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12-*O*-Tetradecanoyl-Phorbol-13-Acetate Induction of the Human Collagenase Gene Is Mediated by an Inducible Enhancer Element Located in the 5'-Flanking Region

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Genomic clones coding for human fibroblast collagenase were isolated. By constructing and transfecting mutants with 5' and 3' deletion mutations of the 5' control region of the gene into human or murine cells, we delimited a 32-base-pair sequence at positions −73 to −42 which is required for the induction of transcription by the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate. The DNA element behaves as a 12-*O*-tetradecanoyl-phorbol-13-acetate-inducible enhancer: it mediates the stimulation of transcription to the heterologous herpes simplex virus thymidine kinase promoter and acts in a position- and orientation-independent manner. Differences in enhancer efficiency in different cell lines are interpreted to indicate differences in the activity of a *trans*-acting factor.

Inflammatory reactions are the response of an organism to various forms of injury and stress (24). Although ill-defined in molecular terms, the inflammatory process is known to include the action of intercellular mediators and growth factors (15, 29, 50). These affect the expression of genes, the proliferation of cells, and the biochemical phenotype of cells. Beside wound healing, pathologic endpoints such as self-destructive disease and carcinogenesis may be reached (6, 14, 42, 52).

While examining the changes in gene expression following the treatment of human primary skin fibroblasts with the skin irritant and tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) (23), we isolated and identified the gene for fibroblast collagenase (2, 53). This gene appears to have a role in both physiologic and pathologic inflammatory reactions (40, 42, 54). Its product, initially designated XHF1 (33), is the major secreted protein of human skin fibroblasts induced in response to TPA, mitomycin C, or UV irradiation (3, 23, 33). It is also induced by the inflammatory mediator interleukin-1 (12, 23, 37). Collagenase is a member of a larger family of metalloproteases which appear to be regulated similarly (9, 35, 53). Collagenase expression is elevated in certain tumor cells (31) and in the mutant fibroblasts derived from tumor-prone Bloom syndrome patients (33). The expression of stromelysin, another member of the family, has been reported to be elevated in rat cells transformed with polyomavirus, Rous sarcoma virus, or oncogene transfection (34, 35). The collagenase gene and other genes in this family may thus not only serve as exploring probes into the regulation of inflammation but also permit entry into the complex of oncogene-dependent genetic changes.

We describe here the isolation of collagenase-specific genomic clones by use of a full-length cDNA and an analysis of the promoter region by transfection of various chimeric constructs. We found that the 5'-flanking region of the collagenase gene contains a TPA-responsive element capable of activating heterologous promoters in response to the tumor promoter. The element behaves as an inducible enhancer.

MATERIALS AND METHODS

Enzymes and chemicals. Restriction endonucleases, Bal 31 nuclease, T4 DNA ligase, T4 polynucleotide kinase, nuclease S1, *Hin*dIII linkers (8-mer), and the M13 pentadecamer sequencing primer were purchased from Bethesda Research Laboratories Inc. (Neu-Isenburg, Federal Republic of Germany), Boehringer GmbH (Mannheim, Federal Republic of Germany), and New England BioLabs, Inc. (Schwalbach, Federal Republic of Germany). A nick translation kit, [α-^{32}P]dCTP, [γ-^{32}P]ATP, and [^{14}C]chloramphenicol were purchased from Amersham Corp. (Brunswick, Federal Republic of Germany), acetyl coenzyme A was purchased from Pharmacia, Inc. (Freiburg, Federal Republic of Germany), and cycloheximide and TPA were purchased from Sigma Chemical Co. (Taufkirchen, Federal Republic of Germany). All enzymes were used in accordance with the recommendations of the manufacturers.

Cells and culture conditions. Primary human skin fibroblasts were from a healthy 29-year-old male individual in Munich, Federal Republic of Germany (2). Simian virus 40-transformed human skin fibroblasts (GM637) from a normal individual were obtained from J. Cleaver. HeLa-TK$^-$ cells were from Y.-C. Cheng, human HepG2 cells were from R. Cortese, and murine L-TK$^-$ fibroblasts were from N. Hynes.Cells were grown in standard petri dishes in Dulbecco modified Eagle medium (for GM637, L-TK$^-$, and HepG2 cells) or Earle modified Eagle medium (for HeLa-TK$^-$ cells) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). After trypsinization, 8×10^5 cells were seeded per 10-cm dish. Experiments were usually done 24 h later. TPA was added to the medium at a concentration of 60 ng/ml unless specified otherwise. For serum starvation, the primary cells were placed into serum-free Dulbecco modified Eagle medium for 48 h prior to the experiment. UV irradiation was performed, and conditioned medium as a source of extracellular protein

