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Transcriptional Inhibition of Type I Collagen Gene Expression in Scleroderma Fibroblasts by the Antineoplastic Drug Ecteinascidin 743*

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We previously showed that *COL1A1* expression is upregulated at the transcriptional level in systemic sclerosis (SSc) fibroblasts and that the CCAAT-binding factor (CBF) is involved in this increased expression. Ecteinascidin 743 (ET-743) is a chemotherapeutic agent that binds with sequence specificity to the minor groove of DNA and inhibits CBF-mediated transcriptional activation of numerous genes. Therefore, we examined the effects of ET-743 on the increased *COL1A1* expression in SSc fibroblasts. The drug caused a potent and dose-dependent inhibition of type I collagen biosynthesis, which reached 70–90% at 700 pM without affecting cell viability. The same drug concentration caused 60–80% reduction in *COL1A1* mRNA levels. The stability of the corresponding transcripts was not affected. *In vitro* nuclear transcription assays demonstrated a 54% downregulation of *COL1A1* transcription. Transient transfections with *COL1A1* promoter constructs containing the specific CBF binding sequence into SSc cells previously treated with 700 pM ET-743 failed to show an effect on *COL1A1* promoter activity. Furthermore, ET-743 did not affect the binding of CBF or Sp1 transcription factors to their cognate *COL1A1* elements. However, treatment with 700 pM ET-743 of stably transfected NIH 3T3 cells expressing a human type II procollagen gene under the control of the human *COL1A1* promoter caused a greater than 50% reduction in the production of type II procollagen and a similar decrease in the corresponding type II procollagen transcripts. These results indicate that ET-743 is a potent inhibitor of *COL1A1* transcription. However, this effect cannot be explained by a direct effect on CBF binding to the *COL1A1* promoter. Although the exact mechanisms responsible for the transcriptional inhibition of *COL1A1* by ET-743 are not apparent, our observations suggest that the drug may be an effective agent to decrease collagen overproduction in SSc and other fibrotic diseases.

Fibroblast activation and increased extracellular matrix deposition are central to normal processes such as wound healing. Dysregulated fibrosis, however, is a pathologic process resulting in tissue damage and destruction. Scleroderma or systemic sclerosis (SSc)[1] is a connective tissue disease characterized by excessive collagen deposition and severe fibrosis of the skin and various internal organs. There are no curative therapeutic modalities currently available for this disease. Although progress has been made toward defining molecular and cellular factors involved in normal tissue repair and in pathologic fibrosis, it is still not clear what distinguishes the normal reparative process from the uncontrolled destructive one occurring in SSc. Identification of such regulatory pathways could allow the development of effective pharmacological agents for diseases in which abnormal fibrosis is responsible for their clinical manifestations.

Several studies have focused on the regulation of *COL1A1* and *COL1A2*, the genes encoding the two chains of type I collagen, due to the central role that the protein plays in the progressive and uncontrolled fibrotic process of SSc (for reviews, see Refs. 1–3). We previously demonstrated that expression of the *COL1A1* gene is primarily regulated at the transcriptional level and that its highest promoter activity in both normal and SSc fibroblasts resides in the proximal promoter region (4, 5). Numerous important regulatory elements that are targets for transcription factor binding, including Sp1 and CCAAT-binding factor (CBF), have been identified in this region (6, 7). Previous studies showed that purified CBF, also known as NF-Y and CP1, binds to the proximal inverted CCAAT box of the murine *COL1A1* promoter, causing a potent transactivation of its transcription (8). We recently demonstrated that CBF binds the high affinity inverted CCAAT box of the human *COL1A1* promoter and found a marked increase in CBF binding activity in SSc fibroblasts when compared with normal cells (9). Also, another study showed that increased CBF binding to the *COL1A1* proximal promoter is involved in the activation of *COL1A1* transcription by mechanical strain in a process that is dependent on transforming growth factor β (10). The importance of the CCAAT box in the transcriptional activity of numerous genes has been well documented. Indeed, mutations of this motif in the promoters of the mouse *COL1A1* and *COL1A2* (11) and the human *COL1A1* (12) result in a significant decrease in their transcriptional activity (reviewed in Ref. 13). The remarkable evolutionary conservation of the CCAAT box elements and of their flanking regions in the se-

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[1] The abbreviations used are: SSc, systemic sclerosis; CBF, CCAAT-binding factor; ET-743, ecteinascidin 743; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CAT, chloramphenicol acetyltransferase; WT, wild type; MT, mutant; NE, nuclear extracts; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

quence of the *COL1A1* promoter from different species including chickens, rats, mice, and humans (14) further emphasizes its importance in the regulation of collagen gene expression. Thus, selective targeting and inhibition of CBF-mediated activation of the type I collagen genes could provide a successful approach to control the process of fibrosis.

Recent studies of ecteinascidin 743 (ET-743) have shown it to be a selective inhibitor of transcription of several genes and that CBF is one of its targets (15, 16). ET-743 is a DNA-alkylating agent extracted from the marine tunicate *Ecteinascidia turbinata* that has demonstrated potent antineoplastic activity against various human tumors including ovarian and colorectal cancers and advanced sarcoma (17). Numerous clinical trials are currently under way, and the mechanism of antineoplastic action of this unique agent is under intense investigation. Although the precise mechanisms of ET-743 action have not been elucidated completely, it appears that it interferes with the function of CBF in the transcription of numerous genes. There is evidence that ET-743 does not directly block CBF binding to DNA, but instead, it appears to interfere with the ability of CBF to recruit and associate with histone acetylases that are typically required for full gene promoter function. This sequence-specific effect has been demonstrated in genes such as the multidrug resistance gene, *MDR-1*, and the gene encoding heat shock protein 70, *HSP70* (15, 16).

Our previous studies were aimed at defining the role of CBF and other transcription factors in type I collagen regulation under normal conditions and in pathologic fibrosis (9). Here, we have extended these studies by examining the inhibitory effects of ET-743 on type I collagen gene expression in SSc, the prototype of fibrotic diseases. Our data show that the drug causes potent inhibition of type I collagen biosynthesis and that these effects are largely due to a reduction of *COL1A1* transcription. Our results provide strong support to the notion that such a gene transcription targeting approach may prove promising for the treatment of SSc, a currently fatal condition, and of numerous other diseases characterized by pathologic fibrosis.

