Klenow fragment was from Boehringer GmbH, Mannheim, Federal Republic of Germany. Nuclease S1 and terminal deoxynucleotidyl transferase were from P-L Biochemicals, Inc., Milwaukee, Wis. The deoxyguanosine-tailed pBR322 was from New England Nuclear Corp., Boston, Mass. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was from Sigma Chemical Co., St. Louis, Mo. Radiochemicals, the rabbit reticulocyte system, and the nick translation kit were from Amersham International, Little Chalfont, England. Restriction endonucleases were obtained from Boehringer and Bethesda Research Laboratories.

RNA purification. Cells were lysed in 7 M urea–2% sodium dodecyl sulfate (SDS)–0.35 M NaCl–1 mM EDTA–10 mM Tris hydrochloride (pH 8.0), and the nucleic acids were extracted several times with phenol-chloroform, precipitated by the addition of CsCl (0.4 g/ml), and purified by centrifugation through a cushion of 5.7 M CsCl–0.1 M EDTA (27). The RNA pellet under the cushion was taken up in 10 mM Tris hydrochloride (pH 7.5). After ethanol precipitation it was used for dot blot analyses. For cDNA preparation, in vitro translation experiments, and Northern blotting, poly(A)$^+$ RNA was purified by chromatography through an oligo(dT)-cellulose column (21).

cDNA synthesis and construction of the cDNA library. Double-stranded cDNA was synthesized from 10 μg of poly(A)$^+$ RNA isolated from normal human fibroblasts (Berlin-2) which had been treated with 20 ng of TPA per ml for 8 h. The presence of induced mRNAs was tested by in vitro translation. For the first library, we followed the protocol of

* Corresponding author.
† Present address: Institut für Veterinärmedizin, Bundesgesundheitsamt, D-1000 Berlin 65, Federal Republic of Germany.

Maniatis et al. (21). The double-stranded cDNAs were tailed with oligo(dC) and inserted into the deoxyguanosine-tailed *Pst*I site of pBR322. After annealing, the constructs were transformed into competent *E. coli* C600. A second cDNA was prepared by the method of Heidecker and Messing (9a).

Colony hybridization. Individual colonies were grown on L broth agar plates containing tetracycline (15 μg/ml) and transferred to nitrocellulose with toothpicks. Triplicate filters were prepared. The transferred bacterial clones were permitted to grow to colonies of 2 mm in diameter. One filter was stored at 4°C in a sealed bag. The cells on the other two filters were lysed with 10% SDS. The DNA was denatured and adsorbed to the filters with 0.5 M NaOH–1.5 M NaCl. The filters were transferred consecutively to 1.5 M NaCl–0.5 M Tris hydrochloride (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 h, and washed at 65°C in 50 mM Tris hydrochloride (pH 8.0)–1 M NaCl–1 mM EDTA–0.1% SDS for 2 h to remove cell debris. Prehybridization was carried out at 42°C for 6 h. The mixture contained 50% formamide, 0.9 M NaCl, 50 mM NaH_2PO_4 (pH 7.4), 5 mM EDTA, 0.1% SDS, 0.1% bovine serum albumin, 0.1% Ficoll (Pharmacia, Uppsala, Sweden), 0.1% polyvinylpyrrolidone, 100 μg of small denatured salmon sperm DNA per ml, and 1 μg of poly(A) per ml. One filter was hybridized with radioactive single-stranded cDNA prepared from poly(A)$^+$ RNA of nontreated growing cells; the other was hybridized with cDNA to RNA from cells 8 h after TPA treatment. The hybridization mixture contained 2×10^6 cpm of [^{32}P]cDNA in 1.5 ml of prehybridization buffer. Incubation was at 42°C for 14 h. The filters were washed three times at room temperature with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS and twice at 68°C with 1× SSC–0.1% SDS, dried, and put on film (Kodak X-Omat AR) with an intensifier screen.

Dot blot and RNA transfer hybridizations. For dot blot hybridizations the required amount of total or poly(A)$^+$ RNA was dried in a vacuum centrifuge, taken up in 20 μl of 50% formamide–6% formaldehyde–1× TBE (90 mM Tris hydrochloride, 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 of 10× SSC, and spotted onto nitrocellulose filters. For Northern blot hybridizations, 5 μg of poly(A)$^+$ RNA was dried, solubilized in 20 μl of 50% formamide–6.5% formaldehyde in RNA running buffer (40 mM MOPS [morpholinepropanesulfonic acid], 10 mM sodium acetate, 1 mM EDTA [pH 7.0]), heated to 55°C for 15 min, and resolved on a horizontal formaldehyde agarose gel (1.2% agarose, 6.5% formaldehyde) (16). Transfer to nitrocellulose was done by electroblotting. Marker rRNAs (28S and 18S) were stained with ethidium bromide.

