Many putative MAPK nuclear acceptor proteins have been suggested in the Egr, Ets, Fos, or AP-1 families (2–7). The \( \alpha(1) \) collagen gene contains AP-1 sites present in its 5′-UTR and 1st intron. These sites could represent the downstream target of the Ras cascade (8–15).

We evaluated the role of the Ras-Raf-MAPK cascade during collagen gene expression using cultured hepatic stellate cells (HSC), the major collagen-producing effector cell responsible for hepatic fibrogenesis (16–21). This system utilizes activated early passage cells, which recapitulate many features of the diseased stellate cell in vivo. (16–21). This model can serve as a paradigm of the enhanced collagen gene expression, which occurs in vivo during liver injury and fibrogenesis (16–21). Dominant negative inhibitory mutants were used to specifically consider the roles of Ras, Raf, and MAPK. Overexpressing activated constructs were avoided. Oncogenic forms of ras and raf are not involved in hepatic fibrogenesis. In addition, these constructs may abnormally stimulate a pathway with little physiologic relevance (4–7, 20, 21).

It was found that blockage of Ras or Raf activity led to an increase in collagen gene expression. This is consistent with the Ras overexpression studies previously mentioned. Surprisingly, however, blockage of MAPK activity decreased collagen gene expression. When both Raf and MAPK activity were simultaneously blocked, collagen gene expression was decreased. These data suggest a branch point between Ras → Raf and MAPK. A MAPK-stimulatory cascade is balanced with a Ras → Raf-inhibitory cascade. The two separate cascades mapped to two distinct regions of the 5′-UTR, unrelated to AP-1 domains. The MAPK cascade involved the ubiquitous Sp-1 and NF-1 transcription factors in the proximal ∼100 bp domain. The Ras → Raf cascade utilized a more upstream −1680 bp domain. This latter domain involves a novel 60-kDa DNA-binding protein, which is selectively produced by activated stellate cells in culture and following activation in vivo.

**EXPERIMENTAL PROCEDURES**

Cell Culture—HSC were isolated from Sprague-Dawley male rats and subcultured by previously described methods (16, 17). Experimental manipulations were performed with cells at passages 2–6.

Transfection Studies—Stellate cells were transfected using the LipofectAMINE reagent, and cell extract handling, extraction, quantitation, and CAT measurements were performed as described previously (22, 23). Following transfection, the cells were maintained in serum (10% fetal calf, 10% calf serum) for 48 h prior to CAT analysis. The plasmids used for transfection included the intracAT plasmids (−3.6/1.0 or −1.7/1.6), which contain either −3.6 or −1.7 kb of the 5′-UTR of the rat \( \alpha(1) \) collagen linked to the 1st exon and 1.6 kb of the 1st intron and the CAT reporter gene (9). The −1.3/1.6 intcolCAT plasmid and the −0.4/1.6 intcolCAT plasmid were derivatives of the −3.6/1.6 intcolCAT plasmid produced by digestion with NheI/Tth1111 and NheI/MfeI restriction endonucleases, respectively. Ends were then blunted and ligated by T4 DNA ligase. The pde1.3–0.4/1.6 plasmid was a derivative of the −1.7/1.6 plasmid, which was produced by digestion with Tth1111/MfeI. This created a deletion from −1284 bp to −392 bp, and then the ends were blunted and ligated with T4 DNA ligase. The −3.6siteTAE/1.6 plasmid was created by overlap extension and contained the mutated TGFβ activation element (TAE(A)) described below (24). All mutated plasmids were sequenced to confirm the positions of the mutations. All transfections contained equivalent amounts of plasmid DNA (1.5 μg) by using empty vector pMNC plasmid. Cotransfections utilized either pMNC or CMV-driven dominant negative raf (301–1 plasmid), dominant negative ras (N-17), dominant negative or wild type MAPK (pCMVp51/Ala54/55ΔSer, GBCAT (Sp-1 CAT reporter), or BCAT-1 (Sp-1 CAT "control" reporter) plasmids (2–7). Transfection efficacy was monitored by paral-
negative

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cells cotransfected with a collagen reporter gene and either
Stellate

