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A Novel Mechanism of Ha-*ras* Oncogene Action: Regulation of Fibronectin mRNA Levels by a Nuclear Posttranscriptional Event

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Although loss of cell surface fibronectin (FN) is a hallmark of many oncogenically transformed cells, the mechanisms responsible for this phenomenon remain poorly understood. The present study utilized the nontumorigenic human osteosarcoma cell line TE-85 to investigate the effects of induced Ha-*ras* oncogene expression on FN biosynthesis. TE-85 cells were stably transfected with metallothionein–Ha-*ras* fusion genes, and the effects of metal-induced *ras* expression on FN biosynthesis were determined. Induction of the *ras* oncogene, but not proto-oncogene, was accompanied by a decrease in total FN mRNA and protein levels. Transfection experiments indicated that these oncogene effects were not due to reduced FN promoter activity, suggesting that a posttranscriptional mechanism was involved. The most common mechanism of posttranscriptional regulation affects cytoplasmic mRNA stability. However, in this study the down-regulation of FN was identified as a nuclear event. A component of the *ras* effect was due to a mechanism affecting accumulation of processed nuclear FN RNA. Mechanisms that would generate such an effect include altered RNA processing and altered stability of the processed message in the nucleus. There was no effect of *ras* on FN mRNA poly(A) tail length or site of polyadenylation. There was also no evidence for altered splicing at the ED-B domain of FN mRNA. This demonstration of nuclear posttranscriptional down-regulation of FN by the Ha-*ras* oncogene identifies a new level at which *ras* oncoproteins can regulate gene expression and thus contribute to development of the malignant phenotype.

Fibronectins (FNs) are large glycoproteins implicated in a wide variety of cellular properties including cell adhesion, morphology, migration, differentiation, and transformation (21). FNs exist in extracellular matrices (cellular FN) and in soluble form in body fluids (plasma FN) and are composed of two similar but nonidentical subunits held together by disulfide bonds. Alternative splicing of the primary transcript of a single gene located on human chromosome 2 generates numerous distinct FN transcripts which account for the individual subunits and for the plasma and cellular isoforms. Loss of cell surface FN is a hallmark of many oncogenically transformed cells and has been correlated with the acquisition of tumorigenic and metastatic potentials (21). This effect has been observed in cells transformed with a variety of oncogenes; however, there is still little known about the mechanisms by which oncogenes affect FN biosynthesis. In two cases, transformation of chick embryo fibroblasts by *src* and growth stimulation of rat embryo fibroblasts by E1A, the primary defects in FN production are reported to be at the level of transcription (31, 45).

The *ras* family of genes encode related 21-kDa plasma membrane-associated proteins that bind guanine nucleotides and are involved in signal transduction and cell growth (2). *ras* oncogenes are mutated in many types of human tumors. As a result of mutation, there is a loss of control of *ras* function, resulting in altered expression of other cellular genes (2). For example, inverse correlations between expression of *ras* oncogenes and levels of FN biosynthesis have been demonstrated (6, 7, 36, 39). We previously reported that a posttranscriptional mechanism appears to be involved in the reduction of FN biosynthesis in N-*ras*-transformed HT1080 cells compared with flat revertants of HT1080 cells (7). However, in that study the posttranscriptional mechanism was not characterized. Furthermore, since independently grown stable cell lines were being compared, it was not certain that decreased FN expression was due to expression of the *ras* oncogene. Therefore, in the present study a human cell line in which *ras* expression can be specifically induced has been established in order to further and more directly investigate the effects of *ras* oncogene expression on FN biosynthesis.

TE-85 is a nontumorigenic human osteosarcoma cell line that can be efficiently transformed by transfection with cloned oncogenes and thus provides a unique human system for oncogenesis studies (43). The experiments reported here utilize TE-85 cells that have been stably transfected with wild-type and mutated human Ha-*ras* genes placed under control of a human metallothionein promoter. Induction of *ras* expression is achieved by treatment of the transfected cells with heavy metal. Characterization of FN expression in these cells has provided evidence for a link between induced expression of the Ha-*ras* oncogene and posttranscriptional down-regulation of FN. The most commonly observed posttranscriptional mechanism affecting levels of accumulated mRNA involves regulation of cytoplasmic mRNA stability (1). In fact, expression of *ras* oncogenes has been shown to affect the cytoplasmic stability of specific mRNAs (20, 28). The present study demonstrates that nuclear mechanisms are primarily responsible for the down-regulation of FN in *ras*-expressing TE-85 cells. In particular, a *ras*-regulated nuclear event that causes decreased accumulation of processed FN mRNA has been identified. This type of nuclear posttranscriptional regulation is not well documented and represents a previously unidentified level at which *ras* oncoproteins can regulate the expression of cellular

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genes and thus contribute to development of the malignant phenotype.

