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Transcriptional Induction of Stromelysin-3 in Mesodermal Cells Is Mediated by an Upstream CCAAT/Enhancer-binding Protein Element Associated with a DNase I-hypersensitive Site*

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Stromelysin-3 (ST3) is a matrix metalloproteinase whose synthesis is markedly increased in stromal fibroblasts of most invasive human carcinomas. In the present study, we have investigated the molecular mechanisms by which high levels of ST3 expression can be induced. In contrast to the early and transient induction of interstitial collagenase by 12-O-tetradecanoylphorbol-13-acetate (TPA), the fibroblastic induction of ST3 was found to be delayed and to require protein neosynthesis. We demonstrated that this induction is transcriptional and does not result from changes in RNA stability. By looking next to promoter regions accessible to DNase I upon gene induction, we have identified two distal elements and have characterized their role in the transcriptional regulation of ST3. The first one is a TPA-responsive element that controls the base-line ST3 promoter activity but is not required for its activation. We demonstrate that ST3 gene induction is actually mediated by the second element, a C/EBP-binding site, by showing: (i) that this element becomes accessible in cells induced to express ST3, (ii) that endogenous C/EBP β binds to the ST3 promoter, and (iii) that this binding leads to ST3 transcriptional activation. Our study provides new insights into the regulation of ST3 and suggests an additional role for C/EBP transcription factors in tissue remodeling processes associated with this MMP.

Remodeling of the extracellular matrix occurs during the growth and differentiation of normal tissues as well as in pathological conditions involving abnormal growth and tissue destruction. Various members of the matrix metalloproteinase (MMP)¹ family have been shown to play an active role in these

processes by degrading most of the extracellular matrix components (1). Stromelysin-3 (ST3) is a member of this MMP family, and its expression *in vivo* has been associated with physiological situations such as development (2), mammary gland involution (3), cycling endometrium (4), or cutaneous wound healing (5). In pathological situations, ST3 is expressed in the stromal compartment of most invasive human carcinomas (6, 7) and has also been detected to a lesser extent in some types of sarcomas (6, 8). Its expression has been correlated with tumor aggressiveness and poor clinical outcome in breast carcinoma (9, 10). In addition, the contribution of ST3 to tumor progression has been reported in experimental models of tumorigenesis (11, 12). Therefore, this MMP might represent a potential target for therapeutic approaches directed against the stromal compartment of human carcinomas.

At the cellular level, ST3 has been essentially observed in cells of mesodermal origin such as fibroblastic cells both *in vivo* and in culture where it can be induced by growth factors (13, 14), tumor promoter agents (13), and retinoic acid (15). On the other hand, recent studies using “tumor/stroma” coculture assays have shown that the fibroblastic expression of ST3 can be induced when fibroblasts are exposed to epithelial cancer cells (16, 17), as initially suggested by *in situ* hybridization studies of breast carcinomas indicating a predominant expression of ST3 in fibroblastic cells located in close vicinity to epithelial cancer cells (13). However, although a number of *in vivo* studies have emphasized the possible contribution of this enzyme in physiological and pathological processes, the molecular factors and mechanisms controlling its expression are still poorly understood.

Most MMP genes are characterized by the presence of an AP-1-binding site in their proximal promoter that mediates transcriptional activation by growth factors, phorbol esters, and oncogenes (18). In addition, this AP-1-binding site also mediates their transcriptional repression by retinoic acid and glucocorticoids (19, 20), which have been associated with anti-tumoral (21) and anti-inflammatory (22) activity, respectively. In contrast, despite the fact that ST3 can also be induced by phorbol esters, its proximal promoter is characterized by the absence of an AP-1 consensus-binding site and the presence of a retinoic acid-responsive element (23). Accordingly, ST3 expression is induced by retinoic acid through this retinoic acid-responsive element interacting with retinoic acid receptor/retinoid X receptor heterodimers (15). In addition, basic fibroblast growth factor was shown more recently to modulate ST3 expression in osteoblastic cells by controlling the stability of its mRNA as well as its transcriptional rate (14). However, no

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¹ The abbreviations used are: MMP, matrix metalloproteinase; C/EBP, CCAAT/enhancer-binding protein; DRB, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole; ST3, stromelysin-3; TPA, 12-O-tetradecano-

ylphorbol-13-acetate; DHS, DNase I-hypersensitive site(s); PIPES, 1,4-piperazinediethanesulfonic acid; bp, base pair(s); kb, kilobase(s).

regulatory element that could account for basic fibroblast growth factor- or 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-mediated ST3 induction has been identified, and to our knowledge, the proximal retinoic acid-responsive element remains the only regulatory element that has so far been functionally characterized in the ST3 promoter. In the present study, we have further investigated the mechanism by which high levels of ST3 gene expression can be induced. The tumor promoter TPA was used to compare the strong fibroblastic induction of ST3 to that of interstitial collagenase to determine the respective contribution of transcriptional and post-transcriptional mechanisms. By looking to DNase I-hypersensitive sites (DHS) associated with open chromatin in TPA-differentiated cells, we have identified a distal AP-1-binding site and a C/EBP element and determined their respective roles in the ST3 promoter activation. Our data demonstrate that ST3 induction by TPA is associated with a strong increase in C/EBP DNA binding activity and that the isolated C/EBP element and the native ST3 promoter are similarly activated by C/EBP β in a dose response manner.

EXPERIMENTAL PROCEDURES

Cell Cultures—HFL1 embryonic human fibroblasts (CCL 153) and the rhabdomyosarcoma tumor cell line RD (CCL 136) were obtained from the American Tissue Culture Collection (Manassas, VA) and maintained in monolayer culture in Dulbecco's modified Eagle's medium supplemented with 5% calf serum, unless indicated for specific experiments. HeLa cells were cultured in the same medium in the presence of 10% fetal calf serum. TPA was purchased from Sigma, whereas 9-*cis*-retinoic acid was provided by P. F. Sorter, J. F. Grippo, and A. A. Levin (Hoffmann-La Roche, Nutley, NJ). The requirement of newly synthesized protein was addressed by using 100 μ g/ml cycloheximide, a concentration that has been previously shown to efficiently inhibit translation in human fibroblasts without apparent toxicity for a period of 24 h (24).

RNA Isolation and Northern Blot Analysis—Cell cultures were washed with phosphate-buffered saline, and RNA extraction was carried out by the guanidinium thiocyanate phenol/chloroform procedure (25). 10–30 μ g of total RNA was denatured at 65 °C for 5 min and electrophoresed on 1% agarose gel prior to be transferred onto a nylon membrane (Hybond-N; Amersham Pharmacia Biotech). Hybridization conditions and cDNA probes were previously described (15).

Analysis of mRNA Stability—mRNA stability was evaluated by the RNA synthesis inhibitor-chase method (26). Briefly, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB, Sigma) was used to inhibit transcription (27) in fibroblasts, and total RNA was isolated at several time points. The amount of ST3 mRNA and the time course for its decay following DRB addition was measured by Northern blot hybridization. ST3 mRNA half-life was determined after signal quantification using a bio-imaging analyzer (BAS 2000; Fuji Ltd.).