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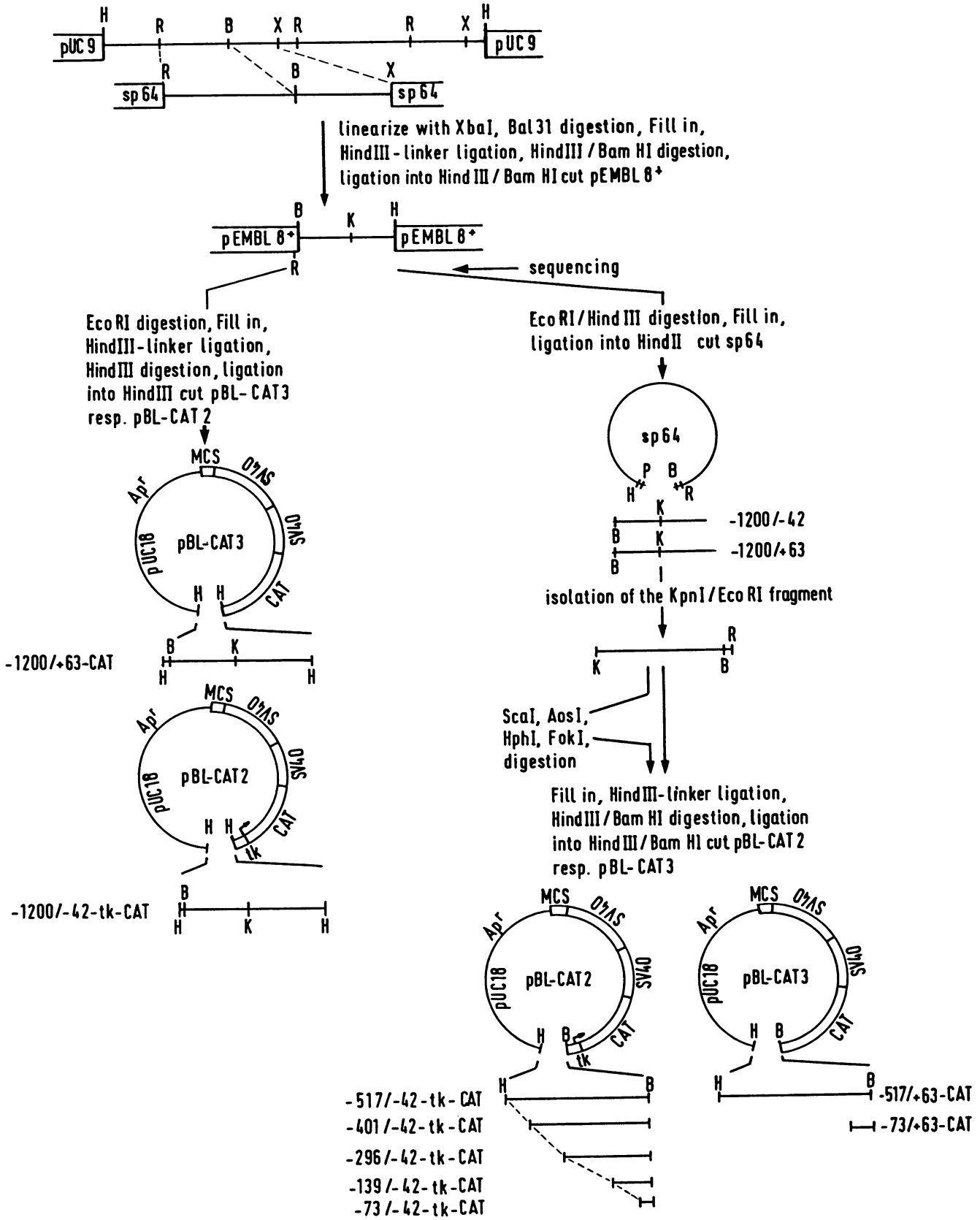


FIG. 1. Construction of 5′ and 3′ deletion mutants of the collagenase 5′-flanking region. Plasmids pBLCAT2, pBLCAT3, and Sp64 are described in Materials and Methods. The parent plasmid for the construction of deletion mutants was a 10.6-kb *Hin*dIII fragment of pCllase 2 (see Fig. 2) cloned in the *Hin*dIII site of pUC9. The numbers beside the different constructs refer to the 5′ and 3′ borders of the collagenase sequence used (compare with Fig. 3). −1200/+63 and −1200/−42 were selected from a series of Bal 31 deletions (cloned into pEMBL8$^+$) starting at the *Xba*I site in the first exon. B, *Bam*HI; R, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; X, *Xba*I. SV40, Simian virus 40.

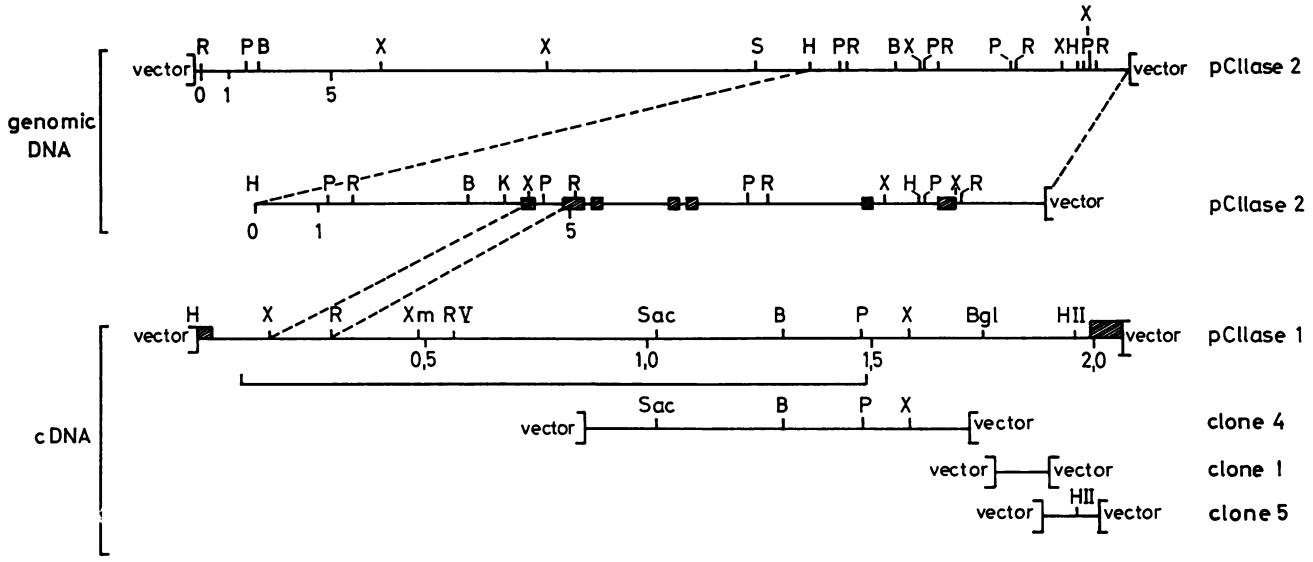


FIG. 2. Structures of the collagenase-specific genomic cosmid clone pCllase 2 and the cDNA clone pCllase 1. The cDNA clone pCllase 1 was isolated from a cDNA library of TPA-treated cells by colony hybridization with nick-translated clone 4 DNA (2, 53). The restriction map was established by single and double restriction enzyme digests and confirmed by sequencing of the cDNA. The cDNA sequence is available from the European Molecular Biology Laboratory data base. The line directly below pCllase 1 indicates the translated part of the mRNA. The hatched boxes indicate the deoxyguanosine-deoxyribosylthymine tails of the cDNA clone. The positions of the partial cDNA clones 1, 4, and 5 (2) are indicated. Counting the first nucleotide after a short cytosine tail as number 1, the pCllase 1 clone reaches the poly(A) tail (75 adenosine residues long) at nucleotide 1969. The first ATG codon (nucleotides 67 to 69) opens a reading frame of 469 amino acids (exact molecular weight, 53,910). The 5'-untranslated region contains the consensus ribosome-binding site CCATC (28) at positions 26 to 30. In the untranslated region, we found the sequence 5'-TTATTTAT-3' (positions 1,597 to 1,604), which also occurs in the RNAs coding for tumor necrosis factor, interleukin-1, fibronectin, colony-stimulating factor, and several interferons (7). The first 19 amino acids of the collagenase form a hydrophobic signal peptide terminating with a consensus cleavage site (51). The secreted proenzyme starts with amino acid 20 (molecular weight, 51,750), and the activated mature enzyme starts with amino acid 100 (molecular weight, 42,550). pCllase 2 is one of the genomic clones isolated from a human cosmid library by using ^{32}P-labeled pCllase 1 DNA for hybridization. The restriction map of pCllase 2 was established by single and double restriction enzyme digests. In the second pCllase 2 line is shown an extended version of pCllase 2 which starts at the *Hin*dIII site. The hatched boxes indicate the exons, as determined by sequence analysis (first exon) or electron-microscopic heteroduplex analysis. The positions of the *Xba*I and *Eco*RI sites in pCllase 1 and pCllase 2 (proven by sequencing) are indicated by broken lines. The numbers underneath the lines are base pairs (in thousands). R, *Eco*RI; P, *Pst*I; B, *Bam*HI; X, *Xba*I; S, *Sma*I; H, *Hin*dIII; Xm, *Xmn*I; RV, *Eco*RV; Sac, *Sac*I; Bgl, *Bgl*II; HII, *Hin*dII; K, *Kpn*I.

