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The c-Jun-Induced Transformation Process Involves Complex Regulation of Tenascin-C Expression†

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In cooperation with an activated *ras* oncogene, the site-dependent AP-1 transcription factor c-Jun transforms primary rat embryo fibroblasts (REF). Although signal transduction pathways leading to activation of c-Jun proteins have been extensively studied, little is known about c-Jun cellular targets. We identified c-Jun-up-regulated cDNA clones homologous to the tenascin-C gene by differential screening of a cDNA library from REF. This tightly regulated gene encodes a rare extracellular matrix protein involved in cell attachment and migration and in the control of cell growth. Transient overexpression of c-Jun induced tenascin-C expression in primary REF and in FR3T3, an established fibroblast cell line. Surprisingly, tenascin-C synthesis was repressed after stable transformation by c-Jun compared to that in the nontransformed parental cells. As assessed by using the tenascin-C (−220 to +79) promoter fragment cloned in a reporter construct, the c-Jun-induced transient activation is mediated by two binding sites: one GCN4/AP-1-like site, at position −146, and one NF-κB site, at position −210. Furthermore, as demonstrated by gel shift experiments and cotransfections of the reporter plasmid and expression vectors encoding the p65 subunit of NF-κB and c-Jun, the two transcription factors bind and synergistically transactivate the tenascin-C promoter. We previously described two other extracellular matrix proteins, SPARC and thrombospondin-1, as c-Jun targets. Thus, our results strongly suggest that the regulation of the extracellular matrix composition plays a central role in c-Jun-induced transformation.

c-*jun* encodes the major component of the sequence-specific AP-1 transcription factor (2–4, 10, 52). Its regulation has been extensively studied, and it appears that the c-Jun protein is a molecular integrator of several signal transduction pathways. The activity of the protein is finely regulated by phosphorylations on serine or threonine residues which are triggered by a variety of stimuli, including tumor promoters, growth factors, UV, or oncogenes (31). These regulations have been mapped to two regions of the protein; three phosphorylation sites in the C-terminal part have an influence on the efficiency of binding to DNA, and two sites in the N-terminal part participate in the transactivation capacity of the protein. c-*jun* expresses a variety of biological activities depending on the cellular background and has been implicated in the control of differentiation, apoptosis, and oncogenesis. The involvement of c-Jun in oncogenic transformation has been assessed in several systems: tumoral progression of bovine papillomavirus-induced fibrosarcomas can be triggered by c-Jun overexpression (13); and overexpression of exogenous c-Jun induces transformation of chicken embryo fibroblasts or established rat fibroblast cell lines and, in cooperation with an activated *ras* gene, primary rat embryo fibroblasts (REF) (12, 17, 38, 44). Deletions of part of the gene encoding the c-Jun oncoprotein indicate that the DNA binding and transactivating regions of the protein are essential for transformation (1). In REF, oncogenic cooperation results in the augmentation of c-Jun-induced transactivation; this Ras-induced effect is mediated by site-specific hyperphosphorylation of the c-Jun activation domain (9, 46).

Although the regulation of c-Jun production and activity is well documented, very little is known about the c-Jun target genes and the outcome of the signal transduction pathways acting via c-Jun. In an attempt to identify these target genes, we differentially screened a cDNA library of REF mRNAs. The transitory overexpression of c-Jun led us to identify several clones the expression of which was either activated or repressed compared to control conditions. Two genes encoding the extracellular matrix proteins thrombospondin-1 and SPARC were repressed by c-Jun both in transient experiments and after stable cellular transformation (38). We report here that one gene was repeatedly found among the activated clones. This gene codes for another extracellular matrix protein, tenascin-C, a member of a multigenic family (25).

Interactions between extracellular matrix proteins and cells have been shown to be important for normal cell growth and development of higher eukaryotes. The most abundant extracellular matrix proteins are structural and have adhesive functions. In contrast, proteins present in lower amounts, including tenascin-C, are thought to modulate cell-matrix interactions. The synthesis of tenascin-C is highly regulated in the adult and is often transient and site restricted, for example, during embryonic development (18, 25, 26, 37), neurite outgrowth (30, 35, 53), or wound healing (36). In the case of tumorigenesis, variable (undetectable and very high) amounts of tenascin-C production have been observed, depending on the cell types studied (11, 16, 29). Tenascin-C is a large modular multimeric protein whose function is still largely unknown. This extracellular matrix protein is implicated in the control of cell attachment, displaying adhesive or antiadhesive properties depend-

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† Dedicated to the memory of Philippe Vigier.

ing on the region of the protein involved. The epidermal growth factor-like repeat region seems to be responsible for the counteradhesive effect (40), whereas several sites of interaction with cell surface molecules (integrins or proteoglycans) have been mapped in the FN-III region and fibrinogen-like knob of the molecule (34, 40). The cell adhesion function of such a complex molecule could depend on the specific receptors expressed on the cell surface. Tenascin-C has also been implicated in the control of cell growth. In NIH 3T3 fibroblasts, DNA synthesis is dose dependently inhibited by tenascin-C (21), whereas a growth-promoting activity was attributed to tenascin-C in Swiss 3T3 fibroblasts (24). These contradictory data and the fact that mice harboring a disrupted tenascin-C gene present a grossly normal phenotype (42, 48) make tenascin-C a protein of undetermined function.

We show here that transient overexpression of the c-Jun or Ha-Ras oncoprotein in primary or established rat fibroblasts stimulates tenascin-C expression. Surprisingly, in cell lines stably transformed by c-Jun and/or Ras, tenascin-C expression is lower than in the normal parental cell line. Thus, the tenascin-C gene is a c-Jun target gene displaying a complex regulation. Together with the previously described targets SPARC and thrombospondin-1, these results suggest an important role of these extracellular matrix proteins in the control of cell growth and carcinogenesis.