Nuclear Run-on Assay—Nuclei isolation and *in vitro* elongation reaction were essentially performed as described previously (15). Briefly, nuclei were prepared from control cells or cells treated with TPA in serum-free medium using Nonidet P-40 lysis buffer. Nascent RNA transcripts from 2×10^7 nuclei were allowed to elongate in the presence of [α -³²P]UTP and were subsequently purified using phenol/chloroform extraction followed by trichloroacetic acid precipitation. Labeled RNA transcripts were hybridized for 3 days to denatured and immobilized cDNAs, and signal quantification was carried out as described above.

Analysis of DNase I-hypersensitive Sites—Nuclei preparation for DNase I-hypersensitive site mapping was performed essentially as described (28). Briefly, control and TPA (50 ng/ml)-treated cells were harvested and washed once with $1 \times$ phosphate-buffered saline. Cell pellets were resuspended in 10 volumes of Buffer D (5 mM PIPES, pH 7.5, 85 mM KCl, 1 mM CaCl₂, 5% sucrose) containing 14 mM β -mercaptoethanol and 0.5% Nonidet P-40 and placed on ice for 10 min. Nuclei were then washed twice by centrifugation in Buffer D, and aliquots were taken for estimating DNA concentration. Finally, purified nuclei were analyzed under phase contrast microscope and were resuspended at a concentration of 0.5 μ g DNA/ μ l in DNase I digestion buffer (Buffer D). Digestion with DNase I (grade I, Roche Molecular Biochemicals) was performed by adding 1, 3, or 6 μ l of diluted enzyme (1 U/ μ l) to 200 μ l (100 μ g) of nuclei suspension for 20 min at 20 °C, and the reaction was stopped by adding an equal volume of stop extraction solution (50

mM Tris-HCl, pH 7.5, 150 mM NaCl, 15 mM EDTA, 0.3% SDS, 1 mg/ml Proteinase K). After an overnight incubation at 50 °C, DNA was extracted by phenol/chloroform and chloroform and precipitated by adding $\frac{1}{3}$ volume of 10 M ammonium acetate and 2.5 volume of ethanol. DNase I digests were then resuspended in 10 mM Tris-HCl, 1 mM EDTA, and 20 μ g were digested with appropriate restriction enzymes. Southern blot was performed as described previously (23) with a polymerase chain reaction generated probe (nucleotides –2708 to –2564) using the 5'-primer 5'-ACAGAGTCTCACTCTGTTGCC-3' and the 3'-primer 5'-TTAGCTGGGCATGGTGGCACGTG-3'.

Nuclear Protein Extracts—Nuclear extracts were prepared essentially as described elsewhere (29). Cells (10^7) that had been either unstimulated or stimulated with TPA for 40 h or with 9-*cis*-retinoic acid for 3 days were collected, washed twice in phosphate-buffered saline, and resuspended in 0.8 ml of ice-cold low salt buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and a proteinase inhibitor mixture (Roche Molecular Biochemicals) including pepstatin, antipain, aprotinin, leupeptin, and chymostatin at 2.5 mg/ml each). After 10 min on ice, the nuclei were centrifuged 10,000 rpm for 2 min, and the pellet was resuspended in high salt buffer C containing 20 mM HEPES-KOH, pH 7.9, 26% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and proteinase inhibitors as in buffer A. Nuclei were incubated on ice for 30 min and centrifuged at 10,000 rpm for 5 min to remove cell debris. The protein concentration of the supernatant was determined, and small aliquots were frozen at –80 °C until required.

Western Analysis of Secreted and Nuclear Proteins—Conditioned media from RD cells cultured in serum free conditions were centrifuged to eliminate cell debris before a 100-fold concentration by 80% ammonium sulfate precipitation and dialysis against 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM CaCl₂, 1 mM ZnCl₂, and 0.005% Brij-35. Nuclear extracts were prepared as described above. Equivalent amounts of secreted or nuclear proteins were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions. After transfer onto nitrocellulose membranes, secreted ST3 was revealed with a monoclonal antibody 5ST-4A9 (30) by using ECL (Amersham Pharmacia Biotech) and a peroxidase-coupled anti-mouse IgG (Jackson). For nuclear extracts, the C/EBP β specific antibody (C-19) (Santa Cruz Biotechnology) was used together with a peroxidase-coupled anti-rabbit IgG (Jackson).

In Vitro DNase I Footprinting—A polymerase chain reaction generated fragment containing 635 bp of the 5' flanking region (spanning –2502 to –1868) was subcloned into pBluescript SK(+) (Stratagene) and then released by *Eco*RI and *Eag*I. The resulting fragment was isolated and purified to label the coding and noncoding strands by incorporating either [α -³²P]dCTP or [α -³²P]dATP with the Klenow enzyme. The purified probes (10,000 cpm) were mixed with 1 μ g of poly d(IC), 40 μ g of nuclear extract and 50 μ l of binding buffer (20 mM HEPES-KOH, pH 7.9, 20% glycerol, 1 mM CaCl₂, 10 mM MgCl₂, 0.2 mM EDTA, 1 mM dithiothreitol) in the presence of 60 mM KCl in a final volume of 100 μ l. Binding was performed for 15 min at room temperature, after which 5 μ l of increasing DNase I concentrations (1, 3, and 5 milliunits/ μ l for naked DNA and 50, 100, and 200 milliunits/ μ l in the presence of protein extract) was added for 2 min. 100 μ l of stop solution containing 1% SDS, 200 mM NaCl, 20 mM EDTA, pH 8, 40 μ g/ml tRNA, 100 μ g/ml proteinase K was added for 15 min at 37 °C. DNA was extracted, precipitated, washed with 70% ethanol, dried, and resuspended in 5 μ l of loading buffer. Digested DNA was then resolved by electrophoresis on a 6% polyacrylamide denaturing sequencing gel.

Electrophoretic Mobility Shift Assay—Double-stranded oligonucleotides with 5'-overhangs were generated and labeled by incorporating [α -³²P]dATP and/or [α -³²P]dCTP with the Klenow enzyme. Labeled probes (20,000 cpm) were added to a 20- μ l binding reaction containing 10 μ l of $2 \times$ binding buffer (20 mM HEPES-KOH, pH 7.9, 20% glycerol, 1 mM CaCl₂, 10 mM MgCl₂, 0.2 mM EDTA, 1 mM dithiothreitol), 0.8 μ g of poly d(IC), and 60 mM KCl and incubated with nuclear extracts (8 μ g) for 15 min at room temperature. Competition assays were performed by adding a 10, 50, or 100 molar excess of unlabeled oligonucleotides. In supershift experiments, the pan-C/EBP (Δ 198) and C/EBP- β (C-19) antibodies (Santa Cruz Biotechnology) were added to the binding reaction for an additional incubation of 30 min. The protein-DNA complexes were separated from the free probes by electrophoresis in a 6% polyacrylamide gel of a low ionic strength (0.5 \times TBE (50 mM Tris borate, 50 mM boric acid, 1 mM EDTA)) and dried gels were exposed for autoradiography. The oligonucleotides used in electrophoretic mobility shift assay were as follows, with the core binding site underlined: C/EBP, 5'-TGCAGATTGGCAATCTG-3' and 3'-GTCTAACGCGTTAGACG-5'; ST3-C/EBP, 5'-CTAGACCATTGAGCAAGACCCT-3' and 3'-TGGTAACTC-GTTCTGGGAGATC-5'; ST3-C/EBP β , 5'-CTAGACCATTGGCAAGACC-

