Posttranscriptional Down-Regulation of Fibronectin in N-ras-transformed Cells

Lois A. Chandler and Suzanne Bourgeois

Regulatory Biology Laboratory, The Salk Institute for Biological Studies, San Diego, California 92186-5800

Abstract
The basal level of fibronectin (FN) biosynthesis is greatly reduced in N-ras-transformed human fibrosarcoma HT1080 cells when compared with normal fibroblasts. The rate of FN biosynthesis can be significantly increased in HT1080 cells by the synthetic glucocorticoid dexamethasone, which has been shown to act by selectively stabilizing the FN message (D. C. Dean, R. F. Newby, and S. Bourgeois. J. Cell Biol., 106: 2159–2170, 1988). The present study demonstrates that the basal levels of FN biosynthesis and accumulated FN mRNA are also reduced in HT1080 cells when compared with nontumorigenic revertants of HT1080 cells in which the mutated N-ras allele is underrepresented. In contrast, the FN promoter is more active in HT1080 cells than in the revertant cells. These results indicate that the down-regulation of FN in HT1080 cells is linked to expression of the N-ras oncogene and that the effect is posttranscriptional. Therefore, the N-ras oncogene and glucocorticoids have opposite posttranscriptional effects on FN biosynthesis in HT1080 cells. However, this study demonstrates that the stimulatory effects of glucocorticoids are not mediated by a transcriptional repression of the N-ras gene.

Introduction
FNs are large glycoproteins composed of two similar but nonidentical subunits joined by disulfide bonds (reviewed in Ref. 1). FN exists in extracellular matrices (cellular FN) and in soluble form in body fluids (plasma FN). Alternative splicing of the primary transcript of a single gene located on human chromosome 2 (2) can generate numerous distinct FN transcripts (3–5). These different FN mRNAs account in particular for the individual subunits and for the plasma and cellular isoforms. FN has multiple functions and has been implicated in cell adhesion, morphology, migration, differentiation, and oncogenic transformation. Oncogenically transformed cells often express reduced levels of cell surface FN when compared with their normal counterparts. This effect has been observed with a variety of oncogenic stimuli including nuclear (T antigen, E1A), cytoplasmic (mos, fes), or plasma membrane-bound (src, abl, ras) oncogenes (reviewed in Ref. 1). Several studies have also demonstrated a correlation between decreased cell surface FN and the acquisition of tumorigenic and metastatic potentials (6, 7). In general, the loss of cellular FN appears closely involved in the changes in cell adhesion and morphology that accompany oncogenic transformation. However, the mechanisms by which oncogenes affect the accumulation of matrix FN remain largely unknown.

N-ras oncogenes are activated by mutation in many types of human tumors and have also been linked to animal carcinogenesis (8). There are three known members of the ras oncogene family: Ha-, N-, and Ki-ras. These three genes encode related 21 kilodalton proteins (p21) that are associated with the cytoplasmic surface of the plasma membrane, bind guanine nucleotides, and are involved in signal transduction (reviewed in Ref. 9). ras proteins exist in equilibrium between active (GTP-bound) and inactive (GDP-bound) forms. A putative cytoplasmic effector of ras action has been identified as GAP (10). GAP stimulates the intrinsic GTPase activity of normal ras protein, converting it to an inactive GDP-bound state. The mutant ras protein is unresponsive to GAP action and is maintained in an active GTP-bound state.