MATERIALS AND METHODS

Cells, plasmids, transfections, and CAT assays. REF were prepared, cultured, and transfected as previously described (9). FR3T3 cells were cultured in Dulbecco modified Eagle medium (Gibco BRL) supplemented with 10% newborn bovine serum and transfected by the calcium phosphate precipitation method as previously described (38). Mock transfections were performed with salmon sperm carrier DNA. The c-*jun* expression vectors used were pRSV-c-Jun (9) for transitory experiments and pMM-c-Jun (46) for stable selection of c-Jun-expressing derivatives. The NF-κB expression vectors used were RcCMV-p50 (33) and RcCMV-p65 (43) for the p50 and p65 human subunits, respectively. The activated Ras expression vector was pZIPneoRas (9). Cells were stimulated with tetradecanoyl phorbol acetate (TPA; Sigma) at 100 ng/ml or anisomycin (Sigma) at 25 ng/ml. Chloramphenicol acetyltransferase (CAT) assays were performed as described in Binétruy et al. (9); cells in 10-cm-diameter petri dishes were transfected with 10 μg of tenascin-C promoter CAT constructs (27). Equal amounts of various expression vectors were cotransfected when indicated. To remove a cryptic AP-1 site in the plasmid backbone, the 246-bp *Eco*O109I-*Nde*I fragment was deleted from the human tenascin-C promoter CAT constructs described by Gherzi et al. (27). Percentages of acetylated [^{14}C]chloramphenicol were measured with a STORM radioimager (Molecular Dynamics).

Differential screening of the cDNA library and Northern blot analysis. Differential screening experiments were previously described in detail by Mettouchi et al. (38). Total RNA was prepared as described by Chomczynski and Sacchi (20). For Northern blot analysis, 20 μg of total RNA per lane was blotted onto nylon membranes (Biotrans; ICN), cross-linked with UV, and hybridized to radiolabeled probes synthesized from purified cDNA inserts. The hybridization signals were quantified by densitometry of autoradiographs.

Deletion and site-directed mutagenesis of the tenascin-C promoter. The −206 and −101 deletion mutants were generated, after linearization of −220TN-CAT with *Hin*dIII, by a 2-min partial *Bal* 31 digestion. Independent isolated clones were then sequenced to check the extension of the deletion. To generate the mGCN4-CAT construct, the GCN4 site was mutagenized by PCR as described elsewhere (7). Briefly, the region was amplified in two PCR steps using one pair of primers: the M13 reverse primer and the specific mutated primer creating a restriction site for *Stu*I. The second pair of primers was the M13 primer and the complementary mutated primer. Each PCR product was digested with *Stu*I and one enzyme with a single site, different for each PCR product, on the other side of the mutation site. The resulting fragments were religated into the parental context, and the presence of the mutation was confirmed by sequencing. The NF-κB–GCN4 double mutant was constructed from the mGCN4-CAT plasmid by deleting the *Hin*dIII/*Eco*RI fragment which includes the NF-κB site. Both restriction sites were blunted to allow the ligation. The corresponding region of the resulting plasmid (m2-CAT) was sequenced; as expected, it is 18 bp shorter than the mGCN4-CAT plasmid and presents a mutated NF-κB site: GCTAAT TCCT instead of GGGAATTCCT.

Gel shift experiments. REF were transfected and collected 24 h after transfection, and nuclear extracts were prepared as described previously (22). Electrophoretic mobility shift assays were performed by incubating for 15 min at 25°C 4 μg of nuclear extract with 1 μg of poly(dI-dC) in 10 μl (final volume) containing glycerol (5% [vol/vol]), EDTA (0.5 mM), dithiothreitol (0.5 mM), NaCl (50 mM), and Tris-HCl (10 mM, pH 7.5). Nonlabeled competitors (4 pmol) or antiserum (0.4 μl) were added at the same time; 40 fmol of 5′ ^{32}P-labeled double-stranded oligonucleotide was added, and the mixture was incubated for 10 min at 25°C. Samples were fractionated by electrophoresis through a 5% 29:1 acrylamide/bisacrylamide gel run in 45 mM Tris base–45 mM boric acid–5 mM EDTA.

The sequences of the sense strand of the oligonucleotides used were the following (binding sites are in boldface, and their mutations are underlined): NF-κB, 5′ GCAGGC**GGGAATTCCT**ACTTTC; NF-κBm, 5′ GCAGGC**GG**<u>TC CG</u>**TCCT**ACTTTC; GCN4, 5′ AGCCAGGAG**TGAGTG**CGTCTTT; GCN4m, 5′ AGCCAGGAG<u>GACC</u>**TG**CGTCTTT; AP-1, 5′ GATCTAGGAAG**TGACTC A**CA; and AP-1m, 5′ GATCTAGGAAG**T**<u>CTG</u>**TCA**CA.

All antisera were rabbit polyclonal. Anti-human p50 (33) and anti-mouse p65 (which recognizes the human protein coded by the RcCMV-p65 vector [14a]) were gifts of A. Israel. Anti-c-Jun antiserum is directed against c-Jun DNA binding domain (19).

Immunoprecipitation experiments. Tenascin-C proteins were immunoprecipitated from cell conditioned medium by using the specific anti-tenascin-C monoclonal antibody Mtn-12 (Biomakor). Briefly, cells were labeled in methionine-cysteine-free Dulbecco modified Eagle medium plus 0.1% serum with 300 μCi of [^{35}S]methionine-cysteine per 10-cm-diameter plate for 12 h. The media were then collected, and proteins were precipitated with an equal volume of acetone and then resuspended in radioimmunoprecipitation assay buffer. Samples containing equal amounts of trichloroacetic acid-precipitable counts were cleared by preincubation for 1 h with protein A-Sepharose (Sigma) at room temperature, and proteins were immunoprecipitated for 2 h at 4°C with monoclonal antibody Mtn-12 (rat immunoglobulin G1 isotype). To enhance binding to protein A-Sepharose, samples were additionally incubated overnight with a rabbit serum against rat immunoglobulin G1. Immunoprecipitates were then incubated for 1 h with protein A-Sepharose and run on sodium dodecyl sulfate–5% polyacrylamide gels.

