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Transcriptional Repression of the α1(I) Collagen Gene by *ras* Is Mediated in Part by an Intronic AP1 Site

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Abstract We have previously shown that transformation of fibroblasts by *ras* results in transcriptional inhibition of the α1(I) gene. An α1(I)-hGH chimeric plasmid containing 3.7 kb of 5′ flanking and 4.4 kb of α1(I) transcribed sequence was regulated appropriately by *ras* in a transient transfection assay. In contrast, a similar plasmid containing α1(I) DNA from −220 to +500 was virtually unresponsive to *ras*. The regions from −3700 to −220 and +500 to +4400 contributed equally to the *ras*-mediated inhibition of the parental plasmid. Deletion analysis indicated that a short fragment, between +500 and +890 in the first intron of the α1(I) gene, was recognized differently in *ras*-transformed and wild-type cells. A previously described AP1 site in this fragment stimulated α1(I) transcription in Rat1 fibroblasts but was inactive in *ras*-transformed cells. Mobility shift assays using nuclear extracts from the two cell types demonstrated differences in binding to the α1(I) AP1 site. We conclude that *ras* transformation suppresses the function of a cell-specific enhancer in the first intron of the α1(I) collagen gene.

Key words: gene regulation, oncogene, cell transformation, transient transfection, DNase hypersensitivity, mobility shift assays

Type I collagen is a heterotrimeric extracellular matrix protein found predominantly in bone, tendon, and other connective tissues. It is composed of two polypeptides, α1(I) and α2(I), in the ratio 2:1; the genes encoding these two peptides are coordinately regulated and expressed in a strict tissue-specific manner [Brenner et al., 1993; Slack et al., 1993]. We have previously shown [Slack et al., 1992] that the α1(I) gene is expressed at much lower levels in *ras*-transformed than in untransformed cells, and that both transcriptional and posttranscriptional mechanisms contribute to this effect. The aim of the present study was to identify *ras*-responsive regions in the α1(I) gene and to identify factors binding to these regions. Our ultimate goal is to understand how *ras* modulates the amount and/or activity of these factors, and how such changes lead to transcriptional inhibition of the α1(I) gene.

The three mammalian Ras proteins (N-, K-, and H-Ras) are members of a large family of homologous guanine nucleotide binding proteins [Bourne et al., 1991], all of which catalyze GTP hydrolysis and transmit effector signals in the GTP-bound form. They function as critical intermediates in signal transduction pathways that link extracellular events to changes in gene expression [Lowy and Willumsen, 1993]. Activating *ras* mutations are common findings in human tumors [Bos, 1989], and oncogenically active Ras proteins are thought to contribute to the proliferative, invasive, and metastatic properties of transformed cells. The transforming properties of Ras are dependent on its interaction with, and activation of, one or more cellular kinases [Ruderman, 1993]; substrates for these kinases include cytoplasmic proteins involved in cell cycle progression as well as nuclear transcription factors such as the AP1 component, c-Jun [Pulverer et al., 1991; Smeal et al., 1992]. Constitutive AP1 activity appears to be a fundamental feature of *ras*-transformation, since interference with c-Jun or c-Fos function in *ras*-trans-

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formed cells blocks many aspects of the neoplastic phenotype [Granger-Schnarr et al., 1992; Lloyd et al., 1991; Wick et al., 1992].

The list of *ras*-inducible genes includes a diverse array of molecules which perform both metabolic and structural functions [Bortner et al., 1993; Chambers and Tuck, 1993], and *ras*-responsive elements have been identified in several of these genes [Bortner et al., 1993]. Imler [Imler et al., 1988] originally identified a short sequence in the polyoma virus enhancer which was inducible by Ras, phorbol esters, and serum. This element contained both ets and AP1 binding sites, and the ets/AP1 combination has proven to be the most common motif involved in *ras*-responsiveness of both cellular genes and viral enhancers [Gutman and Wasylyk, 1990; Owen and Ostrowski, 1990; Pankov et al., 1994; Reddy et al., 1992]. In this report, we show that *ras* transformation paradoxically blocks the function of an intronic AP1 site in the α1(I) gene. We propose that different AP1 factors interact with the AP1 site in untransformed and transformed cells, and that these differences are responsible for at least part of the *ras*-mediated transcriptional inhibition of the α1(I) gene.

MATERIALS AND METHODS

Plasmid Constructs

The plasmid WTC-1 includes the entire mouse α1(I) collagen gene as well as 3.7 kb of 5′- and approximately 3 kb of 3′-flanking DNA [Stacey et al., 1988]. A 21 bp polylinker insert at the *Xba*I site in the 5′ untranslated region allows differentiation between endogenous and transgene-specific α1(I) mRNAs in an S1 nuclease assay [Wu et al., 1990]. The plasmids MT-bGH and MT-hGH, used as cotransfection controls, contain either the bovine (bGH) or human growth hormone (hGH) genes driven by the mouse metallothionein promoter.

Mouse α1(I) collagen-hGH constructs were prepared using standard techniques and were verified by either restriction digestion or direct sequencing. Two different versions of −3700/+4400MCol-hGH were created; each contains sequences from −3700 to +4400 of mouse α1(I) collagen subcloned upstream of a small segment of the hGH gene. In version 1, the α1(I)-hGH fusion is between an *Eag*I site in exon 10 of α1(I) and a *Bgl*II site in the final (fifth) exon of hGH (via intervening polylinker sequences), preserving the hGH polyadenylation region. In version 2, the identical α1(I) fragment was ligated to a *Nar*I site in intron 4 of hGH, generating a similar construct but with an exon-intron fusion and slightly more hGH DNA. These 2 plasmids were regulated identically in transfection assays (data not shown), and thus both plasmids are generically designated as −3700/+4400MCol-hGH. To create −220/+500 MCol-hGH, α1(I) sequences from −220 (*Bgl*II) to +500 (*Stu*I) were ligated through a polylinker to the *Nar*I site in intron 4 of hGH. All subsequent MCol-hGH plasmids contain the indicated amount of α1(I) sequence added to the −220/+500 construct.