UV and TPA treatment, in vitro translation, and two-dimensional gel electrophoresis were performed as described previously (20, 23, 26). TPA was dissolved in dimethyl sulfoxide at a concentration of 200 μg/ml. Cells treated only with dimethyl sulfoxide did not show the genetic changes described here. EPIF-containing medium was prepared as described previously (26). Briefly, 10 petri dishes with logarithmically growing xeroderma pigmentosum GM2994 (group A) cells were irradiated with 2 J of UV light per m^2 or were mock irradiated, complete medium was added, and the cells were cultured for 48 h. The medium was collected from the cells and centrifuged at 400 × *g* for 5 min, and the supernatant was used as a source of EPIF. The action of EPIF was tested with logarithmically growing GM2994 cells as target cells.

DNA sequencing. For DNA sequencing the cDNAs cloned in pBR322 were subcloned in the *Pst*I site of pUC9 (28). The recombinant pUC9 plasmids were digested with *Sal*I and extracted with phenol, and phosphatase was added; the 5′ ends were labeled with ^{32}P by treatment with T4 polynucleotide kinase. The plasmid was cleaved with *Eco*RI, and the DNA was sequenced without further separation by the technique of Maxam and Gilbert (22).

S1 nuclease protection experiments. S1 nuclease protection analyses were performed as described previously (12). The probe stems from a chimeric plasmid (p23/20) containing part of the human metallothionein II A (MTIIA) promoter region was fused to the truncated murine c-*fos* gene by using an artificially introduced *Bam*HI site and then cloned in pSV2-neo (24). Homology of the construct with the MTIIA gene reaches from −67 to +73. The construct carries a unique *Bam*HI site at +73 and a unique *Eco*RI site to the left of this homology region. The 5′ end of the codogenic strand of p23/20 was labeled selectively by cutting the plasmid with the single cutter *Bam*HI, dephosphorylating the ends, and then cutting with *Eco*RI and purifying the *Eco*RI-*Bam*HI fragment. Then, only one end (the *Bam*HI end) could be labeled in the kinase reaction. The probe had a length of 161 base pairs (bp) and was protected by MTIIA mRNA that started from the cap site over a length of 77 bp (overhanging 5′ end of the *Bam*HI site at +73; see Fig. 3).

Hybrid-promoted translation was performed as described previously (7). The selected RNA was translated in a reticulocyte lysate; dog pancreas microsomal membranes were from New England Nuclear.

Limited proteolysis was done as described previously (20).

RESULTS

Isolation of cDNA clones complementary to TPA-induced mRNA species. The tumor promoter TPA transiently inhibits the growth of dividing human skin fibroblasts (20), as it does in various other cell systems. Thus TPA inhibits the growth of keratinocytes (9), affects the cell cycle traverse of HeLa cells (14), and leads to the transient inhibition of DNA synthesis in mouse epidermal cell cultures (29). We have observed earlier that treatment with TPA causes the appearance of at least eight new proteins that are detectable by two-dimensional gel electrophoresis (20) and that other inhibitors of growth such as mitomycin C (MMC) or UV radiation induce the same set of proteins (26). TPA-inducible functional mRNA was readily detectable by in vitro translation (26). We therefore decided to prepare a cDNA library using TPA as the inducer, to isolate the TPA-induced sequences, and later to screen UV- or MMC-treated cells to determine whether they contained increased amounts of these same RNA sequences. Two libraries were obtained; one had 1,200 and the other had 50,000 clones. By differential colony hybridization with radioactive cDNA synthesized on mRNA from control and TPA-treated cells, we isolated those clones which hybridized to RNA species that showed an altered abundance after TPA treatment. We first examined the abundant sequence clones from the smaller library. These were numbered 1 through 7 (11). Clone 7 hybridized equally well to both cDNA mixtures and was used as a standard clone. Clones 1, 2, 4, and 5 hybridized strongly to cDNA prepared from TPA-treated cells, whereas clone 3 was labeled only slightly more intensively than with the control cDNA mixture. Clone 6 showed a reduced hybridization signal with cDNA isolated from TPA-treated cells.

The cDNA clones were characterized by size, cross-

hybridization, sequencing, and transfer hybridization to genomic fragments and RNAs. Some of the results are summarized in Table 1.

TPA-induced mRNA species. The cDNA clones hybridized to defined cytoplasmic transcripts. Poly(A)$^+$ RNA from nontreated and TPA-treated cultures was resolved by electrophoresis in a 1.2% denaturing agarose gel. The RNA was then transferred to a charged nylon membrane (Zeta Probe; Bio-Rad Laboratories, Richmond, Calif.) and hybridized in parallel with ^{32}P-labeled cDNA of the individual clones. With one exception (clone 2), all clones hybridized to only one RNA species. Clone 3 RNA was the smallest (0.6 kilobases [kb]); all others were larger, ranging between 2.0 and 2.4 kb (Fig. 1).