dominant negative raf plasmid or dominant negative MAPK plasmid yielded divergent changes in gene expression (Fig. 1A). Dominant negative raf transfection resulted in a 5-fold increase in reporter expression. Surprisingly, when dominant negative MAPK was transfected, a 3-fold decrease in reporter expression was found. Previous HSC studies used these same dominant negative plasmids to demonstrate raf’s and MAPK’s respective roles in insulin growth factor and 1,25-dihydroxyvitamin D₃ nuclear signaling (23). By varying the amount of input DNA, the optimal dominant negative raf or MAPK plasmid concentration was determined (Fig. 1, B and C). Based on previous studies with these same plasmids, the optimal plasmid concentrations should result in sufficient amounts of the mutated kinase proteins to bind the upstream cascade proteins (either Ras or MEK, respectively) (2–7). However, the precise mechanism associated with the dominant negative plasmid effect is unclear. The dominant negative MAPK suppression of reporter expression was observed in the absence (Fig. 1C) or presence of dominant negative raf (Fig. 1D). This reduction in coCAT expression by dominant negative MAPK (1 base line versus 0.35 (dominant negative MAPK) (CAT units/mg of protein)) contrasts markedly with the increase in coCAT expression (to 5.5 CAT units/mg of protein) obtained by transfecting comparable amounts of the wild type MAPK plasmid (data not shown). In other studies, dominant negative ras transfection caused a 3.3-fold increase in reporter expression (data not shown), which mimics the dominant negative raf effect. These findings imply that the suppressive pathway is likely to involve a Ras → Raf link (2–7). Collectively, these results suggest that Raf activation leads to two distinct effects on type I collagen promoter activity: (i) a suppressive effect via a MAPK-independent pathway and (ii) a stimulatory effect via a MAPK-dependent pathway. The latter effect is also likely to involve Raf-parallel pathways, which utilize other members of the enlarging MAPK kinase kinase family (2, 7). These studies further imply that there is an additional branch point between Raf and MAPK. The Ras-Raf-MAPK cascade could help regulate the expression of the major disease-related collagen gene during fibrogenesis. Accumulated evidence suggests that the diseased stellate cell initially responds to mitogenic stimuli (e.g. platelet-derived growth factor), which would be expected to activate Ras → Raf. The increase in stellate cell collagen gene

RESULTS AND DISCUSSION

Dominant Negative raf Versus Dominant Negative MAPK: Differential Regulation of Collagen Gene Expression—Stellate cells cotransfected with a collagen reporter gene and either dominant negative raf plasmid or dominant negative MAPK plasmid yielded divergent changes in gene expression (Fig. 1A). Dominant negative raf transfection resulted in a 5-fold increase in reporter expression. Surprisingly, when dominant negative MAPK was transfected, a 3-fold decrease in reporter expression was found. Previous HSC studies used these same dominant negative plasmids to demonstrate raf’s and MAPK’s respective roles in insulin growth factor and 1,25-dihydroxyvitamin D₃ nuclear signaling (23). By varying the amount of input DNA, the optimal dominant negative raf or MAPK plasmid concentration was determined (Fig. 1, B and C). Based on previous studies with these same plasmids, the optimal plasmid concentrations should result in sufficient amounts of the mutated kinase proteins to bind the upstream cascade proteins (either Ras or MEK, respectively) (2–7). However, the precise mechanism associated with the dominant negative plasmid effect is unclear. The dominant negative MAPK suppression of reporter expression was observed in the absence (Fig. 1C) or presence of dominant negative raf (Fig. 1D). This reduction in coCAT expression by dominant negative MAPK (1 base line versus 0.35 (dominant negative MAPK) (CAT units/mg of protein)) contrasts markedly with the increase in coCAT expression (to 5.5 CAT units/mg of protein) obtained by transfecting comparable amounts of the wild type MAPK plasmid (data not shown). In other studies, dominant negative ras transfection caused a 3.3-fold increase in reporter expression (data not shown), which mimics the dominant negative raf effect. These findings imply that the suppressive pathway is likely to involve a Ras → Raf link (2–7). Collectively, these results suggest that Raf activation leads to two distinct effects on type I collagen promoter activity: (i) a suppressive effect via a MAPK-independent pathway and (ii) a stimulatory effect via a MAPK-dependent pathway. The latter effect is also likely to involve Raf-parallel pathways, which utilize other members of the enlarging MAPK kinase kinase family (2, 7). These studies further imply that there is an additional branch point between Raf and MAPK. The Ras-Raf-MAPK cascade could help regulate the expression of the major disease-related collagen gene during fibrogenesis. Accumulated evidence suggests that the diseased stellate cell initially responds to mitogenic stimuli (e.g. platelet-derived growth factor), which would be expected to activate Ras → Raf. The increase in stellate cell collagen gene
Ras/ Raf and MAPK Regulate Collagen Gene Expression

