Tissue inhibitor of metalloproteinase 1 (TIMP1) is a contributory factor to fibrosis of a variety of organs including hepatic stellate cells (HSC), the key pro-fibrogenic cells of the liver. In this study we identify RUNX1 and RUNX2 as UTE-1-binding proteins that are induced at the post-transcriptional level during activation of HSC. RUNX1 is expressed in at least two major isoforms, RUNX1B and RUNX1A. Overexpression of full-length RUNX1B isoform in HSC repressed TIMP1 promoter activity, whereas the truncated RUNX1A isoform and RUNX2 functioned as stimulators. To gain further understanding of the way in which RUNX1 isoforms differentially regulate TIMP1 transcription, we investigated the relationship between the UTE-1 site and the adjacent extracellular matrix-responsive element (SRE) in the promoter. The UTE-1 and SRE sites cooperate in a synergistic fashion to stimulate transcription of a heterologous minimal active promoter providing that they are in close proximity. The key regulatory sequence within the SRE is an AP-1 site that in HSC directs high level transcription via its interaction with JunD. RUNX1A was shown to interact directly with JunD, and by contrast RUNX1B failed to interact with JunD. Co-expression studies showed that RUNX1B can repress JunD-stimulated TIMP1 promoter activity. From these observations we propose that JunD and RUNX factors interact at the adjacent SRE and UTE-1 sites in the TIMP1 promoter and form functional interactions that stimulate transcription. However, RUNX1B is unable to interact with JunD, and as such its occupancy at the UTE-1 site disrupts the optimal assembly of transcriptional activators required for directing high level TIMP1 promoter function.

Turnover of extracellular matrix (ECM) is an integral process in embryonic development, morphogenesis, and tissue re-modeling. The maintenance of normal tissue structure is in part dependent on the balance between the expression and activity of the ECM-degrading matrix metalloproteinases (MMPs) and the expression of their naturally occurring tissue inhibitors (TIMPs) (1). Disruption of this finely tuned balance may result in uncontrolled restructuring of the ECM and as a consequence to the disruption of normal tissue architecture and function. Diseases associated with changes in the ratio of the expression/activity of TIMPs and MMPs include arthritis (1), fibrosis (2), tissue ulceration (3), cancer (4, 5), cardiovascular diseases (6), neurological disorders (7), and asthma (8).

The mammalian TIMPs are a family of four (TIMP1, TIMP2, TIMP3, and TIMP4) proteinase inhibitors that suppress ECM degradation by forming an inhibitory 1:1 complex with the MMPs (9). TIMP1 is the prototypic and original ancestral member of the mammalian TIMP family. In addition to acting as an inhibitor of the activity of most of the known MMPs, TIMP1 has several other important biological functions. The ability of TIMP1 to promote cell proliferation has been described for a wide range of cell types, and it is a property that is independent of its MMP-inhibitory activity but may instead result from interaction of TIMP1 with an as yet uncharacterized cell-surface receptor (10). Additionally, studies in a number of cell types including B-cells, human breast epithelial cells, rat kidney mesangial cells, and human and rat hepatic stellate cells (HSC) have revealed an anti-apoptotic function for TIMP1 (11–14).

TIMP1 is a key regulator of fibrogenic events in the liver. This is supported by the finding that the extent of carbon tetrachloride-induced liver fibrosis is elevated in mice that overexpress TIMP1 from a liver-specific TIMP1 transgene (15). The major cellular source of TIMP1 in the injured liver is the activated HSC (aHSC) (16). HSC are found in the normal liver in a quiescent state in which their major function appears to be the storage of vitamin A. After injury the HSC activates or overexpress TIMP1 from a liver-specific TIMP1 transgene (15).

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* The abbreviations used are: ECM, extracellular matrix; TIMP, tissue inhibitor of metalloproteinase; SRE, upstream serum response element; HSC, hepatic stellate cells; aHSC, activated HSC; MMPs, matrix metalloproteinases; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; RT, reverse transcription; CAT, chloramphenicol acetyltransferase; MOPS, 4-morpholinepropanesulfonic acid.
We have reported previously (17) that TIMP1 promoter activity is induced during the culture-induced activation of freshly isolated HSC. The TIMP1 promoter is structurally conserved between rodents and humans and contains functional binding sites for AP-1 (Fos/Jun), Pea3 (Ets), and STATs located within a 22-bp serum-response element (SRE) and motifs for transcriptional activity via the UTE-1 site. A non-canonical TIMP1 promoter is located at the 5' end of the minimal active TIMP1 promoter (−102 to +60) is essential for high level transcriptional activity in aHSC and operates by binding JunD which is the predominant Jun family protein in these cells (17, 18). Mutagenesis studies on the human TIMP1 promoter have also functionally implicated a second, but so far poorly defined, regulatory motif called the upstream TIMP1 element 1 (UTE-1) (19). The UTE-1 motif (−5′-TGAATGTA-3′) is located at −62 to −52, which is 10 bp downstream of the SRE. The UTE-1 element is required for high level promoter activity in a wide variety of cell types, including aHSC, and was shown to bind a 30-kDa nuclear protein. The main goal of the present study was to identify transcription factors that regulate TIMP1 promoter activity via the UTE-1 site.