Clones 1 through 5 hybridized to sequences which were enhanced by TPA. The degree of induction at 8 h after TPA treatment ranged from 3- (clone 3) to about 16-fold (clone 4). The sequence complementary to clone 6 was decreased in abundance after TPA treatment; the clone 7 sequence was unchanged.

cDNA clone 2 hybridized to two RNA species (Fig. 1). The smaller RNA of 2.2 kb seemed to be constitutively expressed, while the larger species of about 2.4 kb was much more abundant after TPA treatment.

cDNA clones 1, 4, and 5 select RNA coding for the major secreted protein XHF1. To characterize the proteins encoded by the cDNA sequences, RNAs were hybrid selected and used as templates in a reticulocyte lysate in vitro system. The proteins synthesized in vitro were resolved by one-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 2) and by two-dimensional gel electrophoresis (data not shown) for comparison with the pattern of TPA-induced proteins (20). cDNA clone 6 selected an RNA which coded for a 45-kilodalton (kDa) protein. The protein band was visible in the one-dimensional gel resolution of protein synthesized with total poly(A)$^+$ RNA as the template. The RNA from cells at 8 h after TPA treatment contained less of the RNA species (Fig. 2) as expected from the Northern blot experiment (Fig. 1). The addition of microsomes to the reticulocyte system did not seem to change the 45-kDa protein. The protein was not identified in the two-dimensional gels. We have as yet failed to select by hybridization sufficient RNA with cDNA clone 2. This may be due to the small insert, at least half of which is a bacterial insertion, and the hybridization conditions may need to be adjusted.

cDNA clones 1, 4, and 5 selected the same RNA species. The RNA gave rise to three protein bands of about 54, 43, and 38 kDa (Fig. 2A). cDNA clones 1 and 5, indeed, shared a small section of overlapping sequence. All three hybridized to similar genomic DNA fragments (data not shown). Therefore, they must have been derived from different sections of the same RNA.

The 54-kDa protein is most probably the precursor form of the TPA-inducible protein XHF1, a major secreted protein of as yet unknown function (20, 26). (i) The 54-kDa protein comigrates in one-dimensional PAGE (Fig. 2A) and two-dimensional PAGE (data not shown) with XHF1 synthesized from mRNA of TPA-treated cells. (ii) The addition of microsomes to the reticulocyte system could, at least partially, convert the 54-kDa protein to the mature form of XHF1 (Fig. 2B). (iii) XHF1 synthesized and labeled in cell culture was resolved by two-dimensional gel electrophoresis, and the spot was cut out and subjected to limited proteolysis in SDS. The fragment pattern was almost completely identical to the one generated from the 54-kDa protein band which had been synthesized in vitro with hybrid-selected RNA as a template (Fig. 2C).

The two smaller protein bands of 43 and 38 kDa are most likely proteolytic cleavage products of the largest protein. This was shown by limited proteolysis cleavage patterns (Fig. 2C). The bands were cut out of the SDS gel and subjected to digestion by V8 staphylococcal protease in the presence of SDS. Parts of the fragments generated were identical. The fragments of the two smaller proteins were all represented in the pattern from the 54-kDa protein.

cDNA clone 3 is part of the human MTIIA gene sequence. The sequence of cDNA clone 3 was found to correspond to part of the sequence of the MTIIA gene (13). The homology reaches from nucleotides 718 to 904 of the MTIIA coding strand sequence. There was just a one-base difference. In addition to the poly(A) signal, the cDNA contained part of the poly(A) tail. RNA size and human genomic fragments hybridizing to clone 3 were compatible with previously published data (13).

TPA induces MTIIA RNA started at the normal cap site. The increase in abundance of RNA hybridizing to cDNA clone 3 after TPA treatment was the least impressive of all clones described here. To confirm the threefold enhancement and to examine whether the normal MTIIA promoter was utilized, quantitative S1 nuclease protection experiments were carried out. A 161-bp *Eco*RI-*Bam*HI fragment of the human MTIIA gene which spans the cap site was 5′ end labeled at the transcribed *Bam*HI end. A 77-bp radioactive fragment should be protected from S1 nuclease digestion if hybridized to RNA that had initiated at the cap site. This is indeed the major fragment that was protected (Fig. 3). In both normal human skin fibroblasts and in fibroblasts from a patient with xeroderma pigmentosum, MTIIA RNA was induced by TPA, and the majority of RNA molecules were initiated at the correct and predicted site (Fig. 3, lanes 2 and 4). Other starts, most of which were 5′ of the major start, were also used and enhanced by TPA. One of these appeared to yield RNA that spanned and protected the total 161-bp probe. The enhancement factor was similar to the one observed in the Northern quantification (Fig. 1).

