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# Sp1 and Sp3 Transcription Factors Mediate Malondialdehyde-induced Collagen α1(I) Gene Expression in Cultured Hepatic Stellate Cells*

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**Malondialdehyde, the end product of lipid peroxidation, has been shown to stimulate collagen α1(I) (Col1a1) gene expression. However, mechanisms of this effect are unclear. The purpose of this study was to clarify these mechanisms. Rat hepatic stellate cells were cultured in the presence of 200 μM malondialdehyde, and the effects on collagen gene expression and the binding of nuclear proteins to the *col1a1* promoter were analyzed. Malondialdehyde treatment induced an increase in the cellular levels of *col1a1* mRNA that was abrogated by pretreating cells with cycloheximide, *p*-hydroxymercuribenzoate, pyridoxal 5′-phosphate, and mithramycin. Transient transfections showed that malondialdehyde exerted its effect through regulatory elements located between −220 and −110 bp of the *col1a1* promoter. Gel retardation assays demonstrated that malondialdehyde increased the binding of nuclear proteins to two elements located between −161 and −110 bp of the *col1a1* promoter. These bindings were supershifted with Sp1 and Sp3 antibodies. Finally, malondialdehyde increased cellular levels of the Sp1 and Sp3 proteins and *Sp1* mRNA. Our data indicated that treatment of hepatic stellate cells with malondialdehyde stimulated *col1a1* gene expression by inducing the synthesis of Sp1 and Sp3 and their binding to two regulatory elements located between −161 and −110 bp of the *col1a1* promoter.**

Lipid peroxidation by-products, particularly reactive aldehydes, such as malondialdehyde (MDA)[1] and 4-hydroxy-2-nonenal (4HNE), have been incriminated in the pathogenesis of liver fibrosis. Thus, Chojkier *et al.* (1) first showed that MDA increased significantly the collagen α1(I) (Col1a1) mRNA in cultured human fetal fibroblasts, and Maher *et al.* (2) found that collagen synthesis doubled in response to MDA in rat kidney fibroblasts. Likewise, Parola *et al.* (3, 4) showed that 4HNE and other 4-hydroxy-2,3-alkenals, aldehydic end products of lipid peroxidations, were able to stimulate *col1a1* gene expression and collagen synthesis in cultured hepatic stellate cells (HSC). Similar results have been reported by Tsukamoto and co-workers (5, 6), who also observed a significant correlation between the liver MDA and 4HNE levels and the hepatic collagen accumulation in a rat model of alcoholic liver disease.

Mechanisms by which these reactive aldehydes induce *col1a1* gene expression and synthesis are still unclear. However, a number of studies have provided evidence supporting the role of aldehyde-protein adducts in the regulation of *col1a1* gene expression. These aldehydes are known to react with sulfhydryl or amino groups to form aldehyde-protein adducts (7). These adducts have been found in alcoholic liver disease (8, 9) and other clinical conditions of chronic liver injury associated with active fibrogenesis, as well as in animal models of lipid peroxidation (6, 10–14). Moreover, antioxidant treatment significantly decreased the production of these adducts and prevented the fibrogenesis cascade (10, 13, 15). The purpose of this study was to clarify the mechanisms by which MDA induces *col1a1* gene expression, particularly the transcription factors and *cis*-acting elements involved in the mediation of this effect.

## EXPERIMENTAL PROCEDURES

*Reagents*—Minimum essential Eagle's medium with Hanks' balanced salt solution (HMEM), collagenase type VII, *p*-hydroxymercuribenzoate (*p*HMB), mithramycin, MDA, pyridoxal 5′-phosphate (P5P), and cycloheximide were from Sigma. Fetal bovine serum (FBS), L-glutamine, nonessential amino acids, and penicillin/streptomycin were purchased from Boehringer Ingelheim and Bio-Whittaker, Verviers, Belgium. Plastic cell culture flasks and dishes were from Nunc (Roskilde, Denmark). Agarose and Bradford protein reagent were from Bio-Rad. Nylon filters Hybond™, L-[$^{3}$H]proline (specific activity, 43 Ci/mmol), [$^{14}$H]chloramphenicol (specific activity, 56 mCi/mmol), and [α-$^{32}$P]dCTP (specific activity, 3000 Ci/mmol) were obtained from Amersham Biosciences. Klenow fragment of the *Escherichia coli* DNA polymerase I, restriction enzymes, and poly(dI-dC) were from Roche Molecular Biochemicals. pBluescript SK+ was obtained from Stratagene, Heidelberg, Germany. Synthetic oligonucleotides were supplied by Amersham Biosciences; silica plates for thin layer chromatography were from Scharlau, Barcelona, Spain; and Sp1, Sp3, NF-1, BTEB, Zf9, AP2, and C/EBPβ antibodies were from Santa Cruz Biotechnology Inc, Santa Cruz, CA. p(−3700)ColCAT, p(−220)ColCAT (16), pRSVβ-Gal (17), pΔSluc, and p(−126)Luc reporter plasmids (18) were generously provided by A. Tugores (University of California, San Diego). c-Krox protein and c-Krox antibody was a gift by Dr. Philippe Galéra (Laboratoire de Biochimie du Tissu Conjonctif, Faculté de Médecine, Caen, France).

*HSC Isolation and Culture*—HSC were isolated from adult Sprague-

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[1] The abbreviations used are: MDA, malondialdehyde; CAT, chloramphenicol acetyltransferase; Col1a1, collagen α1(I); FBS, fetal bovine serum; FP1, sequences between −82 and −103 bp of the collagen α1(I) promoter; FP2, sequences between −110 and −129 bp; FP3, sequences between −133 and −161 bp; FP4, sequences between −170 and −190 bp; HMEM, minimum essential Eagle's medium with Hanks' balanced salt solution; 4HNE, 4-hydroxy-2-nonenal; HSC, hepatic stellate cells; *p*HMB, *p*-hydroxymercuribenzoate; P5P, pyridoxal 5′-phosphate; DMEM, Dulbecco's modified Eagle's medium.

Dawley rats as described originally by Friedman and Roll (19), with the minor modifications introduced by Rippe *et al.* (20). Cells were cultured at 37 °C in DMEM supplemented with 10% horse serum, 10% FBS, and 2% L-glutamine in a 95% air, 5% $CO_2$-humidified atmosphere. Growth medium was changed on a daily basis for the 1st week in culture and then every 3rd day thereafter. By using this procedure, we obtained an HSC population 95–99% pure. The effect of MDA was examined by addition of this agent to cells cultured in serum-free medium.