By using a yeast one-hybrid approach, we have identified members of the RUNX transcription factor family as UTE-1-binding proteins and for the first time as regulators of TIMP1 gene transcription. The regulation and role of RUNX1 and -2 as controllers of TIMP1 expression in HSC is described.

Table I

| Oligonucleotides used for construction of promoter constructs |
|---------------------------------------------------------------|
| UTE-1 probe                                                  |
| Upper                                                        |
| Lower                                                        |
| C/EBP probe                                                  |
| Upper                                                        |
| Lower                                                        |
| pSU                                                          |
| Upper                                                        |
| Lower                                                        |
| pSUmU                                                        |
| Upper                                                        |
| Lower                                                        |
| pUS                                                          |
| Upper                                                        |
| Lower                                                        |
| pSUdU                                                        |
| Upper                                                        |
| Lower                                                        |
| 1xUTE-1-CAT                                                  |
| Upper                                                        |
| Lower                                                        |
| 5′-AGGCCCTGTTGTTTCCGCACC-3′                                   |
| 5′-GGTTGGGAAACCACACGCTC-3′                                   |
| 5′-GCCCTGTTGTTTCCGCACC-3′                                    |
| 5′-GGTTGGGAAACCACACGCTC-3′                                   |
| 5′-GCCCTGTTGTTTCCGCACC-3′                                    |
| 5′-GGTTGGGAAACCACACGCTC-3′                                   |

**MATERIALS AND METHODS**

**Cell Isolation and Culture—**COS1, HeLa, and the human LX2 hepatic stellate cell line (20) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 10% (v/v) fetal calf serum. Primary HSC were isolated from normal fetal liver of 6-month-old, 500-g male Sprague-Dawley rats by sequential perfusion with collagenase and Pronase, followed by purification by discontinuous density centrifugation in 11.5% (v/v) OptiPrep (Invitrogen). Human HSC were isolated from the liver of adult males following partial hepatectomy and were isolated and purified essentially as described for rat HSC. Isolated HSC were cultured on plastic or Matrigel (BD Biosciences)-coated culture dishes in Dulbecco’s modified Eagle’s medium supplemented with 16% (v/v) fetal calf serum and were maintained at 37 °C in an atmosphere of 5% (v/v) CO₂. HSC were routinely detached from plastic or coated dishes by a non-enzymatic method that involved incubation of cells with Matrisperse (BD Biosciences) for 5–10 min at 37 °C. Detached cells were then recovered by centrifugation at 1000 × g for 5 min prior to resuspension in media and seeding onto either plastic or coated dishes. For coating culture dishes with Matrigel (BD Biosciences), 1.5 µl of growth factor-reduced Matrigel (in a 2:1 mixture with serum-free Dulbecco’s modified Eagle’s medium) was evenly spread across a 90-mm plastic dish to generate a thin layer (~0.5 mm) of matrix. All cells were maintained at 37 °C in an atmosphere of 5% (v/v) CO₂.