Kinetics of TPA-induced mRNA accumulation. To explore

TABLE 1. Some properties of the cDNA clones used in this study

Clone	Insert size (bp)[a]	mRNA size (bases)[a]	Fold induction or repression[b] by TPA bases
1	127	2,200	10–15
2	700[c]	2,200, 2,400[f]	4.8[f]
3 (MT-IIA)	265[d,e]	600	3.0
4	850	2,400	16.5
5	125[e]	2,300	12.1
6	1,000[e]	2,300	2.1
7	1,200	2,000	1.0

[a] Insert and mRNA sizes are approximate and were calculated from electrophoretic separations, except for cDNAs 1, 3, and 5, for which the complete sequence exists. The available sequences of clones 1 through 6 were fed into the data base of the European Molecular Biology Laboratory. They are also available from us on request. Induction and repression factors were derived from densitometric scanning of fluorograms (Fig. 1). The cDNA clones did not cross-hybridize.

[b] At 8 h after TPA treatment.

[c] Includes one IS*1* fragment that was picked up during cloning.

[d] Homology (100%) to human MTIIA gene (13) from bases 718 to 904 of the coding strand and from bases 791 to 851 of the opposite noncoding strand, as an artifact during double strand synthesis.

[e] Includes pA and AATAA.

[f] Two RNA species; only the 2.4-kb RNA is regulated.

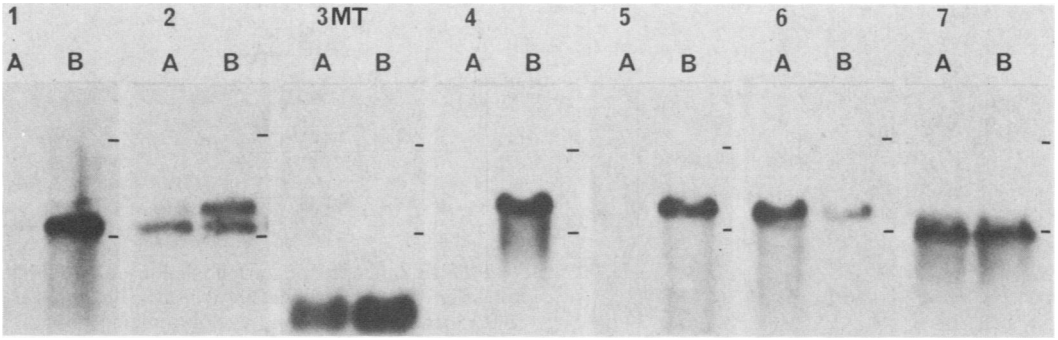

FIG. 1. TPA-induced mRNA species. Poly(A)$^+$ RNA was prepared from nontreated (A) and TPA-treated (B) (20 ng/ml, 8 h) normal human fibroblasts (Berlin-2), resolved by electrophoresis in 1.2% agarose–6.5% formaldehyde, and transferred electrophoretically onto a charged modified nylon membrane (Zeta-Probe; Bio-Rad). The membranes were hybridized with nick-translated cDNA clones 1 through 7. The markers (–) are 28S and 18S RNA resolved in the same gels and detected by ethidium bromide staining.

whether the increase of RNA abundance by TPA could be a direct response, the kinetics of induction was measured and proved to be fairly rapid (Fig. 4). An increase in MTIIA RNA levels could be detected after 2 h, without a detectable lag phase. XHF1 RNA appeared after a lag period of about 2 h. Maximal RNA levels were reached at 8 to 16 h. The response was transient for both MTIIA and XHF1 RNA. Clone 6 RNA was reduced by 50% at about 20 h (Fig. 4). The