TABLE 1. Characteristics of tenascin-C clones differentially activated in c-*jun* transfections

Clone	Insert size (bp)	Similar tenascin-C mouse gene sequence[a]
1	94	3′ noncoding region (6442–6537)
6	73	3′ noncoding region (6307–6380)
7	107	Coding + 3′ noncoding region (6188–6296)
22a	167	3′ noncoding region (6392–6559)
22b	77	Coding region (3590–3667)
23	72	3′ noncoding region (6323–6391)
30	89	3′ noncoding region (6339–6429)
32	87	3′ noncoding region (6590–6675)

[a] The region of similarity with the tenascin-C mRNA sequence is indicated, and the sequence position is given in parentheses.

RESULTS

Differential screening after transitory overexpression of c-Jun identifies the tenascin-C gene as a new cellular target gene. We used differential screening to characterize the panel of genes regulated by c-Jun. A subtracted cDNA library was constructed with mRNA isolated from REF transiently cotransfected by c-*jun–ras* expression vectors. Differential screening was performed with subtracted radiolabeled probes synthesized from either mock-transfected or c-*jun* transiently transfected REF cells. The two resulting hybridization signals were compared, and 39 clones (activated and repressed) were thereby identified as genes potentially regulated by c-Jun (for details, see reference 38). Complete or partial sequences of the inserts and computer analysis showed that a majority of them are previously unknown sequences. These clones are currently being characterized. We describe herein an analysis of cDNA inserts from eight clones, activated in response to c-Jun overexpression, displaying sequence similarities with the mouse tenascin-C gene (41). The characteristics of these clones are described in Table 1. The small size of the cDNA inserts results probably from the thermal degradation effect of the subtraction procedure used during construction of the library.

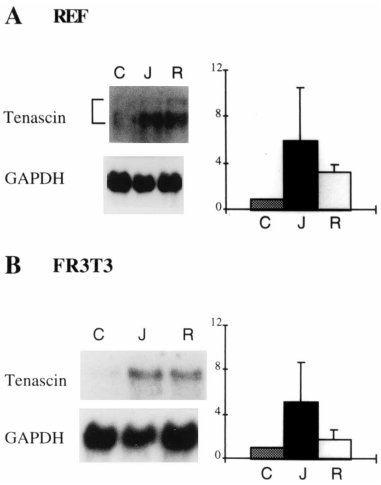

FIG. 1. Levels of tenascin-C mRNA after transient transfection of REF (A) or FR3T3 cells (B) with control (C), c-*jun* (J), and *ras* (R) expression vectors. Cells were transfected overnight and incubated for 24 h prior to RNA isolation. Twenty micrograms of total RNA was loaded per lane and blotted. Blots were probed with the tenascin-C cDNA insert purified from clone 6 and *GAPDH* cDNA as the control. In each case, the histograms show mean hybridization signals (± standards errors of the means) obtained from three different Northern blots, corrected for the corresponding *GAPDH* signal and normalized to 1 for signals from the control lane.

Northern blotting was used to confirm the regulation observed by the differential screening approach. Total RNAs isolated from REF either mock, c-*jun* or *ras* transfected were probed with the insert from clone 6. Two bands that hybridized migrated at around 6 and 8 kb, corresponding to the differentially spliced mRNAs of the tenascin-C gene (Fig. 1A). The same hybridization pattern was obtained (data not shown) with a probe from a tenascin-C cDNA expression vector (6). As a control for RNA loading, the same blots were then hybridized with a *GAPDH* probe. The intensities of bands on three independent Northern blots were quantified and indicated that transitory overexpression of c-*jun* induced the level of tenascin-C mRNAs sixfold. When the cells were transfected with *ras*, a lower but significant level of induction was obtained. This induction is likely to be due to the Ras activation of endogenous c-Jun via posttranslational modifications. We performed similar experiments with FR3T3, an immortalized rat fibroblast cell line. As observed in REF, transient overexpressions of c-Jun or Ras up-regulated tenascin-C expression in FR3T3 cells (Fig. 1B).

As suggested by the results obtained with Ras, we investigated whether tenascin-C expression can be stimulated in FR3T3 cells by inducers of endogenous c-Jun proteins. Phorbol esters like TPA lead, via the activation of protein kinase C, to the dephosphorylation of serine residues located close to the c-Jun DNA binding domain and thereby augment the DNA binding capacity of c-Jun (14). Anisomycin is a specific inducer of the JNK/SAPK kinases, members of the mitogen-activated protein kinase family, which hyperphosphorylate the activation domain of c-Jun and lead to a stimulation of c-Jun transactivation (15). TPA treatment induced tenascin-C expression after 2 h, and the peak of activation was observed after 4 h (Fig. 2A). Anisomycin treatment gave a similar high activation of tenascin-C expression (Fig. 2B). The induction kinetics were repeatable (not shown). To test whether c-Jun regulates tenascin-C transcription by triggering the synthesis of an intermediate transcriptional regulator, a concomitant treatment with cycloheximide, an inhibitor of protein synthesis, was performed. Cycloheximide did not block the induction observed in response to anisomycin (Fig. 2C).

These results show that tenascin-C is a new cellular target gene of c-Jun. The rapid induction kinetics of tenascin-C expression following treatment with c-Jun inducers and the fact that protein synthesis is not required suggest a direct mechanism of activation.