EXPERIMENTAL PROCEDURES

Culture of Human Dermal Fibroblasts—Dermal fibroblast cell lines were established from patients with diffuse SSc of recent onset and rapid progression, as previously reported (4, 5, 9). All patients studied were followed at the Scleroderma Center of Thomas Jefferson University and fulfilled published criteria for the classification of SSc (18). Only untreated cases were studied to avoid spurious results caused by various therapeutic agents. In all cases, the cell lines were obtained from full thickness skin biopsies surgically excised from the leading edge of clinically apparent SSc lesions. Control fibroblasts were obtained from age- and sex-matched individuals undergoing surgical procedures for unrelated purposes. For all studies, only early passage fibroblasts (<10) were employed to avoid possible phenotypic changes during extended subculture. Cells were routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen), 1% vitamins, 2 mM L-glutamine, and antibiotics and incubated at 37 °C in a 5% CO_2 humidified atmosphere. All studies were performed in parallel with normal and SSc fibroblasts. ET-743 was obtained from Dr. R. Schultz (Drug Synthesis and Chemistry Branch, NCI, National Institutes of Health, Bethesda, MD) and from PharmaMar S.A. (Trescantos, Madrid, Spain) and was prepared as a stock solution (1 mg/ml) in absolute ethanol and stored at −20 °C until use. For experiments, the stock solution was diluted in double-distilled sterile H_2O to the desired concentrations.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Cell Viability Assays—Cytotoxicity of ET-743 for normal and SSc fibroblasts was assessed with the MTT TOX-1 kit assay (Sigma) as described previously (19). This assay measures the ability of mitochondrial dehydrogenase enzymes to convert the soluble yellow MTT salt into an insoluble purple formazan salt. Only live cells are able to carry out this reaction. Cells were grown to confluence in 6-well plates with Dulbec-

co's modified Eagle's medium containing 10% fetal bovine serum and treated with L-ascorbic acid phosphate magnesium salt *n*-hydrate (Waco Biochemical, Osaka, Japan) for 24 h and then incubated with various concentrations of ET-743 for 24, 48, and 72 h. MTT was added to each well and incubated for 2 h, the resulting formasan was dissolved in acidified isopropyl alcohol, and optical densities were determined at 570 nm using a Micro Plate Reader (Molecular Dynamics, Inc., Sunnyvale, CA). Colorimetric assays were performed in triplicate.

Determination of Type I Collagen Production—Normal and SSc cells were plated in 6-well dishes, cultured until confluent, and then pretreated for 24 h with 40 μg/ml L-ascorbic acid phosphate magnesium salt *n*-hydrate in order to optimize their level of collagen production. For dose-response studies, the cells were incubated with ET-743 (70, 140, and 700 pM) in 10% fetal bovine serum for 48 h. Following treatment, the media were changed with fresh media containing the same concentrations of ET-743, and the cells were cultured for an additional 24 h. The type I collagen present in the media of the cell cultures was quantified by an enzyme-linked immusorbent assay using an anti-human type I collagen polyclonal rabbit antibody (Rockland, Gilbertsville, PA). A standard curve was established that related the inhibition of color development to the concentration of added human type I collagen. The concentration of type I collagen in the culture media was analyzed in duplicate and determined directly from the standard curve, which was linear between 30 ng/ml and 2 μg/ml type I collagen.

Fibroblast Collagen Biosynthesis—Fibroblast collagen biosynthesis was examined essentially as described previously (20). Briefly, normal and SSc fibroblasts were plated in 6-well plates, grown to confluence, and pretreated with L-ascorbic acid as described above. The cells were incubated with ET-743 for 24 and 48 h, and then the media were changed and 600 μl of serum-free medium containing the same ET-743 concentrations was added. The cultures were labeled with 4 μCi/ml ^{14}C-labeled proline (ICN Biomedical, Costa Mesa, CA). After 3 h, the media were harvested from each well, and the cells were trypsinized and counted. Aliquots of media were taken for immunoprecipitation. The volume of medium for each immunoprecipitation assay was normalized according to the number of cells present in each well. The medium was incubated with the type I collagen rabbit polyclonal antibody for 2 h, and the antigen-antibody complexes were precipitated overnight with Protein G-agarose (Roche Applied Science). The precipitates were resuspended in 2× SDS sample buffer containing β-mercaptoethanol and boiled for 5 min. Samples were electrophoresed in 6% polyacrylamide gels, and the radioactive bands were visualized employing a Storm PhosphorImager and analyzed with ImageQuant version 5.1 software (Amersham Biosciences).

Northern Hybridizations—Fibroblasts were grown to confluence in 100-mm dishes and treated with ET-743 under the same conditions as those used for the protein experiments. Total RNA was isolated by an Rneasy Mini Kit (Qiagen, Valencia, CA), and aliquots (6 μg/well) were electrophoresed on formalin 1% agarose gels. The RNA was then transferred to Hybond™ N+ filters (Amersham Biosciences) and hybridized to ^{32}P-radiolabeled human cDNA for *COL1A1*, *COL2A1*, and GAPDH. The filters were analyzed using densitometry equipment and software (ImageQuant version 5.1 software; Amersham Biosciences). The hybridization signals were normalized to those of GAPDH.

Assessment of Steady-state mRNA Levels and mRNA Stability—Confluent SSc fibroblasts were incubated for 24 h with or without ET-743 (700 pM), and mRNA synthesis was inhibited by the addition of 1 μg/ml α−amanitin as described previously (21). Cells were harvested every 6 h following the addition of α−amanitin, and total RNA was isolated and analyzed by Northern hybridizations using specific cDNA probes for *COL1A1* and GAPDH. All data were normalized for the levels of GAPDH mRNA.

In Vitro Nuclear Transcription Assay—The transcription rates of *COL1A1* were measured by an *in vitro* nuclear run-off assay. Confluent SSc fibroblasts were cultured in T-175 flasks in the presence or absence of 700 pM ET-743 for 48 h. At the end of the incubation, cells were trypsinized, nuclei were isolated, and transcription reactions were carried out as described previously (21). The nuclei were labeled with 125 μCi of [α-^{32}P]UTP (3000 Ci/mmol), and samples were extracted following the sequential addition of 2.0 M sodium acetate (pH 4.0), phenol, and chloroform/isoamyl alcohol-saturated H_2O and then precipitated with isopropyl alcohol. The RNA pellets were dissolved in 50 μl of STE buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 mM NaCl) and passed through a Probe-Quant G-50 microspin column (Amersham Biosciences). One set of plasmids (10 μg each) was prepared for each experimental condition and dotted onto a nitrocellulose membrane using a Schleicher & Schuell dot blot minifold. Hybridization and washing
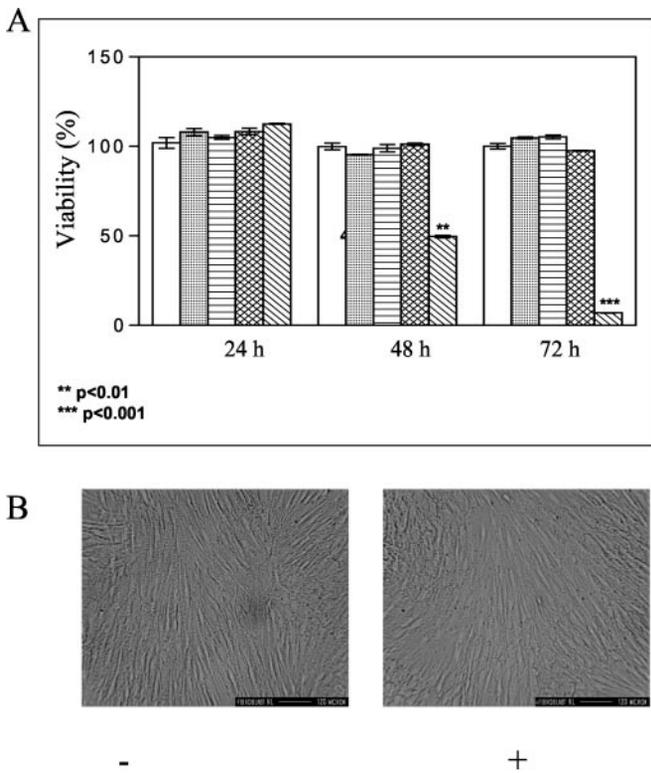
FIG. 1. **Effects of ET-743 on normal and SSc fibroblast viability and morphology.** Cytotoxicity of ET-743 was tested by a MTT-based cytotoxicity assay as described under "Experimental Procedures." *A*, values shown represent the percentage of viable cells following 24, 48, or 72 h of treatment with a given drug concentration compared with untreated cells. The results are shown as means ± S.E. of three independent experiments. *Open bars*, control; *dotted bars*, 70 pM ET-743; *horizontally striped bars*, 140 pM ET-743; *criss-crossed bars*, 700 pM ET-743; *hatched bars*, 3 nM ET-743. *B*, photomicrograph of cultured fibroblasts; −, untreated cells; +, cells treated with 700 pM ET-743 for 72 h.