*Plasmids*—The p(−3700)ColCAT, p(−905)ColCAT, and p(−220)-ColCAT (17) constructs contain the *Xba*I-*Xba*I (nucleotides −3700 to +116), the *Pvu*II-*Xba*I (nucleotides −905to +116), or the *Bgl*II-*Xba*I (nucleotides −220 to +116) fragment, respectively, and the 5′-untranslated region of the first exon of the *col1a1* gene fused to the bacterial chloramphenicol acetyltransferase (CAT) gene. The vector plasmid pUCCAT, also containing the CAT gene, was obtained by digesting p(−3700)ColCAT (16) with *Xba*I, removing the *Xba*I-*Xba*I fragment, and ligating both ends. The reporter plasmid p(−111)ColCAT was constructed as described elsewhere (21). The reporter plasmid p(−126)Luc was constructed by subcloning the fragment −126 to +58 of the T-cell-specific *mal* gene promoter into the promoterless plasmid p19Luc. This plasmid contains two consensus sites for nuclear factor Sp1 (18). In the reporter plasmid pΔSLuc, Sp1 *cis*-acting elements of the p(−126)Luc had been deleted (18). The mutated plasmids p(−905ΔFP2)ColCAT and p(-905ΔFP2/ΔFP3)ColCAT were constructed using the technique of site-directed mutagenesis described by Ho *et al.* (22). The PCR products were inserted into the p(−905)ColCAT vector after removing the fragment *Pvu*II-*Xba*I. In the p(−905ΔFP2)ColCAT construct, the binding site for Sp1 and Sp3 extending from −129 to −110 bp had been converted from GGGGGCCGGGCC into GGG**AA**CC**T**GGCC. In addition to this mutation, in the p(−905ΔFP2/ΔFP3)ColCAT construct, the sequence between −161 and −133 bp, the binding site for Sp1 and Sp3, had been converted from CCCCCCTCTT to CC**AAA**CTCTT. Mobility shift assays confirmed that recombinant Sp1 does not bind oligonucleotides containing these mutated sequences (data not shown). All constructions and the orientation of the inserts were tested by restriction analysis and limited DNA sequencing by using an ABI PRISM 310 Genetic Analyzer (PerkinElmer Life Sciences).

*Northern Blotting*—HSC were incubated as described above in DMEM without FBS in the presence or absence of MDA and processed as described previously (21, 23). Rat complementary DNA for the α1 chain of type I collagen (1.6-kb *Pst*I fragment) was generously provided by Dr. D. Rowe (Farmington, CT). The *col1a1* cDNA probe was radiolabeled with [α-$^{32}$P]deoxycytidine triphosphate (specific activity, 3000 Ci/mmol) using a primer extension kit (Multiprime DNA labeling system; from Amersham Biosciences) to a specific activity of $1 \times 10^9$ cpm per mg of DNA.

*Preparation of Nuclear Extracts and Gel Retardation Assays*—Nuclear proteins from HSC untreated and treated with 200 μM MDA were extracted according to the method of Dignam *et al.* (24). The pellet was resuspended in 50 μl of Dignam C buffer, and protein concentration was determined by using the Bradford protein assay reagent (25). Gel retardation assays were performed as described by Nehls *et al.* (26). The oligonucleotides used in these assays are shown in Table I. For competition experiments, 200-fold excess of unlabeled oligonucleotide was added to binding reactions. Supershift assays were performed as a standard mobility shift assay, except that 1 μl of specific polyclonal antiserum raised against Sp1, Sp3, NF-1, BTEB, Zf9, AP2, βC/EBP, or c-Krox was added to the binding reaction for 1 h at 0 °C. Sp1 antibody is specific for Sp1 and does not cross-react with Sp3 or other members of the Sp family. Likewise, Sp3 antibody is specific for Sp3 protein and does not cross-react with Sp1.

*Transient Transfection and Chloramphenicol Acetyltransferase (CAT) Assay*—In general, $5 \times 10^6$ HSC were transiently transfected prior to confluence by the LipofectAMINE technique (27). Cell lysates were prepared, and CAT activity was determined as described elsewhere (28). Luciferase activity was determined using the enhanced luciferase assay kit according to the manufacturer's protocol (Analytical Luminescence, San Diego). Cell lysates were prepared in 125 μl of cell lysis buffer. Luciferase activity was determined using 50-μl aliquots, and protein concentrations were determined with 5-μg aliquots using the Bradford protein assay (Bio-Rad) (25). All reporter genes were normalized for transfection efficiency by cotransfecting a constant amount of *pRSVβ-Gal* reporter gene and determining β-galactosidase activity (29).

*Statistical Analysis*—All results are expressed as mean ± S.D. unless otherwise mentioned. Student's *t* test was used to evaluate the difference of means between groups, accepting a *p* value of $<0.05$ as the level of significance (30).

TABLE I
*Oligonucleotides used in gel retardation assays*

| Name | Sequence |
|---|---|
| FP1 | 5'-CTAGCTGATTGGCTGGGGGCCGGGCT-3'<br>3'-GACTAACCGACCCCCGGCCCGACTAG-5' |
| FP2 | 5'-AGCTTCCAAATTGGGGGCCGGGCCAG-3'<br>3'-AGGTTTAACCCCCGGCCCGGTCCTAG-5' |
| FP3 | 5'-CCTTCCTTTCCCTCCTCCCCCCTCTTCG-3'<br>3'-GGAAGGAAAGGGAGGAGGGGGGAGAAGC-5' |
| FP4 | 5'-TTGCGGGAGGGGGGGCGCGCTGGGTGGAC<br>3'-AACGCCCTCCCCCCCGCGCGACCCACCTG-5' |
| Sp1 | 5'-GGATTCGATCGGGGCGGGGCGAGC-3'<br>3'-TAAGCTAGCCCCGCCCCGCTCGTT-5' |
| NF-1 | 5'-GTTTTGGATTGAAGCCAATATGAG-3'<br>3'-AAAACCTAACTTCGGTTATACTCG-5' |

## RESULTS

*Malondialdehyde Induces col1a1 Gene Expression in Primary Culture of HSC*—Treatment of cells with MDA led to a significant increase in the steady-state level of *col1a1* mRNA without change in the 18 S RNA. This effect was time- and dose-dependent. Northern blots showed that *col1a1* mRNA levels in HSC incubated in HMEM without FBS were increased 1.5-, 2-, 2.3-, 2.8-, and 3.3-fold over the control level after incubation with 25, 50, 100, 200, and 300 μM MDA, respectively, for 24 h (Fig. 1*A*). Likewise, *col1a1* mRNA levels increased 1.1-, 1.0-, 1.8-, 2.2-, and 2.6-fold over the control level in cells treated with 200 μM MDA for 1, 2, 6, 16, and 24 h, respectively (Fig. 1*B*). This effect of MDA was prevented by pretreating cells with 0.1 mM cycloheximide (Fig. 2*A*) and with 4 μM *p*HMB or 10 mM P5P, two inhibitors of the aldehyde-protein adduct formation (Fig. 2*B*). None of these agents was toxic to the HSC at these concentrations.