Two sites, a GCN4/AP-1-like site and a NF-κB site, of the tenascin-C promoter are the targets of c-Jun activation. We investigated whether the tenascin-C promoter was responsive to c-Jun. In transient-transfection experiments, c-Jun was able to transactivate the tenascin-C promoter, as assessed by using fusions to the CAT reporter gene (Fig. 3). In parallel experiments, neither the thymidine kinase promoter nor the Rous sarcoma virus long terminal repeat promoter, cloned in front

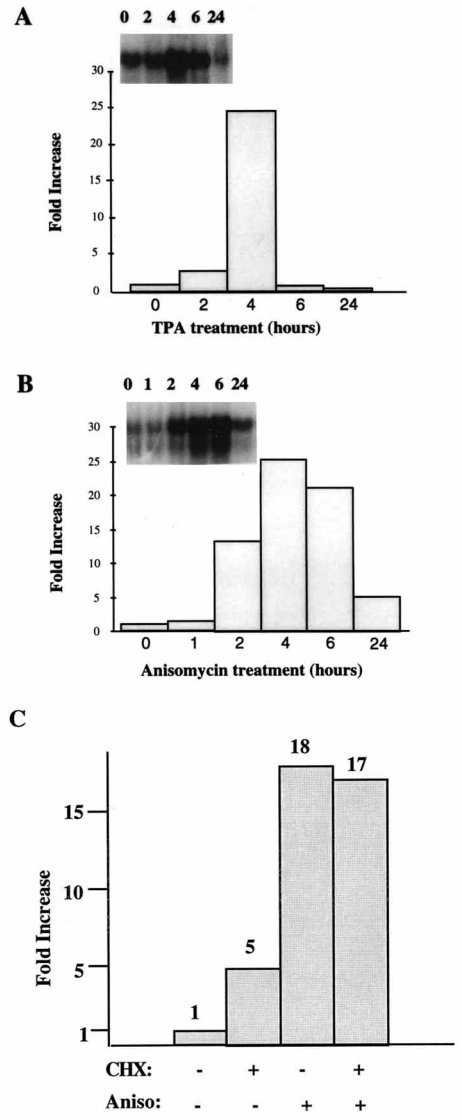

FIG. 2. TPA (A) and anisomycin (B) responsiveness of tenascin-C expression in FR3T3 cells. Fibroblasts were treated for the indicated time with either TPA (100 ng/ml) or anisomycin (25 ng/ml). In panel C, the effect of cycloheximide (CHX) on tenascin-C was tested, either alone or after a 6-h concomitant anisomycin (Aniso) treatment. Total RNA was analyzed by Northern blotting as indicated for Fig. 1. Representative Northern blots are shown in panels A and B. Histograms show the amounts of mRNA after correction for the control *GAPDH* signal and normalization to 1 for untreated cells.

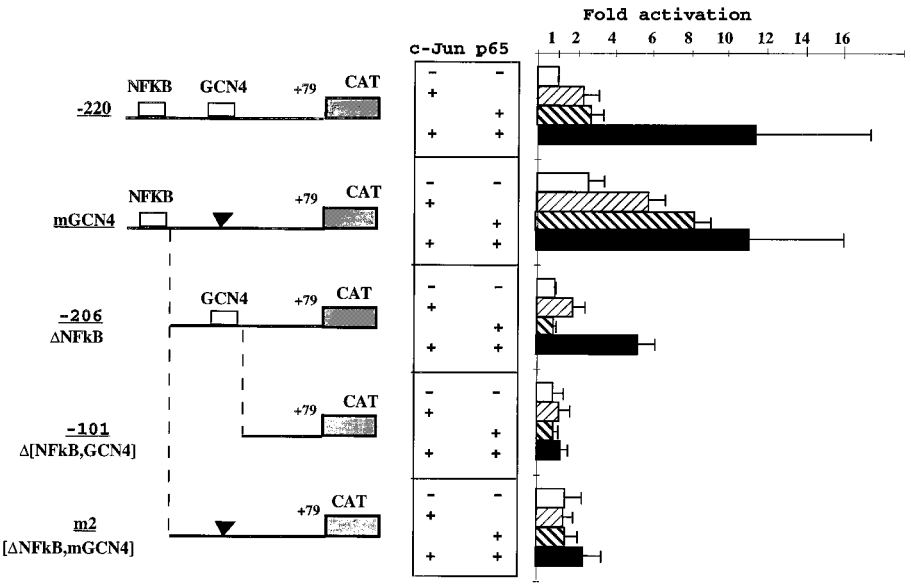

FIG. 3. Expression of tenascin-C promoter–CAT constructs transfected alone or cotransfected with a combination of c-Jun and NF-κB/p65 expression vectors. REF were transiently transfected with 10 μg of the different CAT recombinants in the presence (+) or absence (−) of 10 μg of pRSV-c-Jun (c-Jun) and 10 μg of p65 expression vectors. Histograms are mean values of at least three independent experiments and are normalized to 1 for the −220-CAT activity in mock-transfected cells.

of a luciferase gene reporter, was transactivated by c-Jun (data not shown), indicating the specificity of the tenascin-C activation. Specific c-Jun transactivation of gene promoters has been shown to be dependent on its binding to AP-1 (or TPA response element [TRE]) sites at the consensus sequence TGA (C/G)TCA. Specific point mutations of this recognition site abolish c-Jun DNA binding and, consequently, c-Jun-induced transactivation. The human tenascin-C promoter contains a consensus AP-1 site (TGACTCA) at position −875, the function of which has not been demonstrated (27). To determine whether this AP-1 element mediates the response, various fragments of the tenascin-C promoter in front of the CAT reporter gene were tested after overexpression of c-Jun by transient transfections in REF. The (−1175,+79) CAT reporter construct, containing the consensus AP-1 site, displayed a very low basal activity and was stimulated twofold by overexpression of c-Jun (data not shown). A shorter promoter segment (−220,+79), displaying a higher basal activity, was also transactivated by c-Jun (Fig. 3). These results indicate that the AP-1 sequence at position −875 is not necessary for the c-Jun activation in REF. We then analyzed the response of a 119-bp 5′ deletion of the (−220,+79) fragment of the tenascin promoter (−101-CAT construct). No stimulation by c-Jun was obtained in this case, indicating that the c-Jun response was included in the (−220,−101) fragment of the promoter (Fig. 3). No consensus AP1 site, even with one mismatch, is present in this fragment, but computer analysis detected two potential binding sites: a GCN4 binding site at position −146 with the sequence $T_1G_2A_3G_4T_5G_6$ (5) and an NF-κB consensus sequence ($G_1R_2G_3R_4N_5N_6Y_7Y_8Y_9Y_{10}$ [39]) at position −210 with the sequence GGGAATTCCT. GCN4 is a yeast transcription factor containing a DNA binding region very similar to the c-Jun DNA binding domain. Their DNA binding domains can be swapped without affecting the transactivation properties of the proteins, and both c-Jun and GCN4 wild-type proteins can transactivate by binding to either site (49). A physical interaction between c-Jun and the p65 subunit of the transcription factor NF-κB has been described, and the resulting protein complex displayed synergistic transactivation of reporters under the control of either the NF-κB site or the TRE site (47).