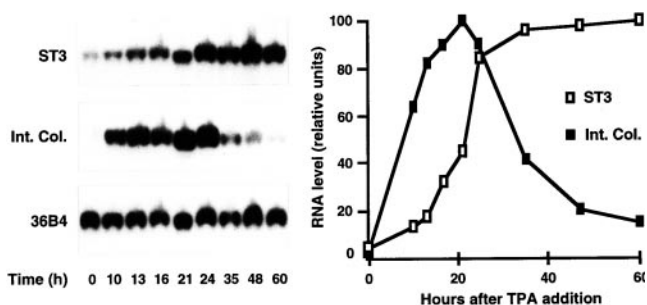


FIG. 1. Time course of ST3 and interstitial collagenase (*Int. Col.*) mRNA expression in fibroblasts exposed to TPA. HFL1 fibroblasts were exposed to TPA (10 ng/ml) for various time periods in serum-free conditions. Levels of ST3 and interstitial collagenase transcripts were analyzed by Northern blot performed with 10 μ g of total RNA. Hybridization signals were estimated by using a bioimaging analyzer (BAS 2000; Fuji Ltd). In the quantitative representation, ST3 and interstitial collagenase mRNA levels are expressed relative to their highest expression levels (100%), as observed after 60 and 20 h of TPA treatment, respectively. Normalization was performed with respect to 36B4 mRNA (32) levels.

T-3' and 3'-TGGTAACCGTTCTGGGAGATC-5'; and AP-1, 5'-AGCTTATG-AGTCACA-3' and 3'-ATACTCAGTGTGATC-5'.

Plasmid Construction—Luciferase constructs containing various lengths of ST3 promoter were generated by polymerase chain reaction amplification from a 5.5-kb *XhoI/NotI* genomic template (23) and insertion into the firefly luciferase reporter plasmid (pGL3-Basic, Promega Corp.). Sequences of the three 5'-primers used are as follows (with restriction sites underlined): 5'-CGGGGTACCTGGCCTGAAATGCCT-AC-3' for 2.5-ST3-LUC (-2447 to +15), 5'-CGGGGTACCAAGATCAC-ACCATTGAGCAAG-3' for 2.5- Δ AP1-LUC (-2179 to +15) and 5'-CGGGGTACCGGGCCGGCTGCTAGGAGAGTTC-3' for 0.1-ST3-LUC (-110 to +15). The 3'-primer 5'-CCATCCGCCTCGAGGCTGCTGGGCTTGCTGG-3' was used for the amplification of all three ST3 promoter fragments, which were digested with *KpnI* and *XhoI* and ligated into pGL3-Basic plasmid digested with the same restriction enzymes. Isolated ST3-C/EBP and ST3-C/EBPm sequences were cloned into the *NheI* restriction site of the luciferase pGL3-Promoter (Promega Corp.) containing the SV40 promoter and were designated ST3-C/EBP-SV40-LUC and ST3-C/EBPm-SV40-LUC. The double-stranded oligonucleotides with 5' *XbaI* overhangs used for both constructs are indicated above.

Transient Transfection and Luciferase Assay—RD cells were transiently transfected in 6-well plates by the calcium phosphate procedure, as described previously (23) using 2.5 μ g of luciferase reporter plasmids and 0.25 μ g of pCH110 plasmid as an internal control for transfection efficiency. The total amount of DNA transfected in each culture well was made up to 5 μ g with pBluescribe plasmid DNA. 6 h after transfection, the DNA precipitate was eliminated, and the cells were further incubated for 24 or 48 h and lysed in 150 μ l of Passive Lysis buffer (Promega), whereas cell debris were removed by centrifugation. Cell extracts (50 μ l) were assayed for Luciferase activity using the luciferin reagent (Promega). Light intensity was measured in a EG&G Berthold luminometer. All transfections were performed in triplicate and repeated three or four times. Results were normalized for protein content (light units/ μ g of protein).

RESULTS

Time Course of ST3 and Interstitial Collagenase mRNA Expression Following Fibroblast Exposure to TPA—To determine the mechanism by which high levels of ST3 can be induced, we first examined the induction kinetics of ST3 mRNA in human fibroblasts exposed to TPA. As a variety of MMPs are expressed in this cellular type *in vivo* and *in vitro*, this kinetics was compared with that of interstitial collagenase whose AP-1-mediated transcriptional activation has been well documented (31). As shown in Fig. 1, the induction pattern of both genes is clearly different. The interstitial collagenase gene is rapidly and highly induced within the first hours following fibroblast exposure to TPA, with a 40-fold increase measured after 21 h of incubation. This induction is transient, and maximal levels of interstitial collagenase mRNA levels fall off after 24 h. In

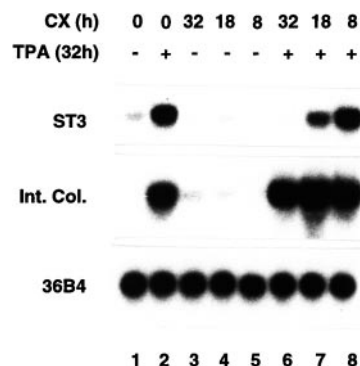


FIG. 2. Comparison of protein synthesis requirement for ST3 and interstitial collagenase (*Int. Col.*) mRNA induction in fibroblasts exposed to TPA. The effect of 100 μ g/ml cycloheximide was analyzed on steady-state mRNA levels. HFL1 fibroblasts were cultured in 5% calf serum in the absence (-) or presence (+) of 10 ng/ml TPA for 32 h, and cycloheximide (CX) was added for either the whole incubation time (32 h) or only during the last 18 or 8 h, as indicated. Northern blot analysis was performed with 10 μ g of total RNA. 36B4 mRNA (32) was used as loading control.

contrast, ST3 gene induction by TPA is delayed, leading to maximal mRNA levels after 35 h where a 20-fold increase was observed. This stimulatory effect is sustained, and the levels of ST3 transcripts remain stable at least until 60 h.

Differential Protein Synthesis Requirement for ST3 and Interstitial Collagenase mRNA Induction by TPA—The prolonged lag period of about 10 h during which low levels of ST3 transcripts were detected following exposure to TPA suggested the requirement of *de novo* protein synthesis. To test this hypothesis, HFL1 fibroblasts were cultured in the presence or absence of TPA for a period of 32 h, and the protein synthesis inhibitor cycloheximide was added either during the whole incubation time (32 h) or during the last 18 or 8 h. As shown in Fig. 2, the induction of ST3 mRNA expression by TPA (lane 2) was not observed when cycloheximide was added together with TPA for 32 h (lane 6). Consistent with this observation, the levels of ST3 mRNA observed after 14 h exposure to TPA (Fig. 1, right panel) were not further induced when cycloheximide was added for an extra period of 18 h (lane 7). In marked contrast, we found by hybridizing the same Northern blot that the stimulation of interstitial collagenase mRNA was not affected by the addition of cycloheximide (compare lane 2 with lanes 6-8). These results demonstrate that protein synthesis is required for ST3 but not for interstitial collagenase mRNA induction by TPA, indicating that these genes are regulated by different mechanisms.