*MDA Induces the Binding of trans-Acting Factors Sp1 and Sp3 to FP2 (−129 to −110 bp) and FP3 (−161 to −133 bp) of the col1a1 Promoter*—Addition of 200 μM MDA to confluent HSC transiently transfected with a reporter-CAT plasmid driven by the whole *col1a1* promoter resulted in a 3.4 ± 0.1-fold increase in the CAT activity (Fig. 3*A*). This effect was totally abrogated by pretreating HSC with 4 μM *p*HMB. Deletion of promoter sequences upstream of −220 bp relative to the transcription start site increased the basal CAT activity 4.4-fold (Fig. 3*E*) but did not abrogate the stimulatory effect of MDA on *col1a1* gene expression (Fig. 3*B*). By contrast, HSC transfected with the construct p(−111)Col1a1, lacking sequences upstream of −110 bp, did not respond to MDA (Fig. 3*C*), suggesting that the MDA-responsive element is located in sequences between −220 and −111 bp relative to the transcription start site of the *col1a1* promoter.

Because the 220 bp upstream of the transcription start point in the *col1a1* promoter contains four regions protected from DNase I digestion by nuclear proteins from a number of cell types (16, 17, 26, 31), including HSC (20, 32), we studied whether treatment of HSC with 200 μM MDA induced any change in the binding of nuclear proteins to these elements. Gel retardation experiments showed that incubation of a 5′-end-labeled oligonucleotide containing sequences between −103 and −82 bp (FP1) with nuclear protein extracts from either untreated cells or cells treated with 200 μM MDA for 1–24 h led to the formation of two major specific DNA-protein complexes (Fig. 4). However, few changes in the pattern or intensity of the retarded bands were observed whether the nuclear protein

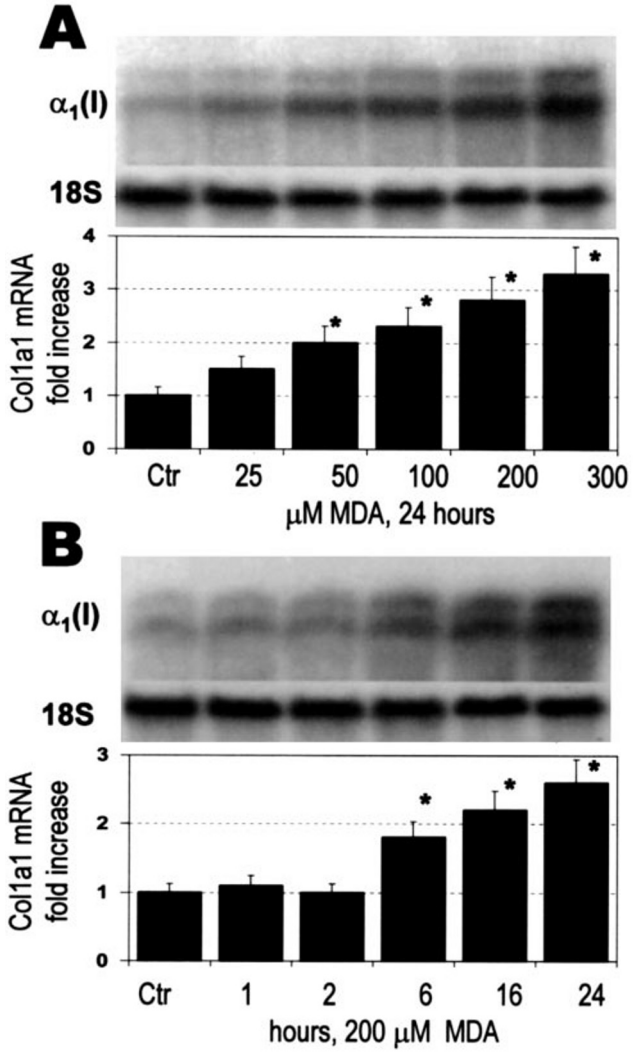


FIG. 1. **Effect of MDA on the expression of *col1a1* mRNA in primary culture of HSC.** *A*, dose-response effect of MDA on *col1a1* mRNA. Confluent HSCs were incubated for 24 h in the absence (*Ctr*) or presence of 25–300 $\mu$M MDA. *B*, time-response effect of MDA on *col1a1* mRNA. Confluent HSC cultured in DMEM without fetal calf serum were incubated with 200 $\mu$M MDA for 1–24 h. Total *col1a1* RNA was electrophoresed, transferred to a nylon filter, and hybridized as described under "Experimental Procedures." *Histograms* represent relative *col1a1* mRNA expression with control assigned a value of 1 and refer to mean ± S.E. The level of *col1a1* mRNA ($\alpha_I(I)$) in each sample was normalized to the level of 18 S RNA (*18 S*). These blots are representative of three separate experiments. *, $p$ less than 0.05.

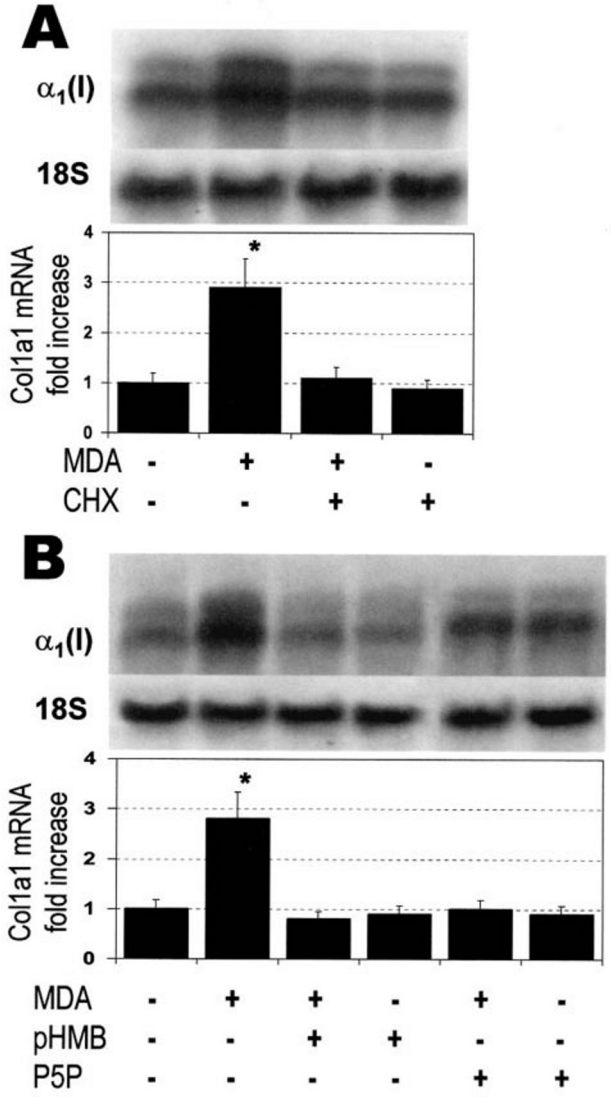


FIG. 2. **Effects of cycloheximide, *p*-hydroxymercuribenzoate, and pyridoxal 5′-phosphate on MDA-activated *col1a1* gene expression.** *A*, confluent cells were incubated with 100 $\mu$M cycloheximide (*CHX*) for 1 h prior to the addition of 200 $\mu$M MDA. Treatment was maintained for another 24 h. *B*, cells were treated with 4 $\mu$M *p*HMB for 1 h. After this time, cells were washed three times with phosphate-buffered saline and treated with 200 $\mu$M MDA for another 23 h. Pyridoxal 5′-phosphate (10 mM) and MDA (200 $\mu$M) were added to the cells at the same time. This treatment was maintained for 24 h. *Histograms* represent relative *col1a1* mRNA expression with control assigned a value of 1 and refer to mean ± S.E. The level of *col1a1* mRNA ($\alpha_I(I)$) in each sample was normalized to the level of 18 S RNA (*18S*). These blots are representative of three separate experiments. *, $p$ less than 0.05.