To test the involvement of NF-κB and GCN4 sites of the tenascin-C promoter in the c-Jun activation, we independently mutated these sites. Starting from the (−220,+79) CAT construct, the GCN4 site was mutated at positions 1, 2, 3, and 4 into the sequence GCCTTG by site-directed mutagenesis to create mGCN4-CAT (see Materials and Methods). The mutation of the NF-κB site was obtained by deleting 14-bp from the 5′ end of the (−220,+79) fragment to the nucleotide at position −206 (see Materials and Methods). The sequence of the resulting construct (−206-CAT construct) in the region of interest is TGCTTCCCCT, introducing mutations at positions 1, 3, and 4 of the NF-κB consensus sequence. Both mGCN4-CAT and −206-CAT plasmids retained c-Jun inducibility similar to that of the wild-type construct (Fig. 3). We then constructed the NF-κB–GCN4 double mutant of the tenascin-C promoter by deleting the NF-κB site from the mGNC4-CAT plasmid (called m2-CAT [see Materials and Methods]). Similarly to the −101-CAT plasmid, the m2-CAT construct was no longer responsive to c-Jun (Fig. 3), demonstrating that both sites are involved in the c-Jun-mediated activation. Along with the data obtained with the single-site mutants, these results indicate that the two sites can function independently.

c-Jun and NF-κB act synergistically to transactivate the tenascin-C promoter. The role of the NF-κB site evidenced by these experiments suggested that c-Jun and NF-κB could act synergistically to transactivate the tenascin-C promoter. To test this hypothesis, we cotransfected into REF expression vectors for c-Jun and NF-κB together with the different tenascin-CAT reporters. Expression vectors for NF-κB p50 and p65 subunits (independently or together) and c-Rel (another member of the NF-κB family) were used. A strong synergy between c-Jun and p65 was obtained on the activation of the (−220, +79) CAT construct: c-Jun and p65 transactivate 2.3- and 2.7-fold, respectively, alone but 11.5-fold when cotransfected (Fig. 3). A similar synergy was observed between c-Jun and p50

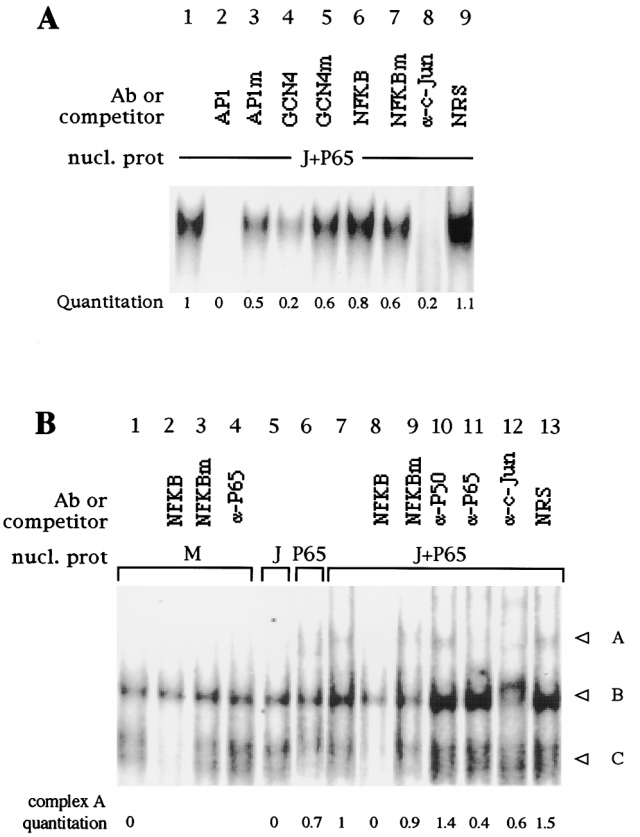

FIG. 4. Gel shift analysis using AP-1 (A) or NF-κB (B) radiolabeled DNA probes incubated with protein nuclear from mock-transfected REF (M) and REF transiently transfected with c-Jun (J), NF-κB/p65 (P65), or c-Jun–NF-κB/p65 (J+P65). The use of competitors and antibodies is described in the text. Specific band shifts were quantitated with a radioimager. Ab, antibody; nucl. prot, nuclear protein.

plus p65 (data not shown). No effect was seen in the case of p50, which does not transactivate by itself, or c-Rel, which transactivates threefold the tenascin-C promoter (data not shown). The −206-CAT, −101-CAT, and m2-CAT constructs were not transactivated by p65 (Fig. 3), demonstrating that the NF-κB transactivation was dependent on the integrity of the NF-κB site. As expected for the unresponsive mutants of the tenascin promoter −101-CAT and m2-CAT, the c-Jun–p65 cotransfections did not affect, or slightly affected, their expression (Fig. 3). A c-Jun–NF-κB synergy was also observed on the mGCN4-CAT and −206-CAT single-mutant constructs, although twofold weaker for the latter (Fig. 3). These results probably reflect the ability of the c-Jun–p65 protein complex (see below) to be active on both sites.