**Chromatin Immunoprecipitation—**Chromatin immunoprecipitation (ChIP) assays were performed using a ChIP assay kit (Upstate Biotechnology, Inc.). Formaldehyde cross-linking was performed for 5 min in LX2 cells, and sonication of chromatin was carried out in a sonication water bath twice for 3 min. 4 µl of anti-RUNX1 (active motif) or 4 µl of anti-Notch2 antibody (Santa Cruz Biotechnology) was used in the immunoprecipitation. PCR primers used to amplify the minimal promoter region (−102 to +60) of the human TIMP1 promoter are as follows: forward 5′-TGAATGTA-3′ and reverse 3′-CAAGAAGCTTCCCAGCTCCGGTCCCTGCTG-5′ of the human TIMP1 promoter are as follows: forward 5′-TGAATGTA-3′ and reverse 3′-CAAGAAGCTTCCCAGCTCCGGTCCCTGCTG-5′ of the human TIMP1 promoter are as follows: forward 5′-TGAATGTA-3′ and reverse 3′-CAAGAAGCTTCCCAGCTCCGGTCCCTGCTG-5′ of the human TIMP1 promoter are as follows: forward 5′-TGAATGTA-3′ and reverse 3′-CAAGAAGCTTCCCAGCTCCGGTCCCTGCTG-5′ of the human TIMP1 promoter are as follows: forward 5′-TGAATGTA-3′ and reverse 3′-CAAGAAGCTTCCCAGCTCCGGTCCCTGCTG-5′ of the human TIMP1 promoter are as follows: forward 5′-TGAATGTA-3′ and reverse 3′-CAAGAAGCTTCCCAGCTCCGGTCCCTGCTG-5′ of the human TIMP1 promoter are as follows: forward 5′-TGAATGTA-3′ and reverse 3′-CAAGAAGCTTCCCAGCTCCGGTCCCTGCTG-5′ of the human TIMP1 promoter are as follows: forward 5′-TGAATGTA-3′ and reverse 3′-CAAGAAGCTTCCCAGCTCCGGTCCCTGCTG-5′ of the human TIMP1 promoter are as follows: forward 5′-TGAATGTA-3′ and reverse 3′-CAAGAAGCTTCCCAGCTCCGGTCCCTGCTG-5′ of the human TIMP1 promoter are as follows: forward

**Electrophoretic Mobility Shift Assay (EMSA)—**UTE-1 DNA binding activities were determined as described previously (19). Briefly, standard EMSA reactions consisted of an initial incubation (at 4 °C) of nuclear extract (5 µg) with 1 µg of poly(dI-dC) in a total volume of 18 µl for 10 min. Annealed double-stranded 5′-end-labeled oligonucleotide probes (2 µl; 0.1 ng µl⁻¹) were then added to the EMSA reaction and incubated for a further 20 min at 4 °C. The UTE-1 probe was generated by annealing a sense strand oligonucleotide with its antisense strand (Table I). For detection of specificity of DNA binding, competitive EMSAs were used in which binding of proteins to the UTE-1 probe were challenged by a 10-min pre-incubation (prior to addition of the probe) with 200 ng (100-fold excess) of unlabeled double-stranded specific (UTE-1) or nonspecific (C/EBP) oligonucleotides. To generate the double-stranded C/EBP competitor, a sense strand oligonucleotide was annealed with its antisense strand (Table I). For detection of specific transcription factors as components of retardation complexes, EMSA reactions were incubated in the presence of 4 µg of anti-RUNX1 (Santa Cruz Biotechnology), anti-RUNX2 (active motif), or anti-Notch-D (Santa Cruz Biotechnology).

**IMMUNOPRECIPITATION—**FLAG immunoprecipitation was carried out by using FLAG® immunoprecipitation kit (Sigma) according to the manufacturer’s protocol. Briefly, 1 × 10⁵ (5) COS-1 cells were transfected with 3 µg of pLNCX-FLAG-RUNX1A or pLNCX-FLAG-RUNX1B (both gift from I. Kitabayashi, National Cancer Center Research Institute, Japan) and 1 µg of PCM2-JunD or PCM2. 48 h later, cells were lysed, and the lysate was incubated overnight with 30 µl of prewashed FLAG-agarose beads with shaking at 4 °C. Beads were then washed four times in 1× wash buffer (Sigma), resuspended in 1 volume of Western loading buffer (Invitrogen), and boiled for 10 min at 90 °C. The presence of immunoprecipitated proteins was detected by SDS-PAGE and immunoblotting by using anti-FLAG (Sigma) or anti-Jun-D (Sigma) antibodies.
TIMP1 Transcription by RUNX1 and RUNX2

| Primers | AT °C | Amplicon size bp |
|---------|-------|-----------------|
| Rat gene | RUNX1 Upper | 5’-AGGCCCAGATGGGGGGCCTTG-3’ | 58.9 | 657 |
| RUNX1 Lower | 5’-TGAGGGTAAAGGCGATGGT-3’ | | |
| RUNX2 Upper | 5’-GCTCGGAAATGGTTCTGTTAT-3’ | 59.2 | 590,420 |
| RUNX2 Lower | 5’-CACAGGCTGCCCCTTCTGTA-3’ | | |
| RUNX3 Upper | 5’-GAGCCCACCGCGACCCAAAATC-3’ | 66.6 | 535 |
| RUNX3 Lower | 5’-GTCGACTGGGCGTTGCTTACG-3’ | | |
| Human gene | RUNX1 Upper | 5’-GACCTGCGTGGACAGGTTG-3’ | 60.1 | 687 |
| RUNX1 Lower | 5’-GGGGGAGAGGGACAGGAGGAG-3’ | | |
| RUNX1B Upper | 5’-AGATGAGGCTAGAGGGGTGAGG-3’ | 59.7 | 674 |
| RUNX1B Lower | 5’-TTGCGGGTGGTTGGAAGA-3’ | | |
| RUNX2 Upper | 5’-GCTCGGAAATGGTTCTGTTAT-3’ | 59.2 | 590,420 |
| RUNX2 Lower | 5’-CACAGGCTGCCCCTTCTGTA-3’ | | |
| RUNX3 Upper | 5’-TCCAGGGCCACAGAGGATTAGTC-3’ | 58.2 | 563 |
| RUNX3 Lower | 5’-AGAGGGGCGGGAGATGGTCTTAT-3’ | | |