conditions were carried out as previously described (21). The membranes were exposed for 72 h in a phosphor imaging cassette and analyzed with phosphor imaging equipment.

Transient Transfections of Human Dermal Fibroblasts—Cells were transfected employing the FuGene 6 kit (Roche Applied Science) as described previously (9). Control and SSc fibroblasts were plated and cultured to 85% confluence in 60-mm dishes and then transfected with 2.0 μg of the −804 bp, −675 bp, −174 bp, or −84 bp *COL1A1* CAT constructs described previously (6) or with an SV40 CAT construct. Transfection experiments were in triplicate and were repeated at least twice, employing two different SSc fibroblast cell lines. Following 6 h of incubation, fresh medium containing 700 pM ET-743 was added. The cells were harvested 48 h after transfection and treated with a lysis buffer (Roche Applied Science). The protein concentration of the extracts was determined using the BCA protein assay kit (Pierce). The cell lysates were assayed for CAT protein employing a CAT enzyme-linked immusorbent assay kit (Roche Applied Science). CAT activity was determined in cell extracts in triplicate experiments in at least two different cell lines. The concentration of CAT in supernatants was determined directly from the standard curve of CAT protein.

Electrophoretic Gel Mobility Shift Assay—Confluent cultures of normal and SSc fibroblasts from three 175-mm flasks were washed with PBS, and nuclear extracts were prepared as described previously (22). Electrophoretic gel mobility shift assays using nuclear extracts were performed as described previously (6, 9, 19). The protein concentration of the extracts was determined using the Coomassie Plus protein assay kit (Pierce). Radioactive probes were generated by phosphorylating the 5′-ends with polynucleotide kinase (Roche Applied Science) and [γ-^{32}P]ATP (ICN, Costa Mesa, CA). Binding reactions containing 10 μg of nuclear extracts, 4 μg of poly(dI-dC), and 5–8 × 10^4 cpm (~0.2 ng) of radiolabeled probes were incubated for 15 min at room temperature in a buffer containing 40 mM KCl, 15 mM HEPES, pH 7.9, 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM MgCl$_2$, and 5% glycerol in a total volume of 25 μl. To determine the *in vitro* effects of ET-743 on CBF and Sp1 binding
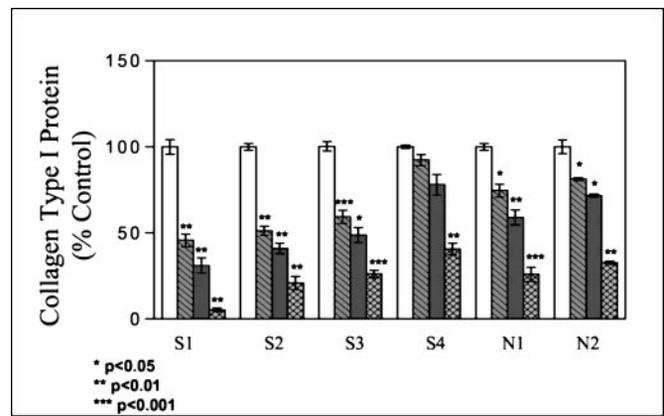
FIG. 2. **Effects of ET-743 on normal and SSc fibroblast collagen production.** Confluent cultures of four SSc (S1–S4) and two normal fibroblast (N1 and N2) cell lines were treated under control conditions or with 70, 140, or 700 pM ET-743 for 48 h. Collagen type I production was examined by an indirect enzyme-linked immusorbent assay as described under "Experimental Procedures." The values represent the percentage of collagen in the culture media following treatment with ET-743 compared with untreated cells. The results shown are the means ± S.E. from three separate experiments. *Open bars*, control; *hatched bars*, 70 pM; *dotted bars*, 140 pM; *criss-crossed bars*, 700 pM.
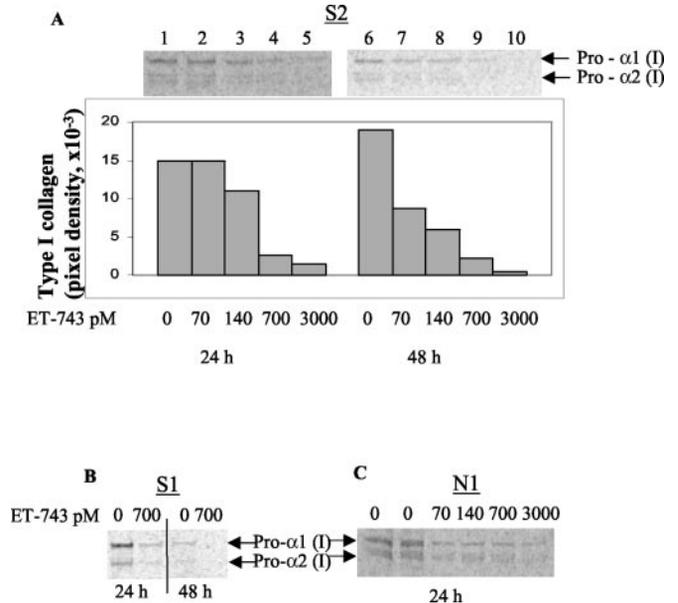
FIG. 3. **Effect of ET-743 on collagen biosynthesis.** Confluent fibroblast cultures were incubated under control conditions or with various drug concentrations (from 0 to 3000 pM) for 24 and 48 h and were then labeled with [^{14}C]proline for 3 h as described under "Experimental Procedures." Following labeling, the culture media were harvested, and the radiolabeled procollagen in the media representing newly synthesized protein was immunoprecipitated with a specific anti-type I collagen antibody. Aliquots of the media corresponding to equal numbers of cells were employed for immunoprecipitation. The immunoprecipitates were solubilized in SDS sample buffer, and equal volumes were electrophoresed in SDS-polyacrylamide gels. The bands corresponding to radiolabeled procollagen chains were visualized by fluorography and quantitated. *A*, representative experiment from one SSc cell line (S2). *B* and *C* representative results from an additional SSc cell line (S1) and a normal cell line (N1). The migration of proα1(I) and proα2(I) chains is shown.