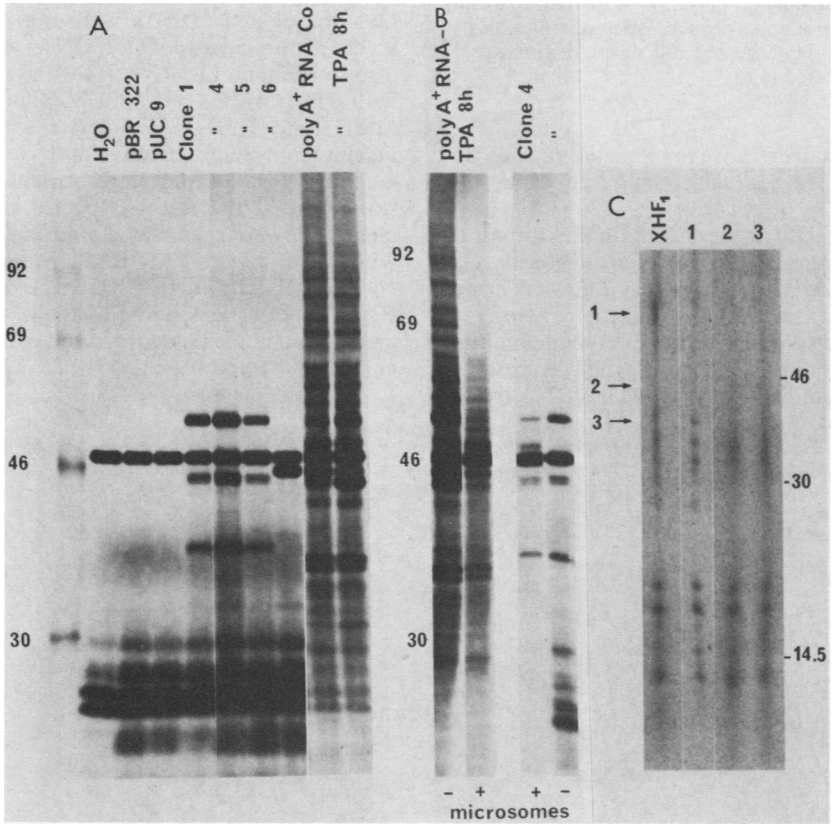

FIG. 2. In vitro translation with mRNAs hybrid selected by TPA-regulated cDNAs and identification of XHF1-specific clones. (A and B) cDNA clones 1, 4, 5, and 6 (10 μg each) and the control plasmids pBR322 and pUC9 were linearized and bound to nitrocellulose filters (diameter, 0.5 cm). The filters containing clones 1, 4, and 5; pBR322; and pUC9 were hybridized to 30 μg of poly(A)$^+$ RNA from TPA-treated cells (20 ng/ml, 8 h); clone 6 was hybridized to mRNA from control (Co) cells. After elution, the specific mRNAs were translated in a reticulocyte system in the presence of 15 μCi of [^{35}S]methionine and 2 U of placental RNase inhibitor (Bethesda Research Laboratories). The proteins were separated in a 10% polyacrylamide gel. Total poly(A)$^+$ mRNAs (0.3 μg) and dog pancreatic microsomes were included in the reaction where indicated. (C) In-vivo-labeled XHF1 protein was cut out of a two-dimensional PAGE (avoiding fluorography) and subjected to limited proteolysis with *Staphylococcus aureus* V8 protease. mRNA was hybrid selected with clone 4 cDNA and translated in vitro; and the protein products were separated by one-dimensional PAGE. The 54- (lane 1), 43- (lane 2), and 38-kDa (lane 3) protein bands were cut out and subjected to limited proteolysis. The peptides generated were separated by 15% SDS-PAGE and visualized by fluorography. The arrows point to the position of the nondigested proteins (1, 54, kDa; 2, 43 kDa; 3, 38 kDa). Numbers to the right of the gel are in kilodaltons.

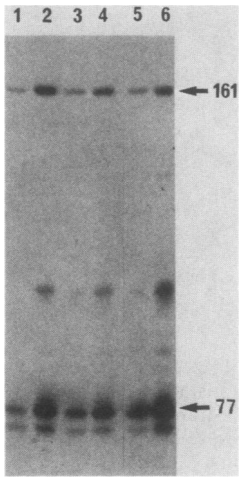

FIG. 3. S1 nuclease protection analyses of endogenous MTIIA transcripts. S1 nuclease analyses in DNA excess of total RNA from various sources were performed. The indicative fragment is 77 bp; the probe is 161 bp (see text). The probe stems from the chimeric gene p23/20 and mismatches at three positions when hybridized to the endogenous human MTIIa RNA. Still, the hybrids were stable under the conditions used. Lane 1, normal human fibroblasts, untreated; lane 2, normal human fibroblasts, TPA treated (20 ng/ml, 8 h); lanes 3 through 6, xeroderma pigmentosum skin fibroblasts; lane 3, untreated; lane 4, TPA treated (20 ng/ml, 8 h); lane 5, untreated; lane 6, EPIF treated (6 h).

data shown in Fig. 1 suggest an even greater rate of decrease. Clone 7 RNA levels were unaltered (data not shown).

The fast kinetics suggest that the induction and turnoff of RNA by TPA do not require prior protein synthesis. To investigate this point, the TPA action was followed in the presence of cycloheximide. By incorporation of [^{35}S]methionine in a control culture, the effective inhibition of translation was monitored (data not shown). The presence of cycloheximide indeed did not prevent MTIIA RNA induction (Fig. 5, rows b and d) and clone 6 RNA turnoff (data not shown) to any extent. MTIIA RNA was enhanced further by the combined treatment (Fig. 5). Thus TPA acts on the

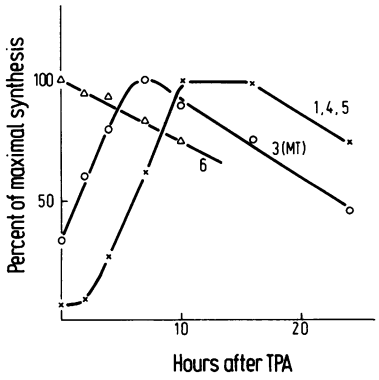

FIG. 4. Time course of appearance of TPA-induced RNAs. Normal human fibroblasts (Berlin 2) were treated with 20 ng of TPA per ml and incubated for various periods of time. Total RNA was isolated, and dot blot hybridizations were performed. Autoradiograms were evaluated by microdensitometry, and RNA levels were blotted as the percentage of maximal levels.