synthesis-inducing factor (EPIF) was obtained as described previously (48). The conditioned medium was transferred to new cells without dilution.

Cosmid library and screening. To isolate collagenase-specific genomic clones, we screened a genomic cosmid library from *Sau*3A-digested human lymphocyte DNA cloned into pcos 2 EMBL (44) with the complete collagenase cDNA clone pCllasel (see Fig. 2 and reference 53). The cosmid library was kindly provided by H. Lehrach. Hybridization conditions were described previously (10).

DNA sequencing. DNA sequencing was performed by dideoxynucleoside triphosphate-dependent premature termination (13, 45) and by the method described in reference 36. Electron-microscopic heteroduplex analysis was done as described previously (5).

Plasmid construction. The generation of most of the deletion mutants is described in Fig. 1. Plasmid Sp64 has been described previously (39). Plasmid pBLCAT2 (containing the herpes simplex virus thymidine kinase [TK] promoter from positions −105 to +56 in front of the chloramphenicol acetyltransferase [CAT] structural gene) and plasmid pBLCAT3 (without the promoter), both constructed by B. Luckow and G. Schütz (unpublished data; see also reference 41), were kindly provided by L. Klein-Hitpass (27). For Bal 31 deletion, the −139/−42 TK-CAT construct was linearized with *Hin*dIII and digested with Bal 31. After a fill-in reaction, *Hin*dIII linker ligation, and digestion with *Hin*dIII and *Pst*I (located in the TK promoter), the fragment containing the desired deletion was exchanged for the corresponding *Hin*dIII/*Pst*I fragment in pEMBL8$^+$ CAT2 (27; see also legend to Fig. 5). Endpoints of the Bal 31 deletions were determined by sequencing (13).

The insertion mutants were constructed by inserting a 326-base-pair (bp) *Bgl*II-*Bam*HI fragment from the 3'-untranslated region of pCllase 1 into the unique *Bam*HI site of the −139/−42 TK-CAT construct (Fig. 1, designated "a" in Fig. 7A; yielding −139/−42 ↔ TK) or pBLCAT2 (yielding ↔ TK). For construction of mutants −139/−42 TK-CAT$_B$, −42/−139 TK-CAT, and TK-CAT −139/−42 (Fig. 7A), the −139/−42 *Hin*dIII-*Bam*HI fragment of −139/−42 TK-CAT$_A$ was isolated, and the ends were filled in and blunt-end ligated into the filled-in *Bam*HI site 5' of the TK promoter of TK CAT (yielding −139/−42 TK-CAT$_B$ and −42/−139 TK-CAT) or the *Sma*I site of pBLCAT2 (polylinker sequence 3' of the CAT gene; yielding TK-CAT −139/−42) (Luckow and Schütz, unpublished data; see also reference 41).

Transient transfection and CAT assay. GM637 cells, HeLa-TK$^-$ cells, L-TK$^-$ cells, or HepG2 cells were seeded at 8 × 10^5 cells per 10-cm petri dish. After 24 h the cells were washed twice with Tris-buffered saline (TBS [4]). Subsequently, a 1-ml transfection solution containing 10 μg of CsCl-purified plasmid DNA and 500 μg of DEAE-dextran

(molecular weight, 2×10^6; Pharmacia) in TBS was added to the cells. After 30 min at room temperature, the cells were washed once with TBS and incubated in culture medium containing 0.1 μM chloroquine diphosphate for 10 h. The cells were washed twice with TBS and incubated in culture medium in the presence or absence of TPA (60 ng/ml) for 48 h. For GM637 fibroblasts, the chloroquine treatment was omitted. After treatment, the cells were washed twice with TBS, scraped into ice-cold phosphate-buffered saline (PBS), pelleted, suspended in 100 μl of 0.25 M Tris hydrochloride (pH 7.8), and broken by three cycles of freezing and thawing. The extracts were centrifuged in a microcentrifuge at 4°C for 10 min, and the protein content of the supernatants was determined as described previously (32). CAT activity was measured as described previously (19).

RNA preparation and nuclease S1 analysis. RNA was prepared as described previously (2). The probe for nuclease S1 analysis was a collagenase-specific genomic subclone containing collagenase sequences from about position −3000 (*Eco*RI) to the *Xba*I site located in the first exon (position +139). The plasmid was linearized with *Xba*I, dephosphorylated, and cut with the single-site cutter *Kpn*I at position −517. After separation on a low-melting-temperature agarose gel, the 656-bp *Kpn*I-*Xba*I fragment was isolated and labeled at the *Xba*I end in a kinase reaction. Nuclease S1 analysis was performed as described previously (25), except that the hybridization temperature was 47°C. The probe was protected by collagenase mRNA over a length of 139 nucleotides (major start) or 144 nucleotides (minor start).

RESULTS

Isolation of collagenase-specific genomic clones. A cosmid library carrying 35- to 45-kilobase (kb) inserts of human lymphocyte DNA (44) was screened for genomic fragments hybridizing to collagenase cDNA. We used as a probe a full-length collagenase cDNA clone (pCllase 1) which we had isolated from a cDNA library prepared with poly(A)$^+$ RNA from TPA-treated fibroblasts (Fig. 2 and reference 53) (the complete sequence is available from the European Molecular Biology Laboratory data base). Two clones, pCllase 2 and pCllase 3, were isolated. pCllase 2 covers most of the 5′ portion of the gene plus about 28 kb of 5′-flanking DNA (Fig. 2). pCllase 3 contains the utmost 3′ end of the cDNA and 3′-flanking sequences of the gene (data not shown).