to their corresponding binding sites in the *COL1A1* promoter, the probes were incubated with either 700 pM or 3 nM of the drug for 10 min, and then nuclear extracts were added to the mixture for an additional 15 min. To test the specificity of transcription factor binding to the DNA probes, competition experiments using a 150-fold molar excess of wild type (WT) or mutated (MT) oligonucleotides were performed. Oligonucleotides containing consensus WT sequences for Sp1 and CBF and

A

B

C

D
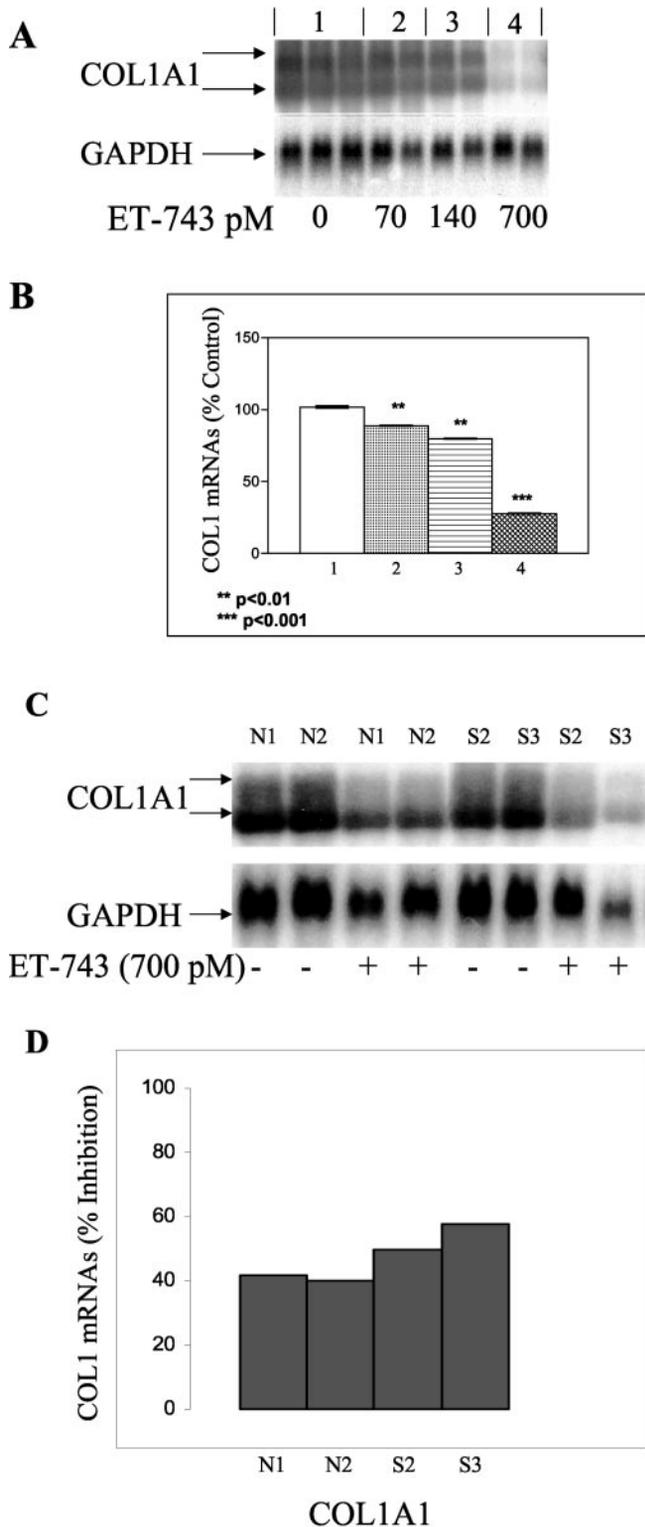
FIG. 4. **Effects of ET-743 on *COL1A1* mRNA steady-state levels.** Confluent cultures of one SSc cell line (S1) were incubated with the ET-743 concentrations shown for 48 h, and the *COL1A1* mRNA levels were analyzed by Northern hybridizations as described under "Experimental Procedures." *A*, representative Northern hybridization; *B*, densitometric analysis. The results represent three independent experiments, and values are expressed as mean ± S.E. percentage relative to untreated fibroblasts. *Lane 1*, control; *lane 2*, 70 pM; *lane 3*, 140 pM; *lane 4*, 700 pM. Confluent fibroblast cultures of two normal (N1 and N2) and two SSc (S2 and S3) cell lines were incubated for 48 h with or without 700 pM ET-743, and *COL1A1* mRNA levels were determined by Northern hybridization analysis. *C*, representative autoradiograph of Northern blot. *D*, densitometric analysis of Northern blots. The results represent two independent experiments, and values are expressed as a percentage relative to untreated fibroblasts.
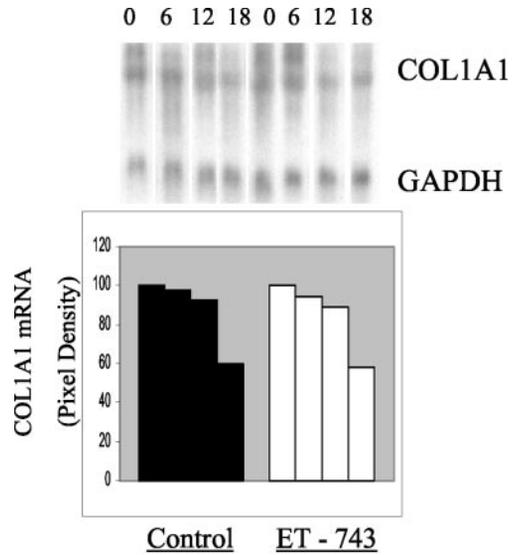
FIG. 5. **Effects of ET-743 on the stability of the *COL1A1* transcripts.** Confluent fibroblasts were incubated in T-175 flasks in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and L-ascorbic acid (40 μg/ml) with or without 700 pM ET-743 for 24 h, and then α-amanitin (1 μg/ml) was added to arrest further transcription. Cells were harvested at the intervals shown following the addition of α-amanitin, total RNA was isolated, and equal amounts were analyzed by Northern hybridizations and densitometry as described under "Experimental Procedures." Densitometric analyses are shown in arbitrary densitometric units, and the results represent two independent experiments.

their mutated forms were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The *COL1A1* promoter-specific WT oligonucleotides employed were as follows: oligonucleotide 9/10 (CTCCCAAATT-GGGGGGCCGGGCCAG) containing the Sp1 site and oligonucleotide 13/14 (CAGCTCTGATTGGCTGGGGCA) containing the proximal CCAAT box. Their mutated forms were as follows: oligonucleotide 9/10 M (CTCCCAAATTGGG**TTT**CGGGCCAG) containing a mutation in the Sp1 site and oligonucleotide 13/14 M (CAGCTCTGAT**CAA**CT-GGGGCA) containing a mutation in the CAAT box. These oligonucleotides were obtained from the Nucleic Acid Facility at Thomas Jefferson University (Philadelphia, PA). DNA-protein complexes were electrophoresed through 5% nondenaturing polyacrylamide gels using low ionic strength buffers. The gels were dried under vacuum and exposed to x-ray film (Eastman Kodak Co.) at -70 °C using intensifying screens.