**Northern Blotting**—Total cellular RNA was isolated from rat HSCs subcultured on either plastic or Matrigel-coated plates using the RNeasy mini kit (Qiagen). 5 μg of each sample RNA was then resolved on a MOPS/formaldehyde 1.2% (w/v) agarose gel and transferred under negative pressure to Hybond XL (Amersham Biosciences). Membranes were then subjected to Northern analysis by using the human TIMP1 partial (0.6 kb) cDNA probe labeled with [32P]dCTP using an oligonucleotide labeling kit (Amersham Biosciences). Following hybridization, membranes were washed first in 2× SSC, 0.1% (w/v) SDS at room temperature (two washes) and at 52 °C (two washes). Membranes were then washed at 52 °C in 1× SSC, 0.1% (w/v) SDS followed by one final rinse in 0.5× SSC. 0.1% (w/v) SDS also at 52 °C. Membranes were then subjected to autoradiography using Kodak XMB autoradiography film (GRI) for 72 h at ~80 °C.

**Plasmid DNAs, Transfections, and Reporter Gene Assay**—Heterologous synthetic promoter constructs were generated by T4 DNA ligase (Promega)-mediated recombination of phosphorylated and annealed oligonucleotides carrying Xhol and BamHI overhangs into Xhol/BamHI-digested pBlCAT2. SRE-UTE-1-CAT (pSU), SRE-mutUTE-1-CAT (pSmU), UTE-1-SRE-CAT (pUS), SRE-revUTE-1-CAT (pRSU), and SRE-distalUTE-1-CAT (pSUd) were generated by using sense and antisense oligonucleotides listed Table I, 1×, 2×, and 4× UTE-1-CAT vectors were generated by insertion of double-stranded oligonucleotides (Table I) carrying 1, 2, or 4 tandem UTE-1 sites into Xhol/BamHI-digested pBlCAT2. pSUd was generated by insertion of double-stranded oligonucleotides into the Small/ SaeI site located at position 2239 downstream of the t intron and polycadenyl signals from SV40 in pBlCAT2. Four tandem UTE-1 sites were also cloned into Smal/SacI-digested pg2 promoter vector (Promega). Verification of all synthetic promoter constructs was made by DNA sequence analysis. Determination of TIMP1 promoter activity was carried out by using a previously described minimal active 162-bp human TIMP1 promoter upstream of a CAT gene (17). Expression of RUNX1 and transcriptional co-activator proteins in cells was achieved by transfection of expression vectors for pCMV5-RUNX1B (gift from S. Hiebert, Vanderbilt Cancer Center), pLNCX-FLAG-RUNX1A (gift from I. Kitabayashi, National Cancer Center), pLNCX-FLAG-RUNXA (gift from I. Kitabayashi, National Cancer Center), or pCMV2-JunD (18). Cells were transfected using Effectene (Qiagen) and were harvested 48 h later, and CAT assays were determined as described previously (17). Luciferase assays were performed according to the manufacturer’s protocol (Promega).

**Reverse Transcription (RT)-PCR—DNAse I-treated whole cell RNA was used for synthesis of by using Moloney murine leukemia virus reverse transcriptase (Promega) and random hexamer primers (Promega).** PCR amplification of RUNX1, RUNX2, RUNX3, and β-actin cDNA species was carried out by using oligonucleotide primers selected within the coding regions of the genes and are listed in Table II. PCR products were resolved by electrophoresis through a 1% (w/v) agarose gel, detected by ethidium bromide staining, and verified as being amplified from the predicted cDNA species by DNA sequencing.

**SDS-PAGE and Immunoblotting**—Whole cell protein extracts were prepared, processed, and fractionated by electrophoresis through a 9% (v/v) SDS-polyacrylamide gel and subsequently subjected to immunoblotting as described previously (17, 18). Detection of specific proteins by immunoblotting was achieved by using antibodies specifically recognizing RUNX1 (active motif), RUNX2 (active motif), or FLAG (Sigma) tag.