used in this assay was extracted from control or MDA-treated cells. A 200-fold molar excess of unlabeled Sp1 oligonucleotide abrogated the formation of the upper complex, whereas the same excess of unlabeled NF1 oligonucleotide failed to compete significantly with nuclear proteins for the binding to the FP1 probe (Fig. 4). These results suggest that treatment of HSC with the MDA does not influence the interaction of nuclear proteins with FP1.

To examine the effect of the treatment of cells with MDA on the interaction of nuclear proteins with the element extending from −129 bp to −110 bp (FP2) of the *col1a1* promoter, we performed DNA mobility assays using as a radiolabeled probe the FP2 oligonucleotide. Incubation of this probe with nuclear protein extracts from either untreated or treated cells with 200 $\mu$M MDA for 1–24 h also led to the formation of two specific DNA-protein complexes. However, the intensity of these bands was clearly more pronounced when nuclear extract was prepared from cells treated with MDA for 6 or more hours (Fig. 5*A*). The FP2 contains a reverse CCAAT motif and a 12-bp G + C-rich direct repeat (26). Because it has been shown that the *trans*-acting factors NF-1 and Sp1 interact with this footprinted region in NIH 3T3 fibroblasts (26), we performed competition assays with 200-fold molar excess of unlabeled oligonucleotides with high affinity for either NF-1 or Sp1. As Fig. 5*A* demonstrates, the excess of NF-1 unlabeled oligonucleotide did not compete significantly with the formation of any of these bands, whereas the 200-fold molar excess of Sp1 oligonucleotide totally competed with the formation of the upper DNA-protein complex and partially with the lower one. To confirm that Sp1 is involved in the formation of the FP2-protein complexes, we performed supershift assays by including in the binding reaction an Sp1-specific polyclonal antibody. This in-

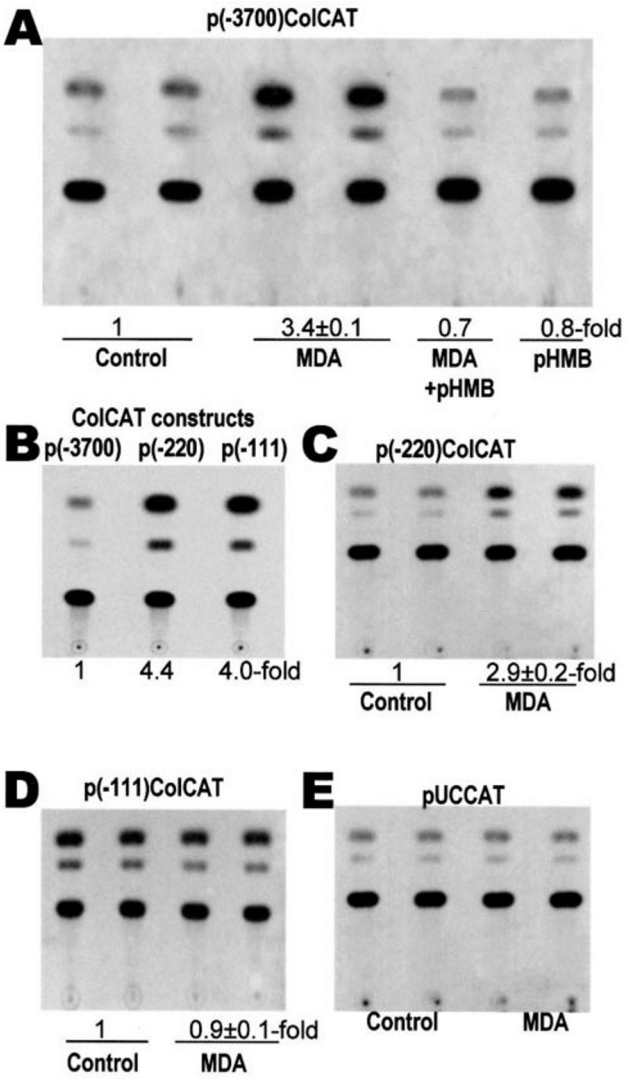


FIG. 3. **Deletion analysis of the *col1a1* promoter.** HSC were transiently cotransfected by the LipofectAMINE technique with pRSV$\beta$-Gal plasmid and ColCAT constructs obtained by removing progressively more 5′-flanking sequences of the *col1a1* promoter. Two hundred micromolar MDA was added to confluent cells after 12 h of starvation, and this treatment was maintained for another 24 h. *p*HMB (4 $\mu$M) was added to the cells as described in Fig. 1. Cell lysates were assayed for CAT and $\beta$-galactosidase activities as described under "Experimental Procedures." Autoradiograms were quantitated by scanning spectrophotometry. Individual spots were localized and excised, and their $^{14}$C-labeled content was measured by liquid scintillation spectrophotometry. Panels show the effect of MDA on the CAT activity of the lysate from cells transfected with p(−3700)ColCAT (*A*), p(−220)ColCAT (*C*), p(−111)ColCAT (*D*), or pUCCAT (*E*). *B* shows basal CAT activity of the three ColCAT fusion gene constructs transfected into HSC. The p(−3700)ColCAT contained *col1a1* promoter fragment *Xba*I-*Xba*I (nucleotides −3700 to +116) and p(−220)ColCAT, the fragment *Bgl*II-*Xba*I (nucleotides −220 to +116). The p(−111)ColCAT, containing the fragment between −111 and +116 of the *col1a1* gene, was constructed as described elsewhere (21). The vector plasmid pUCCAT was obtained by removing the *Xba*I-*Xba*I fragment from p(−3700)ColCAT. Figure shows representative results of three independent experiments that were performed in duplicate. The CAT activity (normalized to $\beta$-galactosidase activity) in MDA-treated cells is expressed relative to the CAT activity in untreated cells.