Cell Culture, Transfections, and Assays of Transgene Activity

Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated calf serum. All transfections were performed using the calcium-phosphate method. Cells were plated at 5×10^5 (*ras*-transformed Rat1) or 2.5×10^5 (Rat1) cells per 60 mm dish, exposed to DNA-containing precipitate overnight, and washed once the following morning with phosphate-buffered saline (PBS). Total cellular RNA was harvested [Chomczynski and Sacchi, 1987] 48 h after transfection, and quantitation of transgene activity was accomplished with an S1 nuclease assay as described [Wu et al., 1990]. In each experiment, an internal cotransfection control plasmid (either MT-hGH or MT-bGH) was included, and expression of the control plasmid was assayed by RNase protection using previously described probes and techniques [Bornstein and McKay, 1988; Bornstein et al., 1988a]. Gels were quantitated by phosphorimage analysis.

Dnase Hypersensitivity Analysis

Rat1 and *ras*-transformed Rat1 cells were grown to 70% confluency in DMEM/10% calf serum, harvested by trypsinization, washed extensively in PBS, and resuspended to a final concentration of 1×10^7 cells/ml in hypotonic buffer (10 mM Tris/HCl, pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$) containing 0.15% Triton ×100. The suspensions were incubated at 4°C for 15 min and then homogenized with 10 strokes of a type B pestle in a Dounce homogenizer. Nuclei were pelleted, washed twice in hypotonic buffer, and resuspended at 5×10^7 nuclei/ml in 15 mM Tris/HCl, pH 7.4, 60 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiotreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 5%

glycerol. The nuclei were then digested with increasing concentrations of DNase I (BRL) at 37°C for 15 min, followed by addition of EDTA and SDS to 10 mM and 1%, respectively. Proteinase K was added to a final concentration of 200 μg/ml and incubation continued for 1 h at 37°C. Samples were extracted twice with buffer-saturated phenol, once with chloroform:isoamyl alcohol, and DNA was precipitated by the addition of 0.1 volume of 3M sodium acetate and 2.5 volumes of ethanol. After restriction digestion, DNA was fractionated on a 1% agarose gel, transferred to nitrocellulose, and hybridized using standard procedures.

PCR-Mediated Site-Directed Mutagenesis

The AP1 site at +555 in intron 1 of mouse α1(I) was mutated from TGATTCA to TAGTAGA using a two-step polymerase chain reaction strategy [Zhao et al., 1993]. One nanogram of wild-type template DNA (−220/+890MColhGH) was mixed with 100 pmol each of mutant oligonucleotide (5′ GCTGCGGGAT**AGTAGA**TAAGGAAAG 3′) and a downstream antisense oligonucleotide (5′ ACTTTCTCATCCAGCAACCC 3′; α1[I] sequence from +681 to +662) in a 100 μl reaction that included 200 μM dNTPs, 5 U of *Taq* DNA polymerase (Gibco/BRL, Gaithersburg, MD), and 10 μl of 10× *Taq* buffer (BRL). The reaction was incubated at 94°C for 2.5 min prior to 25 rounds of cycling (denature 1.5 min at 94°C, anneal 2.5 min at 45°C, polymerize 2 min at 72°C, final extension 10 min at 72°C) in a Perkin Elmer Cetus (Norwalk, CT) DNA Thermal Cycler. In the second reaction (final volume 100 μl), 2.5 μl of product from reaction 1 (about 150 fmol of the expected 141 bp fragment) was added to 1 ng (0.29 fmol) of wild-type template along with 200 μM dNTPs, 5 U of *Taq* polymerase, and 10× buffer as before. After 7 cycles (with identical parameters as for reaction 1), 100 pmol each of upstream sense (5′ GGGCAGAGTAGTCGAAAATG 3′; from +469 to +488 of α1[I]) and downstream antisense primers were added and the reaction was continued for 18 more cycles. The resultant 212 bp product was digested with *Stu*I and *Pst*I to release a 90 bp fragment containing the desired mutation; this fragment was then substituted for the wild-type sequence in −220/+890MColhGH and the mutation confirmed by direct sequencing. The mutated sequence is identified with an asterisk in Table I.

Preparation of Nuclear Extracts and Mobility Shift Assays

Exponentially growing Rat1 and *ras*-transformed Rat1 cells were harvested by scraping, washed once each with PBS and hypotonic buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCL, 0.1 mM PMSF, 100 μg/ml leupeptin, 5 mM sodium fluoride, 1 mM sodium vanadate, 0.5 mM DTT), and then swollen for 10 min in 3 packed cell volumes of hypotonic buffer. All steps were performed at 4°C. Cells were homogenized with 10 strokes of a type B pestle, nuclei were pelleted, and then resuspended in one packed nuclear volume of nuclear extract buffer (20 mM Hepes, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.7 M KCl, 0.5 mM PMSF, 100 μg/ml leupeptin, 5 mM sodium fluoride, 1 mM sodium vanadate, 1 mM DTT). Nuclear proteins were extracted with gentle rocking at 4°C for 30 min, followed by centrifugation at 25,000*g*. The resultant supernatant was dialyzed against nuclear dialysis buffer (20 mM Hepes, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.1 mM PMSF, 0.4 mM sodium fluoride, 0.4 mM sodium vanadate, 1 mM DTT) for 2 h at 4°C, recentrifuged, and supernatant stored in aliquots at −80°C. Protein concentrations were determined by the Bradford assay.

For mobility shift assays, nuclear extract (generally 10–30 μg) was mixed with specific and non-specific [poly (dI-dC) · poly(dI-dC), 1 μg] competitor DNAs in a final reaction volume of 20 μl in nuclear dialysis buffer with 5 mM $MgCl_2$. After incubation for 5 min at room temperature, end-labeled probe (approximately 0.1 ng, 30,000 CPM) was added and the incubation continued for another 20 min at room temperature. Reaction products were electrophoresed in a 5% native polyacrylamide gel in 0.4× Tris-borate EDTA for 2 h at 150 V. Gels were dried and exposed to Kodak X-OMAT AR scientific imaging film.

Oligonucleotides were synthesized using an Applied Biosystems (Foster City, CA) DNA synthesizer, and complementary strands annealed by heating to 95°C and slow cooling to room temperature. Double-stranded oligonucleotides were end-labeled with [γ-^{32}P] ATP (6,000 Ci/mMol) and gel-purified prior to use. SP1 and AP1 consensus oligonucleotides were obtained from Promega (Madison, WI). The sequence of the wild-type α1(I) AP1 oligonucleotide was 5′ GCTGCGGGATGATTCATAAGGAAAG 3′

(AP1 site underlined), while that for the mutant α1(I) AP1 oligonucleotide was 5′ GCTGCGGG-ATAGTAGATAAGGAAAG 3′ (introduced base changes in bold letters). The sequence of the consensus AP1 oligonucleotide was 5′ CGCTT-GATGAGTCAGCCGGAA 3′ (AP1 site underlined).