Analysis of ST3 mRNA Stability—The maintenance of high levels of ST3 transcripts after a long exposure of HFL1 fibroblasts to TPA (Fig. 1) prompted us to evaluate ST3 mRNA stability in transcriptionally arrested cells. The transcription elongation inhibitor DRB was first tested on the fibroblastic expression of ST3 to establish the optimal concentration required to arrest transcription. The time interval between 15 and 30 h of fibroblast exposure to TPA was chosen for this analysis, because it corresponds to a maximal increase in ST3 mRNA levels (Fig. 1). DRB was found to completely block ST3 mRNA induction at concentrations over 25 μ M (Fig. 3). Interestingly, the degradation of interstitial collagenase mRNA occurring normally after its induction by TPA was not observed in the presence of DRB concentration over 25 μ M, indicating that it was also under the control of a transcriptional mechanism.

We next used 75 μ M DRB to arrest transcription to determine whether the stability of ST3 mRNA was modified in HFL1 fibroblasts exposed to TPA. Fibroblasts were cultured for 32 h in the presence or absence of TPA, and DRB was then added for

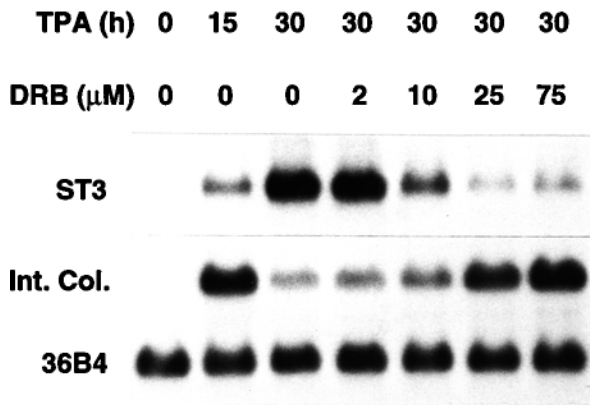


FIG. 3. Effect of increasing doses of DRB on *ST3* and interstitial collagenase (*Int. Col.*) induction in fibroblasts exposed to TPA. HFL1 fibroblasts were first incubated for 15 h in serum-free conditions with or without TPA (10 ng/ml). After 15 h, TPA-stimulated fibroblasts were further cultured in the absence or presence of increasing concentrations of DRB for an additional period of 15 h. Northern blot analysis was performed with 10 μ g of total RNA. 36B4 mRNA (32) was used as loading control.

up to an additional 40 h. The decay of *ST3* mRNA was monitored by Northern blot analysis. Because of the low constitutive levels of *ST3* mRNA in control cells, 30 μ g of total RNA was analyzed for each evaluation performed in the absence of TPA. We found that the slow decay of *ST3* mRNA observed in control cells (Fig. 4A) was very similar to that observed in cells stimulated with TPA (Fig. 4B). Indeed, the half-life of *ST3* mRNA estimated during the first 28 h following DRB addition was approximately 18 h in both conditions. We therefore concluded that *ST3* mRNA induction by TPA could not be attributed to a mechanism involving mRNA stabilization.

Transcriptional Activation of *ST3* and Interstitial Collagenase Gene Expression by TPA—The lack of TPA effect on *ST3* mRNA stability suggested that TPA was inducing *ST3* gene expression by a transcriptional mechanism. We therefore performed nuclear run-on assays to directly assess the rate of *ST3* gene transcription and compared it to that of the interstitial collagenase gene. HFL1 fibroblasts were exposed to TPA for 0–48 h. Nuclei were isolated, and radiolabeled RNAs resulting from the *in vitro* elongation of nascent nuclear RNA transcripts were hybridized to blotted denatured cDNAs cloned into the pBluescript II vector and corresponding to interstitial collagenase, *ST3*, the housekeeping gene 36B4 (32), and the plasmid alone as a control for nonspecific hybridization. As shown in Fig. 5A, TPA was found to increase the transcription rate of both *ST3* and interstitial collagenase genes with kinetics consistent with those observed for their mRNA levels evaluated simultaneously (Fig. 5B). The transcriptional rate of the interstitial collagenase gene was transiently increased and peaked at 16 h of TPA treatment. In contrast, whereas the basal transcription rate of the *ST3* gene was similar to that of the 36B4 gene, its transcription rate was higher at all incubation times in the presence of TPA. Taken together, these data demonstrate that the induction of both *ST3* and interstitial collagenase genes by TPA occurs through a transcriptional mechanism in HFL1 fibroblasts.

Induction of *ST3* Expression Is Associated with an Inducible DNase I-hypersensitive Site in TPA-differentiated RD Cells—Having established that the mechanism by which TPA induces *ST3* gene expression was transcriptional, we were interested in the identification of regulatory sequences that could account for this induction. However, because HFL1 fibroblasts are resistant to transient transfection, we looked for alternative cells expressing *ST3* that could be transfected for promoter studies.

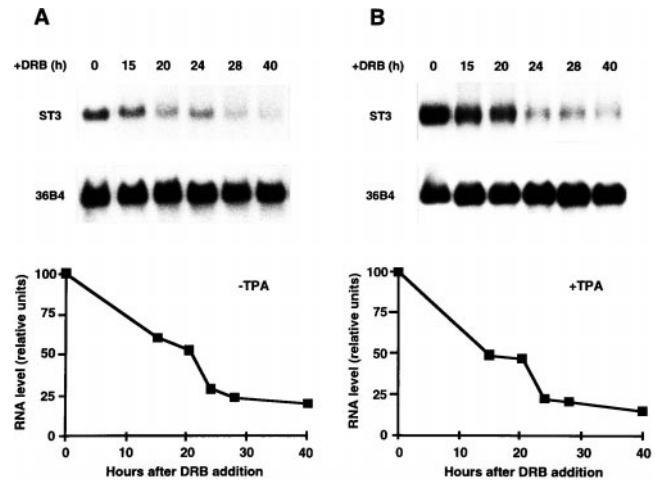


FIG. 4. *ST3* mRNA decay in transcriptionally arrested fibroblasts. HFL1 fibroblasts were first exposed for 32 h to serum-free medium in the absence (A) or in the presence (B) of 10 ng/ml TPA. DRB (75 μ M) was then added to all cultures for various incubation times before evaluation of *ST3* mRNA levels (*ST3*) by Northern blot analysis performed with 30 (A) or 10 (B) μ g of total RNA. Hybridization signals were estimated by using a bioimaging analyzer (BAS 2000; Fuji Ltd). In the quantitative representations, *ST3* mRNA levels are expressed relative to their initial levels (100%) prior to DRB addition and after normalization with respect to 36B4 (32) mRNA levels.

As the transcriptional activation of *ST3* by retinoic acid in HFL1 has been recently shown to be similar in rhabdomyosarcoma (RD) cells (15), we first tested whether TPA could also induce expression of *ST3* in these cells. Fig. 6A shows that levels of *ST3* mRNA are strongly induced in RD cells exposed to TPA with a similar induction pattern as that in HFL1 fibroblasts, indicating that similar transcriptional mechanisms are involved in both cell types. Likewise, the secretion of the mature form of *ST3* is also increased by TPA in the conditioned medium of RD cells (Fig. 6B).