Because of the primary interest in gene regulation, efforts were concentrated on pCllase 2. By electron-microscopic analysis of heteroduplex molecules between the cDNA clone pCllase 1 and the genomic clone pCllase 2, the approximate positions of exons and introns were determined (Fig. 2 and data not shown). pCllase 2 carries about 1.2 kb of coding sequences. These are spread over 7 kb of genomic DNA, divided into seven exons of 113 to 327 bp.

The region between the *Xba*I site within the first exon and the first *Kpn*I site 5′ of the exon was sequenced (Fig. 3). The sequence features will be described below.

Start of transcription. Nuclease S1 protection analysis was used to map the transcriptional start site. RNA from TPA-treated human fibroblasts was hybridized to a *Kpn*I-*Xba*I fragment of pCllase 2 labeled at the *Xba*I site. Two protected fragments were obtained: a major one of 139 nucleotides and a minor one of 144 nucleotides (Fig. 4). The 5′ end of the major transcript corresponded exactly to the 5′ end of the cDNA clone. The minor start is indicated as −5 in Fig. 3. Suitable TATA box sequences are located between −27 to −24 and −31 to −25. A third, very faint protected fragment which was seven nucleotides shorter than the major transcript was also observed.

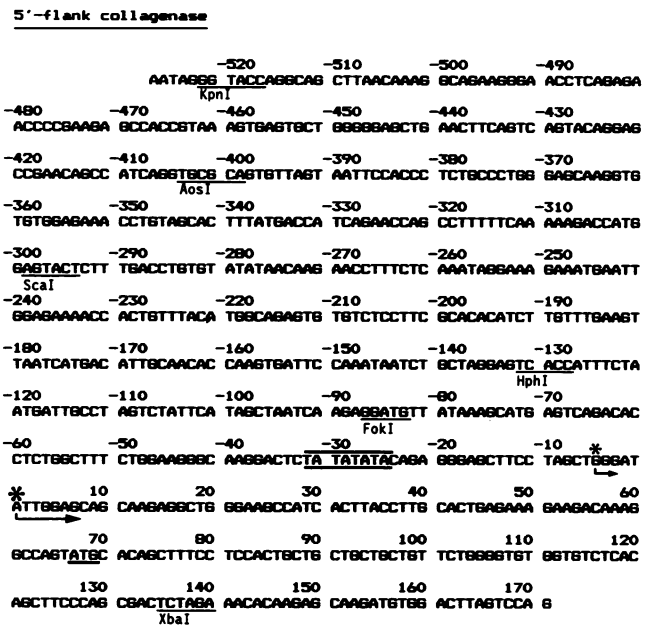


FIG. 3. Sequence of the 5′-flanking region of pCllase2. The sequence of the 5′-flanking region was established as described previously (45) by using pEMBL8$^+$ (13) as a vector for a series of overlapping Bal 31 deletions generated from the *Xba*I site in the first exon. The restriction sites used for the generation of 5′ deletion mutations are indicated. The major start site of transcription is indicated by a large asterisk and a large arrow, and the minor one (at position −5) is indicated by small symbols. The two potential TATA boxes are outlined, and the start site of translation is underlined.

Regulation of mRNA abundance. The same type of nuclease S1 analysis (in excess of the DNA probe) served to quantitate collagenase mRNA levels (Fig. 4). Both the major and the minor transcripts were regulated coordinately. The basal level of RNA varied with growth conditions. In the absence of serum (Fig. 4A, lane 10), transcripts were barely detectable. Cells supplied with 10% fetal calf serum contained fourfold higher levels (Fig. 4A, lane 2). Upon the addition of TPA, the abundance of collagenase mRNA species increased rapidly to a maximal level that was 15- to 20-fold higher than that in nontreated cells (Fig. 4A, lanes 2 to 7). The lag period for this response was less than 30 min (Fig. 4B). The effect of TPA was observed in the presence or absence of serum factors. The gene was induced better in the absence of serum (Fig. 4A, lanes 10 and 11).

Adding cycloheximide (at doses which blocked protein synthesis to more than 95%) together with TPA reduced the total yield of collagenase mRNA (Fig. 4A, lane 9). The induction of mRNA by TPA, however, was not abolished.

The abundance of collagenase transcripts was also enhanced by UV irradiation and by the UV-induced factor EPIF (48) (Fig. 4). Note the long lag period of the UV induction response, which suggests an indirect activation mechanism, possibly through the production of the UV-induced factor EPIF. EPIF itself acts fast (23, 48).

TPA-responsive sequence. The strong enhancement by TPA of collagenase mRNA abundance encouraged us to examine the mechanism of induction. Since collagenase mRNA is stable, transcriptional activation seemed likely.