**Yeast One-hybrid System**—Screening for cDNA clones that express UTE-1-binding proteins was carried out according to the manufacturer’s protocol (Matchmaker One-hybrid System, Clontech). Briefly, four tandem copies of the 11-bp UTE-1 motif (5’-TGTGGTTTCCC-3’) were inserted upstream of the HIS3 or lacZ reporter gene by designing two antiparallel 4xUTE-1 oligonucleotides with appropriate restriction sites at the ends. Annealed oligonucleotides were cloned into pHIS and pLacZi reporter plasmids (Clontech). Both UTE-1 reporter constructs were integrated into the genome of YM4271 strain to generate UTE-1 reporter strains. Background HIS3 activity was ablated using 45 μm 3-aminostriozole (Sigma). Screening UTE-1 reporter strains with HeLa pACT2 cDNA library (Clontech) identified genes encoding UTE-1-binding proteins. Plasmids were isolated from yeast clones capable of growing in the presence of 45 μm 3-aminostriozole in YPD media (Clontech) lacking histidine and showing a positive signal in the β-galactosidase assay. Plasmids from the positive clones were transformed into competent HB101 bacteria (Promega) and purified using Maxiprep kit (Qiagen). They were transformed into three different yeast reporter strains that contained within their genome the lacZ gene under control of either four copies of UTE-1 or mutated UTE-1 (5’-TGTTAGTTCCG-C’) or three copies of the pS3-binding sequence. The activation of the lacZ gene was determined using the β-galactosidase assay. Briefly, after 3 days of growth at 30 °C, the transformed colonies were lifted up by rubbing on a sterile Whatman paper No. 5 filter (Fisher) and transferred to a pool of liquid nitrogen for a freeze-thaw cycle. Another filter was pre-soaked in buffer Z (Na2HPO4.7H2O 60 mM, NaH2PO4.H2O 40 mM, KCl 10 mM, MgSO4.7H2O 0.1 mM, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) 33 mg/ml, galactose 0.27% (v/v)) and placed beneath the previous filter. The filters were incubated at room temperature until the colonies turned blue because of the activity of β-galactosidase.

**RESULTS**

**UTE-1 DNA Binding Activity Is Associated with the Activated Phenotype of HSC and TIMP1 Gene Transcription—**
Freshly isolated HSC can be effectively maintained in a quiescent state for several days when they are cultured on Matrigel (21). Moreover, when culture-activated HSC are detached from plastic and seeded onto Matrigel, several classic morphological and biochemical markers of HSC activation are repressed indicative of a reversal to the quiescent phenotype (22). We reasoned that if UTE-1 DNA binding activity is a phenotypic feature of aHSC, then we should observe suppression of the induction of UTE-1 DNA binding in freshly isolated HSC cultured on Matrigel and inhibition of UTE-1 DNA binding in aHSC seeded onto Matrigel. As reported previously, Fig. 1a shows that culture activation of rat HSC is associated with induction of TIMP1 mRNA expression at day 5 of culture which correlates with the time at which HSC begins to adopt an activated phenotype (16). Culture of freshly isolated rat HSC on Matrigel suppressed induction of UTE-1 DNA binding activity, whereas Fig. 1b confirms that this effect was accompanied by suppression of the induction of TIMP1 mRNA expression. The eventual appearance of UTE-1 DNA binding activity in cells cultured for 8 days is most probably because of the fact that the HSC in these long term cultures migrated beneath the gel and contacted plastic. aHSC seeded on Matrigel underwent a rapid diminution of both UTE-1 binding activity (Fig. 1c) and TIMP1 mRNA expression (Fig. 1d). These data suggest that there is a close association between the activated phenotype of HSC and UTE-1 activity.

The UTE-1 Site Functions Cooperatively with the SRE of the TIMP1 Promoter—To characterize further the function of the UTE-1 site, a series of synthetic heterologous reporter constructs were designed in which the UTE-1 site was located either singly or in multiple tandem copies immediately upstream of the minimal thymidine kinase promoter. Transfection experiments in either 3T3 fibroblasts or rat aHSC showed that the UTE-1 site is unable to stimulate transcription either alone or when present as two tandem copies (Fig. 2a). However, a construct containing four tandem copies of the UTE-1 site was substantially more active than the control minimal thymidine kinase promoter construct. These data suggest that the UTE-1 site is unable to function in isolation, and in the context of the TIMP1 promoter probably operates in cooperation with multiple cis-acting regulatory elements. As the UTE-1 motif is located just 10 bp downstream from the SRE in the TIMP1 promoter, a series of constructs were designed to test whether the UTE-1 and SRE sites are functionally associated. Transfections in 3T3 fibroblasts and aHSC showed that combination of the TIMP1 SRE with a single UTE-1 site resulted in a synergistic enhancement of transcription (Fig. 2b). In the case of aHSC, a significant increase in transcription over that detected from the thymidine kinase promoter alone was only observed when both the SRE and UTE-1 elements were present. To determine whether there is a positional requirement for these effects, the UTE-1 site was relocated either 10 bp up-
stream of the SRE site (pUS) or at a more distal site from the SRE (pSdU) (Fig. 2c). A construct was also designed in which the UTE-1 site was located in its wild type position relative to the SRE only in reverse orientation (pSrU). UTE-1 operated synergistically with the SRE site when located upstream or in reverse orientation. However, synergism was lost when the UTE-1 site was distal to the SRE (pSdU). Taken together these data indicate that factors binding to the UTE-1 site may functionally interact with members of the AP-1 and Ets family of transcription factors at the SRE to regulate TIMP1 promoter activity. Identification of UTE-1 factors was therefore a priority for understanding how TIMP1 transcription is controlled.