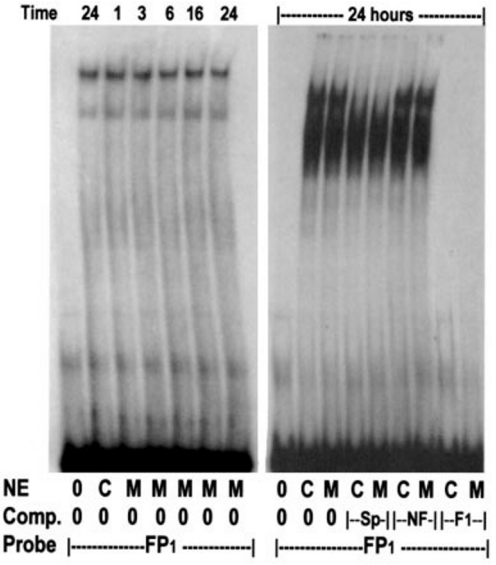


FIG. 4. **Effects of treatment of HSC with MDA on the binding of nuclear factors to FP1.** DNA binding was analyzed by gel retardation assays as described under "Experimental Procedures." Double-stranded oligonucleotide corresponding to the FP1 (−103 to −82 bp) of the *col1a1* promoter was radiolabeled and incubated with 5 $\mu$g of nuclear protein extracts from confluent untreated (*C*) or treated cells with 200 $\mu$M MDA (*M*) for 1–24 h. A 200-fold molar excess of unlabeled Sp1 (*Sp*), NF-1 (*NF*), FP1 (*F1*) oligonucleotide was used as competitors (*Comp.*) in a competition assay. *NE*, nuclear extract.

cubation led to the formation of a supershifted complex and to a marked decrease in the intensity of the upper band (Fig. 5*B*). In an attempt to identify other nuclear factors bound to FP2, supershift assays using polyclonal antisera against Sp3, BTEB, NF1, Zf9, AP2, C/EBP$\beta$, or c-Krox were also performed. Whereas BTEB, NF1, Zf9, AP2, C/EBP$\beta$, and c-Krox antibodies failed to supershift any band, Sp3 antiserum supershifted almost completely the lower band (Fig. 5*B*). In addition, incubation of nuclear extracts and FP2 probe with Sp1 and Sp3 antibodies simultaneously supershifted both bands.

Gel retardation experiments were performed to analyze the effect of MDA treatment on the binding of nuclear proteins to the remaining two DNase I-protected regions. Incubation of nuclear extracts from confluent HSC with a radiolabeled FP3 oligonucleotide (−161 to −133 bp) led to the formation of two major DNA-protein complexes, and the intensity increased markedly with nuclear extracts from cells treated with 200 $\mu$M MDA for 6–24 h (Fig. 6*A*). Competition with a 200-fold molar excess of unlabeled FP3 oligonucleotide demonstrated the specificity of these complexes. To determine the identity of the *trans*-acting factors involved in the formation of these DNA-protein complexes, competition and supershift assays were performed. Whereas a 200-fold molar excess of unlabeled consensus Sp1 oligonucleotide competed totally the formation of these complexes, the same molar excess of unlabeled NF-1 oligonucleotide failed to compete the formation of any of these complexes. Supershift assay using specific Sp1 antibody resulted in the formation of a supershifted band and in a decrease in the intensity of the upper band (Fig. 6*B*). Likewise, inclusion of specific Sp3 antibody in the binding reaction led to the complete disappearance of the lower complex and the formation of a supershifted band. Finally, incubation of nuclear extracts and FP3 probe with both antisera, anti-Sp1 and anti-Sp3, supershifted almost completely the two complexes. Inclusion of antisera against BTEB, NF-1, Zf9, or AP2 transcription factors in the binding reaction failed to supershift any band. Supershift assay using c-Krox antiserum led to the formation of a supershifted band. However, the intensity of this band decreased after incubation of cells with MDA (Fig. 6*B*). Western blot using specific polyclonal c-Krox antibody showed that MDA treatment did not induce any significant change in c-Krox protein content in HSC (Fig. 7*A*).

By contrast with the results obtained with FP2 and FP3 oligonucleotides, gel retardation experiments with FP4 oligo-

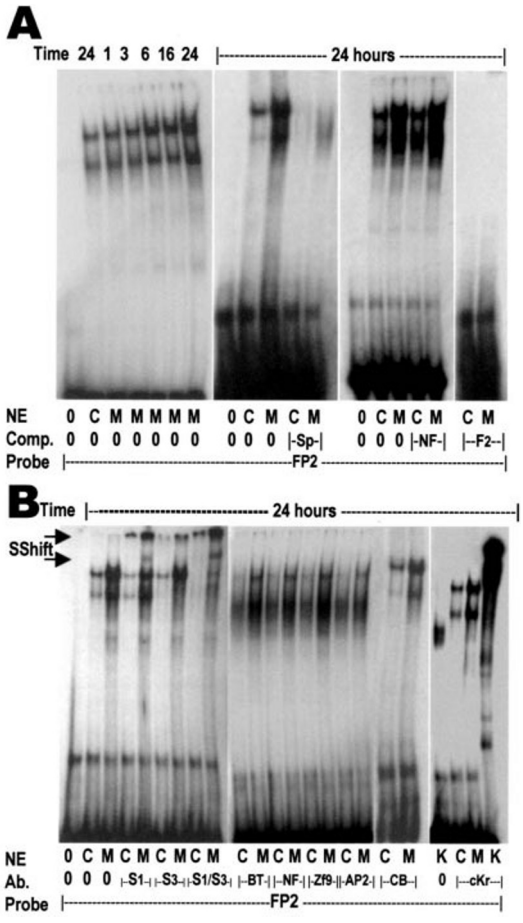


FIG. 5. **Treatment of HSC with MDA increases the binding of Sp1 and Sp3 to FP2.** DNA binding was analyzed by gel retardation assays as described under "Experimental Procedures." *A*, double-stranded oligonucleotide corresponding to the FP2 (−129 to −110 bp) was radiolabeled and incubated with 5 $\mu$g of nuclear protein extracts from confluent untreated (*C*) or treated cells with 200 $\mu$M MDA (*M*) for 1–24 h. A 200-fold molar excess of unlabeled Sp1 (*Sp*), NF-1 (*NF*), or FP2 (*F2*) oligonucleotide was used as competitors (*Comp.*) in a competition assay. *NE,* nuclear extract. *B*, 1 $\mu$l of specific antibodies against Sp1 (*S1*), Sp3 (*S3*), BTEB (*BT*), NF1 (*NF*), Zf9, AP2, C/EBP$\beta$ (*CB*), or c-Krox (*cKr*) proteins was included in the binding reaction. c-Krox recombinant protein was also added to the assay as control of mobility (*lanes K*). *NE*, nuclear extract; *Ab*, antibody; *Sshift*, supershifted proteins.