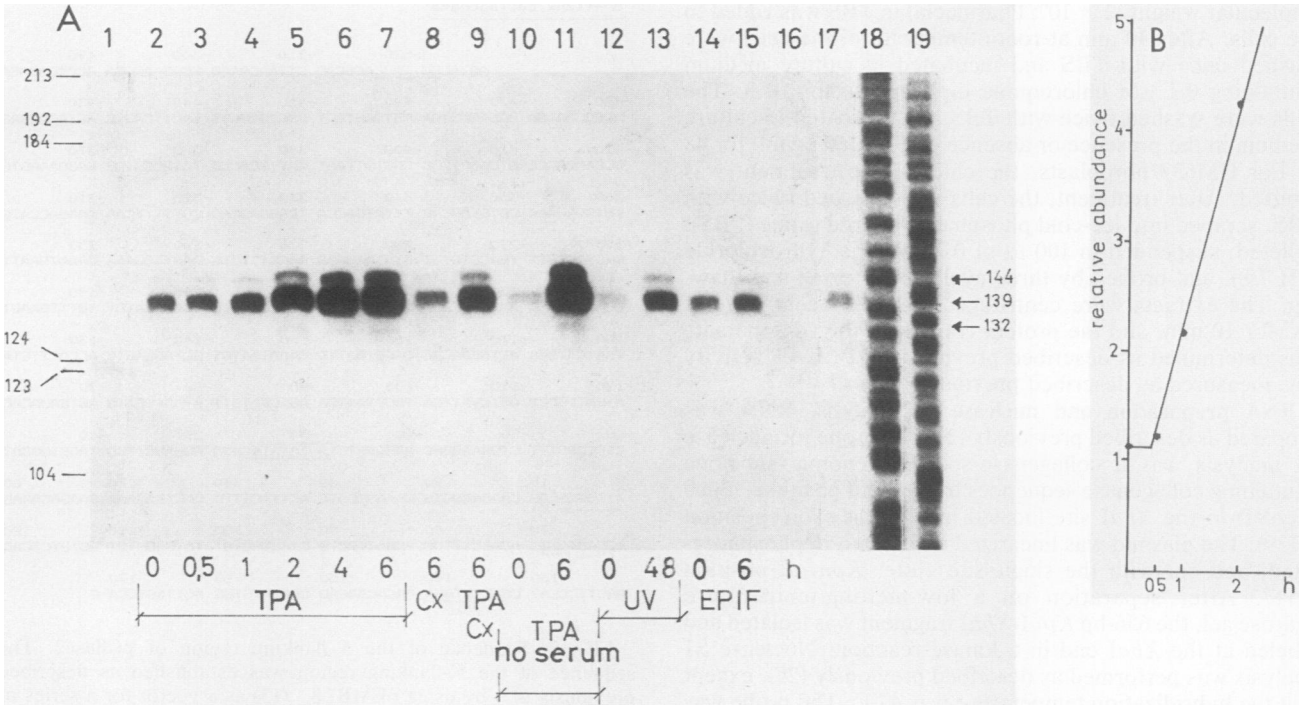


FIG. 4. Regulation of human collagenase expression. (A) Total RNA (20 μg) from normal human fibroblasts was hybridized to an excess of the *Kpn*I-*Xba*I fragment (positions −517 to +139) labeled at the *Xba*I site. Lanes: 1, end-labeled *Hae*III-cut pBR322; 2 to 7, RNA from logarithmically growing normal human fibroblasts treated with TPA (20 ng/ml) for 0, 0.5, 1, 2, 4, or 6 h, respectively; 8, RNA from cells treated with cycloheximide (Cx) (10 μg/ml) for 6 h; 9, same as lane 8, but in the presence of TPA (20 ng/ml); 10, RNA from quiescent cells (serum starvation for 48 h); 11, same as lane 10, but treated with TPA for 6 h; 12, RNA from mock-irradiated cells; 13, RNA from cells irradiated with UV light (30 J/m^2) and harvested 48 h later; 14, cells treated with conditioned medium from nonirradiated cells; 15, same as lane 14, but treated for 6 h with EPIF-containing conditioned medium harvested from UV-irradiated cells; 16, *Saccharomyces cerevisiae* RNA; 17, 1 μg of poly(A)$^+$ RNA from TPA-treated fibroblasts; 18 and 19, sequencing reactions (36) with the known sequence of the mouse mammary tumor virus long terminal repeat (25) serving as markers for the accurate size determination of the protected fragments (lane 18, adenine-plus-guanine reaction; lane 19, cytosine-plus-thymine reaction). The numbers on the left and right sides of panel A are lengths in base pairs. (B) Densitometric evaluation of the kinetics of TPA-mediated collagenase induction (lanes 2 to 5 in panel A).

The ability of the 5′-flanking area of the collagenase gene to stimulate transcription from a heterologous promoter in the presence of TPA indicates this point. This type of experiment also served to delimit the sequence responsible for the stimulation. We constructed chimeric genes consisting of various portions of the collagenase promoter region 5′ to position −42, the herpes simplex virus TK promoter, and the CAT-coding sequence (Fig. 1). This series complemented a similar one in which the collagenase promoter instead of the TK promoter was used (fused to CAT at position +63). To determine the effect of TPA on CAT expression, we assayed these constructs in transient transfections into HeLa-TK$^-$ cells, GM637 human fibroblasts, HepG2 human hepatoma cells, and murine L-TK$^-$ fibroblasts.

The basal level of CAT expression from the collagenase promoter in the absence or presence of serum was very low in most cells. In the absence of TPA, however, the sequence −1200 to −42 or −517 to −42 increased basal transcription from the TK promoter two- to fourfold (Table 1). This was also true for L-TK$^-$ cells, which had a relatively high spontaneous TK promoter activity (Table 1). We did not test whether serum starvation would reduce the spontaneous activity. (Note also that comparisons between cell lines are not valid without a measure of transfection efficiency.) Thus, in the cell systems used, the 5′-flanking region does contain a sequence with basal-level enhancer activity, although the activity is low in comparison to that in the presence of TPA, and in comparison to that of other basal-level enhancers (26). In the presence of TPA, the sequences −1200 to −42 or −1200 to +63 as well as −517 to −42 or −517 to +63 stimulated expression from either promoter (Fig. 5 and Table 1). As demonstrated by primer extension, the increased CAT activity was due to an increased level of transcripts which started at the natural (major) cap site of the collagenase promoter (Fig. 6) or the TK promoter (data not shown). The enhancement was 2-fold in L-TK$^-$ cells, 2-fold in rat XC cells (data not shown), 25-fold in GM637 cells, 50-fold in HepG2 cells, and 250-fold in HeLa-TK$^-$ cells. Several deletion mutants, including that containing the sequence −73 to −42, were tested and showed these cell-dependent differences (Table 1 and data not shown). The sequence −4300 to −1200 conferred no TPA responsiveness (Table l).

To delimit the TPA-responsive sequence element, we analyzed various deletion mutants (Fig. 5). Care was taken that the same vector sequence always flanked the deletion endpoints. The series shown (Table 1) indicated that a TPA-responsive element was found between positions −73 and −42. The TPA response was lost when deleting beyond position −73, e.g., mutant −66/−42 was not inducible (Fig. 5). This result indicates that the left end of the element is at position −73 and that the 3′ end is 5′ of position −42. Because TPA responsiveness decreased about fivefold after