The UTE-1 Site Binds RUNX Family Transcription Factors—The yeast one-hybrid system was employed to isolate cDNAs encoding human UTE-1 DNA-binding proteins utilizing four UTE-1 sites upstream of the His and LacZ yeast one-hybrid selection vectors. By using a HeLa cDNA library we selected three clones that survived in media lacking histidine and were able to transactivate a 4x-UTE-1-LacZ vector but were unable to transactivate control 4x-mutated UTE-1- and p53-LacZ vectors (Fig. 3a). Nuclear extract from clone B, which displayed the strongest LacZ expression, generated a sequence-specific UTE-1 protein-DNA complex (Fig. 3b). By contrast the remaining two clones which only generated weak LacZ expression lacked sequence-specific UTE-1 DNA binding activity. Sequence analysis of clone B revealed perfect identity with the published cDNA sequence for human RUNX1 (GenBank accession number NM_001754). To determine whether RUNX factors are stimulators of UTE-1-dependent gene transcription, 4X-UTE-1-Luc was co-transfected with expression vectors for RUNX1 (1B) and RUNX2. Western blotting of transfected COS1 cells confirmed protein expression from the vectors, and promoter studies showed that both RUNX factors stimulate 4X-UTE-1-Luc activity (Fig. 3c). To confirm that RUNX factors are UTE-1-binding proteins in aHSC, we initially used EMSAs to show that UTE-1-binding complexes assembled from rat aHSC nuclear extracts were supershifted by antisera recognizing RUNX1 and -2 (Fig. 4a). We next employed ChIP assays to determine in situ binding of RUNX proteins to the TIMP1 promoter. For these studies a well characterized human HSC cell line LX2 (20) was used, which we confirmed expressed both RUNX1 and RUNX2 (data not shown). RUNX1 was consistently found to be associated with the TIMP1 promoter by ChIP assay (Fig. 4b). RUNX2 ChIP assays were also attempted; however, these did not generate reproducible data indicating technical problems most probably related to RUNX2 antibodies (data not shown).

Expression of RUNX Transcription Factors in Rat and Human HSC—RT-PCR assays were initially carried out on mRNA isolated from rat HSC using primers specific for rat RUNX1, RUNX2, and RUNX3 (Fig. 5a). Expression of RUNX1 and RUNX2 mRNA was detected in rat HSC with no significant change in levels of message detected during culture activation. By contrast, RUNX3 mRNA was only observed at detectable levels in quiescent HSC indicating that HSC activation is associated with loss of RUNX3 expression. Similar RT-PCR studies in human aHSC (Fig. 5b) demonstrated expression of the two major human isoforms of RUNX1 (RUNX1A and RUNX1B)
RT-PCR for RUNX2 generated two products with both human and rat HSC mRNA. Sequencing confirmed that the smaller product is generated from amplification of an alternatively spliced RUNX2 transcript. RUNX3 expression was not detected in human aHSC (data not shown). In contrast to the mRNA studies, changes in the expression of RUNX1 and RUNX2 proteins were observed upon culture activation of rat HSC. Freshly isolated HSC lacked significant RUNX1 protein expression. By contrast rat and human aHSC expressed two anti-RUNX1 reactive proteins (Fig. 6a). The estimated molecular weights of these proteins were similar to those determined in COS1 cells transfected with a RUNX1B expression vector (Fig. 3b). Quiescent rat HSC expressed a 30-kDa protein recognized by anti-RUNX2 antibody that was also expressed in human and rat aHSC (Fig. 6b). However, aHSC differed from quiescent cells in that they also expressed a higher molecular weight protein, which from previous reports (24) and comparison with RUNX2-transfected COS cells (Fig. 3b) is likely to correspond to full-length RUNX2. From these data we conclude that HSC express RUNX1 and RUNX2 mRNAs and that post-transcriptional events occurring during HSC activation generate qualitative and quantitative changes in RUNX1 and RUNX2 isoform expression.