MATERIALS AND METHODS

Plasmids. The plasmid pMT-Ha-*ras*(onc) contains the oncogenic human Ha-*ras*$^{Val\text{-}12}$ gene under transcriptional control of the heavy metal-inducible human metallothionein II_A (hMT-II_A) promoter. To make this plasmid, the Ha-*ras* promoter was deleted from a genomic clone of the oncogene (pT24-C3; American Type Culture Collection [ATCC], Rockville, Md.) by digestion at a *Sma*I site located just upstream of the translation initiation (ATG) codon (3). The remaining Ha-*ras* sequences included all four exons and extended to a *Bgl*II site located 3′ beyond both the poly(A) addition site and the variable tandem repeat region. A 770-bp *Hin*dIII-to-*Bam*HI fragment containing the hMT-II_A promoter was excised from the plasmid pHS1 (kindly provided by Michael Karin, University of California, San Diego) (22) and was fused to the *Sma*I site of the *ras* oncogene. The control plasmid pMT-Ha-*ras*(wt) contains the human Ha-*ras* proto-oncogene (Gly-12) under the control of the hMT-II_A promoter and was similarly constructed. The presence of the wild-type or mutated *ras* codon 12 was confirmed by sequencing of the constructs using a primer from the metallothionein promoter.

Cell lines and culture conditions. The human osteogenic sarcoma cell line TE-85 (29) was obtained from the ATCC, and the cells were grown in Eagle's minimal essential medium (GIBCO-BRL; Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (Tissue Culture Biologicals, Tulare, Calif.), penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml; GIBCO-BRL). Cultures were maintained in humidified 87% air–13% CO_2. TE-85 cells containing the pMT-Ha-*ras* plasmids were obtained by stable calcium phosphate cotransfection (46) with pSV2neo followed by selection for resistance to G418 (600 μg/ml; GIBCO-BRL). Individual colonies of G418-resistant cells were isolated by trypsinization using glass cloning rings.

Metabolic labeling and immunoprecipitation. [^{35}S]methionine labeling of cells and immunoprecipitation and electrophoresis of FN were carried out as previously described (7). Results were quantified by liquid scintillation counting of the FN bands cut from dried gels.

RNA isolation and Northern (RNA) analyses. Total cellular RNAs were extracted from cells using an acid guanidinium thiocyanate-phenol-chloroform procedure (10). RNAs were fractionated through 1% agarose–2.2 M formaldehyde gels and transferred to Hybond-N (Amersham, Arlington Heights, Ill.). Inserts from the human FN cDNA clone pFH154 (26), the human Ha-*ras* genomic clone pT24-C3 (ATCC) (35), and the human glyceraldehyde-3-phosphate dehydrogenase (GAPD) clone pHcGAP (ATCC) (44) were labeled with [α-^{32}P]dATP by the random primer method (16). The blots were prehybridized, hybridized, and washed as previously described (7) and exposed to Kodak XAR-5 film with two Dupont Lightning-Plus intensifying screens at −70°C. Results were quantified with an LKB Ultroscan XL densitometer, and the GAPD band intensities were used to normalize for equal sample loading.

To isolate nuclear RNAs, cells were lysed in 10 mM Tris-HCl (pH 7.5)–10 mM NaCl–3 mM $MgCl_2$–0.5% Nonidet P-40. Nuclei were pelleted at 800 × *g* and washed extensively with cold lysis buffer (without Nonidet P-40). RNA was then extracted by the acid guanidinium thiocyanate-phenol-chloroform procedure used to isolate total cellular RNAs (10). The integrity of the nuclear RNAs was demonstrated by fractionation through agarose gels and ethidium bromide staining. This analysis also demonstrated that the nuclear samples were enriched (compared with total or cytoplasmic RNAs) in precursor rRNAs (data not shown). Nuclear RNAs were transferred to a Hybond-N membrane by using the Schleicher and Schuell Minifold II Slot-Blotter. Filters were hybridized to labeled probes as described above. The human FN intron 1 probe was isolated as a *Sac*II (+430)-to-*Eco*RI (+1000) fragment of the human FN genomic clone pgHF3.7 (12). The FN exon 1 probe was isolated as a *Nae*I (+13)-to-*Esp*I (+368) fragment of pgHF3.7 (12).