TABLE 1. Effect of TPA on CAT expression

Construct transfected into[a]:	CAT level (pmol/min per mg of protein) in:		Induction (fold)
	Control cells	TPA-treated cells	
HeLa-TK$^-$ cells			
None	6	6	
pBLCAT3	6	6	1
−1200/+63 (in pBLCAT3)	16	2,001	200
−517/+63 (in pBLCAT3)	23	3,811	224
−73/+63 (in pBLCAT3)	41	1,569	45
pBLCAT2 (TK promoter)	32	139	5
−4300/−1200 (in pBLCAT2)	11	24	4
−1200/−42 (in pBLCAT2)	47	4,316	105
−517/−42 (in pBLCAT2)	63	3,938	69
−401/−42 (in pBLCAT2)	112	4,599	43
−296/−42 (in pBLCAT2)	94	4,057	46
−139/−42 (in pBLCAT2)	189	9,293	51
−73/−42 (in pBLCAT2)	61	1,144	21
None	5	5	
pBLCAT2	8	25	7
↔ (in pBLCAT2)	8	20	5
−139/−42 (in pBLCAT2)	11	1,345	220
↔ (in −139/−42 in pBLCAT2)	8	319	105
−139/−42 TK-CAT	12	1,098	150
−42/−139 TK-CAT	20	1,104	73
TK-CAT −139/−42	12	412	60
None	5	5	1
−131/−42 [in pEMBL8$^+$ CAT2]	19	1,323	94
−91/−42 [in pEMBL8$^+$ CAT2]	20	893	60
−90/−42 [in pEMBL8$^+$ CAT2]	16	935	84
−66/−42 [in pEMBL8$^+$ CAT2]	9	28	6
−61/−42 [in pEMBL8$^+$ CAT2]	11	24	3
−56/−42 [in pEMBL8$^+$ CAT2]	11	34	5
−47/−42 [in pEMBL8$^+$ CAT2]	9	35	7
−44/−42 [in pEMBL8$^+$ CAT2]	8	22	6
GM637 cells			
None	2	2	
pBLCAT3	2	2	1
−1200/+63 (in pBLCAT3)	9	186	26
pBLCAT2	25	16	0.6
−1200/−42 (in pBLCAT2)	63	461	8
HepG2 cells			
None	2	2	
pBLCAT3	2	2	1
−1200/+63 (in pBLCAT3)	3	49	47
L-TK$^-$ cells			
pBLCAT2	460	586	1.3
−1200/−42 (in pBLCAT2)	965	2,515	2.6
−139/−42 (in pBLCAT2)	1,882	3,748	2.0

[a] All cells were growing (no starvation). ↔, Designation for a 326-bp insert (see Fig. 7).

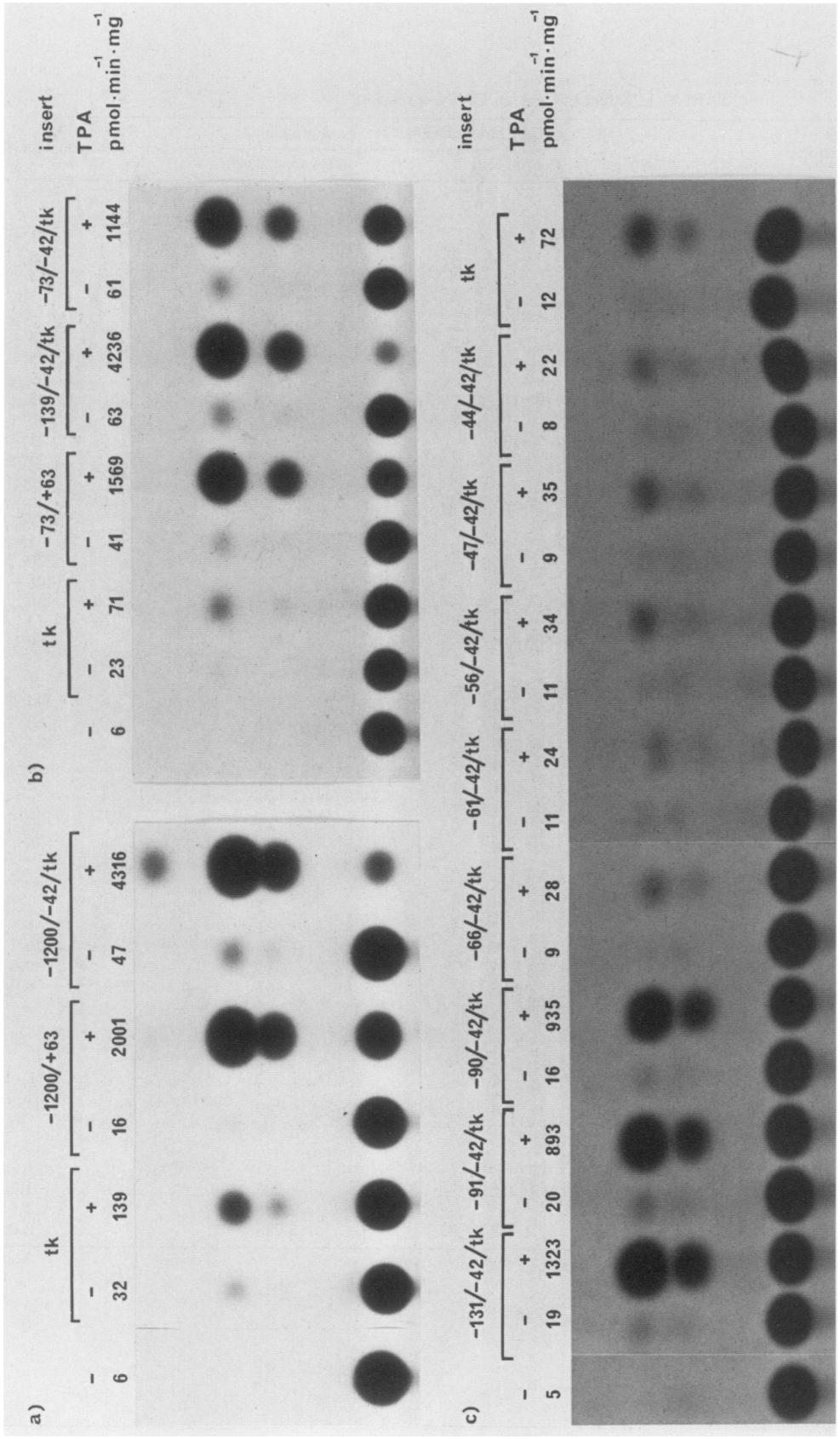


FIG. 5. TPA-responsive element of the human collagenase gene. Transient transfection experiments in HeLa-TK$^-$ cells are shown. The 5′-flanking sequences cloned in front of the CAT-coding sequences (see also Fig. 1) or in front of the TK promoter plus the CAT coding region are indicated above the brackets, e.g., −1200/+63, meaning that the 5′ flank and promoter of the collagenase gene from positions −1200 to +63 were placed in front of the CAT sequences, or −1200/−42/tk, meaning that the 5′ flank of the collagenase gene from positions −1200 to −42 was placed in front of the TK promoter. tk above the brackets represents the promoter of the HSV-TK gene from positions −105 to +56. Bal 31 deletions were cloned in front of the TK-promoter of pEMBL8$^+$ CAT2, in which the pUC18 sequences of pBLCAT2 are replaced by pEMBL8$^+$. In the first row of each independent group of CAT assays (a, b, c), [^{14}C]chloramphenicol was incubated with a protein extract from nontransfected HeLa-TK$^-$ cells. The specific activity (picomoles of acetylated chloramphenicol per milligram of protein extract per minute) was calculated by excising the radioactive spots and determining the radioactivity in a liquid scintillation counter. When the substrate was depleted, the assay was repeated with a smaller amount of cell extract and the conversion rate was determined.