Despite the high frequency of occurrence in human tumors, little is known about the regulation of the transformed phenotype by the mutated ras genes. An inverse correlation between expression of a mutated Ha-ras gene and levels of accumulated FN has been demonstrated in NIH3T3 cells (11). However, the mechanism(s) for altered FN accumulation was not determined in that study. Our laboratory has studied the regulation of FN biosynthesis in N-ras-transformed human HT1080 fibrosarcoma cells (12, 13). HT1080 cells contain both normal and mutated (at codon 61) N-ras alleles (14, 15). Nontumorigenic “flat” revertants of HT1080 cells have been isolated in which underrepresentation of the mutated N-ras allele and its protein product correlates with reversion of the transformed phenotype (16). This evidence indicates that expression of the mutated N-ras allele controls the transformed phenotype of HT1080 cells. The basal level of FN biosynthesis is greatly reduced in HT1080 cells when compared with normal fibroblasts, and a posttranscriptional mechanism contributes to this difference (13). Dexamethasone (a synthetic glucocorticoid) induces a large increase in FN biosynthesis in HT1080 cells which correlates with increased stability of FN mRNA (13). In contrast, only a small induction of FN biosynthesis is seen when normal fibroblasts are treated with dexamethasone.
Taken together, these findings led us to speculate that the relative instability of FN mRNA in HT1080 cells may be due to expression of the N-ras oncogene. We have used HT1080 cells and one of the “flat” revertant cell lines isolated by Paterson et al. (16) to further investigate the effects of N-ras transformation on FN biosynthesis. Our results indicate that the low level of accumulation of FN in HT1080 cells is linked to expression of the N-ras oncogene and that the effect is posttranscriptional. Therefore, the N-ras oncogene and glucocorticoids have opposite posttranscriptional effects on FN biosynthesis in HT1080 cells. However, our data demonstrate that glucocorticoids do not act by decreasing N-ras expression.

Results
FN Biosynthesis in Parental and Revertant HT1080 Cells.
The HT1080 fibrosarcoma subclone used in this study, HT1080sc2 (16), will hereafter be referred to as HT1080. These cells contain both normal and mutated N-ras alleles, they grow in soft agar, and they readily form tumors in nude mice. The flat revertant of HT1080 used in this study has been previously described (16) and is referred to as 10a. 10a cells display a flatter morphology and have a saturation density approximately 3 times lower than HT1080 cells. Furthermore, 10a cells grow poorly in soft agar and do not form tumors in nude mice. The mutated N-ras allele is underrepresented in 10a cells when compared with HT1080 cells (16). Furthermore, the revertants can be fully retransformed by transfection with the cloned N-ras oncogene. These results provide strong evidence that the N-ras oncogene controls the transformed phenotype of HT1080 cells. The revertant cells and the transformed parental HT1080 cells provide a valuable system for investigating the effects of ras transformation on FN biosynthesis.

The relative rates of FN biosynthesis in HT1080 and 10a cells were measured by pulse labeling the cells for 2 h with [35S]methionine followed by immunoprecipitation of FN from the culture medium and cell layer in combination. Immune precipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography, and the radioactivity in the FN-containing portion of the gel was quantified by liquid scintillation counting. This analysis demonstrated that the basal rate of FN biosynthesis was approximately 3 times greater in 10a cells than in HT1080 cells (Fig. 1). Thus, increased biosynthesis of FN correlated with underrepresentation of the mutated N-ras allele and reversion to a flatter, non-tumorigenic phenotype. It has previously been demonstrated that the rate of FN biosynthesis is increased in HT1080 cells by the synthetic glucocorticoid dexamethasone (12, 13). Accordingly, we found that the rate of FN biosynthesis was also increased (~7x) by dexamethasone in the subclone of HT1080 cells used in this study. An increase (~4x) in FN biosynthesis was also observed following dexamethasone treatment of the revertant 10a cells (Fig. 1). Therefore, although the revertant cell line has a higher basal rate of FN biosynthesis, the cells are still responsive to glucocorticoid regulation.

Steady-State Levels of FN mRNA in Parental and Revertant HT1080 Cells. Northern analyses were carried out to determine whether the observed differences in basal FN biosynthetic rates could be accounted for at the RNA level. Northern blots of total cellular RNAs were hybridized to FN and GAPD probes. The FN band intensities, which represent the relative steady-state levels of FN mRNA, were normalized to GAPD band intensities. The results revealed that the accumulated level of FN mRNA was approximately 6 times higher in the revertant 10a cells than in HT1080 cells (Fig. 2). Therefore, the higher FN biosynthetic rate in the revertant cells (Fig. 1) was more than accounted for at the RNA level. This indicates that the increased rate of FN biosynthesis in 10a cells was due to a transcriptional or posttranscriptional mechanism rather than to a translational effect.
Our earlier work has demonstrated that the effect of dexamethasone on FN biosynthesis in HT1080 cells involves selective stabilization of FN mRNA (13). Consistent with this observation, the steady-state level of FN mRNA was higher in both HT1080 and 10a cells following exposure to dexamethasone (Fig. 2), and the magnitude of induction for each cell line approximated that seen at the protein level (Fig. 1). Another interesting observation from these experiments was that following hormone treatment, the magnitude of induction of FN mRNA was consistently less in the revertant cells (~4X) than in HT1080 cells (~10X) (Fig. 2). In other words, a decreased responsiveness to glucocorticoids accompanies under-representation of the N-ras oncogene.