Treatment of Stably Transfected NIH 3T3 Cells with ET-743—Stably transfected NIH 3T3 cells expressing the human type II procollagen gene (*COL2A1*) under the control of the *COL1A1* promoter were employed for these studies. The establishment of these stable transfectants was described by Ala-Kokko *et al.* (26). The cells express a *COL1A1/COL2A1* hybrid gene construct comprising ~560 bp of the *COL1A1* promoter and the first exon (222 bp) and 1218 bp of the first intron of the gene driving the transcription of a full-length type II procollagen gene. The cells were grown to confluence and then were treated with 0.7 nM ET-743 for 48 h under the same conditions as those used for the protein experiments with human skin fibroblasts. In separate experiments, it was found that this concentration of ET-743 did not cause cytotoxicity to the NIH 3T3 cells.

Western Blot Analysis—For Western blot analysis of type II procollagen production by control or ET-743-treated NIH 3T3 stably transfected cell lines, the cell culture medium was removed and precipitated with 5% polyethylene glycol (27). The precipitates were collected by centrifugation and resuspended in a buffer containing 0.4 M NaCl, 25 nM EDTA, 0.4% NaN$_3$, 0.1 M Tris-HCl, pH 7.4. Equal aliquots of the samples were boiled for 5 min in SDS buffer with 1% β-mercaptoethanol and then applied to 6% polyacrylamide gels and electrophoresed as described previously (19, 20). The separated proteins were transferred to nitrocellulose membranes, and following transfer, the membranes were stained with 0.5% Ponceau S to assure equal loading and transfer of the proteins. The transferred proteins were reacted for 2 h with a 1:500 dilution of SJ 441 antibody, which is a polyclonal rabbit antibody specific for the COOH-terminal telopeptide of human type II collagen (28). After incubation with horseradish peroxidase-conjugated anti-

S4

A

ET- 743   -   +

B
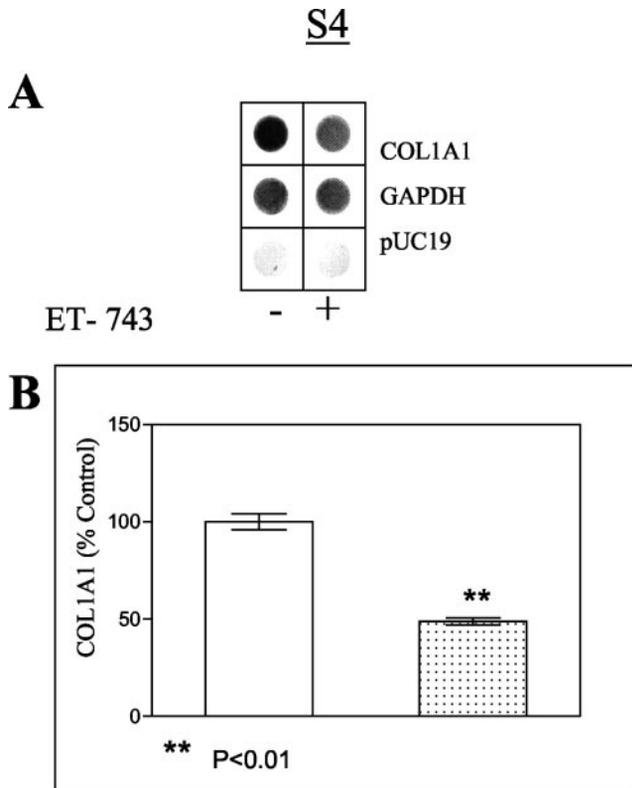
FIG. 6. **Effects of ET-743 on *in vitro* nuclear transcription.** Confluent fibroblast cultures of one SSc cell line (S4) were incubated in T-175 flasks either under control conditions or treated with 700 pM ET-743. After 48 h, nuclei were isolated and processed for a nuclear run-off assay as described under "Experimental Procedures." *A*, aliquots of ^{32}P-labeled RNA containing equal cpm were hybridized to dot-blotted cDNAs for α1(I) collagen, GAPDH, and control pUC19 plasmid and, following hybridization, were analyzed using phosphor storage technology. *B*, densitometric analysis showing the percentage of *COL1A1* transcripts in nuclei from treated cells compared with control. The values are means + S.E. of results obtained in three independent experiments. The values obtained were corrected for the transcription of GAPDH following subtraction of the background represented by pUC19. *Open bar*, untreated; *dotted bar*, ET-743-treated.

rabbit antibody (Amersham Biosciences), signals were detected using the ECL Western blot detection system kit (Amersham Biosciences) according to the manufacturer's recommended protocol.

Statistical Analysis—Statistical differences between untreated (control) and treated cells were assessed employing analysis of variance with the GraphPad Software Prism 2.01. Values of $p < 0.05$ were considered significant. The unpaired t test was used to determine statistical significance.

RESULTS

Effects of ET-743 on Normal and SSc Fibroblast Viability and Morphology—In order to examine cytotoxic effects of ET-743 on human adult dermal fibroblasts, confluent cultures of normal and SSc cells were treated with various ET-743 concentrations for 24, 48, and 72 h, and cytotoxicity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Fig. 1*A*). The data demonstrated that at concentrations of up to 700 pM, ET-743 did not cause any detectable cytotoxic effect following 72 h of continuous treatment. However, a concentration of 3 nM of ET-743 decreased cell viability by about 50% during 48 h of culture and by about 90% during 72 h of culture, but no cytotoxicity was observed during the first 24 h (Fig. 1*A*). Cellular morphology of untreated and treated confluent fibroblast cultures exposed to various ET-743 concentrations showed that cell morphology was not affected by concentrations up to 700 pM during 72 h of culture (Fig. 1*B*).