We next decided to look for DHS in the chromatin of RD cells induced to express high levels of *ST3* in response to TPA. DHS are nucleosome-free regions representing “open windows” that allow enhanced access of *cis*-acting DNA sequences to transcription factors. Accordingly, these sites are often associated with regulatory regions such as promoters or enhancers of actively transcribed genes (33). Based on the time course of *ST3* response to TPA, nuclei were prepared from RD cells treated or not with TPA for 40 h and were exposed to DNase I. DNA was then extracted and Southern blot analysis was performed using genomic probes generated near the end of the restriction fragments of interest. Although several DHS have been found within a 4-kb region of *ST3* gene 5'-flanking sequences (data not shown), one DNase I-hypersensitive region was specifically observed in TPA-differentiated cells and was mapped about 2.3 kb upstream of the gene within a *Ban*I restriction fragment. As shown on the representative Southern blot in Fig. 6C, digestion of nuclei from control cells by increasing concentrations of DNase I only decrease the signal corresponding to this *Ban*I fragment, and no additional band was detected. However, when nuclei from TPA-treated cells were exposed to DNase I, an additional band of about 0.8 kb was observed with the lowest DNase I concentration revealing the presence of a DHS. Higher DNase I concentrations further decrease the size of this 0.8-kb band signal. Taken together these observations demonstrate the presence of a hypersensitive region of about 200–250 bp that is specifically associated with TPA-induced *ST3* gene expression.

Footprint Analysis of the TPA-induced DNase I-hypersensitive Region—The presence of an accessible region associated

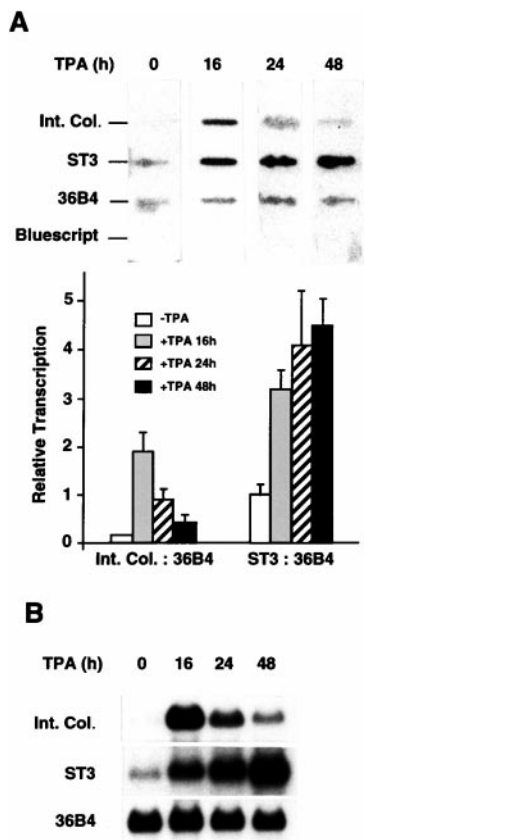


FIG. 5. Run-on transcription assays with nuclei prepared from fibroblasts exposed to TPA. HFL1 fibroblasts were treated with 10 ng/ml TPA for various times in serum-free medium, as indicated, and then processed for nuclear run-on analysis (A) and Northern blot hybridization (B). A, nuclei were isolated and transcripts were labeled by *in vitro* elongation reaction in the presence of [α - 32 P]UTP. Equal amounts of newly synthesized RNA (10^7 cpm) were hybridized to cDNAs immobilized on nylon filters and corresponding to interstitial collagenase (*Int. Col.*), ST3, or 36B4 (32). Band intensity was quantified by using a bioimaging analyzer (BAS 2000; Fuji Ltd). Background hybridization was subtracted from gene-specific hybridization, and the data in the histogram are expressed relative to the signal evaluated for 36B4. Three run-on experiments were performed for each time points and the results are shown as the means \pm S.E. B, Northern blot hybridization was performed using 10 μ g of total RNA extracted from dishes separate from those used for nuclear run-on assays.

with ST3 gene induction suggested that specific sequences in this region might exert an effect on ST3 transcription by binding to transcription factors present in RD cells exposed to TPA. To identify such sequences and to ascertain whether the ST3 gene promoter contains binding sites for nuclear factors near this hypersensitive region, we performed footprinting experiments on a 635-bp DNA fragment spanning nucleotides -2502 to -1868 and including this DNase I-hypersensitive region. Footprint reactions were carried out on both the sense (Fig. 7A) and the antisense (Fig. 7B) strands in the presence of nuclear extracts from HeLa cells that do not transcribe the gene and from untreated RD cells or RD cells induced to express the gene by TPA or retinoic acid. One footprint between bases -2422 and -2408 (AAAATGAGTCACTTT) was seen with nuclear extracts from both cell lines using the sense strand (Fig. 7A). This region was strongly protected in HeLa and control RD cells and includes a consensus AP-1-binding site. Whereas appearance of this footprint was similar with nuclear extracts from either control or retinoic acid-treated RD cells, the protection of this binding site was slightly stronger in RD cells exposed to TPA. More importantly, a second footprint was specifically observed with nuclear extracts from TPA-differen-

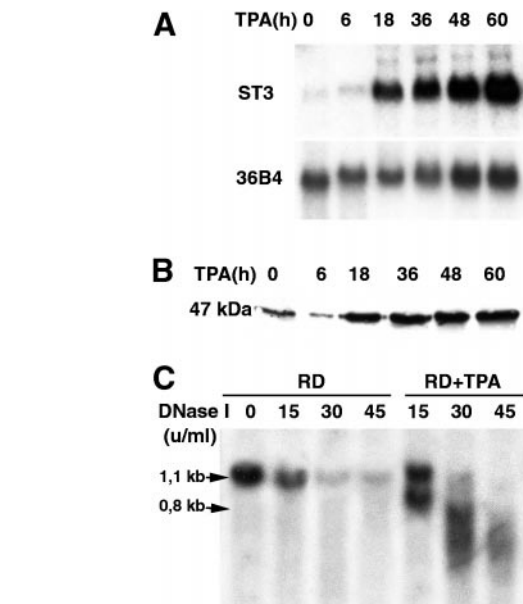


FIG. 6. The delayed ST3 expression in TPA-differentiated cells is associated with an inducible DNase I-hypersensitive site. RD cells were exposed to TPA treatment (50 ng/ml) for 6–60 h as indicated and then processed for Northern (A) and Western (B) blot analyses. A, total RNA was isolated and 10 μ g were evaluated by Northern blot hybridization with 32 P-labeled cDNA probes for ST3 and 36B4. B, culture media conditioned by RD cells in serum-free conditions were collected and concentrated 100-fold by ammonium sulfate precipitation. The secreted mature form of ST3 was detected by Western blot analysis by using a monoclonal antibody 5ST-4A9 and enhanced chemiluminescence. C, DNase I hypersensitivity in ST3 chromatin following RD cells exposure to TPA. Freshly prepared nuclei from control and 40 h TPA-treated RD cells were incubated with increasing amounts of nuclease. DNA was then extracted and analyzed by Southern blot. A single 1.1-kb *Ban*I restriction fragment was detected in control cells, whereas an additional 0.8-kb fragment was seen in nuclei from TPA-treated cells.

tiated cells (Fig. 7, A and B). This protected region was mapped between nucleotides -2169 and -2151 by using the antisense probe resolved on longer run gels. Its corresponding sequence (ACCATTGAGCAAGACCCTG) was found to contain a putative C/EBP-binding site differing by only 1 bp from the consensus sequence previously reported (34). The positions of both protected elements relative to that of the DHS within the ST3 promoter are indicated in Fig. 7C.