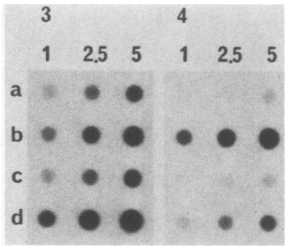

FIG. 5. Influence of cycloheximide on TPA-induced gene expression. Normal human fibroblasts (Munich) were treated with TPA (row b; 20 ng/ml), cycloheximide (row c; 10 μg/ml), or TPA-cycloheximide (row d). Row a, Nontreated. All treatments were for 6 h. The indicated amount (in micrograms) of total RNA from these cells was dotted on nitrocellulose and probed with cDNA clones 3 and 4.

MTIIA promoter without requiring intermediate protein synthesis. The appearance of XHF1 RNA, however, was reduced or even eliminated (Fig. 5). This suggests that a TPA-induced protein at least enhances TPA action on XHF1 RNA levels.

Coordinate regulation by TPA, UV, MMC, and EPIF. Following earlier observations on TPA-induced proteins (26), the cloned cDNAs were not only used to probe for levels of the corresponding RNAs in response to TPA but also to examine the RNA levels after treatment of the cells with UV, MMC, or the UV-induced factor EPIF. UV, MMC, and EPIF stimulated the synthesis of all TPA-inducible proteins. RNA dot blot (Fig. 6), S1 nuclease mapping (Fig. 3), and Northern analyses (data not shown) revealed that the mRNA species identified by the cDNA clones showed a similar altered abundance after treatment with these agents. All RNA sequences behaved largely coordinately, no matter which of the agents was applied; e.g., RNA corresponding to clone 6 was always reduced, and RNA complementary to clones 1, 3, 4, and 5 was always enhanced.

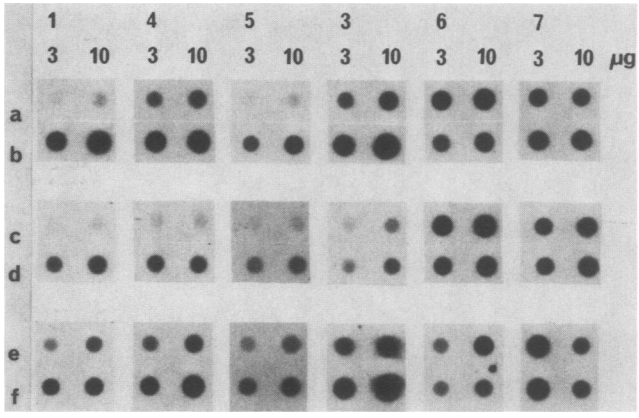

FIG. 6. Coordinate regulation by UV, MMC, and EPIF. xeroderma pigmentosum skin fibroblasts GM2294 were mock irradiated (a) or UV irradiated (b; 2 J/m^2). RNA was prepared 48 h later. Logarithmically growing normal human fibroblasts (Munich) were nontreated (c) or treated with MMC (d; 1 μg/ml) for 72 h. xeroderma pigmentosum skin fibroblasts GM2994 were treated with culture medium from nonirradiated GM2994 cells (e) or with culture medium from UV-irradiated GM2994 cells (f; EPIF) for 8 h. The indicated amount of total RNA was dotted and probed with the cDNA clones.

EPIF induced the mRNAs with a lag period shorter than 4 h. On the basis of earlier experiments, the kinetics of RNA appearance after UV and MMC treatment was also relatively fast (data not shown; compare references 1a and 26). However, no accurate kinetic determinations nor cycloheximide studies have been done, and it is possible that UV and MMC act through the induction of EPIF. In contrast to TPA induction, the UV- and MMC-induced RNAs accumulated for 48 to 72 h.

DISCUSSION

Our approach of interfering with proliferation of cells in culture has revealed genes that code for RNAs, the steady-state levels of which are increased or decreased soon after treatment with TPA, UV, or MMC. Among the subsets examined, one gene product (clone 6) showed a decrease on treatment. All other sequences were increased: the gene or gene family represented by cDNA clones 1, 4, and 5; the MTIIA gene; and clone 2. The small cDNA library probably does not contain rare sequences, e.g., those coding for functions we described earlier. The induced gene-amplifying function (C. Lücke-Huhle and P. Herrlich, Rad. Carcinogenesis, in press) and the UV-induced factor EPIF (26) may not be among the isolated clones. Still, this library was not exhausted.