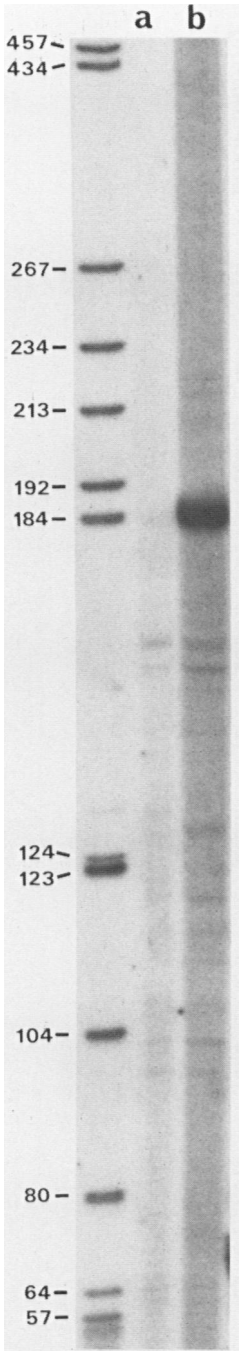


FIG. 6. Primer extension analysis of RNA extracted from human fibroblasts stably transfected with collagenase-CAT chimeric genes. RNA was prepared from a mass culture (80 clones) of G418-resistant human fibroblasts (GM637) stably cotransfected with the −1200/+63 CAT construct and pSV2neo. A 5′ ^{32}P-labeled 30-mer oligonucleotide (starting at the ATG codon of the CAT-coding region) was hybridized to the RNA, extended with reverse transcriptase, and fractionated on a 6% polyacrylamide gel containing 8 M urea. With the authentic start of transcription utilized in vivo, the product is predicted to be 183 bp long. Lanes: a, RNA from nontreated cells; b, RNA from cells treated with TPA (20 ng/ml) for 12 h. With RNA from nontransfected cells, no extension products were observed (data not shown). We made sure that equal amounts of RNA were applied to each lane by analyzing the content of actin RNA on Northern blots. Numbers at left are lengths in nucleotides. The leftmost lane shows size markers (*Hae*III-digested pBR322).

a deletion of sequences upstream of position −73, other TPA-responsive elements may be present upstream of that position.

Inducible enhancer. The TPA-responsive sequence is a TPA-dependent enhancer element. Our finding that the DNA element at positions −73 to −42 conferred TPA inducibility to the collagenase and TK promoters suggested that this element is a TPA-inducible enhancer. To investigate this point, we examined whether this element acted in a position- and orientation-independent manner. Insertion of a 326-bp DNA segment between the TPA-responsive element and the TK promoter at position −42 did not diminish its enhancing capability (Fig. 7). Moreover, when ligated in both orientations in front of or behind the TK-CAT gene, the TPA-responsive element conferred similar TPA inducibility in HeLa-TK$^-$ cells. Even at 2 kb 3′ of the gene, the element still worked efficiently (Fig. 7 and Table 1).

DISCUSSION

Collagenase mRNA is the major inducible transcript in human fibroblasts treated with tumor promoters. Other agents also induce the mRNA and the enzyme, e.g., UV irradiation (Fig. 4 and reference 23), urate crystals (20), cytochalasin B (1), interleukin-1 (23, 37), the UV-induced factor EPIF (Fig. 4 and reference 48), and agents which inhibit replication (unpublished data). The physiologic inducers are not known.

Using a full-length collagenase cDNA clone, we isolated and characterized collagenase-specific genomic clones. Two nonoverlapping cosmid clones hybridizing to the cDNA were obtained. Clone pCllase 2, which carries the 5′ portion of the gene, including 1.2 kb of exons, also carries about 28 kb of flanking DNA. The start sites for the major and minor transcripts detected are probably defined by two TATA boxes which overlap. There are no typical upstream elements such as the CAAT box (17) or the GC box specific for the SP1 factor (16).

We used pCllase 2 to study regulation by one of the inducing agents, TPA. Upon transfection of chimeric collagenase-flanking region–CAT constructs into various cells, these genes were inducible by TPA. Using deletion mutants, we defined the 5′ border and the approximate 3′ end of the TPA-responsive element.

Several interesting features characterize the TPA-responsive element. First of all, at least in HeLa-TK$^-$ cells, there is only a low basal activity of the TPA-responsive element in any of the collagenase-CAT constructs in the absence of TPA, with either the collagenase promoter or the TK promoter. In the same cells, metallothionein II_A is expressed at a rather high basal level (data not shown). If both collagenase and metallothionein II_A contained functionally equivalent regulatory elements, perhaps their spatial arrangement differs, possibly determining the difference in basal expression (21). After removing the 5′-flanking DNA of collagenase, we found no evidence for negatively regulating elements (18, 30, 49). The same observation has been made for metallothionein II_A (26).

Although located fairly close to the TATA box, the TPA-responsive element has enhancer properties and thus belongs to the class of inducible enhancers (8, 25, 27, 47). It can be moved and turned around without loss of enhancing activity. It activates the collagenase promoter or the herpes simplex virus TK promoter with similar efficiency and, as shown by primer extension, the enhanced transcription starts at the same sites which are used in the authentic