RESULTS

In a previous report [Slack et al., 1992], we demonstrated that the transcription rate of the α1(I) gene was 5- to 10-fold lower in *ras*-transformed Rat1 fibroblasts than in control cells. We wished to determine whether this difference in transcription was accompanied by differences in chromatin structure, as assayed by DNase I hypersensitivity. DNase I hypersensitive sites are often associated with gene activation or repression and can thus identify potential transcriptional regulatory elements [Elgin, 1988; Gross and Garrard, 1988]. Nuclei were isolated from exponentially growing Rat1 and *ras*-transformed Rat1 fibroblasts and subjected to digestion with increasing amounts of DNase I; areas of increased sensitivity to DNase I were detected by hybridization with a 260 bp probe from the 5′ end of an 8.4 kb *Eco*R1-*Eco*R1 fragment, encompassing 3.4 kb of α1(I) 5′ flanking sequence and 5 kb of α1(I) coding sequence. Several distinct differences were observed in the DNase I hypersensitivity patterns between Rat1 cells and their *ras*-transformed counterparts (Fig. 1). In Rat1 cells, there were 3 sites in the α1(I) 5′ flanking region (Fig. 1B, sites A, B, and C), at approximately −2600, −1400, and −200, which were hypersensitive to DNase I digestion. Sites A and B were not present in *ras*-transformed cells, while site C was present but less sensitive to DNase I. While the proximal promoter site (C) has been previously identified as a

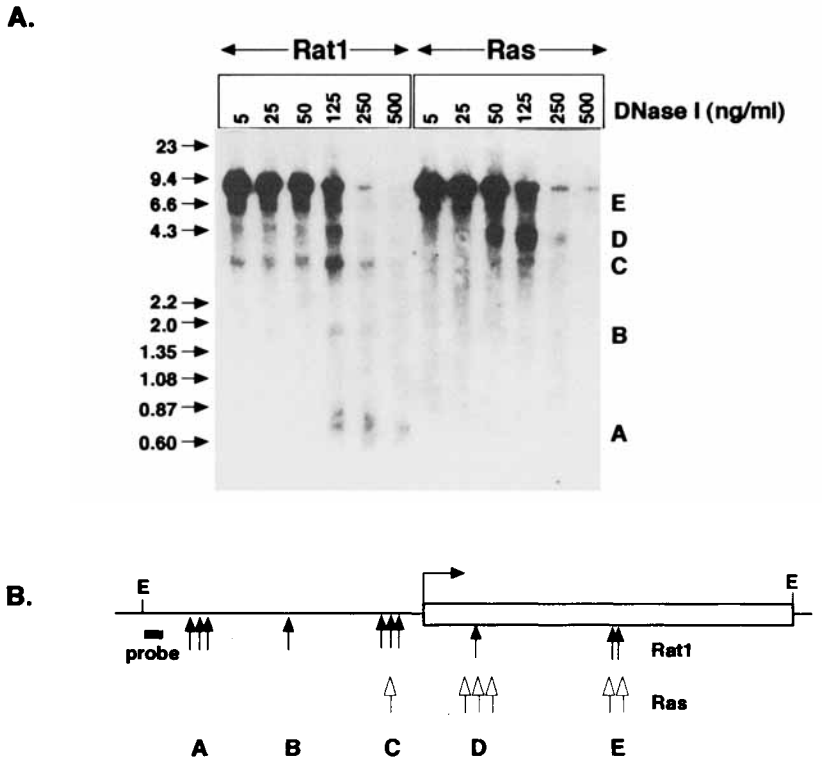

Fig. 1. DNase hypersensitivity analysis of the rat α1(I) gene. **A:** Nuclei from exponentially growing Rat1 fibroblasts (Rat1) and *ras*-transformed Rat1 fibroblasts (Ras) were subjected to digestion with increasing amounts of DNase I as described in Materials and Methods. Following digestion with *Eco*RI, DNA was electrophoresed, transferred to nitrocellulose, and hybridized to a 260 bp *Eco*RI/*Bam*HI fragment ("probe" in **B**) which extends from −3407 to −3147 in the rat α1(I) 5′ flanking region. Regions sensitive to DNase I produce fragments of the parental 8.4 kb band; their approximate locations and relative intensities are indicated schematically in B. Depicted in B is the 8.4 kb *Eco*RI (E) fragment from the 5′ region of the rat α1(I) gene, with the open rectangle representing transcribed DNA. Open (Ras) and closed (Rat1) vertical arrows indicate regions of DNase I sensitivity in the two cell types; the number of arrows in each case reflects the relative intensities of the bands seen in the autoradiogram in A.

region specific for collagen-expressing cells and tissues [Breindl et al., 1984; Brenner et al., 1993], the sites at −2600 and −1400 have not been described previously. In both the Rat1 and *ras*-transformed cells, there was a broad area of DNase I sensitivity in the proximal to mid portion of the first intron, at approximately +900 (Fig. 1, site D); this site was somewhat less sensitive to DNase I digestion in Rat1 cells. Site D has also been described in human and mouse, and has been reported to be independent of the levels of collagen gene expression [Barsh et al., 1984; Breindl et al., 1984]. Finally, there was a region at approximately +2600, equally sensitive to DNase I digestion in both cell types (Fig. 1, site E), which correlates with an ubiquitous site previously described in the mouse α1(I) gene [Breindl et al., 1984; Brenner et al., 1993]. The function of this site, if any, is unknown.

The results of the DNase I hypersensitivity analysis suggested the presence of regulatory elements in the 5′ flanking region and first intron of the α1(I) gene which might be mediating the response of the gene to *ras*. Previous attempts to define such elements using human α1(I) reporter constructs in transient transfection assays had been unsuccessful [Slack et al., 1992]. However, a plasmid containing the mouse α1(I) gene driven by 3.7 kb of 5′ flanking DNA (termed WTC1) was appropriately regulated by *ras* in a stable transfection assay [Slack et al., 1992]. Figure 2 shows that the WTC1 construct was regulated appropriately by *ras* in a transient transfection assay as well. For these and subsequent experiments, parental Rat 1 fibroblasts and *ras*-transformed Rat1 fibroblasts were simultaneously transfected with an experimental plasmid (WTC1 or a derivative) and a cotransfection control (either MT-hGH or MT-bGH), followed 48 h later by harvest of total cellular RNA. Levels of WTC1-specific transcripts in each cell type were quantitated by S1 nuclease assay [Wu et al., 1990], while RNase protection was used to measure activity of the cotransfected control plasmid (Fig. 2). After correction for transfection efficiency, the relative expression of WTC1 or its derivatives in *ras* vs. Rat1 cells was expressed as a ratio; for endogenous α1(I), this ratio was approximately 0.1, indicating that steady-state α1(I) mRNA levels in *ras*-transformed cells were, on average, 10% of the levels seen in control cells. For WTC1 (Fig. 2), this ratio averaged 0.18 ± .04 in five independent experiments, indicating that the regulation of the transfected gene closely resembles, in a transient transfection assay, the regulation of the endogenous α1(I) gene.