Increased expression of MTIIA at very long periods after UV treatment was first described by Lieberman et al. (17). In their system, MTIIA genes were methylated and UV caused demethylation, while in our primary cells, MTIIA may be demethylated to begin with and, therefore, responds immediately.

Data on kinetics and treatment with cycloheximide were compatible with transcriptional regulation data. Other mechanisms that influenced RNA abundance cannot be excluded. Whatever the level of regulation, we need to explain how very different agents lead to the same response. These include TPA, UV, MMC, and the UV-induced factor EPIF as shown here and agents that we have shown to induce on the protein level, e.g., alpha irradiation (L. Hieber, U. Mallick, C. Lücke-Huhle, and P. Herrlich, Proceedings of the 7th International Congress on Radiation Research, B4-17, Nijhoff, Amsterdam), gamma irradiation (20), and cycloheximide (P. Herrlich, P. Angel, H. J. Rahmsdorf, U. Mallick, A. Pöting, L. Hieber, C. Lücke-Huhle, and M. Schorpp, Adv. Enzyme Reg., in press). With the exception of EPIF, all treatments resulted in the retardation of growth. Although for UV we showed that DNA damage could be one of the initiating events (26), it is too early for a unifying hypothesis.

Examinations by cDNA cloning and differential screening for the study of the cell cycle, to our knowledge, have always involved the converse approach: stimulation of cells in G_0 to growth (2, 5, 10, 19). The sequences isolated here may thus have no relationship to those described by others. In the 3T3 cell library of Linzer and Nathans (19) no clones, in fact, have been identified that hybridized better to the resting cell probe than to the probe from serum-activated cells.

One of the sequences (clone 6) showed a negative response to inhibition of growth. This type of reaction is already known for the histone and calmodulin mRNAs (1, 6, 8) and, on the protein level, for cyclin and other protein spots in two-dimensional gels (3, 4, 20).

The response described in this study is transient. It nevertheless may have physiologic consequences. A good example may be the triggering of gene amplification (15, 25; Lücke-Huhle and Herrlich, in press). Among the genes addressed here, MTIIA may fulfill the requirement of a stress or SOS function by serving as a protective molecule against heavy metals and, possibly, radicals. A physiologic evaluation of the TPA- and UV-induced responses must await the identification of additional functions.

ACKNOWLEDGMENTS

We thank H. Ponta, E. Wagner, and B. Salmons for helpful discussions; Petra Butkeraitis for technical assistance; H. Kiesel for photographic work; and C. Heinold for the computer typescript. Part of this study will be included in the Ph.D. theses of P.A. and A.P.

This work was supported by Fonds der Chemischen Industrie.

ADDENDUM IN PROOF

A complete cDNA clone for XHF1 has been isolated. The protein sequence derived suggests that XHF1 is a metalloprotease of a previously undescribed primary structure (our data in collaboration with A. J. P. Docherty and G. Murphy). The precursor protein adds up to 53,637 daltons.

LITERATURE CITED

1. **Alterman, R.-B. M., S. Ganguly, D. H. Schulze, W. F. Marzluff, C. L. Schildkraut, and A. I. Skoultchi.** 1984. Cell cycle regulation of mouse H3 histone mRNA metabolism. Mol. Cell. Biol. **4**:123–132.

1a. **Angel, P., H. J. Rahmsdorf, A. Pöting, C. Lücke-Hule, and P. Herrlich.** 1985. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA)-induced gene sequences in human primary diploid fibroblasts and their expression in SV40 transformed fibroblasts. J. Cell. Biochem. **29**:351-360.

2. **Arya, S. K., F. Wong-Staal, and R. C. Gallo.** 1984. Transcriptional regulation of a tumor promoter and mitogen-inducible gene in human lymphocytes. Mol. Cell. Biol. **4**:2540–2542.

3. **Barque, J. P., F. Danon, L. Peraudeau, P. Yeni, and C. J. Larsen.** 1983. Characterization by human autoantibody of a nuclear antigen related to the cell cycle. EMBO J. **2**:743–749.

4. **Bellatin, J., R. Bravo, and J. E. Celis.** 1982. Changes in the relative proportion of transformation-sensitive polypeptides in giant HeLa cells produced by irradiation with lethal doses of x-rays. Proc. Natl. Acad. Sci. USA **79**:4367–4370.

5. **Campisi, J., H. E. Gray, A. B. Pardee, M. Dean, and G. E. Sonenshein.** 1984. Cell-cycle control of c-myc but not c-ras expression is lost following chemical transformation. Cell **36**:241–247.

6. **Chafouleas, J. G., L. Lagace, W. E. Bolton, A. E. Boyd III, and A. R. Means.** 1984. Changes in calmodulin and its mRNA accompany reentry of quiescent (G0) cells into the cell cycle. Cell **36**:73–81.