CAT assays. The plasmid −1.3FNCAT contains a fragment of the human FN gene from −1.3 kb to +69 bp (12) fused to the coding sequence for the bacterial chloramphenicol acetyltransferase (CAT) gene. TE-MT-*ras*(onc) cells were transfected with −1.3FNCAT by electroporation with an Electro Cell Manipulator 600 (BTX, San Diego, Calif.). Briefly, 100 μg of DNA was added to 3 × 10^6 cells in 0.4 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline in a 2-mm-gap cuvette. Electroporation conditions were 800 μF, 120 V, and 48 Ω, with a 6- to 7-ms pulse length. For background measurements, cells were transfected with a promoterless CAT construct. Following electroporation, like samples were pooled in complete medium and split into 100-mm-diameter dishes at 3 × 10^6 cells per dish. Dead cells were removed by changing the culture medium 5 to 6 h later. When desired, cells were treated with heavy metal the next morning. After 48 h of metal treatment, cell extracts were prepared by freeze-thawing, and CAT activities were measured by using the xylene phase-extraction assay described by Seed and Sheen (37). Relative CAT activities were determined by liquid scintillation counting of the xylene phases and normalized on the basis of the amount of protein added to each reaction. Control experiments demonstrated that there was no effect of heavy metal treatment on the protein content of TE-MT-*ras*(onc) cells.

Polyadenylation (H-blot) analysis. An 18-mer oligonucleotide (GCA-GAA-CAG-GCA-ATG-TGC) that was complementary to a region 200 nucleotides downstream of the translation termination codon and 470 nucleotides upstream of the polyadenylation site in FN mRNA was synthesized (25). This FN oligonucleotide was hybridized, in the absence or presence of oligo(dT)$_{12-18}$ (Pharmacia, Piscataway, N.J.), to total cellular RNAs, and the samples were digested with RNase H as described by Carrazana et al. (5). RNAs were then phenol-chloroform extracted, fractionated through 2% agarose–2.2 M formaldehyde gels, and transferred to Hybond-N. Blots were hybridized to an *Xmn*I-*Hin*dIII FN probe from the cDNA clone pFH1 (25) that spans the 3′ untranslated RNA fragment generated by RNase H digestion. Samples hybridized to oligo(dT) and to the FN oligonucleotide generated deadenylated fragments that were detected by the 3′ untranslated region probe, whereas samples hybridized to the FN oligonucleotide alone generated fragments with poly(A) tails.

RNase protection assays. An antisense riboprobe corresponding to intron 1 of human FN RNA was generated by first subcloning a *Sac*II (+430)-to-*Eco*RI (+1000) fragment of the human FN genomic clone pgHF3.7 (12) into pBluescript SK− (Stratagene, La Jolla, Calif.). The resulting plasmid was digested with *Pvu*II, and ^{32}P-labeled antisense RNA was prepared by transcription with T7 RNA polymerase in the presence of [^{32}P]UTP as specified by Promega (Madison, Wis.). A riboprobe corresponding to exon sequence in human FN RNA was generated by first subcloning a *Pst*I (+3526)-to-*Pvu*II (+4669) fragment from the human cDNA clone pFH154 (26) into pBluescript SK−. The resulting plasmid was digested with *Bam*HI, and the riboprobe was prepared with T3 RNA poly-