To further identify the regions in the α1(I) gene that were responsive to *ras*, a series of deletion plasmids were prepared and tested in transient transfection assays as described above. To provide for polyadenylation and processing, each α1(I) sequence was linked to a small fragment of the hGH gene (containing part of intron 4, all of exon 5, and the polyadenylation region). Splice sites were preserved in all gene fusion constructs. Deletion of a large segment of the 3′ portion of WTC1 (a 16 kb stretch of DNA from +4400 to beyond the native polyadenylation signals) resulted in a construct which was regulated essentially identically to both the parental WTC1 plasmid and the endogenous α1(I) gene (Fig. 3A,B: −3700/+4400 construct). In contrast, a plasmid which contained only 220 bp of α1(I) promoter and 500 bp downstream of the transcriptional initiation site (−220/+500) was expressed at nearly identical levels in both *ras* and Rat1 cells (Fig. 3A,B). Thus, although the region between −220 and +500 in the α1(I) gene may retain a minor degree of *ras*-responsiveness, the above results suggested that the bulk of the inhibition by *ras* was mediated by elements located between either −220 and −3700 or between +500 and +4400. When these two regions were tested independently in conjunction with the −220/+500 construct, each was found to confer partial *ras*-mediated inhibition on the basal construct (Fig. 3A,B), suggesting that independent *ras*-responsive elements were located in each of these regions.

As previously discussed, the regulation of the −3700/+4400 MCol-hGH plasmid by *ras* essentially mirrored that of the endogenous α1(I) gene (Fig. 3). A deletion of the region between +1390 to +4400 in this plasmid had essentially no effect on its regulation by *ras* (Fig. 4A), a finding which suggested that the putative 3′ *ras* inhibitory element was located between +500 and +1390. Similar results were seen in the context of a −220 promoter (Fig. 4B). Further deletions were therefore made in the +500 to +1390 fragment and tested in the context of the −220 promoter (Fig. 4C). The relative expression in Rat1 and *ras* cells decreased significantly when constructs −220/+500 and −220/+890 were compared, suggesting that the stretch of DNA between +500 and +890 contained sequences that were differentially regulated by *ras*

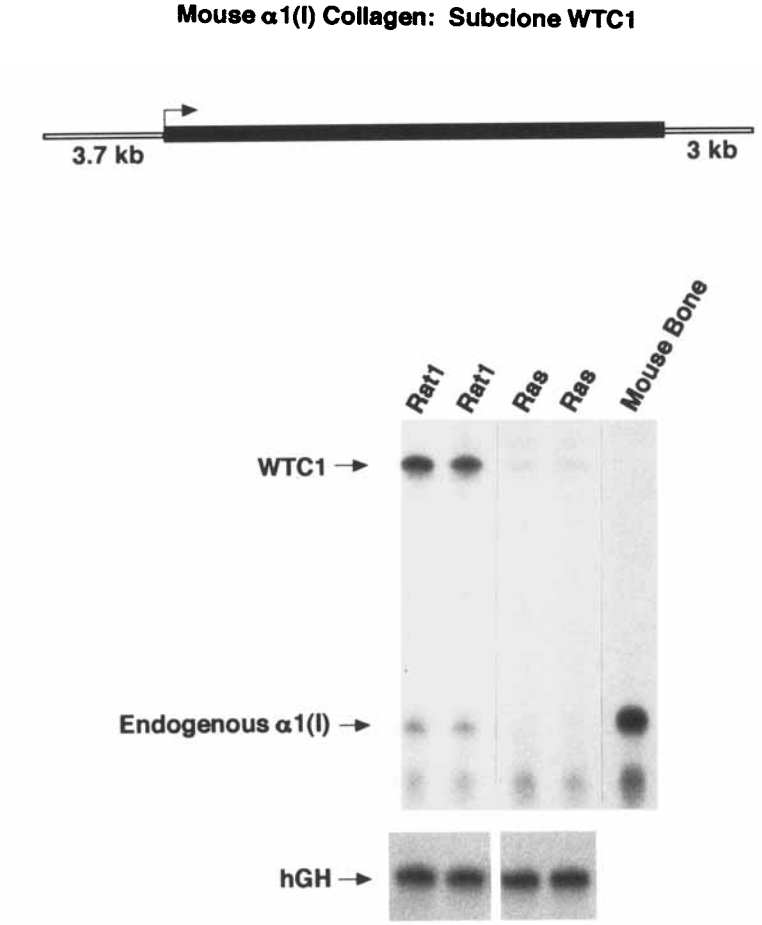

Fig. 2. Expression of mouse α1(I) subclone WTC1 in Rat1 and *ras*-transformed Rat1 fibroblasts. **Top:** Schematic representation of mouse α1(I) collagen subclone WTC1, which contains approximately 3.7 kb of 5'- and 3 kb of 3'-flanking sequence (open rectangles), as well as the entire mouse α1(I) gene (filled rectangle). There is a polylinker insert in the first exon (not shown), which allows differentiation between WTC1-specific and endogenous α1(I) transcripts in an S1 nuclease assay [Wu et al., 1990]. **Bottom:** Rat 1 and *ras*-transformed Rat1 fibroblasts were transfected with WTC1 and MT-hGH (which served as a contransfection control) using the calcium phosphate technique. After 48 h, total cellular RNA was prepared and expression of each plasmid was analyzed by subjecting equal amounts of RNA (in duplicate) to S1 (WTC1) or RNase protection (hGH) analysis as outlined in Materials and Methods. The two cell types were equivalent in their ability to take up and process plasmid DNA, as indicated by their equal expression of MT-hGH (hGH band) in this and other experiments. However, like the endogenous gene, WTC1 was expressed at much lower levels in the *ras*-transformed than in the parental cells (WTC1 and endogenous bands). This experiment was repeated 5 times with essentially identical results (see text). S1 analysis of mouse bone RNA, included as a control, demonstrated the protection only of a probe fragment of the expected size for endogenous α1(I). The band below the endogenous α1(I) band is a nonspecific degradation product.

in the two cell types. Since the values in Figure 4C are reported as ratios, it was possible that this intronic fragment was acting as a positive control element in Rat1 cells (hence increasing the denominator) or as a negative element in *ras* cells (hence decreasing the numerator). Analysis of the activity of this fragment in each cell type indicates that the former scenario is correct, i.e., the +500 to +890 intronic fragment functions as a positive element in Rat1 fibroblasts but is completely inactive in *ras*-transformed cells (Table I).