7. **Cochran, B. H., A. C. Reffel, and C. D. Stiles.** 1983. Molecular cloning of gene sequences regulated by platelet-derived growth factor. Cell **33**:939–947.

8. **DeLisle, A. J., R. A. Graves, W. F. Marzluff, and L. F. Johnson.** 1983. Regulation of histone mRNA production and stability in serum-stimulated mouse 3T6 fibroblasts. Mol. Cell. Biol. **3**: 1920–1929.

9. **Eisinger, M., and O. Marko.** 1982. Selective proliferation of normal human melanocytes in vitro in the presence of phorbol ester and cholera toxin. Proc. Natl. Acad. Sci. USA **79**: 2018–2022.

9a. **Heidecker, G., and J. Messing.** 1983. Sequence analysis of zein cDNAs obtained by an efficient mRNA cloning method. Nucleic Acids Res. **11**:4891-4906.

10. **Hendrickson, S. L., and C. D. Scher.** 1983. Platelet-derived growth factor-modulated translatable mRNAs. Mol. Cell. Biol. **3**:1478–1487.

11. **Herrlich, P., P. Angel, A. Pöting, M. Schorpp, U. Mallick, and H. J. Rahmsdorf.** 1985. Proliferation-controlled and -controlling genes, p. 50–57. *In* S. Ebashi (ed.), Cellular regulation and

malignant growth, Lipmann Symposium. Springer-Verlag, Berlin.
12. **Hynes, N. E., A. J. J. van Ooyen, N. Kennedy, P. Herrlich, H. Ponta, and B. Groner.** 1983. Subfragments of the long terminal repeat cause glucocorticoid responsive expression of mouse mammary tumor virus and of an adjacent gene. Proc. Natl. Acad. Sci. USA **80**:3637–3641.
13. **Karin, M., and R. I. Richards.** 1982. Human metallothionein genes—primary structure of the metallothionein-II gene and a related processed gene. Nature (London) **299**:797–802.
14. **Kinzel, V., J. Richards, and M. Stöhr.** 1981. Early effects of the tumor-promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate on the cell cycle traverse of asynchronous Hela cells. Cancer Res. **41**:300–305.
15. **Lavi, S.** 1981. Carcinogen-mediated amplification of viral DNA sequences in simian virus 40-transformed Chinese hamster embryo cells. Proc. Natl. Acad. Sci. USA **78**:6144–6148.
16. **Lehrach, H., D. Diamond, J. M. Wozney, and H. Boedtker.** 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16**:4743–4751.
17. **Lieberman, M. W., and L. R. Beach, and R. D. Palmiter.** 1983. Ultraviolet radiation-induced metallothionein-I gene activation is associated with extensive DNA demethylation. Cell **35**: 207–214.
18. **Little, J. W., and D. W. Mount.** 1982. The SOS regulatory system of Escherichia coli. Cell **29**:11–22.
19. **Linzer, D. I. H., and D. Nathans.** 1983. Growth-related changes in specific mRNAs of cultured mouse cells. Proc. Natl. Acad. Sci. USA **80**:4271–4275.
20. **Mallick, U., H. J. Rahmsdorf, N. Yamamoto, H. Ponta, R.-D. Wegner, and P. Herrlich.** 1982. 12-*O*-Tetra-decanoylphorbol 13-acetate-inducible proteins are synthesized at an increased rate in Bloom syndrome fibroblasts. Proc. Natl. Acad. Sci. USA **79**:7886–7890.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
23. **Rahmsdorf, H. J., U. Mallick, H. Ponta, and P. Herrlich.** 1982. A B-lymphocyte-specific high turnover protein: constitutive expression in resting B-cells and induction of synthesis in proliferating cells. Cell **29**:459–468.
24. **Rüther, U., E. F. Wagner, and R. Müller.** 1985. Analysis of the differentiation-promoting potential of inducible c-fos genes introduced into embryonal carcinoma cells. EMBO J. **4**:1775–1781.
25. **Schimke, R. T.** 1984. Gene amplification in cultured animal cells. Cell **37**:705–713.
26. **Schorpp, M., U. Mallick, H. J. Rahmsdorf, and P. Herrlich.** 1984. UV-induced extracellular factor from human fibroblasts communicates the UV response to nonirradiated cells. Cell **37**:861–868.
27. **Ullrich, A., J. Shine, J. Chirgwin, R. Pictet, E. Tischer, W. J. Rutter, and H. M. Goodman.** 1977. Rat insulin genes: construction of plasmids containing the coding sequences. Science **196**:1313–1319.
28. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
29. **Yuspa, S. H., T. Ben, E. Patterson, D. Michael, K. Elgjo and H. Hennings.** 1976. Stimulated DNA synthesis in mouse epidermal cell cultures treated with 12-*O*-tetradecanoylphorbol-13-acetate. Cancer Res. **36**:4062–4068.