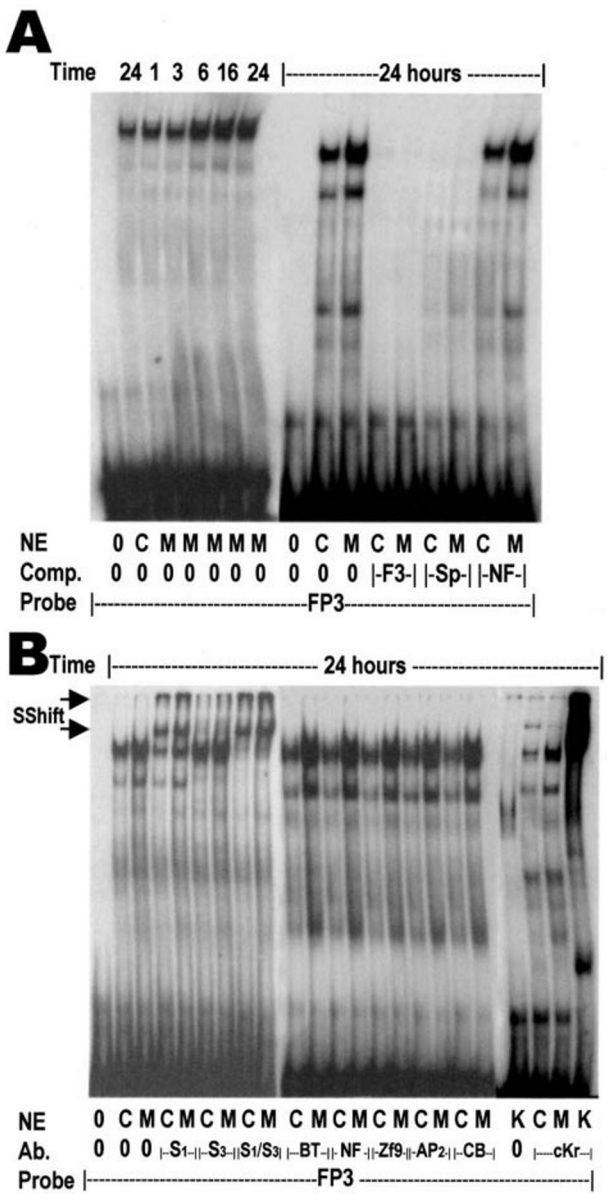


FIG. 6. **Treatment of HSC with MDA increases the binding of Sp1 and Sp3 to FP3.** DNA binding was analyzed by gel retardation assays as described under "Experimental Procedures." *A*, double-stranded oligonucleotide corresponding to the FP3 (−161 to −133 bp) was radiolabeled and incubated with 5 $\mu$g of nuclear protein extracts from confluent untreated (*C*) or treated cells with 200 $\mu$M MDA (*M*) for 1–24 h. A 200-fold molar excess of unlabeled FP3 (*F3*), Sp1 (*Sp*), or NF-1 (*NF*) oligonucleotide was used as competitors (*Comp.*) in a competition assay. *B*, 1 $\mu$l of specific antibodies against Sp1 (*S1*), Sp3 (*S3*), BTEB (*BT*), NF-1 (*NF*), ZfB, AP2, C/EBP$\beta$ (*BP*), or c-Krox proteins was included in the binding reaction. c-Krox recombinant protein was added to the assay as control of mobility (*lanes K*). *NE*, nuclear extract; *Ab*, antibody; *Sshift*, supershifted proteins.

nucleotide containing sequence spanning nucleotides −190 to −170 of the *col1a1* promoter as a labeled probe also demonstrated two major complexes formed with nuclear extract from HSC. However, the pattern and the intensity of these complexes were not modified by the treatment of cells with 200 $\mu$M MDA for 1–24 h (Fig. 7*B*).

To assess the role of the FP2 and FP3 in the stimulation of *col1a1* gene expression, we transfected HSC with plasmids containing mutations either at FP2 [p(−905ΔFP2)ColCAT] or at FP2 and FP3 [p(−905ΔFP2/ΔFP3)ColCAT] or no mutations [p(−905)ColCAT]. As Fig. 8*A* shows, the stimulatory effect of MDA on p(−905)ColCAT expression disappeared in cells transfected with mutant constructs in which the Sp1/Sp3-binding sites in FP2 have been converted from GGGGGCCGGG into GGG**AA**CC**T**GG and the Sp1/Sp3-binding sites in FP3 from CCCCCCTCTT into CC**AAA**CTCTT. To determine whether the binding of Sp1 and Sp3 to the *col1a1* promoter is involved in the mediation of the MDA-induced expression of the *col1a1* gene, we pretreated HSC with 0.1 $\mu$M mithramycin for 2 h, a drug that interferes with the binding of transcription factors to G + C-rich promoters (33). These experiments confirmed not only that mithramycin reduced the binding of nuclear proteins to FP2 and FP3 oligonucleotides (Fig. 8*B*), but also that this drug decreased the basal *col1a1* mRNA levels and abrogated the effect of MDA on *col1a1* gene expression (Fig. 8*C*). This drug was not toxic to HSC at the used concentration.

Because these data suggested that the stimulatory effect of MDA was mediated through the binding of Sp proteins to the *col1a1* promoter, we wanted to determine whether this effect of MDA could be reproduced in other genes containing Sp1-binding sites. Therefore, we transiently transfected HSC using either pΔSLuc or p(−126)Luc reporter plasmids. Whereas addition of 200 $\mu$M MDA to HSC transfected with pΔSLuc, a deletion construct lacking Sp1-binding site, did not change

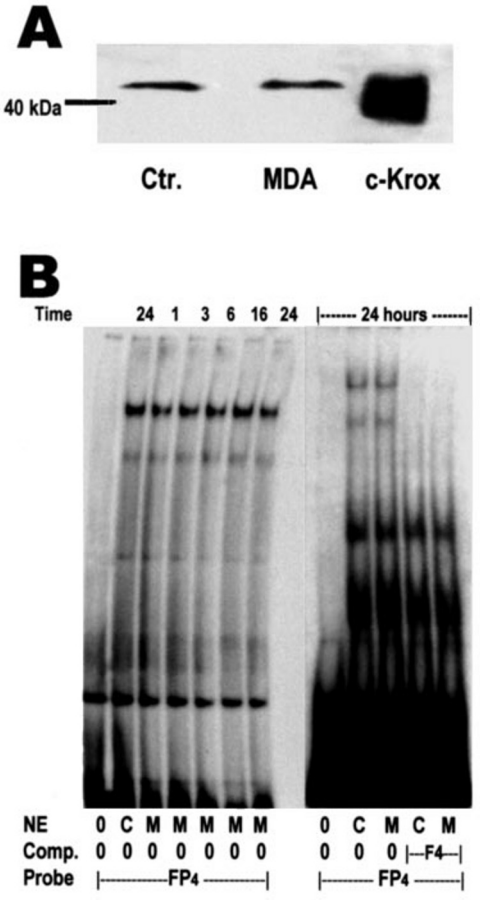


FIG. 7. **Treatment of HSC with MDA neither affects the cellular content in c-Krox protein nor the binding of nuclear factors to FP4.** *A*, 25 $\mu$g of nuclear protein extracts from cells incubated in the absence (*Ctr*) or presence of 200 $\mu$M MDA for 24 h (*MDA*) and 0.45 $\mu$g of recombinant c-Krox protein (*c-Krox*) were separated by 8% SDS-PAGE and transferred to a membrane for immunoblot analysis as described under "Experimental Procedures." Ponceau S staining was used to confirm equal loading. Immunoblot was then probed with specific antibodies against c-Krox protein and detected by enhanced chemiluminescence. *B*, double-stranded oligonucleotide corresponding to the FP4 (−190 to −170 bp) of the *col1a1* promoter was radiolabeled and incubated with 5 $\mu$g of nuclear protein extracts from untreated (*C*) or treated cells with 200 $\mu$M MDA (*M*) for 1–24 h. A 200-fold molar excess of unlabeled FP4 (*F4*) oligonucleotide was used as competitor (*Comp.*) in a competition assay. *NE*, nuclear extract.