Previous work from our laboratory, and others, has established that a functional AP1 site (TGATTCA) exists in the first intron of the human α1(I) gene [Katai et al., 1992; Liska et al., 1990]. This site is conserved among human, mouse, and rat [Liska et al., 1990], although its function has not been demonstrated in the latter 2 species. Based on these considerations, it seemed reasonable to ask whether the positive effect of the 500–890 fragment in Rat1 fibroblasts was mediated by the AP-1 site at +555. The AP1 site was thus altered by site-directed

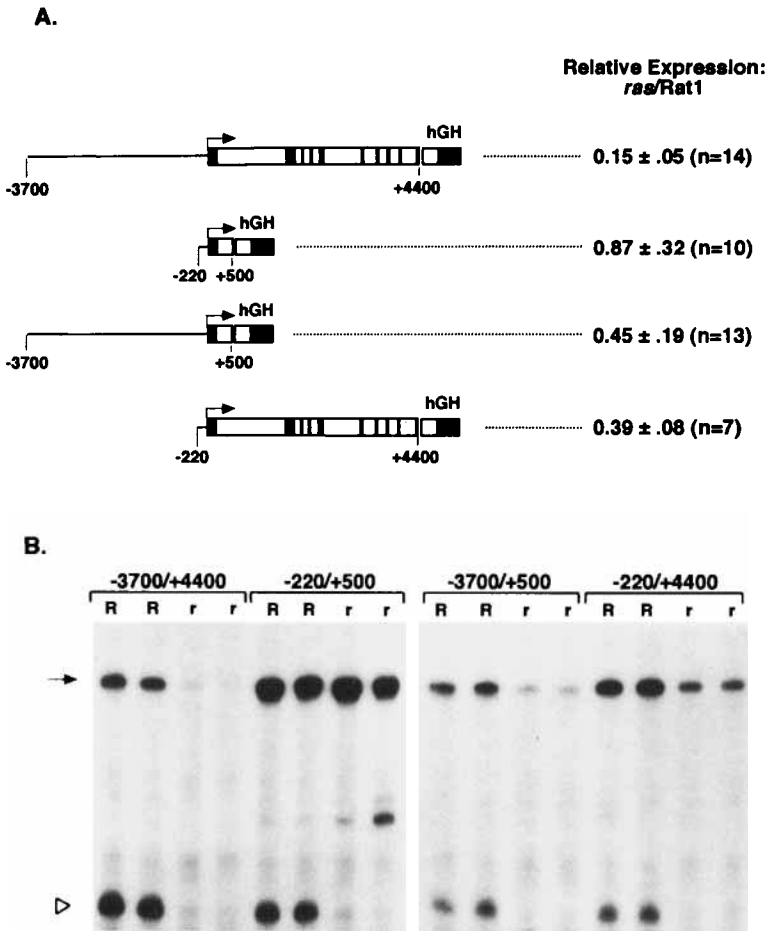

Fig. 3. Expression of α1(I)-hGH chimeric plasmids in Rat1 and *ras*-transformed Rat1 fibroblasts. **A:** Schematic representation of α1(I)-hGH constructs and their relative expression in transiently transfected *ras*-transformed and parental cells (see text for discussion). Numbering is relative to the start of transcription of the α1(I) gene (indicated by the bent arrow). Indicated in the drawing are α1(I) 5′-flanking DNA (solid thin line), α1(I) and hGH exons (black rectangles), α1(I) and hGH introns (open rectangles), and hGH 3′ untranslated sequences (shaded rectangle). **B:** Representative S1 assay of the constructs depicted in A. Duplicate analyses were performed as outlined in Materials and Methods and in the legend to Figure 2. R = Rat1; r = *ras*-transformed Rat1 cells. The arrow (top) depicts the signal generated by transgene-specific RNA, while the open arrowhead (bottom) indicates that of endogenous α1(I) RNA.

mutagenesis (from TGATTCA to TAGTAGA) and the mutant construct tested in transient transfection assays. Table I shows that the AP1 site was responsible for the modest but reproducible enhancing activity of the 500–890 fragment.

The absence of function of the α1(I) AP1 site in the *ras*-transformed cells could be explained either by quantitative or qualitative differences in the factors that interact with this element. AP1 sites bind members of the *fos* and *jun* transcription factor families [Curran and Franza, Jr., 1988] either as homo- (*jun-jun*) or hetero- (*jun-fos*) dimers. We prepared nuclear extracts from Rat1 and *ras*-transformed Rat1 cells and tested binding to the α1(I) AP1 site by mobility shift assays. Figure 5A demonstrates specific binding to the α1(I) AP1 oligonucleotide by nuclear proteins from Rat1 and *ras* cells (bands 1 and 2). Although both extracts gave rise to a similar pattern of DNA/protein binding, complex 2 migrated more slowly when generated with nuclear extract from *ras*-transformed cells (Fig. 5A,B). With both extracts, formation of complex 2 was inhibited completely by the wild-type α1(I) AP1 oligonucleotide (Fig. 5B, lanes 2 and 7) and by a consensus AP1 oligonucleotide (Fig. 5B, lanes 4 and 9), but not by the mutant α1(I) AP1 oligonucleotide (Fig. 5B, lanes 3 and 8) or by an SP1 control oligonucleotide (Fig. 5B,