Our results evidenced a nonclassical mechanism for c-Jun activation. We hypothesized that the GCN4 site of the tenascin-C promoter could function by binding directly c-Jun proteins. Gel shift experiments were performed using GCN4 and AP-1 DNA probes incubated with protein nuclear extracts from transfected REF. A barely detectable specific band shift of the radiolabeled GCN4 probe was observed (data not shown). The capacity of the tenascin-C GCN4 site to bind c-Jun proteins was clearly revealed by the displacement of c-Jun binding to a radiolabeled AP-1 probe (Fig. 4A). A single band shift migrating at the same position was observed in assays using protein nuclear extracts from either mock-transfected REF, c-Jun-transfected cells (data not shown), or cells transfected with expression vectors for c-Jun and NF-κB/p65 (lane 1). This shifted complex was efficiently competed by an excess of unlabeled AP-1 DNA (lane 2) but not by a mutated AP-1 (lane 3). The intensity of this band shift was 2.6-fold higher than in mock-transfected cells, revealing the activity of the c-Jun exogenous protein over the activity of the endogenous AP-1. This protein-DNA complex was recognized by anti-c-Jun antibodies (lane 8) and not by normal rabbit serum (lane 9), confirming the contribution of c-Jun proteins to the complex. The band shift was efficiently competed (to 21% of the intensity of the shift of lane 1) by an excess of unlabeled GCN4 DNA (lane 4) but only marginally competed by the mutated GCN4 DNA (lane 5) or NF-κB-derived sequences (lanes 6 and 7), between 58 and 85% of the shift of lane 1. These results suggest that the binding of c-Jun proteins to the GCN4 site of the tenascin-C promoter is weak but that the GCN4 sequences of the tenascin-C promoter can efficiently compete for the c-Jun binding on an AP-1 DNA probe. This effect is very likely the result of a weaker affinity of c-Jun proteins for the GCN4 site than for a consensus AP-1 site.

Our *in vivo* data on the NF-κB site suggested that it is regulated by p65 and c-Jun similarly to the human immunodeficiency virus (HIV) NF-κB site (47). In this case, the NF-κB sequence is recognized by a c-Jun–p65 protein complex. Gel shift experiments were performed with the NF-κB sequences of the tenascin-C promoter as radiolabeled probes (Fig. 4B). After incubation with protein nuclear extracts from either mock-, c-Jun-, NF-κB/p65-, or c-Jun–NF-κB/p65-transfected REF, three band shifts, A, B, and C, were observed. No competition with an excess of unlabeled NF-κB probe was observed for band shift B, indicating that it is a nonspecific shift (lanes 2 and 8). Complexes A and C were efficiently competed by an excess of unlabeled NF-κB DNA (compare lane 1 to lane 2 and lane 7 to lane 8) and not by a mutated NF-κB (lanes 3 and 9), demonstrating their specificity. Band shift C was observed with each protein extract, even from mock-transfected cells, and therefore corresponds to the endogenous NF-κB activity of REF (lanes 1, 5, 6, and 7). Likely due to species specificity, this rat protein complex was not recognized by anti-human p50 or anti-mouse p65 antibodies (lanes 4, 10, and 11). By contrast, band shift A was present only with NF-κB p65 (lane 6)- and c-Jun–NF-κB/p65 (lane 7)-transfected cells, suggesting that it might include p65 proteins. Complex A was efficiently and specifically displaced by incubation with anti-p65 antibodies (lane 11) and with anti-c-Jun antibodies (lane 12), to 40 and 60%, respectively, of the intensity of band shift A in lane 7. Incubation with either normal rabbit serum or anti-p50 antibodies did not affect the band shift (lanes 13 and 10). These results demonstrate that the NF-κB site of the tenascin-C promoter is recognized by the NF-κB p65 subunit and can form a protein-DNA complex including both p65 and c-Jun proteins.

Together, our *in vivo* and *in vitro* data indicate that c-Jun is capable of activating the tenascin-C promoter in synergy with the p65 subunit of NF-κB. These effects are mediated by a GCN4 site, recognized by c-Jun proteins, and by an NF-κB site which binds a c-Jun–p65 protein complex.

Tenascin-C expression is repressed in c-*jun*- and/or *ras*-transformed cells. We then assessed the level of tenascin-C expression after stable transformation by c-*jun* and/or *ras* oncogenes. REF cell lines cotransformed by c-*jun* and *ras* were isolated and the exogenous oncoprotein production evaluated by Western blotting (50). Tenascin-C expression was investigated by Northern blot experiments in which S26, a gene encoding a ribosomal protein whose expression is not regulated (51), was used as the RNA loading control. Surprisingly, tena-