The FN Promoter Is More Active in HT1080 Cells than in 10a Cells. We previously observed that FN promoter-

CAT gene constructs are expressed in HT1080 cells despite the fact that the cells express very low levels of FN mRNA (13, 17). This apparent discrepancy could be explained by an N-ras oncogene-mediated destabilization of FN mRNA in HT1080 cells, and thus there would be no inhibitory effect of oncogene expression on FN promoter activity. To further test this hypothesis, we assayed FN promoter activity in the HT1080 and revertant 10a cells. Each cell line was transfected with a plasmid containing human FN gene promoter sequences from -510 to +69 base pairs fused to the CAT gene (-510FCAT). We have demonstrated that properly initiated transcripts are generated from the FN cap site within this plasmid when it is used as a template for in vitro transcription (17). Fig. 3 shows that the FN promoter was about 3 times more active in the parental HT1080 cells than in the "flat" revertant cells. Therefore, the reduced level of accumulated FN mRNA in HT1080 cells (Fig. 2) could not be accounted for by decreased FN promoter activity. This indicates that a posttranscriptional mechanism is responsible for the reduced level of FN mRNA in HT1080 cells as compared to the revertant cells. Since the mutated N-ras allele controls the transformed phenotype of HT1080 cells (16), and since the oncogene is underrepresented in the revertant cells, it seems likely that the oncogene mediates this posttranscriptional down-regulation of FN.

Dexamethasone Treatment Does Not Affect N-ras Expression in HT1080 Cells. We have previously dem-
effects onto the laboratory treatment mechanism increases Poly(A)" HT1O8O (20). We also noted that dexamethasone-mediated destabilization of FN mRNA appears as the most likely explanation for the reduced level of FN mRNA in HT1O80 cells. We have attempted to measure the half-life of FN mRNA in the transformed and revertant cell lines using the transcription inhibitors actinomycin D and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (21). However, with each inhibitor, FN mRNA decay was only transient when assayed by Northern analysis, thus prohibiting half-life determinations (data not shown). One possible interpretation of these results is that a short-lived protein encoded by a short-lived mRNA mediates degradation of the FN message. Half-life measurements by pulse-chase methods were also attempted but were hindered by the fact that the FN mRNA is very low in abundance in the HT1O80 subclone used in this study. In the future, we hope to overcome some of these difficulties by adopting a strategy involving transfection of a transiently inducible FN gene into the cell line of interest, as has been described for other systems (22). ras proteins are associated with the plasma membrane and are thus not likely to be directly involved in the cytoplasmic degradation of FN mRNA. Instead, a cascade of events is probably required for the ras oncoproteins to exert their effects on FN mRNA. The N-ras oncogene and glucocorticoids have opposite posttranscriptional effects on FN biosynthesis in HT1O80 cells. However, our Northern analysis data demonstrate that the stimulatory effects of glucocorticoids are not mediated by a transcriptional repression of the N-ras gene.

Discussion
An early observation that stimulated much interest in FN is that this important matrix glycoprotein was lost or dramatically reduced following oncogenic transformation of cells (reviewed in Ref. 1). The detailed mechanisms by which oncogenes affect the various steps in the accumulation of cellular FN are still largely unknown. It is likely that different oncogenes have different effects because their respective proteins have different cellular locations and functions. In one case, src-transformation of chick embryo fibroblasts, the primary defect in FN production appears to be at the level of transcription (20). In a few other systems, reduced levels of FN mRNA have also been reported, but no attempts were made to identify the mechanisms involved.

In this paper, we utilize N-ras-transformed HT1O80 cells and “flat” revertants of these cells to investigate the effects of the N-ras oncogene on FN biosynthesis. The mutated N-ras allele is underrepresented in the revertant cells, thus providing evidence that the oncogene controls the transformed phenotype of HT1O80 cells (16). The HT1O80 and revertant cell lines provide a valuable experimental system with which to study the effects of the oncogene on FN biosynthesis.