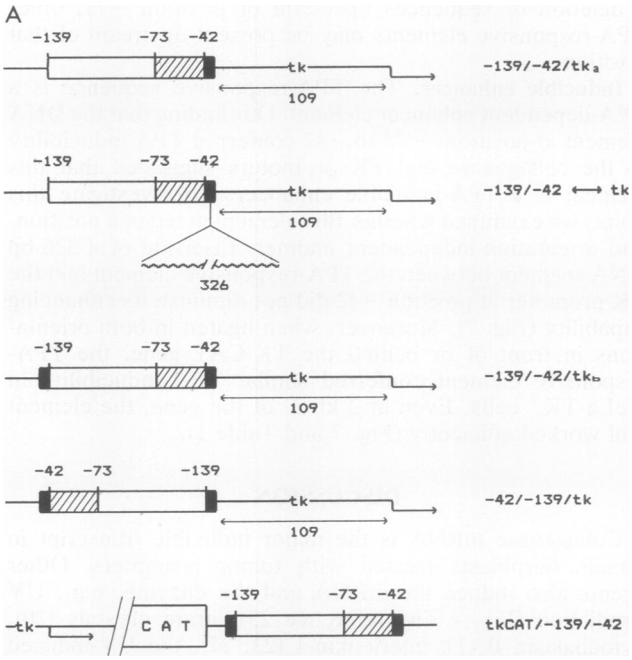
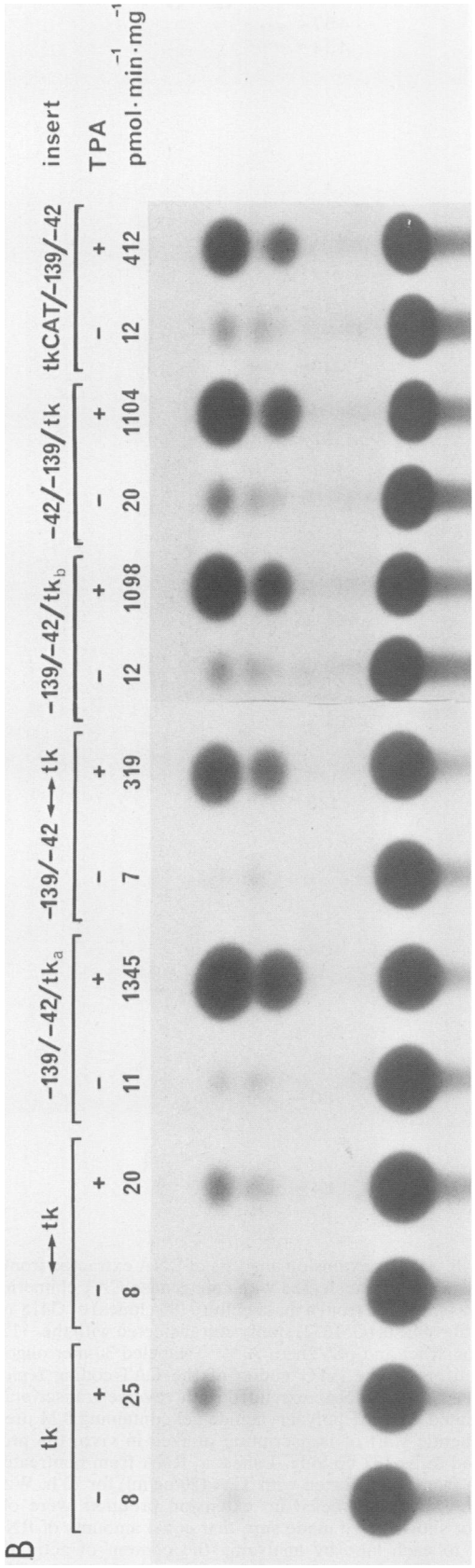


FIG. 7. Behavior of the TPA-responsive element as a conditional enhancer. The following gene constructs were transfected transiently into HeLa-TK$^-$ cells and probed for TPA inducibility: tk, pBLCAT2; ↔ tk, pBLCAT2 containing a 326-bp insert (described in Materials and Methods) in the *Bam*HI site (5′ of the TK promoter); −139/−42/tk$_a$, pBLCAT2 containing collagenase sequences from positions −139 to −42 in front of the TK promoter; −139/−42 ↔ tk, same as −139/−42/tk$_a$, but with a 326-bp insert between the collagenase sequences and the TK promoter; −139/−42/tk$_b$, pBLCAT2 containing collagenase sequences from positions −139 to −42 cloned in the *Bam*HI site in syn orientation (blunt-end ligation after filling in of the overhangs); −42/−139/tk, same as −139/−42/tk$_b$, but in anti orientation; tkCAT/−139/−42, same as −139/−42/tk$_b$, but cloned in syn orientation in the *Sma*I site of pBLCAT2 located 3′ of the CAT gene. (A) Schematic representation of the chimeric constructs. Solid box, Linker sequences; open box, collagenase sequence −139 to −73; hatched box, collagenase sequence from position −73 to position −42. —tk—, TK promoter. (B) Expression of the constructs as determined by CAT activity (see legend to Fig. 5).

promoters. In terms of the repeat structure or redundancy of other enhancers (22, 46), the residual TPA-responsive collagenase promoter and the TPA-responsive element itself appear less complex. In the 5′ control region of the collagenase gene there is no exact duplication of the TPA-responsive element. However, it is possible that this region carries an additional TPA-responsive element to the left of position −73, because the enhancement of the −73/−42 TK-CAT construct by TPA was always reduced in comparison to that of all the longer constructs tested.

In the rat stromelysin gene, a nonanucleotide, 5′-ATGAGTCAG-3′, occurs at almost exactly the same position (−72 to −64) with respect to the TATA box and to the start of transcription as in the human collagenase gene (35). The human proenkephalin gene, which is also inducible by TPA, contains a similar sequence at positions −93 to −85 when two base-pair changes are permitted (11). We are currently examining whether the nonanucleotide in fact suffices as an enhancer element, and we are testing permissive alterations in the sequence to predict the sites in other TPA-inducible genes, e.g., c-*fos* and metallothionein II$_A$.



In HeLa-TK$^-$ cells, the TK-promoter itself seemed to be weakly inducible by TPA, although it does not contain the TPA-responsive element described here (Fig. 5 and 7B and Table 1). Here, the SP1 factor or the GCAAT transcription factor may be influenced (38). In contrast, in HepG2 human hepatoma cells and GM637 human fibroblasts, in which the collagenase-TK-CAT constructs were inducible by tumor promoters, the TK promoter itself was not stimulated by TPA (Table 1 and data not shown).

For communication to occur between TPA and the TPA-responsive element of the collagenase gene, at least one *trans*-acting protein factor is necessary. The rapid induction of collagenase mRNA and its relative insensitivity to cycloheximide suggest that a preexisting protein is involved and that this protein is activated posttranslationally. It is possible that a short-lived second component is required for full induction by TPA. It is tempting to speculate that the activation of the stable factor acting on the TPA-responsive element is due to phosphorylation by protein kinase C, the major TPA receptor (43). The phosphorylation could (i) enhance the binding of the protein to the TPA-responsive element, (ii) modify the activity of the protein already bound to DNA prior to TPA treatment, or (iii) influence protein-protein interactions (P. Herrlich, C. Jonat, H. J. Rahmsdorf, P. Angel, A. Haslinger, M. Imagawa, and M. Karin, J. Cell. Biochem., in press). The involvement of protein kinase C would form an attractive hypothesis unifying genetic responses to adverse agents and mitogenic reactions, both of which need to occur in inflammatory processes.

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