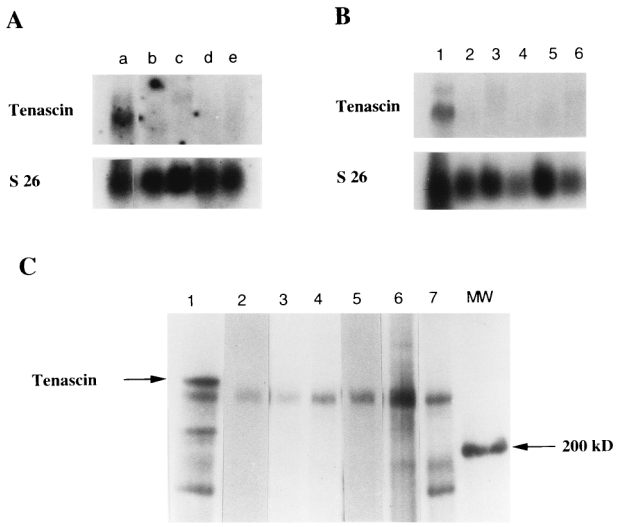

FIG. 5. Tenascin-C expression after stable transformation by c-*jun* and/or *ras* oncogenes. (A) Northern blot analysis of c-*jun*–ras-cotransformed REF cell lines. Twenty micrograms of total RNA from each of the following cell lines was analyzed by Northern blotting as described for Fig. 1: primary REF (lane a) and c-*jun*–*ras*-cotransformed REF clones 2 (lane b), 3 (lane c), 4 (lane d), and 5 (lane e). (B) Northern blot analysis of FR3T3 cells (lane 1), c-*jun*-transformed FR3T3 derivatives FRcJ-1 (lane 2), FRcJ-3 (lane 3), FRcJ-4 (lane 4), and FRcJ-8 (lane 5), and *ras*-transformed FR3T3 cell line FREJRas (lane 6). Filters were probed with either tenascin-C cDNA or S26 cDNA as the control. (C) Immunoprecipitation of tenascin-C proteins with monoclonal antibody Mtn-12 from conditioned media of FR3T3 (lane 1), FRcJ-1 (lane 2), FRcJ-3 (lane 3), FRcJ-4 (lane 4), FRcJ-8 (lane 5), and FREJRas (lane 6) cells. In lane 7 a nonrelated monoclonal antibody was incubated with FR3T3 conditioned medium as the control. Lane MW, ^{14}C-methylated molecular weight marker.

scin-C mRNA was not detected in any of four independent transformed REF clones (Fig. 5A). Similarly, four cell lines obtained from FR3T3 cells stably transformed by c-Jun alone and one cell line transformed by an activated Ras (38) were tested for tenascin-C expression (Fig. 5B and C). Quantitative analysis of Northern blots showed that in the c-Jun-transformed cells, the amount of tenascin-C mRNA was 80 to 90% lower than in parental FR3T3 cells. The Ras-transformed cell line also displayed a repressed tenascin-C expression. In contrast, the same transformed cell lines displayed a much higher level of transin mRNA, a classical c-Jun-induced gene, than the nontransformed parental cells (reference 38 and data not shown). Thus, in contrast to transient-expression experiments, we found that long-term expression of c-Jun and the resulting cellular transformation led to almost extinction of tenascin-C expression. This down-regulation of tenascin-C expression in c-Jun- or Ras-transformed cells was confirmed at the protein level. The different cell lines were metabolically labeled, and secreted tenascin-C proteins were immunoprecipitated with a specific monoclonal antibody. Tenascin-C proteins, mostly the larger 230-kDa form in the parental FR3T3 cell line, were undetectable in FR3T3 cells transformed by c-Jun or Ras (Fig. 5C).

To study the specificity of the tenascin-C regulation, cells transformed by other oncogenes acting through pathways not involving c-Jun and Ras were analyzed. As assessed by both mRNA and protein analyses, FR3T3 cells transformed by bovine papillomavirus type 1 oncogenes (RV145-4 cell line), by simian virus 40 large T antigen (SVWT-N1 cell line), or by polyomavirus middle T antigen (MTT4 cell line) (38) displayed tenascin-C expression levels similar to that of the parental FR3T3 cell line (Fig. 6). Thus, tenascin-C down-regulation seems to be a phenomenon specific to the c-Jun and Ras transformation pathways or, at least, not a general phenomenon associated with cellular transformation.

DISCUSSION

Decisive breakthroughs in the understanding of mechanisms leading to cellular transformation by oncogene cooperation have recently been made. Although some links are still missing, the transduction pathways from diverse external stimuli to nuclear oncoproteins like c-Jun are almost completely deciphered. For example, the cascade of kinases from Ras to the nucleus is now characterized at the molecular level. The regulation of c-Jun protein activity by phosphorylation can be considered as a model not only for oncogenesis but also for other biological processes such as apoptosis or the cellular response to stress (28, 32, 54). In contrast, events downstream from the activation of nuclear oncoproteins remain to be elucidated. In particular, the cellular target genes of c-Jun relevant to its transforming activities are unknown. We used differential screening of a cDNA library to isolate c-Jun target genes in primary REF. Previously, these experiments led us to identify two genes, encoding the extracellular matrix proteins SPARC and thrombospondin-1, as c-Jun-repressed targets (38).

We now describe another c-Jun-regulated gene which also encodes extracellular matrix protein: the tenascin-C gene. Transient overexpression of c-Jun, in both the differential screening and Northern blot experiments, led to activation of tenascin-C expression in primary or established fibroblasts (Table 1 and Fig. 1). We found that transient expression of Ras results also in the activation of tenascin-C expression, suggesting that the

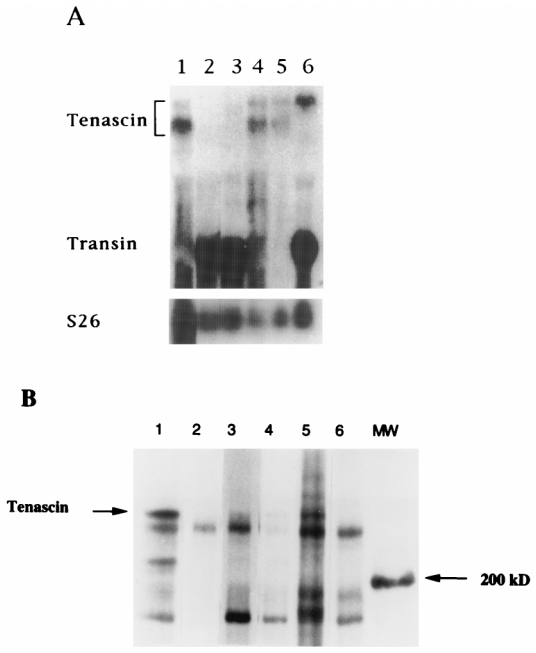

FIG. 6. Tenascin-C expression after stable transformation by different viral oncogenes. (A) Northern blot analysis of FR3T3 (lane 1), FRcJ-4 (lane 2), FREJRas (lane 3), RV145-4 (lane 4), SVWT-N1 (lane 5), and MTT4 (lane 6) cells. Filters were probed with either tenascin-C cDNA or S26 cDNA as the control. (B) Immunoprecipitation of tenascin-C proteins with specific monoclonal antibodies from media conditioned by FR3T3 (lane 1), FRcJ-4 (lane 2), RV145-4 (lane 3), SVWT-N2 (lane 4), and MTT4 (lane 5) cells. In lane 6, conditioned medium from FR3T3 cells was incubated with an irrelevant monoclonal antibody. Lane MW, molecular weight marker.