Effect of ET-743 on Collagen I Production and Biosynthesis—
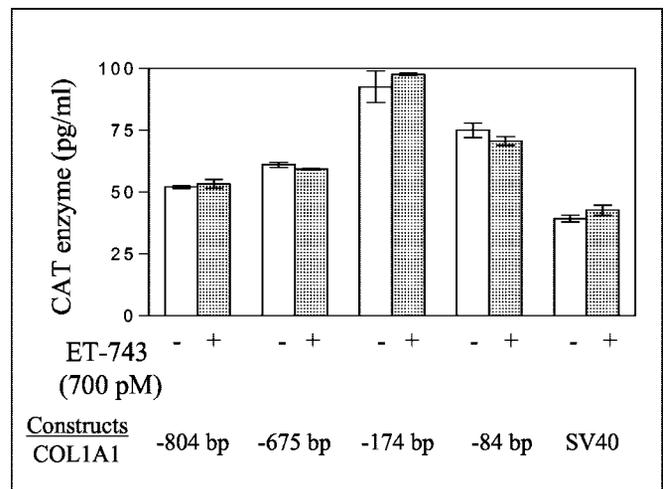
FIG. 7. **Transient transfection of *COL1A1* promoter into untreated or ET-743-treated SSc fibroblasts.** CAT assays were performed with extracts from two SSc cell lines (S2 and S3) that had been transfected with 2 μg of various *COL1A1* promoter CAT constructs and incubated with 700 pM ET-743 for 48 h following transfection as described under "Experimental Procedures." The amount of CAT protein driven by each of the promoter CAT constructs was assayed by an enzyme-linked immunosorbent assay. Results from an illustrative experiment are shown. The values represent the means ± S.E. of triplicate wells from representative experiment. At least three experiments were done, and the values are expressed as concentrations of CAT enzyme.

The effects of ET-743 on collagen I expression were examined in four different cell lines of SSc and two different cell lines of normal fibroblasts treated for up to 72 h with 70, 140, or 700 pM ET-743. The results are shown in Fig. 2. ET-743 reduced type I collagen production in both types of cells in a dose-dependent manner. At concentrations of 70 and 140 pM, ET-743 collagen production was reduced by an average of 50 and 65%, respectively, in four SSc cell lines (S1–S4) and by an average of 30 and 40%, respectively, in two normal fibroblast cell lines (N1 and N2). The higher ET-743 concentration (700 pM) had a more potent inhibitory effect (~70–90%) in both normal and SSc fibroblasts. The experiments were carried out in triplicate, and the variation between samples was 5–10%. Biosynthetic studies in SSc fibroblasts treated with 70, 140, 700, and 3000 pM ET-743 showed a strong inhibition of production of newly synthesized type I procollagen (Fig. 3*A*). At a concentration of 700 pM, ET-743 caused an 85% inhibition during a 24-h treatment (Fig. 3*A*, *lane 4*) and greater than 90% inhibition during a 48-h treatment (Fig. 3*A*, *lane 9*). Studies using 700 pM ET-743 were also performed in other SSc (Fig. 3*B*) and normal fibroblast (Fig. 3*C*) cell lines with similar results.

ET-743 Down-regulation of COL1A1 Steady-state mRNA Levels—To investigate the effects of ET-743 on *COL1A1* mRNA levels, confluent cultures of one SSc fibroblast cell line (S1) were treated for 48 h with 70, 140, and 700 pM ET-743. A 75% reduction of *COL1A1* transcripts was observed during 48-h treatment with 700 pM ET-743 (Fig. 4, *A* and *B*), whereas the control GAPDH mRNA levels were not affected. Two other SSc fibroblast cell lines (S2 and S3) and two normal fibroblast cell lines (N1 and N2) were incubated with 700 pM ET-743, and *COL1A1* mRNA was analyzed following 48 h of treatment (Fig. 4, *C* and *D*). A 58% reduction of *COL1A1* was observed in the two SSc fibroblast cell lines (Fig. 4*C*, *lanes 7* and *8*). Inhibition of up to 40% was observed for *COL1A1* mRNAs in the two normal fibroblast cell lines (Fig. 4*D*, *lanes 3* and *4*). Further studies showed that ET-743 did not affect the stability of the *COL1A1* mRNA in either normal or SSc dermal fibroblasts (Fig. 5).
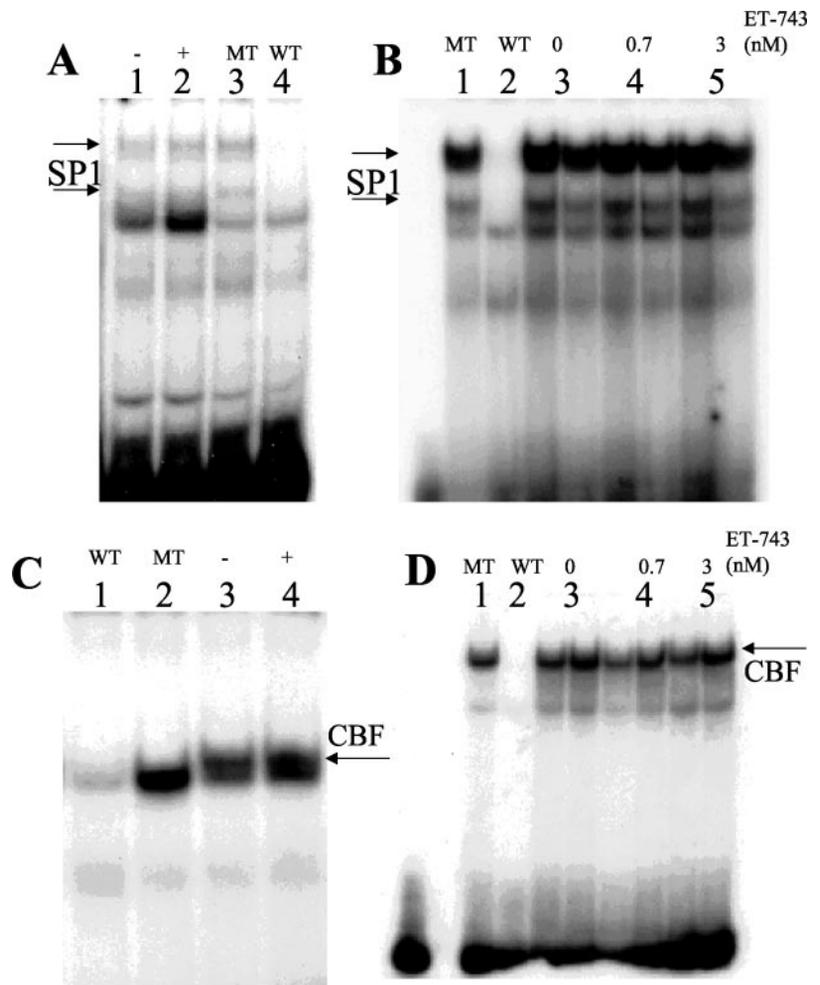
FIG. 8. **Electrophoretic gel mobility shift assay of nuclear extracts (NE) from SSc fibroblasts interacting with the Sp1 and CBF consensus after ET-743 treatment.** *A*, *lane 1*, NE were incubated with an Sp1 probe; *lane 2*, NE prepared from fibroblasts treated for 48 h with 700 pM ET-743 incubated with an Sp1 probe; *lanes 3 and 4*, competition with a 150-fold molar excess of either MT or WT Sp1 oligonucleotides, respectively. *B*, Sp1 consensus oligonucleotide was incubated with 0, 0.7, and 3 nM (*lanes 3–5* in duplicate) ET-743 and analyzed by gel shift with NE from untreated fibroblasts. *Lanes 1* and *2*, competition with MT and WT Sp1 oligonucleotides. *C*, *lane 3*, interaction of CBF probe with NE from untreated cells. *Lane 4*, interaction of CBF probe with NE from fibroblasts treated for 48 h with 700 pM ET-743; *lanes 1 and 2*, competition with WT and MT CBF oligonucleotides. *D*, CBF consensus oligonucleotide was incubated with 0, 0.7, and 3 nM (*lanes 3–5* in duplicate) ET-743 and analyzed with NE from untreated fibroblasts. *Lanes 1* and *2*, competition with MT and WT CBF oligonucleotide.