Our results demonstrate an increased rate of FN biosynthesis and increased accumulation of FN mRNA in the revertants as compared to the parental HT1O80 cells. Furthermore, transfection experiments to analyze FN promoter activity indicate that the effect of the N-ras oncogene on FN biosynthesis is at the posttranscriptional level. N-ras-mediated destabilization of FN mRNA appears as the most likely explanation for the reduced level of FN mRNA in HT1O80 cells. We have attempted to measure the half-life of FN mRNA in the transformed and revertant cell lines using the transcription inhibitors actinomycin D and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (21). However, with each inhibitor, FN mRNA decay was only transient when assayed by Northern analysis, thus prohibiting half-life determinations (data not shown). One possible interpretation of these results is that a short-lived protein encoded by a short-lived mRNA mediates degradation of the FN message. Half-life measurements by pulse-chase methods were also attempted but were hindered by the fact that the FN mRNA is very low in abundance in the HT1O80 subclone used in this study. In the future, we hope to overcome some of these difficulties by adopting a strategy involving transfection of a transiently inducible FN gene into the cell line of interest, as has been described for other systems (22). ras proteins are associated with the plasma membrane and are thus not likely to be directly involved in the cytoplasmic degradation of FN mRNA. Instead, a cascade of events is probably required for the ras oncoproteins to exert their effects on FN mRNA. The N-ras oncogene and glucocorticoids have opposite posttranscriptional effects on FN biosynthesis in HT1O80 cells. However, our Northern analysis data demonstrate that the stimulatory effects of glucocorticoids are not mediated by a transcriptional repression of the N-ras gene.

Fibronectin Down-Regulation in ras-transformed Cells

![Image](image-url)

Fig. 4. Steady-state levels of N-ras mRNA in HT1O80 and 10a cells. Poly(A)* mRNA was isolated from cells grown for 72 h in the absence (−) or presence (+) of 0.2 mM dexamethasone (DEX). Aliquots of mRNA (10 μg/mlane) were fractionated through formaldehyde-agarose gels, blotted onto Hybond-N, and hybridized to the 32P-labeled human N-ras probe pNRSac. The blot was then rehybridized to FN and GAPD probes. The data presented are representative of three independent experiments. A, the positions of the 4.3- and 2 kb N-ras transcripts are indicated by the arrows. B, the positions of the FN and GAPD transcripts are indicated by the arrows. Migration of the 285 (5 kb) and 185 (2 kb) ribosomal RNAs are indicated by the bars between the two panels.

The utility of N-ras-transformed HT1O80 cells to increase levels of FN mRNA via a posttranscriptional mechanism (13). In this paper, we provide evidence that expression of the N-ras oncogene mediates a posttranscriptional down-regulation of FN in HT1O80 cells. Transcriptional repression of several genes by glucocorticoids has been reported in recent years (18). Therefore, one possible explanation of our results would be that induction of FN mRNA levels by dexamethasone is mediated by a decrease in N-ras expression. To test this possibility, poly(A)* mRNA was isolated from untreated and dexamethasone-treated HT1O80 cells, and the steady-state levels of N-ras mRNA were determined by Northern analysis. Two N-ras transcripts (approximately 4.3 and 2 kb) that differ in the length of their 3' untranslated regions have previously been detected in HT1O80 cells (19). Our experiments revealed no effect on the abundance of either N-ras mRNA following 72 h of dexamethasone treatment (Fig. 4A). In contrast, strong induction of FN mRNA was observed when the same blot was rehybridized with the FN probe (Fig. 4B). Therefore, the stimulatory effect of glucocorticoids on FN biosynthesis in HT1O80 cells is not due to a transcriptional repression of the N-ras gene.
tional activity has been observed for transforming growth factor β (23), β-polymersase (24), c-fos (25), and γ-glutamyltransferase (26) genes. ras has also been shown to increase the stability of ornithine decarboxylase (27), c-sis (28), and glutathione-S-transferase-P (26) mRNAs. The posttranscriptional down-regulation of FN of that we demonstrate in this study is clearly different and adds a new dimension to the repertoire of effects induced by ras transformation. It is a particularly interesting finding given the important role that FN plays in maintaining cell morphology and cell adhesion, two aspects of the cellular phenotype that are often dramatically altered upon oncogenic transformation. Our determination of posttranscriptional down-regulation of FN in N-ras-transformed cells complements the studies of Radinsky et al. (29) which demonstrate altered FN-mediated adhesion mechanisms in revertants of Ki-ras transformed cells. Further analysis of FN biosynthesis in other transformed cells promises to yield more information regarding the process by which production of this important matrix protein is disrupted as a function of oncogenic transformation.