activation of endogenous c-Jun proteins, which is observed in this case, can lead to tenascin-C induction. This hypothesis was strengthened by the fact that treatment with TPA or anisomycin, two different inducers of endogenous c-Jun activity, led to rapid and strong induction of tenascin-C mRNA (Fig. 2A and B). Cycloheximide treatment did not interfere with the anisomycin activation, indicating that the c-Jun induction of tenascin-C does not require the synthesis of an intermediate transcriptional regulator (Fig. 2C). Our findings are consistent with recent studies of tenascin-C activation by angiotensin II (45), a hormone specifically activating JNK (56). Thus, angiotensin II, via an activation cascade involving JNK and c-Jun, may specifically induce tenascin-C expression.

Transient cotransfections of CAT constructs under the control of the tenascin-C promoter demonstrated that c-Jun was able to transactivate the promoter of tenascin-C (Fig. 3). A region of more than 2 kb of this promoter is known and carries potential binding sites for a variety of transcription factors. However, its regulation has been only roughly characterized; in particular, the function of the different recognition sites is still unknown (27). Classical transactivation of c-Jun-responsive promoters is dependent on the binding of c-Jun to AP-1 consensus sequences, and single-point mutations in these sequences can abolish both DNA binding and transactivation. By studying the response to c-Jun of successive deletion mutants of the promoter, we first ruled out the role of a perfect TRE site present at position −875 and second mapped the responsive region between positions −220 and −101. Computer analysis of these latter sequences detected two potential transcription factor binding sites: one for GCN4 and one for NF-κB. Analysis by site-directed mutagenesis demonstrated that the c-Jun transactivation was mediated by these two sites, which can function independently (Fig. 3). As shown by gel shift assays using nuclear extracts from c-Jun-transfected cells, the GCN4 site is recognized directly by c-Jun proteins (Fig. 4A). It has been shown that c-Jun can bind to and transactivate via a GCN4 site in yeast cells (49). Our results clearly indicated that such a divergent TRE sequence can also function in mammalian cells in the context of an endogenous cellular promoter. Studies of the HIV promoter by Stein et al. (47) demonstrated that p65 subunits of NF-κB can interact with c-Jun proteins, leading to a transcription factor complex able to bind and strongly transactivate a promoter via either NF-κB or TRE sites. Here we show that the NF-κB site of the tenascin-C promoter is recognized by a protein complex composed of both c-Jun and NF-κB proteins (Fig. 4B). Therefore, our results indicate that the regulation of the tenascin-C promoter by c-Jun and NF-κB is very similar to that described by Stein et al. for the HIV promoter. This activation of the tenascin-C promoter represents another example of regulation by the superfamily of transcription factor supracomplexes comprising Rel and bZIP families. Indeed, several complexes between proteins containing Rel DNA binding/dimerization domain and bZIP proteins, such as NFAT and AP-1 or p50 and C/EBPβ, have been described (for a review, see reference 39). c-Jun is known to interact specifically with numerous transcription factors, thereby modulating the expression of cellular genes independently of its DNA binding to AP-1 sequences (8, 23, 55). This phenomenon, by widening the spectrum of c-Jun target genes and consequently affecting the regulation of potential critical targets, could be relevant to cellular transformation.

In contrast to SPARC and thrombospondin-1, whose c-Jun-induced repression is mediated by a secreted factor, our results suggest that the initial differential screening experiment identified tenascin-C as a direct c-Jun target.

In direct opposition with the activation evidenced in transient experiments, tenascin-C expression was repressed after stable transformation by c-*jun* and/or *ras* oncogenes. Both REF cell lines transformed by c-*jun* plus *ras* and FR3T3 cell lines transformed by c-*jun* or *ras* displayed much lower levels of tenascin-C mRNA and protein than the parental cells (Fig. 5). Cells transformed by other oncogenes expressed tenascin-C to normal levels, indicating that these repressions are not a general phenomenon associated with cellular transformation and likely specific to the Ras–c-Jun transforming pathway (Fig. 6). Further studies are necessary to understand the molecular mechanisms involved in this repression and the likely role of c-Jun in this process.

The biological role for the transitory activation and the long-term repression of the tenascin-C expression evidenced in the c-Jun transformation process is not clear. Possibly by facilitating the emergence of transformed cells, antiadhesive properties displayed by tenascin-C could be implicated in the early step of transformation. Conversely, the repression of tenascin-C after the establishment of transformation could be correlated to the growth-inhibitory effect described in some cases for tenascin-C treatment.

Tenascin-C is the third example, after thrombospondin-1 and SPARC, of an extracellular matrix protein specifically repressed in c-Jun-transformed cells. These three secreted proteins display complex biological activities, including regulatory roles in cell division and adhesion. Moreover, thrombospondin-1 has been described as an antiangiogenic factor *in vivo*. Therefore it is likely that these genes constitute a class of c-Jun targets relevant to transformation. Together with collagenase and stromelysin-1/transin, which are activated by c-Jun, our results strongly suggest that the regulation of the extracellular matrix compounds plays a central role in c-Jun-induced transformation. Use of constitutive or inducible expression vectors for the genes encoding these different proteins to restore their expression in c-Jun-transformed cells or to obtain a c-Jun-independent expression in normal cells will contribute to an understanding of their roles in cellular transformation.

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