ET-743 Causes a Reduction in COL1A1 Transcription Rates—To determine whether the changes in *COL1A1* mRNA levels resulted from the modulation of the transcription rates of the corresponding gene, *in vitro* transcription assays were performed in SSc fibroblasts treated with 700 pM ET-743 (Fig. 6). Three experiments were performed using nuclei from SSc cells. The results showed that *COL1A1* transcription was down-regulated by an average of 50%. The level of *COL1A1* transcription inhibition was similar to the level of inhibition of mRNA steady-state levels found by Northern hybridization (see Fig. 4). The transcription of the control GADPH was not affected by ET-743 treatment.

COL1A1 Promoter Activity Is Not Affected by ET-743 in Transient Transfections—Transient transfection experiments were performed to determine whether the *COL1A1* promoter region that contains the CBF response element was involved in the transcriptional down-regulation of *COL1A1* activity observed in the *in vitro* nuclear run-off assays. Various *COL1A1* promoter constructs were transfected into normal and SSc fibroblasts, and then the cells were treated with 700 pM ET-743. When concentrations of the drug shown in the *in vitro* transcription experiments to cause inhibition of *COL1A1* transcription (700 pM) were employed, the promoter activity of the *COL1A1* constructs spanning −804 to −84 bp was not down-regulated (Fig. 7).

To determine whether the transcriptional down-regulation of *COL1A1* expression by ET-743 was due to competition of CBF binding to its cognate elements within *COL1A1*, the interaction of ET-743 with gene promoter sequences known to be involved in the regulation of *COL1A1* transcriptional activity was investigated. Also, the effects of ET-743 on binding of the regulatory transcription factors Sp1 and CBF to *COL1A1* proximal promoter sequences were examined. Nuclear extracts from untreated and ET-743 (700 pM)-treated fibroblasts were examined by an electrophoretic gel mobility shift assay with consensus oligonucleotides for Sp1 and CBF and with DNA fragments from the *COL1A1* promoter containing Sp1 or CBF binding sites. As shown in Fig. 8, nuclear extracts from fibroblasts treated with ET-743 displayed the same level of Sp1 and CBF binding activity to consensus Sp1 or CBF oligonucleotides as untreated cells (Fig. 8, *A* and *C*). The specificity of the binding was confirmed by specific displacement and inhibition of binding employing an excess of unlabeled oligonucleotides. Similar binding assays with oligonucleotides corresponding to Sp1 and CBF binding sites from the *COL1A1* promoter also failed to show any differences between untreated and ET-743-treated cells (data not shown).

A direct interaction of ET-743 with Sp1 or CBF binding sites preventing or blocking the subsequent binding of the transcription factors was then examined. For this purpose, oligonucleotides containing Sp1 and CBF consensus sequences were incubated *in vitro* with ET-743, and DNA-protein complex formation was analyzed by an electrophoretic gel mobility shift assay. As shown in Fig. 8, *B* and *D*, ET-743 at a 700 pM concentration and at 3 nM did not change the pattern of transcription factor binding obtained without the drug. The results for Sp1 consensus oligonucleotide are shown in Fig. 8*B*, and those for CBF are shown in Fig. 8*D*. Similar results were obtained with oligonucleotides corresponding to the Sp1 and CBF sequences from the *COL1A1* promoter (data now shown).

COL1A1 Promoter Activity Is Affected by ET-743 in Stably Transfected NIH 3T3 Cells—Experiments with stably transfected cells were performed to confirm the transcriptional down-regulation of *COL1A1* activity by ET-743 observed in the *in vitro* nuclear run-off assays. NIH 3T3 cells stably transfected with a cosmid containing a chimeric *COL1A1/COL2A1* gene, encompassing a 2-kb *SphI/Hind*III fragment from the 5′-end of the human *COL1A1* gene, which included the promoter region (~560 bp), the first exon (222 bp), and 1218 bp of the first intron, and a full-length type II procollagen gene were employed. The stable transfectants were prepared by Ala-Kokko *et al.* (26) and express effectively the cartilage-specific type II procollagen gene under the control of *COL1A1* regulatory elements. Confluent cultures of NIH 3T3 cells expressing type II procollagen were treated with 0.7 nM ET-743 for 48 h, and medium proteins were assayed for type II procollagen by Western blot (Fig. 9). Separate experiments indicated that these conditions did not cause cytotoxic effects on the stable transfectants (data not shown). Collagen II protein production by these cells displayed a significant decrease (~50%) in response to ET-743 treatment (Fig. 9*A*, *lanes 3* and *5*) compared with control untreated cells (Fig. 9*A*, *lanes 4* and *6*). In order to confirm the data at the transcription level, total RNA was isolated from treated and untreated cells, and Northern hybridizations with a *COL2A1* cDNA were performed (Fig. 9, *C* and *D*). The level of inhibition of *COL2A1* mRNA was similar to the level of inhibition of type II procollagen production found by Western blot, and both levels of inhibition were close to those of *COL1A1* transcription inhibition observed in the nuclear run-off assays (Fig. 6).

DISCUSSION

Here, we describe the first demonstration that the antineoplastic drug ET-743 is a potent inhibitor of the expression of *COL1A1* in normal human dermal fibroblasts and, more importantly, that the drug also inhibits the exaggerated overexpression of this collagen gene in SSc fibroblasts. These effects were apparent both at the protein (Figs. 2 and 3) and at the mRNA levels (Fig. 4). Notably, these effects occurred without affecting the viability and morphology of normal and SSc fibroblasts (Fig. 1). We also show that the drug is effective at low concentrations (70–700 pM), much lower than those required to exert antineoplastic effects (15, 16). With a run-off assay employing isolated fibroblast nuclei, we determined that at this low concentration (700 pM), ET-743 has a direct transcriptional effect on *COL1A1* (Fig. 6).