Regulation of TIMP1 Promoter Activity by RUNX1 and RUNX2

To evaluate the potential of RUNX factors to regulate TIMP1 gene transcription, rat aHSC were transfected with a human TIMP1 promoter-CAT reporter together with expression vectors for RUNX1 and RUNX2. Co-transfection of the

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**FIG. 3.** Identification of RUNX factors as UTE-1 DNA-binding proteins. a, yeast one-hybrid screening of a HeLa cDNA library identified three positive (lacZ expressing) clones designated clones A–C. These clones were able to transactivate a 4xUTE-1-driven lacZ gene in yeast but were unable to transactivate LacZ under control of mutant 4xUTE-1 (containing a 3-nucleotide mismatch) or control 3x53 promoters. As controls for the screening assay, yeast were transformed with 3x53-LacZ with either a p53 expression vector (positive control) or an empty vector (negative control) as supplied by the manufacturer. b, to determine whether clones A–C express a sequence-specific UTE-1-binding protein, 5 µg of nuclear extract from each yeast clone were used in EMSA reactions in the presence and absence of 100-fold molar excess of specific (UTE-1) and nonspecific (C/EBP) competitor DNAs. Note expression of sequence-specific binding protein in clone B which, upon sequencing, was found to be identical to human RUNX1. c, expression vectors (2 µg) for pCMV-RUNX1 and pCMV-RUNX2 were initially tested for production of proteins by transfection into COS1 cells followed by Western blot analysis of the RUNX factors on 20 µg of whole cell protein extracts using anti-RUNX1 and RUNX2 antisera. RUNX1B and RUNX2 expression vectors were then co-transfected in increasing amounts with a fixed concentration of human 4xUTE-reporter into HeLa cells, and activities were determined after 48 h. Data are presented as reporter activity relative to control (reporter alone) and represent the mean (± S.E.) of two independent experiments done in triplicate.
full-length isoform of RUNX1 (1B) resulted in a dose-dependent repression of TIMP1 promoter activity (Fig. 7a), which was in contrast to the stimulatory effect of RUNX1B on the heterologous 4X-UTE promoter construct (Fig. 3c). A shorter isoform of RUNX1 (1A) has been described that lacks C-terminal transcriptional repression domains (23). Expression of RUNX1A
produced a dose-dependent stimulation of TIMP1 promoter activity (Fig. 7b). A similar effect was also observed in cells transfected with a RUNX2 expression vector (Fig. 7c) indicating that RUNX factors have the potential to either repress or activate TIMP1 transcription in HSC.

Functional Interaction between RUNX1 and JunD—To further investigate the mechanism underlying RUNX1B-mediated repression of the TIMP1 promoter, we investigated the effects of RUNX1B on JunD-induced transcription. As described previously (18), expression of JunD stimulated TIMP1 promoter activity; however, co-expression of RUNX1B blunted this effect (Fig. 8a). By contrast RUNX1A did not repress JunD-stimulated transcription (data not shown). We next determined whether RUNX1 can interact with JunD by carrying out immunoprecipitation of transfected RUNX1B-FLAG and RUNX1A-FLAG followed by immunoblot detection of JunD. As shown in Fig. 8b, JunD was co-immunoprecipitated with RUNX1A but not with RUNX1B. Hence, the repressive effects of RUNX1B on the TIMP1 promoter are associated with loss of interaction of RUNX with JunD.

DISCUSSION

This study shows that the mammalian RUNX proteins bind to the previously described essential UTE-1 regulatory sequence in the human TIMP1 promoter and regulate transcription. In the study that originally described the UTE-1 site, we also reported its association with a 30-kDa nuclear protein (19). The precise identity of this 30-kDa protein remains unknown; however, it should be noted that RUNX2 antisera detected a protein of ~30 kDa that was strongly expressed in HSC. This protein may be a truncated form of full-length RUNX2 generated either by alternative splicing or due to the susceptibility of RUNX proteins to proteolytic degradation (25).