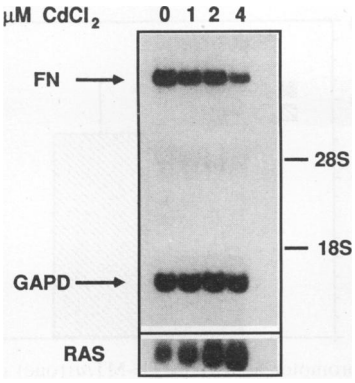

FIG. 1. Dose-responsive heavy metal induction of Ha-*ras* in TE-MT*ras* cells. Total cellular RNAs were isolated from TE-MT*ras*(onc) cells grown for 72 h in the presence of 0, 1, 2, or 4 μM $CdCl_2$. Aliquots of RNA (10 μg per lane) were fractionated through a formaldehyde-agarose gel, blotted onto Hybond-N, and hybridized to a ^{32}P-labeled human FN cDNA probe and a human GAPD probe. The relative positions of the 18S (2-kb) and 28S (5-kb) rRNAs, the FN mRNA, and the GAPD mRNA are indicated. The same blot was stripped and rehybridized to a human Ha-*ras* probe. The box at the bottom shows a cut-out portion of the autoradiogram containing the *ras* bands.

merase. A 620-bp *Hin*dIII-to-*Bam*HI fragment containing the alternatively spliced FN exon ED-B was excised from the human cDNA construct pCEF103 (kindly provided by Alberto Kornblihtt) (15) and was subcloned into the *Hin*dIII-*Bam*HI sites of pGEM-4 (Promega). The resulting plasmid was digested with *Eco*RV, and ^{32}P-labeled antisense RNA was prepared by transcription with SP6 RNA polymerase. The RNA probes were purified by gel electrophoresis and electroelution. Ten micrograms of cellular RNAs was hybridized to 5 × 10^5 cpm of riboprobe in 80% formamide for 18 h at 45°C (18). The samples were then digested with RNase One as specified by the manufacturer (Promega), and the protected FN RNA fragments were resolved in 5% sequencing gels which were then subjected to autoradiography. Results were quantified by densitometry.

RESULTS

Isolation of TE-85 cells containing inducible *ras* genes. Our previous studies using tumorigenic HT1080 cells and nontumorigenic revertants of HT1080 cells provided correlations between *ras* transformation and posttranscriptional down-regulation of FN (7). We have now established a human cell line in which Ha-*ras* gene expression can be specifically manipulated in order to further and more directly investigate the effects of *ras* on FN biosynthesis. The human osteosarcoma cell line TE-85 provided a valuable system for such experiments. TE-85 is an immortal, nontumorigenic cell line that can be transformed efficiently by transfection with cloned oncogenes (43). TE-85 cells have several in vitro properties of malignant cells, including chromosome breaks and a mutated p53 gene (34). The p53 gene product has been assigned tumor suppressor functions (27) and thus mutation of the p53 gene may predispose TE-85 cells to oncogenic transformation.

We constructed plasmids containing the heavy metal-inducible hMT-II_A promoter fused upstream of the human Ha-*ras* oncogene or (as a control) proto-oncogene (see Materials and Methods). Induction of the hMT-II_A promoter by heavy metals is mediated by four metal-responsive elements located within 200 bp upstream of the transcription start site (22). The MT-Ha-*ras* constructs were stably transfected into TE-85 cells, and individual clones were screened by Northern analysis for induction of Ha-*ras* mRNA upon treatment with 4 μM $CdCl_2$ (data not shown). Clones with low basal *ras* expression and high metal inducibility were chosen for further analysis. The selected clones were designated TE-MT*ras*(onc) and TE-MT*ras*(wt) for oncogene- and proto-oncogene-containing cells, respectively. As Fig. 1 illustrates for TE-MT*ras*(onc) cells, treatment with increasing concentrations of $CdCl_2$ resulted in a dose-dependent increase in Ha-*ras* mRNA levels. This induction of *ras* was reversible upon removal of heavy metal from the culture medium (data not shown). Therefore, the cells exhibited the desired ability to manipulate *ras* expression. Figure 1 also reveals that a dose-dependent decrease in levels of accumulated FN mRNA accompanied the increase in *ras* oncogene mRNA levels (see below).