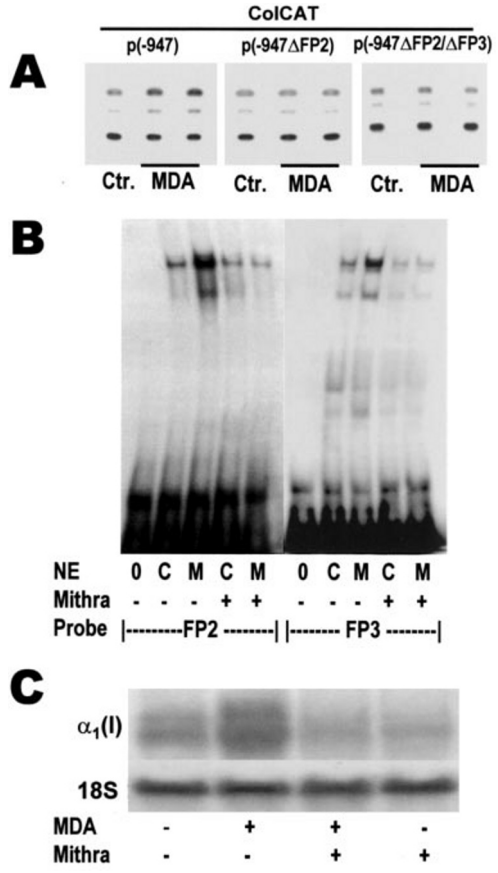


FIG. 8. **Effects of MDA on constructs with mutated Sp1/Sp3 site in FP2 or FP2 and FP3 regions, and effect of mithramycin on the MAD-induced FP2 and FP3 binding and on the increased *col1a1* mRNA.** *A*, HSC were transiently cotransfected by the LipofectAMINE technique with pRSV$\beta$-Gal plasmid and ColCAT constructs containing mutated Sp1/Sp3 site in FP2 [p(−905ΔFP2)ColCAT] or mutated Sp1/Sp3 sites in FP2 and FP3 [p(−905ΔFP2/ΔFP3)ColCAT] regions or no mutations [p(−905)ColCAT]. MDA (200 $\mu$M) was added to the transfected cells as described in Fig. 1. Cells lysates were assayed for CAT and $\beta$-galactosidase activities as described under "Experimental Procedures." *B*, DNA binding was analyzed by gel retardation assays. Radiolabeled FP2 and FP3 oligonucleotides were used as a probe. The HSC were pretreated with 0.1 $\mu$M mithramycin (*Mithra.*) for 2 h and incubated for 24 h with (*M*) or without (*C*) 200 $\mu$M MDA. *NE*, nuclear extract. *C*, the HSC were treated with 0.1 $\mu$M mithramycin for 2 h. Afterward, cells were incubated with 200 $\mu$M MDA for 24 h. Total RNA was isolated and analyzed by Northern blotting as described under "Experimental Procedures." The blot was hybridized with $^{32}$P-labeled probe specific for *col1a1* mRNA ($\alpha_1(I)$) and 18 S RNA (*18S*), which served as a control for sample loading. Autoradiograms were quantitated by scanning laser densitometry. The level of mRNA was normalized to the level of 18 S RNA. Blot is representative of three separate experiments.

significantly the expression of this gene, addition of the same amount of MDA to HSC transfected with p(−126)Luc, a plasmid containing two consensus Sp1-binding elements, resulted in a 3.7-fold increase in gene expression (Fig. 9).

*MDA Increases Sp1 Gene Expression and Immunoreactive Sp1 and Sp3 Proteins in Primary Culture of HSC*—To determine whether the increased binding of Sp1 and Sp3 proteins to the *col1a1* promoter induced by the MDA treatment was due to a change in the amount of these proteins in the cell extract, Western blot assays were performed. These assays showed that treatment of cells with 200 $\mu$M MDA increased 2- and 3-fold the cell content in Sp3 and Sp1 proteins, respectively (Fig. 10*A*). Moreover, treatment of cells with 200 $\mu$M MDA increased the steady-state levels of *Sp1* mRNA 4.8-fold. The maximal expression was reached by 3 h, but declined afterward (Fig. 10*B*).

## DISCUSSION

In the present study, we demonstrate that MDA stimulates significantly *col1a1* gene expression in primary culture of confluent HSC in a dose- and time-dependent manner (Fig. 1). These results concur with those reported by Tsukamoto and co-workers (5, 6) in HSC and by Chojkier *et al.* (1) and Maher *et al.* (2) in human and rat fibroblasts, respectively. On the contrary, Maher *et al.* (2) found that HSC displayed only a modest increase in collagen synthesis in response to MDA or even failed to produce collagen in response to oxidants or their by-products (34). The reasons for this discrepancy are unknown, although differences in the glutathione levels or conditions of HSC culture may have an impact in the response to MDA (34). On the other hand, a number of authors have shown that 4HNE also stimulates the *col1a1* gene in HSC (3–6). Moreover, in a previous study (21), we showed that incubation of HSC with a pro-oxidant combination of iron, ascorbic acid, and citric acid induced a time- and dose-dependent stimulation of the *col1a1* gene expression.

A large number of studies have suggested that aldehyde-

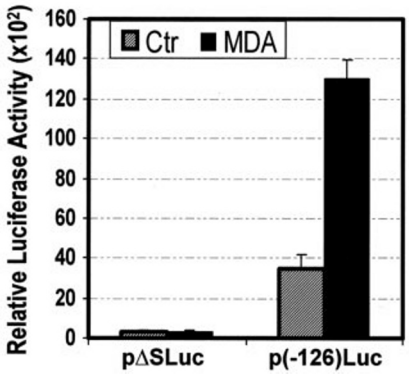


FIG. 9. **MDA treatment increases the expression of genes with Sp1-binding elements.** HSC were transiently cotransfected as indicated under "Experimental Procedures" with 0.5 $\mu$g of pRSV-$\beta$-galactosidase plasmid, 1.1 $\mu$g of pUC19, and either 0.5 $\mu$g of p(−126)Luc, containing two Sp1-binding sites, or 0.5 $\mu$g of pΔSLuc, containing no Sp1-binding site, and cultured without fetal bovine serum in the presence (*MDA*) or absence (*Ctr*) of 200 $\mu$M MDA for 24 h. After the indicated times, luciferase and $\beta$-galactosidase activities were measured. Luciferase values were normalized for differences in transfection efficiencies. Values are given as relative luciferase activity unit. The results presented are representative of three separated experiments.