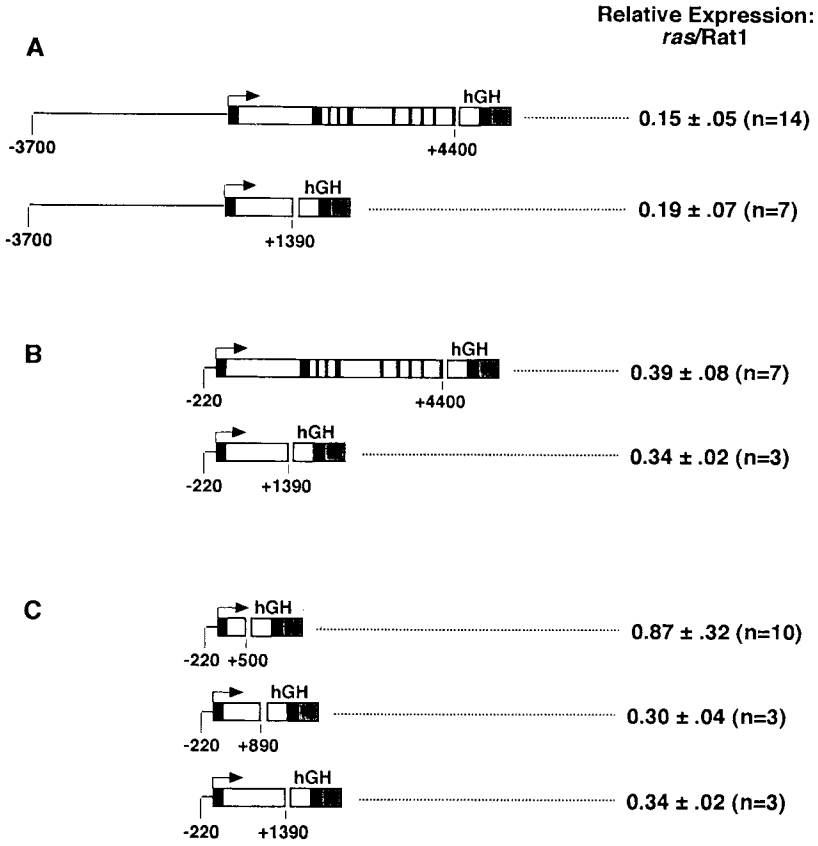

Fig. 4. Deletion analysis of the +500 to +4400 region of the mouse α1(I) gene. Schematic depiction of constructs tested and their relative expression in *ras*-transformed and Rat1 fibroblasts. Analyses were performed by transient transfection as outlined in Materials and Methods and in the legend to Figure 2.

TABLE I. Expression of MCol-hGH Constructs in *ras*-Transformed and RatI Cells*

Construct	*ras*	Rat1
−220/+500 MCol-hGH	1.00	1.00
−220/+890 MCol-hGH	0.86 ± 0.21	1.94 ± 0.33
−220/+890*MCol-hGH[a]	0.60 ± 0.08	0.87 ± 0.17

*Expression of −220/+500 MCol-hGH is set at 1. Results are the average of three independent experiments with the standard deviation noted.
[a]AP1 mutation; see text for details.

lanes 5 and 10). This result strongly suggests that members of the *fos* and/or *jun* transcription factor families are responsible for complex 2 formation. The subtle difference in mobility shift patterns generated by the two extracts further suggests that there are qualitative and/or quantitative differences in AP1 factors in the Rat1 and *ras*-transformed Rat1 cells. Interestingly, the formation of the faster migrating band (1) was competed (partially) only with the wild-type α1(I) AP1 sequence and not with the consensus AP1 sequence (Fig. 5B). This finding suggests that non-AP1 nuclear proteins may also interact with the α1(I) AP1 site. However, in view of the relatively poor competition, even with the α1(I) AP1 oligonucleotide, this band may also be the result of nonspecific binding.

We also tested nuclear extracts from both cell types for their ability to bind to a consensus AP1 oligonucleotide (Fig. 6). The mobility shift pattern was similar but not identical to that seen with the α1(I) AP1 oligonucleotide (compare Fig. 5A with Fig. 6). The predominant shifted band seen in Figure 6 correlated with complex 2 in Figure 5A and B. As with the α1(I) AP1 oligonucleotide, there were differences in binding between the Rat1 and *ras* extracts; whereas only one shifted complex was observed when the Rat1 extract was used (Fig. 6, open arrowhead), two bands were seen with the *ras* extract (Fig. 6, filled arrowheads). In addition, complex 1, seen in Figure 5A and B, was not observed when the consensus AP1 oligonucleotide was used as a

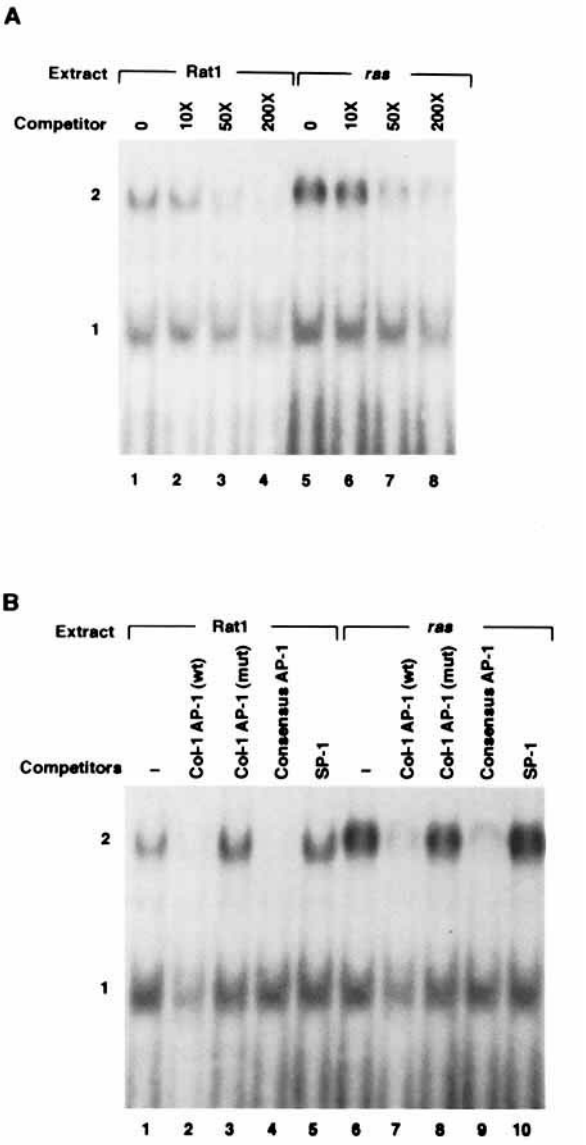

Fig. 5. Analysis of DNA/protein binding interactions at the intronic α1(I) AP1 site. Nuclear extracts were prepared from Rat 1 and *ras*-transformed Rat1 cells and electrophoretic mobility shift assays were performed as described in Materials and Methods. **A:** 30 μg of Rat1 (**lanes 1–4**) or *ras* (**lanes 5–8**) extract was incubated with the end-labeled α1(I) AP1 oligonucleotide in the presence of increasing amounts of unlabeled α1(I) AP1 fragment. Two shifted bands were reproducibly observed (1 and 2). **B:** 30 μg of each extract was incubated with labeled α1(I) AP1 oligonucleotide in the presence of the following competitor oligonucleotides at 200-fold molar excess: wild-type α1(I) AP1 (**lanes 2** and **7**), mutant α1(I) AP1 (**lanes 3** and **8**), consensus AP1 (**lanes 4** and **9**), and SP1 (**lanes 5** and **10**).