Effects of induced *ras* expression on levels of total FN mRNA in TE-85 and TE-MT*ras* cells. The effects of heavy metal

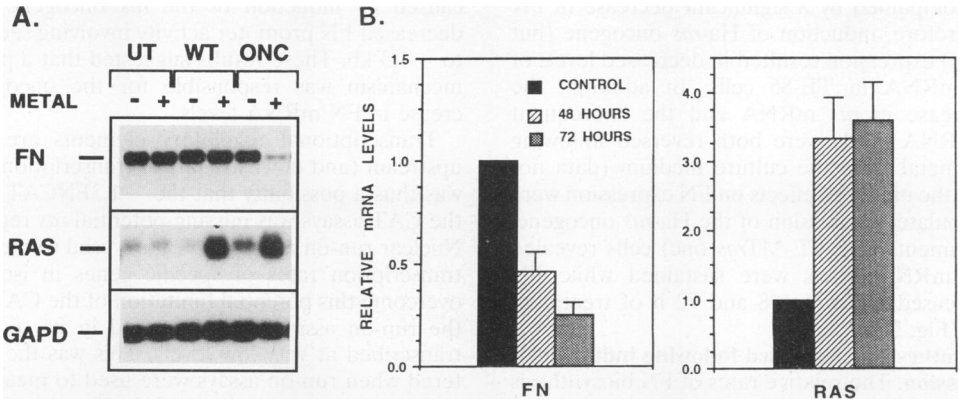

FIG. 2. Modulation of FN mRNA levels by the Ha-*ras* oncogene. (A) Total cellular RNAs were isolated from cells grown for 48 h in the absence (−) or presence (+) of 4 μM $CdCl_2$–100 μM $ZnSO_4$. Aliquots of RNA (10 μg per lane) were fractionated through a formaldehyde-agarose gel, blotted, and sequentially hybridized to FN, Ha-*ras*, and GAPD probes. The data are representative of three independent experiments. UT, untransfected TE-85; WT, TE-MT*ras*(wt); ONC, TE-MT*ras*(onc). (B) The data obtained by Northern analysis of TE-MT*ras*(onc) RNAs were quantified by densitometry, and the FN and *ras* signals were normalized to GAPD signals. The changes in FN and *ras* mRNA levels following 48 and 72 h of treatment with 4 μM $CdCl_2$–100 μM $ZnSO_4$ relative to values for untreated controls are presented. The plotted values represent the means plus standard deviations for three independent experiments.

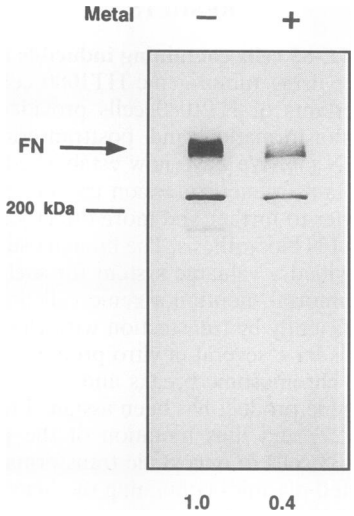

FIG. 3. Modulation of FN protein biosynthesis by the Ha-*ras* oncogene. TE-MT*ras*(onc) cells were grown for 48 h in the absence (–) or presence (+) of 4 μM $CdCl_2$–100 μM $ZnSO_4$. The cells were then labeled for 2 h with [^{35}S]methionine, and FN from the culture medium and cell layer in combination was immunoprecipitated and electrophoresed on a SDS–5% polyacrylamide gel. The relative positions of immunoprecipitated FN (arrow) and a protein marker (200 kDa) are indicated. Results were quantified by liquid scintillation counting of FN bands cut from the dried gels. The mean relative rates of FN biosynthesis obtained from two independent experiments are shown at the bottom. Independent values did not vary from the mean by more than 8%.

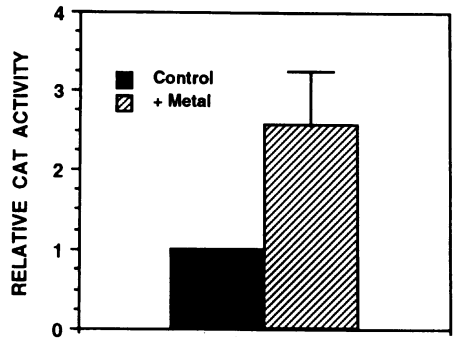

FIG. 4. FN promoter activity in TE-MT*ras*(onc) cells. TE-MT*ras* (onc) cells were transiently transfected with –1.3FNCAT and grown for 48 h in the absence or presence of 4 μM $CdCl_2$–100 μM $ZnSO_4$. Normalized amounts of cell extracts were used in CAT assays, and relative promoter activities were determined by liquid scintillation counting of the reaction products. FN promoter activity in *ras*-expressing (metal-treated) cells relative to activity in untreated controls is presented. The values are the means (plus standard deviations) for four independent experiments.