Expression of both RUNX1 and RUNX2 was induced during HSC activation by a post-transcriptional mechanism. This as yet undefined mechanism is likely to be intimately associated with the HSC activation process because culture of aHSC on Matrigel led to a rapid and complete loss of UTE-1 binding activity and TIMP1 mRNA expression. The concomitant loss of UTE-1 binding and TIMP1 expression in this latter experiment also indicated that RUNX factors may function as positive regulators of TIMP1 gene transcription. This was proved to be the case for the RUNX1A isoform and for RUNX2, both of which stimulated increased TIMP1 promoter activity when overexpressed in aHSC. However, the full-length RUNX1B isoform repressed TIMP1 promoter activity suggesting that the balance between RUNX factors recruited to the promoter will dictate the level of transcription. RUNX1B has been shown previously to have either stimulatory or inhibitory influences on transcription depending on both promoter and cell context (26–28). Of note, in the current study RUNX1B stimulated a heterologous promoter construct containing four tandem UTE-1 sites upstream of a minimal thymidine kinase promoter in HeLa cells. Our data suggest that TIMP1 is among the class of mammalian RUNX gene targets that can be transcriptionally repressed by RUNX1B. This class of genes include p21<sup>WAF1</sup>, HERF1, MRP14, Stefin3, uridine phosphorylase,
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is the predominant Jun protein expressed in activated HSC (17, 18). Moreover, JunD is the only AP-1 factor that significantly stimulates TIMP1 promoter activity in HSC and appears to operate optimally at this promoter in a homodimeric form (18). RUNXIB not only repressed basal TIMP1 promoter activity but also inhibited JunD-stimulated transcription. A key finding in the current study was that although RUNXIA can directly interact with JunD, no interaction was detected between RUNXIB and JunD. Although this is the first study that has described an interaction between JunD and RUNX factors, c-Jun has been reported to interact with both RUNX1 and RUNX2 (31). The reason for this lack of interaction between RUNXIB and JunD is not known but presumably results from properties of the C-terminal regions of RUNX1B that are missing from the truncated RUNX1A isoform. Because the C-terminal region of RUNX1B has been shown to interact with a variety of different proteins including transcriptional corepressors, it is possible that these interactions may prevent the interaction of RUNX1B with JunD (25, 35, 36). Occupancy of the UTE-1 site by RUNX1B may then bring about repression of TIMP1 promoter activity by destabilizing the association of JunD. In addition the recruitment of transcriptional co-repressors by RUNX1B would also be expected to impact on activity of the TIMP1 promoter. We have not yet determined whether the stimulatory effects of RUNX2 are associated with an ability to interact with JunD; however, because RUNX2 can interact with c-Jun, this is a possibility that warrants further investigation (31).

Elevated hepatic expression of TIMP1 promotes fibrosis. The discovery that RUNX proteins are expressed in activated HSC and can exert both repressive and stimulatory effects on the activity of the TIMP1 promoter is an important step toward a full understanding of the mechanisms that regulate high level expression of TIMP1 in the injured liver.

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Pim-2, and bone sialoprotein (29). Because promoter context is an important determinant of the type of influence that RUNXIB exerts on transcription, it is highly relevant to consider the role of transcription factors binding to sites adjacent to RUNX sites. RUNX-binding sites are often found in proximity to Ets or AP-1 sites, and RUNX factors can interact directly with transcription factors binding at these adjacent sites (30–32). These interactions have been proposed to stimulate transcription by stabilizing protein-DNA complexes via conformational changes in the transcription factors that relieve the repressive effects of negative regulatory domains for DNA binding (33, 34). The UTE-1 site is in close proximity to an SRE that consists of overlapping AP-1, STAT, and Ets-binding motifs of which in HSC the AP-1 site is functionally the most important (17). In the present study we have shown that the TIMP1 SRE and UTE-1 can function in synergy to stimulate transcription providing that they remain in close proximity, raising the possibility that RUNX and AP-1 factors may cooperate at the TIMP1 promoter. Jun factors are critical components of transcriptionally active AP-1 dimers, and JunD

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FIG. 8. Functional interaction between JunD and RUNX1. a, effects of RUNXIB on JunD-stimulated TIMP1 promoter activity were determined by transfection of the TIMP1 promoter-CAT reporter together with expression vectors for JunD and RUNXIB into activated rat HSC. After 48 h of culture, cells were harvested and promoter activities determined according to the protocol described under “Materials and Methods.” Data are presented as promoter activity relative to the control (TIMP1 promoter reporter construct alone) and represent the mean ± S.E. of three independent experiments. b, for detection of interaction of JunD and RUNX1, COS-1 cells were transfected with expression vectors for FLAG-tagged RUNX1A or RUNX1B either alone or with a JunD expression vector. 48 h after transfection, cells were harvested, and lysates were used for immunoprecipitation with anti-FLAG antibody which was monitored by immunoblotting of immunoprecipitates for FLAG. Co-immunoprecipitation of JunD was also monitored by immunoblotting using an anti-JunD antisera. The gels shown were representative of two independent experiments.
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