probe. With increasing amounts of competitor DNA, an additional, faster migrating band was often observed (Fig. 6, lanes 3, 4, 7, 8), but the origin or significance of this band is not clear.

DISCUSSION

The α1(I) collagen gene is expressed less efficiently in cells transformed by a variety of agents, including dominantly-acting oncogenes such as *ras* [Eizenberg and Oren, 1991; Slack et al., 1992]. We show here that the decrease in α1(I) transcription in *ras*-transformed cells is accompanied by an altered pattern of DNase I hypersensitivity of the α1(I) gene. Furthermore, we have identified two regions, one 5′ to the α1(I) transcriptional start site and one in the first intron, which mediate the effects of *ras* on α1(I) transcription. Preliminary experiments suggest that a *ras*-specific inhibitory element is located between −2800 and −1050 in the α1(I) 5′-flanking region, and experiments to further characterize this region are currently in progress. The intronic site encompasses a previously-described AP1 motif [Liska et al., 1990] which enhances α1(I) transcription in Rat1 fibroblasts but has no activity in the same cells transformed by *ras*. While this AP1 site binds nuclear proteins from both Rat1 and *ras*-transformed cells, the patterns of binding are qualitatively different, suggesting that differences in relative amounts or activities of Jun and/or Fos family members account for at least part of the inhibition of α1(I) transcription by *ras*.

Several groups have assayed AP1 activity or function in *ras*-transformed cells. Kamada et al. [1994] reported that there were no differences in amounts or phosphorylation states of c-Jun between NIH3T3 cells and *ras*-transformed NIH3T3 cells when both cell types were grown in 5% serum. Nevertheless, the activity of an AP1-responsive reporter plasmid (TRE-CAT) was 5- to 10-fold higher in the *ras*-transformed cells. In mobility shift assays, there were both quantitative and qualitative differences in binding to an AP1 consensus sequence by extracts of exponentially growing, serum-replete normal cells and *ras*-transformed cells [Kamada et al., 1994], although the binding patterns were much different from those reported here. In contrast, Pfarr et al. [1994] reported that *ras*-transformed NIH3T3 cells contained 5-fold more c-Jun protein than untransformed cells when both cell types were grown in 7% fetal calf serum. Our data (Figs. 5 and 6) indicate that, while the quantitative level of AP1 binding is similar with extracts of Rat1 and *ras*-transformed Rat1 fibroblasts (both grown in 10% serum), there are

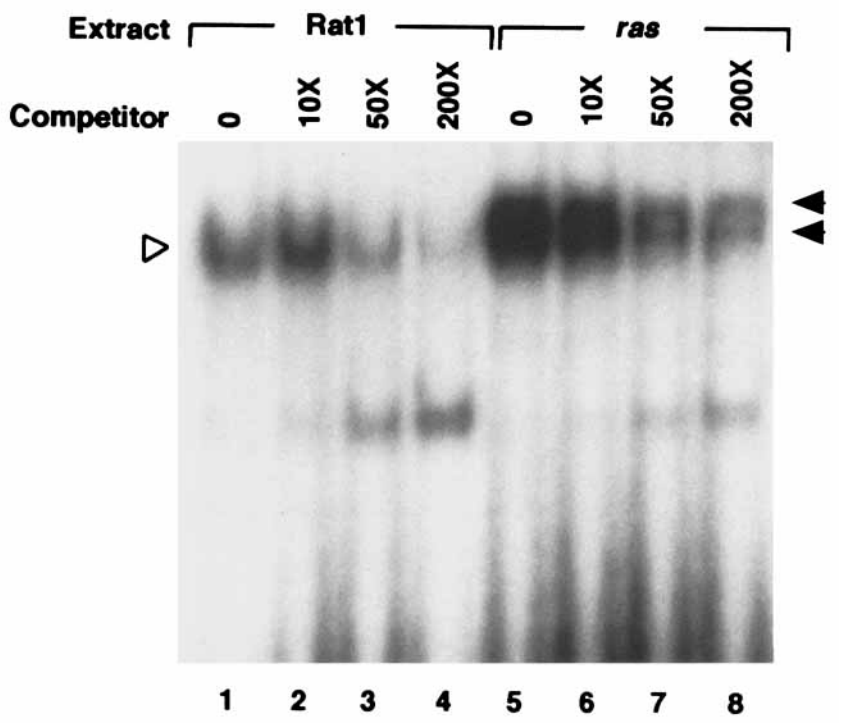

Fig. 6. Nuclear protein binding to a consensus AP1 oligonucleotide with extracts from Rat1 and *ras*-transformed cells. Electrophoretic mobility shift assays were performed as previously outlined, but using a consensus AP1 oligonucleotide whose sequence is given in Materials and Methods. Thirty micrograms of nuclear extract was used in each lane. Competition was with increasing amounts of unlabeled consensus AP1 oligonucleotide.

qualitative differences in the bands seen in mobility shift assays.