Materials and Methods

Cell Lines and Culture Conditions. HT1080sc2 is a subclone of human HT1080 fibrosarcoma cells (30) obtained by picking and propagating a single cell from an HT1080 cell population. 10a is a flat, nonmucromorphic revertant of HT1080sc2 obtained by suicide selection techniques. The isolation and characterization of both cell lines has been described (16), and they were generously provided by Dr. Hugh Paterson (London). Cells were maintained in DMEM (GIBCO) supplemented with 10% heat-inactivated calf serum (HyClone), penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml) (GIBCO). Cultures were maintained in humidified 87% air, 13% CO2. 10a cells are phenotypically unstable in vitro. Therefore, cultures obtained from the original stock were maintained for a limited number (four to five) of passages. Dexamethasone (Sigma) was solubilized at 2 mM concentration in 100% ethanol and diluted in DMEM prior to addition to the cells.

Metabolic Labeling and Immunoprecipitation. Metabolic labeling of cells was achieved by incubating nearconfluent cells from 60-mm diameter dishes for 2 h in 0.4 ml of DMEM (minus methionine) containing 200 μCi/ml of U-[35S]methionine (>1000 Ci/mmol; ICN). FN from both the cell layer and the medium in combination was immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis and fluorography as previously described (13). Results were quantified by liquid scintillation counting of the FN bands cut from the dried gels.

RNA Isolation and Analysis. Total cellular RNA was extracted from cells using an acid guanidinium thiocyanate-phenol-chloroform procedure (31). Where indicated, poly(A)* mRNA was selected by affinity chromatography on oligo(dT) cellulose (32). RNAs were fractionated through 1% agarose-2.2 M formaldehyde gels and transferred to Hybond-N (Amersham). The inserts from the human FN complementary DNA clone pFH154 (provided by A. Kornbluth) (33), the human N-ras clone pNRSac (American Type Culture Collection) (34), or the human GAPD clone pHC GAP (American Type Culture Collection) (35) were labeled to >106 cpm/μg by the random primer method (36) using the Multiprime DNA labeling kit from Amersham. The blots were UV crosslinked and then prehybridized and hybridized at 42°C in 1% bovine serum albumin-1 mM EDTA-500 mM sodium phosphate (pH 7.2)-5% SDS-50% formamide. Following hybridization, blots were washed for 15 min at room temperature in 2X SSC-1% SDS; for 15 min at room temperature in 0.2X SSC-1% SDS; and for 30 min at 55°C in 0.2X SSC-1% SDS with two buffer changes. The membranes were then exposed to preflashed Kodak XAR-5 film with two DuPont Lightning-Plus intensifying screens for 1–2 days at −70°C. Results were quantified using an LKB Ultrosan XL densitometer, and the GAPD band intensities were used to normalize for equal sample loading.

Transfections and CAT Assays. The plasmid p510FNCAT contains a PvuII-PstI fragment of the human FN gene from −510 to +69 base pairs fused to the coding sequence for the bacterial CAT gene (13). Cultured cells were transfected by the calcium phosphate method as previously described (37). Briefly, 10-cm-diameter plates at 30% confluence were cotransfected with 5 μg of p510FNCAT DNA and 2.5 μg of RSV-Bgal DNA (38). The cells were incubated for 5 h in the presence of the precipitated DNA, washed twice with phosphate-buffered saline, and refed with complete DMEM. After 48 h, cell extracts were prepared, and assays for β-galactosidase (38) were used to standardize for transfection efficiencies. Normalized amounts of cell extracts were used for CAT assays, as previously described (39). In general, approximately 50 μg of protein were used for 2-h reactions containing 0.10 μCi [3H]chloramphenicol (NEN). CAT activities were measured by liquid scintillation counting of the relevant spots cut from the thin-layer chromatography plates.

Acknowledgments

The authors are grateful to Dr. Hugh Paterson for generously providing us with the cell lines used in this study. We thank Gail Baughman and John Moffett for critical reading of the manuscript.

References