C/EBP β Binds to the ST3 Promoter in Response to TPA—The TPA-inducible footprint identified in RD cells prompted us to further analyze the binding of nuclear factor(s) to the putative ST3-C/EBP sequence. Gel shift assays were carried out with a double-stranded oligodeoxyribonucleotide probe containing the putative ST3-C/EBP-binding element by using nuclear extracts from either control or TPA-treated RD cells (Fig. 8A). No specific complex was detected by using nuclear extracts from untreated cells (lane 2). However, consistent with our footprint data, a strong binding activity was only observed with nuclear extracts from RD cells exposed to TPA for 40 h (lane 3). The specificity of this interaction was demonstrated by competition experiments showing that increasing amounts of cold ST3-C/EBP (lanes 4–6) or C/EBP consensus sequence (lanes 7–9) at 10–100-fold excess efficiently competed this complex formation. In contrast, no competition was observed when using the same excess of cold ST3-C/EBP oligonucleotide that had been mutated by deleting a single bp (lanes 10–12), and consistent with this observation, no complex was observed by using the ST3-C/EBP as a probe (Fig. 8B, lanes 1–3). We also noted the absence of any binding to the ST3-C/EBP element by using nuclear extracts from cells that had been induced to express ST3 by retinoic acid (lane 6). When either a specific

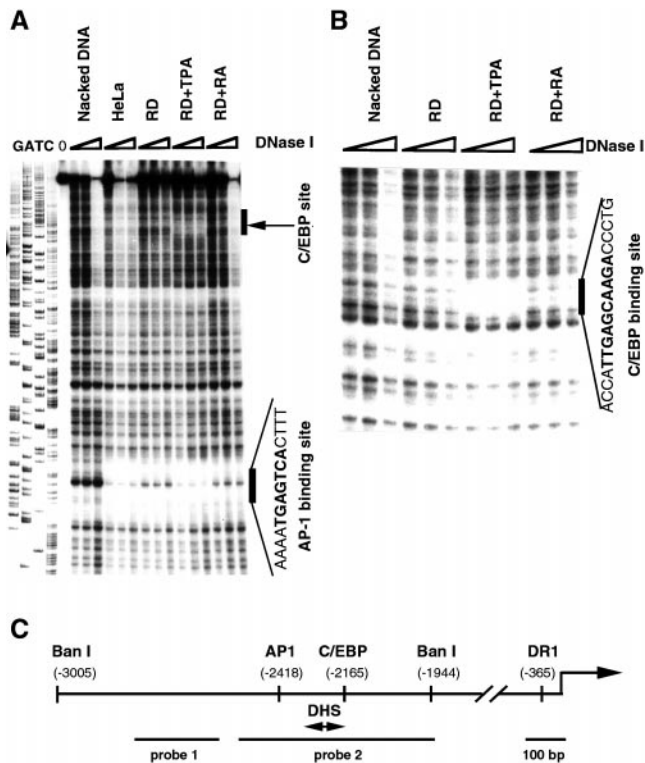


FIG. 7. DNase I footprint analysis of the *ST3* promoter region including TPA-inducible hypersensitive site. The sense (A) and antisense (B) strands of a polymerase chain reaction generated genomic fragment (nucleotides -2502 to -1868) were analyzed by footprint in the absence or in the presence of $40 \mu\text{g}$ of nuclear extracts from control HeLa or RD cells as indicated. For RD cells, nuclear extracts were also prepared from cells treated with 50 ng/ml TPA for 40 h or $1 \mu\text{M}$ 9-*cis*-retinoic acid for 3 days. Naked and bound DNA was then treated with increasing DNase I concentrations and electrophoresed in a 6% polyacrylamide sequencing gel together with sequencing reactions. The positions of protected regions are indicated by *solid rectangles* together with their corresponding sequences. C, schematic representation of the distal *ST3* promoter including the position of DHS and that of the AP-1 and the C/EBP-binding sites relative to the transcription start site. The proximal retinoic acid-responsive element (directly repeated consensus hexameric half-sites (5'-PuG^G_TTCA-3') separated by one base pair; labeled *DR1*) is also indicated as well as the probes 1 and 2 used for Southern blot (Fig. 6) and footprint analyses, respectively.

C/EBP β (lanes 8 and 9) or a pan-*C/EBP* antibody (lanes 10 and 11) was added in the binding reaction, the TPA-inducible complex (lane 7) was totally eliminated and partially supershifted, indicating that *C/EBP* β was present in this complex. The capacity of *C/EBP* β to directly interact with the *ST3*-*C/EBP* element was further demonstrated by showing a strong complex of the same mobility (lane 19) with nuclear extracts from COS-1 cells transiently transfected with *C/EBP* β plasmid, whereas no complex was observed with extracts from control COS-1 cells or when using the *C/EBPm* probe (lanes 12–17). As expected, the *C/EBP* β complex was eliminated with both *C/EBP* antibodies, and the amount of its corresponding supershift was dependent of the nature and the concentration of the antibody (lanes 20–23), as those observed with RD cell nuclear extracts.

The expression of *C/EBP* β was then evaluated in nuclear extracts from RD cells by Western blotting. Nuclear extracts from COS-1 cells transiently transfected with *C/EBP* β expression vector were used as positive control, and the *C/EBP* β -specific antibody revealed a strong band of about 37 kDa that corresponds to the full-length *C/EBP* β isoform (35). This form was also detected in RD cells, but its induction by TPA was quite weak and did not exceed a 2-fold stimulation (Fig. 8C). A second band of about 35 kDa was also detected in RD cells and