Previous studies on the mechanisms of the antineoplastic effects of ET-743 demonstrated that the drug at much higher concentrations than those employed here (10–50 nM) inhibits promoter activation of *MDR-I* and *HSP-70* genes (15, 16). It was also shown that ET-743 at high concentrations (3 nM) suppressed okadaic acid induction of the JUN B CAAT box promoter and that CBF and possibly Sp1 were involved in this process (23). Therefore, we analyzed whether CBF or Sp1 are involved in the transcriptional inhibition of ET-743 on *COL1A1*. We performed gel shift analysis of the interaction of Sp1 and CBF oligonucleotides to examine the binding activity of Sp1 and CBF present in fibroblast nuclear extracts from untreated or ET-743 (700 pM)-treated cells. Nuclear extracts from treated fibroblasts yielded normal CBF and Sp1 protein binding activities, since there was not any detectable quantitative or qualitative change in the Sp1 and CBF complexes detected by an electrophoretic gel mobility shift assay (Fig. 8). However, it is necessary to mention that these experiments may be limited by the size or sequence of the fibre oligonucleotides employed and, according to Hurley *et al.* (24), by the stability of ET-743·DNA complexes. The stability of these complexes is
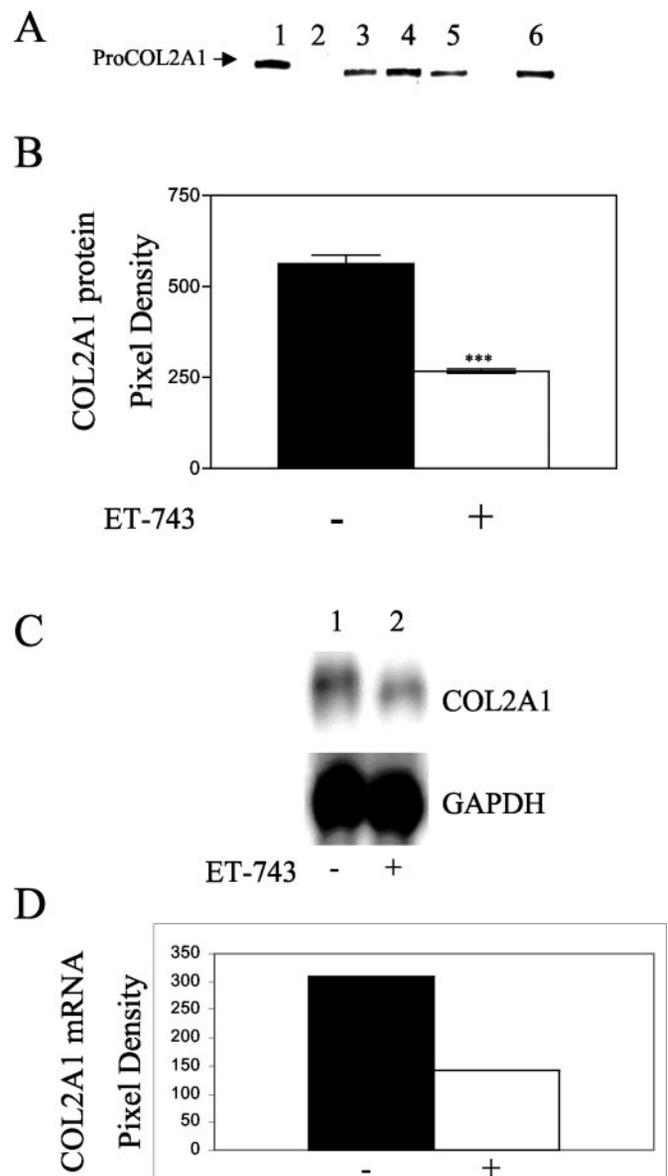
FIG. 9. **Western and Northern blot analysis of type II collagen and type II procollagen mRNA levels in stably transfected NIH 3T3 cells expressing the human type II procollagen gene under the control of the *COL1A1* promoter.** Cells were treated 48 h with 0.7 nM of ET-743. *A*, immunoblotting with anti-collagen II antibody of culture medium. *Lane 1*, type II collagen as positive control; *lane 2*, medium from NIH 3T3 cells that were not transfected as negative control; *lanes 3* and *4*, medium from ET-743-treated (*lane 3*) and untreated cells (*lane 4*) from experiment 1. *Lanes 5* and *6*, medium from ET-743-treated (*lane 5*) and untreated (*lane 6*) cells from experiment 2. Equal loading of the gel was confirmed by Ponceau S staining of the transfer membrane (data not shown). *B*, densitometric analysis of Western blots shown in arbitrary densitometric units as means ± S.E. of three independent experiments. *C*, a representative Northern hybridization of total RNA from ET-743-treated and untreated NIH 3T3 cells with a type II procollagen cDNA. *D*, densitometric analysis of Northern blots shown in arbitrary densitometric units. The results represent the averages of two independent experiments, which differed from each other by less than 10%.

governed by DNA target sequences, since under some conditions the drug is not capable of forming optimally stable bonds with its corresponding recognition sites in the DNA leading to the formation of unstable products. The same authors in other work (25) demonstrated that ET-743 can migrate from a non-favored bonding sequence (5′-AGT) to the favored sequence site (5′-AGC) and that the ET-743-AGT adduct is less stable, displays a higher degree of molecular motion, and produces dif-

ferent conformational changes in DNA than the more stable alkylation product ET-743-AGC.

Our studies employing transient transfections of *COL1A1* constructs containing CBF and Sp1 binding sites showed that in the picomolar concentration range ET-743 is unlikely to target CBF- or Sp1-mediated transcription. Therefore, the mechanisms responsible for the transcriptional down-regulation of *COL1A1* caused by the drug do not appear to be related to competition or prevention of CBF or Sp1 binding to their corresponding sites within the collagen gene promoter. Thus, the putative mechanisms of the drug on *COL1A1* transcription remain elusive but could include interactions of ET-743 with other DNA sites close to transcription factor binding sites within *COL1A1* or to sites recognized by other transcriptional factors. Alternatively, the observed effects could be due to reversible ET-743 alkylation products that, when formed at this low concentration of the drug, are not stable, and the drug could then interact with other preferable, more stable, sequences. The experiments employing stably transfected NIH 3T3 cells expressing a *COL1A1/COL2A1* hybrid gene indicate that ET-743 exerts a negative effect on *COL1A1* transcription at very low concentrations (0.7 nM) without causing cytotoxic effects. A comparison of these results with those obtained employing transient transfections of the human dermal fibroblasts suggests that the drug may be more effective within a more permissive gene context, perhaps due to the formation of more stable alkylation products than those formed with the shorter gene sequences employed in the transient transfection experiments. Alternatively, it is possible that ET-743 might prevent association of histone acetylases with the gene, thus precluding its full promoter function. Therefore, our studies show that ET-743 displays transcriptional inhibition on *COL1A1* at very low concentrations, an effect that cannot be explained appropriately by a direct CBF-mediated mechanism. Thus, although the precise mechanism(s) by which ET-743 affects *COL1A1* transcription in normal and SSc dermal fibroblasts remains to be determined, the results described here indicate that the drug may represent a novel and potentially effective therapy for SSc and other fibrotic diseases.

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Transcriptional Inhibition of Type I Collagen Gene Expression in Scleroderma Fibroblasts by the Antineoplastic Drug Ecteinascidin 743

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