A previous study by Breindl et al. [Breindl et al., 1984] documented a DNase I hypersensitive site in the mouse α1(I) promoter that was specific for collagen expressing cells; an equivalent site in the rat α1(I) promoter (Fig. 1, site C) also correlated with collagen expression, since it was more sensitive to DNase I digestion in Rat1 than in *ras*-transformed cells. This site, given its location and cell-specificity, very likely reflects binding of transcription factors critical for high level α1(I) transcription. Some or all of these factors, which include NF1, SP1 [Nehls et al., 1991], and presumably TATA-binding proteins, are present in both untransformed and *ras*-transformed cells, since a transfected construct which contains the proximal α1(I) promoter is expressed almost equally well in both cell types (Fig. 3, −220/+500 plasmid). Presumably, the relatively closed chromatin conformation in the promoter region of the endogenous α1(I) gene in the *ras*-transformed cells (Fig. 1, site C) prevents access of some or all of these factors to the DNA. The inference of this argument is that nucleosome positioning in the α1(I) basal promoter is sensitive to *ras* transformation, but not because *ras* down-regulates factors which bind to this region. Ras-sensitive factors that bind elsewhere in the gene may therefore determine the chromatin conformation of the α1(I) promoter and in this way dictate the transcriptional activity of the α1(I) gene.

We also observed 2 novel DNase I hypersensitive sites in the 5′ flanking region of the rat α1(I) gene, at −2600 and −1400 (Fig. 1, sites A and B). These sites appeared to correlate with α1(I) transcription, since they were not observed in the *ras*-transformed cells; however, we could identify no strongly positive *cis* elements in this region of the α1(I) gene in transient transfection assays (data not shown). As reported by Nitsch et al. [1990], it is not always possible to correlate cell-specific hypersensitive sites with function in transient transfection assays, and thus a full understanding of the role of these sites in modulating α1(I) gene expression must await further study. It is worthwhile noting, however, that in transgenic mice a strong tissue-specific enhancer(s) has been mapped to this general region

[Krebsbach et al., 1993; Pavlin et al., 1992; Slack et al., 1991].

Our previous results [Slack et al., 1992] had suggested that *ras*-responsive elements in the α1(I) gene would be found either in the body of the gene itself or in the distal 3′ flanking region. We were therefore surprised to find that a mouse α1(I) plasmid, containing 3,700 bp of 5′ flanking sequence and as little as 1,390 bp of DNA downstream of the transcriptional start site, was regulated appropriately by Ras (Fig. 4, −3700/+1390 construct). In particular, we had previously concluded that the first intron of the human α1(I) gene played no role in regulation by *ras* [Slack et al., 1992]. The reasons for the discrepancy with the current study are not clear but most likely relate to the use of human constructs in the previous series of experiments. Thus, although the human, mouse, and rat α1(I) genes respond similarly to transformation by *ras,* the precise mechanisms that are used to effect this change may differ among species.

In transient transfection assays in cultured fibroblasts, deletion of most of the first intron results in an inhibition of α1(I) transcriptional activity [Bornstein et al., 1988b; Liska et al., 1990]. Liska et al. [1990] originally identified a highly conserved AP1 site in the proximal portion of the first intron that is responsible for a modest but reproducible positive effect on α1(I) transcription. Other groups [Katai et al., 1992; Maatta et al., 1993] have now confirmed these observations, suggesting that this AP1 site is a critical determinant of α1(I) transcriptional activity. That this AP1 site can have both positive and negative effects on α1(I) expression was first reported by Katai et al. [1992]; these investigators reported that the AP1 site functioned as a negative element in a human rhabdomyosarcoma cell line, but as a positive element in all other cell lines tested. We report here that this AP1 site is inactive in *ras*-transformed fibroblasts, but has a positive effect in parental cells (Table I), and accounts in part for the decreased level of α1(I) transcription in *ras*-transformed cells. Although modest, this 2-fold effect on collagen expression could be physiologically significant if one considers that type I collagen is synthesized over substantial periods of time in many tissues.

Since our assay measures steady-state mRNA levels rather than transcription directly, it is theoretically possible that the effects of the AP-1 site are on transcript processing or stability, instead of on transcription. Our constructs were designed to preserve the endogenous α1(I) splice sites and orientation of the AP-1 site relative to the α1(I) promoter. We believe it is unlikely that site-specific mutation of an intronic sequence would lead to significant changes in transcript processing or stability. We therefore suggest that the AP-1 site, by binding appropriate transcription factors, modulates transcription of the α1(I) gene.

The critical question raised in this study is how is the α1(I) AP1 site inactivated in *ras*-transformed cells, particularly since, as discussed above, AP1 (c-Jun) levels and/or activity are in general higher in *ras*-transformed as compared with normal cells [Kamada et al., 1994; Pfarr et al., 1994]. One possibility is that the α1(I) AP1 site does not bind c-Jun, and that the binding interactions observed in Figure 5 are due to other Jun and/or Fos family members that may differ from c-Jun in their effects on cell growth and gene expression. It has been recently reported that JunD and c-Jun have opposing effects on cell growth [Castellazzi et al., 1991; Pfarr et al., 1994], with JunD being the primary component of AP1 complexes in quiescent, slowly proliferating cells. It was further suggested [Pfarr et al., 1994] that JunD (probably as a homodimer) enhances the transcription of genes that are up-regulated in quiescent cells and thus potentially involved in growth inhibition. In fact, α1(I) collagen is such a gene [Coppock et al., 1993; Kindy et al., 1988]. It is possible that the α1(I) intronic AP1 site preferentially or exclusively binds JunD, and that JunD activates the transcription of α1(I) collagen through this site. Such a model predicts that the level of JunD would be lower in *ras*-transformed than in untransformed cells, a prediction that is supported by experimental data [Pfarr et al., 1994].

It is also possible that the effects of c-Jun on the α1(I) AP1 site are modulated by other regulatory factors or by post-translational modification of c-Jun itself. In the first scenario, c-Jun binds to the α1(I) AP1 site in both Rat1 and *ras*-transformed Rat1 cells, but fails to transactivate in the transformed cells, due either to the presence of a *ras*-specific inhibitor or to the loss of a Rat1-specific coactivator. Along these lines, it has been reported that increasing levels of c-Fos can convert a glucocorticoid regulatory element from a transcriptional activator into a transcriptional repressor, despite a constant level

of c-Jun [Diamond et al., 1990]. Transformation by *ras* is known to result in phosphorylation of c-Jun on serines 63 and 73, but in the systems studied this has led to increased, rather than decreased, c-Jun activity [Smeal et al., 1992]. In the context of the α1(I) AP1 site, however, phosphorylated c-Jun may be less effective than its unphosphorylated counterpart in stimulating transcription, perhaps due to differences in flanking sequences between the α1(I) AP1 site and previously characterized AP1 sites. A full understanding of the function of the α1(I) intronic AP1 site will clearly require identification of the specific AP1 components that interact with this element and characterization of the functional consequences of these interactions in both transformed and untransformed cells.

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