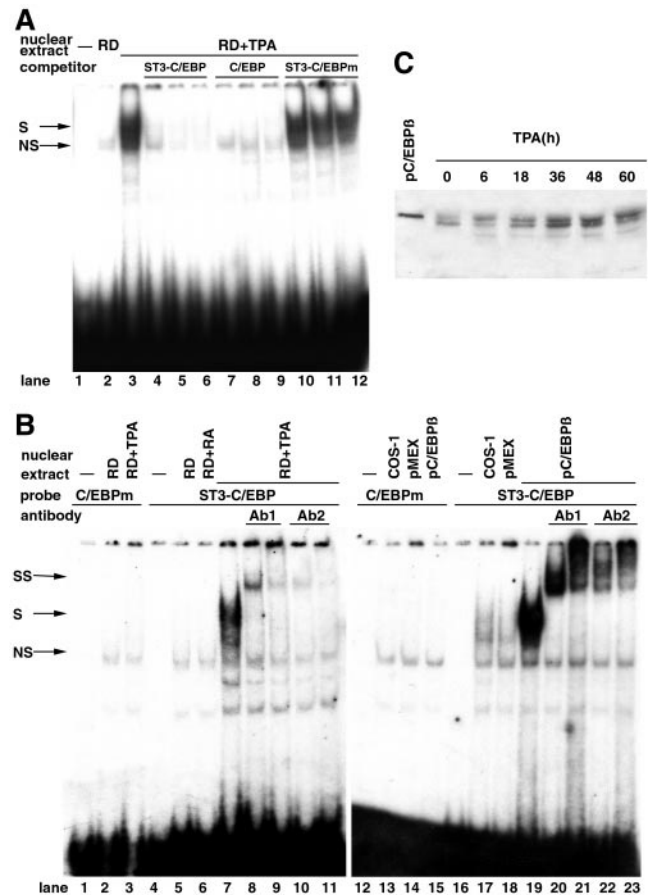


FIG. 8. TPA induces *ST3*-*C/EBP* binding activity in RD cells. A, nuclear extracts ($8 \mu\text{g}$) from either control (lane 2) or 40 h TPA-treated RD cells (lanes 3–12) were incubated with a Klenow end-labeled oligonucleotide corresponding to the *ST3*-*C/EBP*-binding site. Competition experiments were performed by using a 10-, 50-, and 100-fold molar excess of the same *ST3*-*C/EBP* oligonucleotide (lanes 4–6) or of sequences corresponding either to a consensus C/EBP sequence (lanes 7–9) or to a *ST3*-*C/EBP*-binding site with a single bp deletion (*ST3*-*C/EBPm*, lanes 10–12). B, *C/EBP* β is present in the TPA-inducible complex and interacts directly with the *ST3*-*C/EBP*-binding site. Labeled *C/EBPm* or *ST3*-*C/EBP* probes were incubated with nuclear extracts from RD cells (left panel) or from COS-1 cells (right panel) transfected with either the pMEX plasmid (without insert) or the *C/EBP* β expression plasmid as indicated. Addition of $1 \mu\text{l}$ (lanes 8, 10, 20, and 22) or $2 \mu\text{l}$ (lanes 9, 11, 21, and 23) of either a *C/EBP* β specific (Ab1, C19) or a pan-*C/EBP* (Ab2, $\Delta 198$) antibody to the binding reactions resulted to a supershift (SS) of the shifted probes (S). NS indicates nonspecific binding. These data are representative of three experiments. C, various *C/EBP* β isoforms are induced by TPA in RD cells. Nuclear extracts from RD cells treated with TPA at the indicated times were analyzed by Western blot. Extracts from COS-1 cells transiently transfected with an expression plasmid for the full-length *C/EBP* β were used as positive control. Similar results were obtained with protein extracts from cells in three independent experiments.

may correspond to the liver-enriched transcriptional activator (LIP) (36). Lower levels of two additional bands of about 30 and 21 kDa were only detected in TPA treated cells and may represent other *C/EBP* β isoforms generated either by alternative usage of AUG codons or by proteolytic cleavage (35). Thus, taken together, these data further confirm the induced binding activity observed by footprint and demonstrate that *C/EBP* β is the factor produced by RD cells that binds to the *ST3* gene promoter at position -2165 . In addition, the weak induction of the full-length *C/EBP* β indicates that the strong increased DNA binding activities are unlikely to be mediated by increased synthesis of *C/EBP* β .

*The *ST3*-*C/EBP* Element Mediates Promoter Activation by TPA and Dose-dependent Response to *C/EBP* β* —Because both

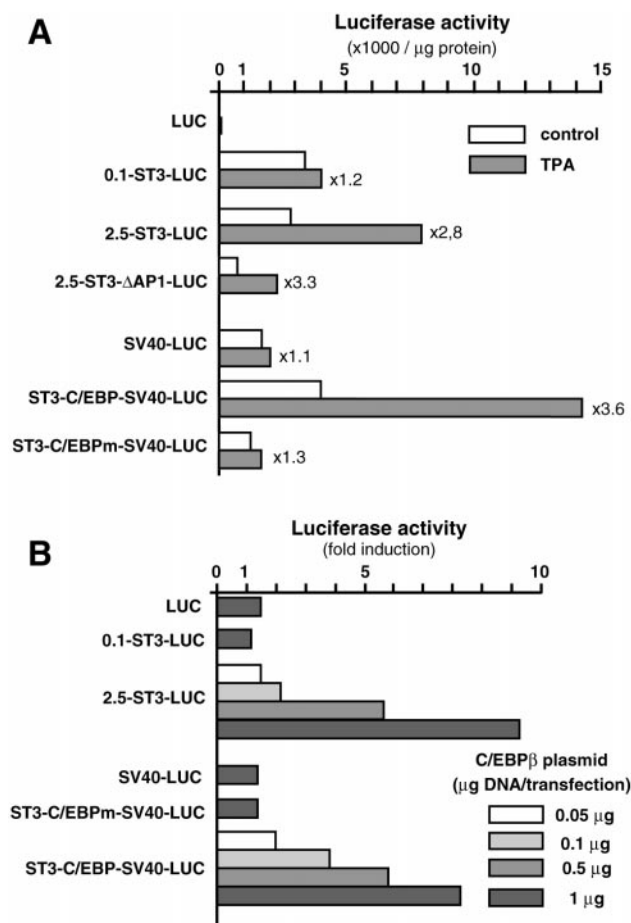


FIG. 9. TPA and C/EBP β dose-dependent responses of the ST3 promoter. A, RD cells were transfected with 2.5 μ g of luciferase reporter plasmids as indicated and further incubated for 48 h in the absence or presence of 25 ng/ml TPA. B, cells were cotransfected with increasing amounts of C/EBP β expression plasmid as indicated and were further incubated for 24 h before being harvested and assayed for luciferase activity. The structures of all constructs are described under "Materials and Methods," and the values are representative of three independent experiments performed in triplicate with interexperimental variation not exceeding 20%.

AP-1 and C/EBP-binding sites were found to be protected by footprint analysis, their respective roles in the control of the ST3 promoter were addressed in transient transfection assays. The promoter construct (2.5-ST3-LUC) including both elements was found to be activated in TPA differentiated RD cells, and this activation was found to be independent of the AP-1-binding site. Indeed, although a decrease of promoter base-line activity was observed by deleting the 5'-AP-1-binding site, the TPA-dependent transactivation of the resulting construct (2.5-ST3- Δ AP1-LUC) was not affected (Fig. 9A). In addition, a 3.6-fold activation was observed for the SV40 heterologous promoter driven by a single copy of the ST3-C/EBP element, whereas its mutation (ST3-C/EBPm) by introducing a single bp deletion completely abolished the TPA response. No significant change was detected by using other control plasmids such as the promoter-less plasmid (LUC), the SV40 promoter (SV40-LUC), or the proximal ST3 promoter (0.1-ST3-LUC).

Because C/EBP β was found to directly interact with the ST3 promoter (Fig. 8B), we next tested its effect on the native ST3 promoter and on the SV40 promoter driven by the ST3-C/EBP-binding site. Fig. 9B shows that transfection of C/EBP β expression plasmid increased the activity of both promoter constructs in a concentration-dependent manner. In contrast, the highest C/EBP β concentration did not affect the activity of other con-

structs lacking this element or containing the C/EBPm sequence. These results therefore demonstrate that C/EBP β activates the native ST3 promoter and that the integrity of the C/EBP motif is required for this activation.

DISCUSSION

ST3 expression has been implicated in a wide number of physiological and pathological processes. However, although its expression in tissues such as human endometrium during the menstrual cycle (4) or intestine during thyroid hormone-mediated *Xenopus laevis* metamorphosis (37) suggests a specific hormonal regulation, very little is known about the factors controlling its expression. At the molecular level, other than a retinoic acid-responsive element, no other regulatory element has been functionally characterized so far in its promoter. In this paper, we provide new insights into the mechanism by which ST3 can be induced, and we characterize regions of the ST3 promoter that are involved in the regulation of ST3 gene transcription.