Fig. 2. Dominant negative MAPK-induced suppression requires the NF-1 site in footprint 1, and not the TAE. HSCs were cotransfected with either empty vector pMNC or dominant negative MAPK plasmid and equivalent amounts of colCAT reporters, as described in Fig. 1. The various colCAT reporters are labeled and schematically depicted on the left. The relative amount of colCAT expression is displayed on the right ([□], pMNC; □, dn-MAPK), with the absolute amount of colCAT expression in the presence of the pMNC plasmid given an arbitrary value = 1 for each individual reporter. The parent pIntcolCAT reporter contains −3.6 kb of 5′-UTR region upstream of the 1st exon (depicted as a black box), which is serially linked to 1.6 kb of the 1st intron, the SV40 splice acceptor (depicted as a striped rectangle), and then the translation start site and the chloramphenicol acetyltransferase gene (depicted as a black box). The mutation of the NF-1 site of footprint 1 ([□], pMNC; □, dn-raf) is expressed as a doted oval in its respective plasmid (−3.6/F1mut/1.6). When this plasmid was used in cotransfections with pMNC, the absolute amount of CAT/mg protein was 4–5-fold reduced versus the −3.6/1.6 IntcolCAT reporter. The −3.6/siteTAE/1.6 plasmid contained the mutated TAE region (depicted as a hatched oval) (mTAE(A)) in situ.

The relative amount of colCAT expression occurs at a later time point. The fibrogenic stimuli responsible for this later increase are incompletely understood, but transforming growth factor β (TGFβ) is a likely mediator (17–21). The TGFβ kinase cascade involves several unique kinases, but their role in collagen gene expression is unknown (26, 27). Previous work suggests TGFβ may block Ras activation and therefore decrease Raf activation (28). During fibrogenesis, the stellate cell may be initially exposed to activated Raf and MAPK (in a platelet-derived growth factor-dominated stage). At the later TGFβ-dominated stage, activated Raf levels may decrease. This would be predicted to lead to an increase in collagen gene expression. The relative duration of Raf versus MAPK activation or the amount of nuclear activated MAPK could then determine the ultimate extent of collagen gene activation. In view of the development of selective MEK inhibitors, these observations may have therapeutic importance (29).

Dominant Negative raf Versus Dominant Negative MAPK Utilize Different DNA Response Elements—To identify the DNA response elements which are sensitive to the dominant negative (dn) MAPK versus dominant negative (dn) Raf effects, truncated reporter constructs were substituted for the parent plasmid. The dn-MAPK-sensitive region of the collagen gene was found to be independent of the 1st intron, which contains AP-1 binding sites (Fig. 2) (12–14). In addition, most of the 5′-UTR appears to be dispensable. Recent studies have suggested that the basal promoter activity lies within the most proximal 200 base pairs, which contain consensus Sp-1 binding sites (within a region termed footprint 2) and consensus NF-1 binding sites (within a region termed footprint 1) (8, 30). Site-directed mutagenesis of the NF-1 site in footprint 1 abolished the response to dn-MAPK (Fig. 2). In addition, cotransfections experiments found that dn-MAPK caused a 50% reduction in a Sp-1-driven CAT reporter versus no effect on a control Sp-1 reporter (data not shown). Therefore, the MAPK-sensitive region appears to involve both NF-1 and Sp-1 sites. Future studies will be needed to determine if this is a direct effect on the phosphorylation state of these transcription factors or an indirect effect involving other MAPK-sensitive nuclear factors.

Using the same approach, the dn-raf-sensitive region was localized to a different upstream region (Fig. 3A). Sequential deletion analysis revealed that the 1st intron is not required, but a region between −1.7 kb and −1.3 kb is needed. When this region was placed adjacent to the −400 bp region of the 5′-UTR, the previously unresponsive −400 bp-containing plasmid (−0.4/1.6) regained its sensitivity to dn-raf stimulation (8, 30).

Previous studies in other cell systems have suggested that this −1.7 to −1.3 kb region contains significant DNA binding activity at the −1.62 kb region, termed the TAE, a region previously shown to be required for TGFβ stimulation of the type I
TAE Binding Is Selective for in Vitro and in Vivo Activated Stellate Cells—TAE characterization studies revealed that HSCs contain a single retarded band when nuclear extracts are incubated with radiolabeled TAE in a gel retardation assay (Fig. 4A). Binding specificity was confirmed by competition binding assays with increasing concentrations of unlabeled TAE but not by similar amounts of unlabeled mutated TAEs (mTAE(A) or mTAE(B)). Southwestern blotting demonstrated a single 60-kDa binding activity (p60) in cultured HSCs (Fig. 4B). This binding activity is likely to have relevance during stellate cell activation and fibrogenesis in vivo because an identical binding activity was found in nuclear extracts from stellate cells activated in vivo after a single injection of CCl₄. Freshly isolated stellate cells have very low levels of collagen gene expression and lack this DNA binding activity (Fig. 4B) (8, 20). CCl₄ treatment increases stellate cell proliferation and collagen gene expression during the immediate 48–72 h post-exposure and induces fibrosis/cirrhosis after 6–12 weeks of chronic exposure (20, 21).

Since the TAE binding domain is dissimilar to known transcription factor binding domains, p60 may represent a novel transcription factor. Future studies will need to characterize this protein further.

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