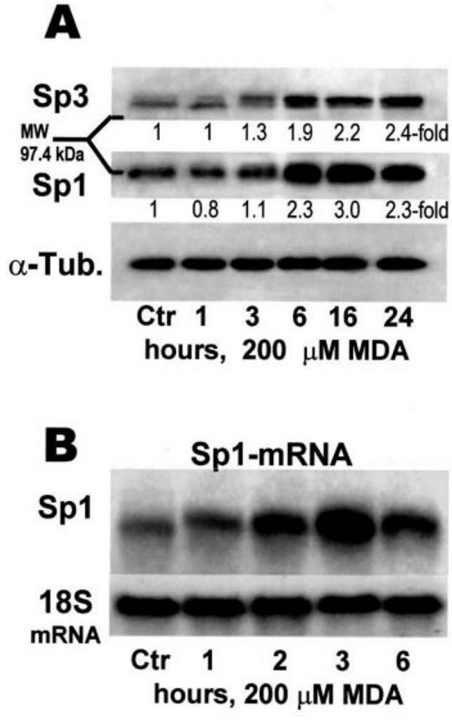


FIG. 10. **Treatment of HSC with MDA increases the intracellular levels of Sp1 and Sp3 proteins and the expression of the *Sp1* gene.** *A*, 25 $\mu$g of whole-cell protein extracts from cells incubated in the absence or presence of 200 $\mu$M MDA for 1–24 h were separated by 8% SDS-PAGE and transferred to a membrane for immunoblot analysis as described under "Experimental Procedures" and Fig. 7. Ponceau S staining was used to confirm equal loading. Immunoblot was then probed with specific antibodies against Sp1, Sp3, or $\alpha$-tubulin ($\alpha$-*Tub.*) and detected by enhanced chemiluminescence. *Ctr*, control cells. *B*, the HSC were treated with 200 $\mu$M MDA for 1–6 h. Total RNA was isolated, analyzed by Northern blotting, and hybridized with specific radiolabeled Sp1 and 18 S probes. *Ctr*, mRNA in untreated cells maintained in culture medium for 24 h.

protein adducts may play a role in the regulation of collagen gene expression and in the pathogenesis of liver fibrosis (1, 6, 12, 14, 15). Our study adds new evidence supporting the role of these adducts in the mediation of these effects, because pretreatment of HSC with *p*HMB or P5P abolished the effect of MDA on *col1a1* mRNA levels (Fig. 2*B*). Likewise, the activating effect of the pro-oxidant combination of iron with ascorbic and citric acids on *col1a1* gene expression was abrogated by pretreating HSC with *p*HMB or P5P (21). These two agents have many biological activities but share a common inhibitory effect on aldehyde-protein adduct formation (35, 36).

We also show that MDA activated *col1a1* gene expression acting on DNA elements located in the 200-bp segment upstream of the transcription start site (Fig. 3). This segment contains four sites protected from DNase I digestion by nuclear proteins from HSC and other cell lines, named footprinting 1 (FP1) to footprinting 4 (FP4) (16, 17, 20, 26, 32, 37). Mobility shift assays suggested that MDA activates *col1a1* gene expression by acting on the footprinted regions, FP2 and FP3 (Fig. 5 and 6), located between nucleotides −110 and −220 bp of the *col1a1* promoter.

The FP2 contains a G + C-rich 12-bp direct repeat and a reverse CCAAT motif that have been shown to be a binding site for transcription factors Sp1 and NF-1 in a variety of cell extracts (16, 26). Supershift and competition assays demonstrated that the increased binding activity of nuclear proteins extracted from MDA-treated cells may be ascribed to Sp1 and Sp3 (Fig. 5). On the contrary, these assays also showed that NF-1, C/EBP$\beta$, two CCAAT box-binding proteins, Zf9, BTEB, AP2, or c-Krox, four transcription factors binding to G + C-rich regions are not involved in the formation of any FP2-protein complex in HSC.

Our study also shows that MDA increased significantly the interaction of nuclear proteins from HSC with a radiolabeled probe containing FP3 sequence (nucleotide −165 to −133) (Fig. 6). This footprinted region contains two consecutive CCCTCC sequences that are considered consensus sequences for the transcription factor c-Krox (38). Although our study confirms an interaction between c-Krox and FP3, we showed that MDA decreases this binding without modifying the cellular content of c-Krox (Figs. 6*B* and 7*A*). Because this factor is considered an inhibitor of collagen gene transcription (37, 39, 40), this reduced DNA binding may contribute to enhance *col1a1* gene expression. On the other hand, supershift assays showed that Sp1 and Sp3 are the main binding proteins responsible for the formation of the two major FP3-protein complexes in HSC (Fig. 6*B*). This interaction of Sp1 with FP3 has also been reported by other authors (41, 42). Although the CCCTCC sequence is an unusual Sp1-binding site, it has also been found in other gene promoters (43, 44). Moreover, Ihn and Tamaki (45) reported that the affinity of Sp1 or Sp3 for the TCCTCC motif found in FP3 was greater that that for the Sp1 consensus sequence.

The MDA-induced binding of Sp1 and Sp3 to FP2 and FP3 seems to be involved in the stimulation of the *col1a1* gene expression as suggested by the abrogation of the effect of MDA on the expression of this gene by mithramycin (Fig. 8). This drug inhibits the binding of transcription factors, including Sp1 and Sp3, to genes containing G + C-rich promoters (33). These effects of MDA were also confirmed on a different gene driven by two Sp1-binding sites (Fig. 9). The role played by the binding of Sp proteins to FP3 and mainly to FP2 in the mediation of the effects of MDA on *col1a1* gene expression was also supported by mutation analysis at the Sp1/Sp3 sites in FP2 and FP3 (Fig. 8*A*).

Transcriptional control of *col1a1* gene expression may be exerted either by varying levels of Sp proteins or by posttranslational modifications of these proteins (46). Our study clearly demonstrates that MDA increases *Sp1* gene expression and Sp1 and Sp3 protein levels (Fig. 10), which might be responsible for both the increased FP2 and FP3 binding activity and the enhanced *col1a1* gene expression observed in MDA-treated

cells. Sp1 and Sp3, like other members of the Sp family, recognize the G + C-rich repeats (47) and act as positive transcription factors on a wide variety of cellular genes (48), including collagen genes (49–51).

The results of the present study led us to consider whether this mechanism of action is specific for MDA or, on the contrary, whether it is shared by other stimulating factors of collagen gene expression such as transforming growth factor-$\beta$1, acetaldehyde, or iron salts (1, 21, 52, 53). The molecular mechanisms by which transforming growth factor-$\beta$1 and acetaldehyde increase *col1a1* gene expression are controversial, but in any case, they seem to be different from those used by MDA (54–57). On the contrary, the results of our study paralleled those obtained when HSCs were exposed to an iron-containing solution (21), which led to the binding of transcription factors Sp1 and Sp3 to the FP2 and FP3 of the *col1a1* promoter and to the activation of this gene.

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