Because fibroblasts express both interstitial collagenase and ST3, we first compared the kinetics of the induction of both genes in response to TPA and found significant differences. Interstitial collagenase was found to be rapidly and transiently induced in fibroblasts, and this induction did not require protein neosynthesis. Similarly, the rapid increase of its transcriptional rate evaluated by run-on assays is consistent with previous reports having shown that its response to TPA requires the rapid activation of members of Fos and Jun transcription factor family (38). In marked contrast, there was a strong but temporally delayed increase in ST3 mRNA that was found to require *de novo* protein synthesis. The maintenance of high and stable ST3 mRNA levels over an extended period of time following TPA treatment suggested that TPA could regulate ST3 expression by controlling mRNA stability, a possibility also supported by observations previously made for other MMPs (24) including ST3 (14). However, when examining the decay of ST3 mRNA levels in transcriptionally arrested fibroblasts, they were found quite stable with a half-life of about 18 h, which was not significantly modified in cells exposed to TPA. Finally, by performing run-on assays, we observed a good correlation between nuclear and messenger RNA expression, demonstrating that the prolonged induction of ST3 gene was resulting from a continuous transcriptional activation. Thus, taken together, these observations clearly demonstrate that both genes are regulated by different transcriptional mechanisms. Furthermore, they indicate that ST3 gene activation requires the involvement of specific transcription factors that are different from those mediating the early interstitial collagenase response to TPA.

To identify such transcription factors, we first looked for regulatory elements that become accessible upon ST3 activation. By mapping DNase I-hypersensitive sites in the ST3 5'-flanking region, one hypersensitive region was specifically observed in cells induced to express ST3. Further footprint analysis of this region revealed the presence of a C/EBP element and an AP-1-binding site raising the question of the respective role of both elements in the control of ST3 expression. Given that the AP-1-binding site is present in most proximal MMP promoters in a very conserved position, its identification in a distal position of the ST3 promoter was intriguing. In fact, we provide evidence that the delayed ST3 activation by TPA is not mediated by the AP-1-binding site and that the C/EBP element is playing a major role in this activation.

Indeed, the AP-1 binding activity evaluated by footprint analysis and electrophoretic mobility shift assay was already observed in control cells expressing base-line level of ST3 and was only slightly induced by TPA (Figs. 7 and 10). Moreover,

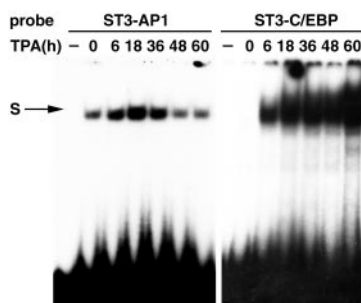


FIG. 10. Time course of AP-1 and C/EBP binding activity in TPA-differentiated RD cells. Nuclear extracts from untreated and TPA-treated RD cells at the indicated times were incubated with a radiolabeled, double-stranded ST3-AP1 or ST3-C/EBP probe. *A*, ST3-AP1 binding activity was detected in nuclear extracts from untreated RD and was transiently increased in TPA-treated cells. *B*, no ST3-C/EBP binding activity was observed in nuclear extracts from untreated RD cells, whereas in TPA-treated cells, there was a strong increase remaining even after 60 h.

high ST3 levels as those observed after 60 h of TPA treatment are no longer associated with increased AP-1 binding activity (Fig. 10). Finally, when the role of this element was addressed in transient transfection assays, consistent with other studies (39, 40), isolated AP-1 failed to confer responsiveness to TPA (data not shown), and the ST3 promoter response to TPA was shown to be independent of the presence of the AP-1-binding site. However, the decrease in ST3 promoter activity associated with the deletion of this element indicates that it rather regulates basal expression of ST3 as reported for other genes (39, 41, 42).

In marked contrast, the ST3-C/EBP binding activity was not detected in resting cells but was strongly induced by TPA even after 60 h (Fig. 10), which was consistent with the kinetics of ST3 induction. This TPA-induced complex observed in RD cells was of the same size as that observed with the full-length recombinant C/EBP β transfected into COS-1 cells and was supershifted by using C/EBP β antibody, indicating that C/EBP β is likely to be the major factor interacting with the ST3-C/EBP-binding site. However, when the expression of C/EBP β was evaluated by Western blot, very little change was observed in TPA-treated cells, indicating the involvement of a post-translation mechanism. In this respect, previous reports have demonstrated that C/EBP β can be phosphorylated by various kinases at different Ser or Thr residues leading to alterations in DNA binding activities, transcriptional activity, and nuclear translocation (see Ref. 43 for review). There are also various isoforms of C/EBP β that can be generated either by alternative usage of AUG codons or by specific proteolytic cleavage, but the binding and transactivation properties of each isoform as well as their *in vivo* targets are not yet fully known (35). Interestingly one study has reported a prolonged induction of C/EBP β DNA binding activity that was not due to increased C/EBP β expression but to its phosphorylation at Ser²⁹⁹ leading to the nuclear translocation of its phosphorylated form (44). However, we found that increasing concentrations of active phosphatase did not affect C/EBP β binding activity in TPA-differentiated cells (data not shown). We therefore concluded that this binding activity could not be attributed to a post-translational mechanism involving its phosphorylation. Alternatively, we propose that additional factors or co-activators may be required to induce the binding of C/EBP β to its cognate element in the ST3 promoter.

Finally, our promoter studies have shown that the integrity of the ST3-C/EBP motif was required for TPA-dependent transactivation and that this motif, isolated or in the context of the native ST3 promoter, was activated by C/EBP β in a dose re-

sponse manner. These data further support the role of C/EBP β in the transcriptional activation of ST3. On the other hand, among various physiological processes in which C/EBP β was reported to play a role (45, 46), it seems important to note that the involution of the mammary gland (47) has also been associated with high levels of ST3 (3). In addition, the most prominent role of C/EBP β in the transcriptional regulation of genes such as those involved in acute phase inflammation appeared to be in the maintenance of the induced state rather than in their initial induction (43). Therefore, these studies suggest that the requirement of C/EBP β in the control of ST3 expression may be physiologically relevant in situations associated with a transcriptional activation of ST3 over long time periods.

Among MMPs, interstitial collagenase was shown to be induced during the differentiation of monocyte into macrophages by TPA in a recent study that has clearly distinguished its early AP-1-mediated response to TPA from its delayed and prolonged accumulation, which was controlled by C/EBP β (41). On the other hand, although C/EBP β has been shown to control the differentiation of macrophages (43), which is associated with the expression of other MMPs (48–50) including ST3 (51), the possible role of C/EBP transcription factors in the regulation of other MMPs in macrophages or in other cell types has not been addressed. However, the expression of the neutrophil elastase, which belongs to the serine proteinase family, has been shown to be controlled by a C/EBP element (52), and its inactivation results to an increased sensitivity to bacterial infections (53), phenotype also reported for C/EBP knock-out mice (43). Taken together, these studies suggest that C/EBP isoforms could control the expression of different proteinases in various cells *in vivo*. Our data have demonstrated that ST3 represents an additional target gene transcriptionally regulated by C/EBP β . Further investigation will be required to define the possible role of C/EBP isoforms in the *in vivo* control of ST3 and/or other proteinases associated with tissue remodeling.

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