treatment on FN expression in TE-MT*ras*(onc) cells and (as controls) in the parental TE-85 and transfected TE-MT*ras*(wt) cells were further characterized. Cells were treated with heavy metals for 48 h, and levels of accumulated FN and Ha-*ras* mRNAs were determined by Northern analysis (Fig. 2A). As expected, treatment with heavy metals had no effect on levels of endogenous FN or Ha-*ras* mRNAs in untransfected TE-85 cells. TE-MT*ras*(wt) cells exhibited significant induction of *ras* proto-oncogene expression without any effect on FN mRNA levels. Finally, treatment of TE-MT*ras*(onc) cells with heavy metals resulted in an increase in Ha-*ras* oncogene mRNA levels that was accompanied by a significant decrease in FN mRNA levels. Therefore, induction of Ha-*ras* oncogene (but not proto-oncogene) expression resulted in decreased levels of accumulated FN mRNA in TE-85 cells. In addition, the metal-induced increase in *ras* mRNA and the consequent decrease in FN mRNA levels were both reversed following removal of heavy metal from the culture medium (data not shown). Therefore, the observed effects on FN expression were linked to the manipulated expression of the Ha-*ras* oncogene. Time course experiments with TE-MT*ras*(onc) cells revealed that elevated *ras* mRNA levels were sustained while FN mRNA levels decreased following 48 and 72 h of treatment with heavy metals (Fig. 2B).

FN protein biosynthesis is decreased following induction of *ras* oncogene expression. The relative rates of FN biosynthesis in untreated and metal-treated TE-MT*ras*(onc) cells were measured by pulse-labeling for 2 h with [^{35}S]methionine followed by immunoprecipitation of FN from the culture medium and cell layer in combination. Immunoprecipitates were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and the data revealed that decreased biosynthesis of FN correlated with induced expression of the Ha-*ras* oncogene (Fig. 3). Therefore, the *ras* effect seen at the FN mRNA level was also evident at the FN protein level. Furthermore, the magnitude of the protein effect was similar to that seen at the total RNA level (Fig. 2B).

FN promoter activity is not inhibited by *ras* oncogene expression. We previously reported that a CAT gene under control of human FN promoter sequences extending 5′ to –510 bp was more actively expressed in N-*ras*-transformed HT1080 cells than in revertant HT1080 cells (7). This result indicated that a posttranscriptional mechanism was involved in the down-regulation of FN mRNA in HT1080 cells compared with the revertant cells. To measure activity of the FN promoter in TE-MT*ras*(onc) cells, a CAT construct containing additional FN sequence extending 5′ to –1.3 kb was used. TE-MT*ras*(onc) cells were transiently transfected with –1.3FNCAT, and the effect of metal treatment (*ras* induction) on CAT activity was determined. CAT activities were increased, and thus the FN promoter was more active in the oncogene-expressing cells than in the uninduced cells (Fig. 4). In other words, the reduced level of accumulated FN mRNA caused by induction of the *ras* oncogene was not due to decreased FN promoter activity involving sequences extending to –1.3 kb. These results suggested that a posttranscriptional mechanism was responsible for the oncogene-induced decrease in FN mRNA levels.

Transcriptional regulatory elements are often found far upstream (and downstream) of transcription start sites. There was thus a possibility that the –1.3FNCAT construct used in the CAT assays was missing potential *ras* regulatory elements. Nuclear run-on assays, which are used to measure the relative transcription rates of specific genes in isolated nuclei, can overcome this potential limitation of the CAT assay. However, the run-on assay is itself limited in its sensitivity for genes transcribed at very low levels. This was the problem encountered when run-on assays were used to measure relative rates of FN gene transcription in TE-MT*ras*(onc) cells. Using standard labeling and hybridization conditions (19), transcription of the metallothionein gene was clearly induced by heavy metals. In contrast, even basal FN signals were not detectably above background levels, thus prohibiting quantitative analysis of FN gene transcription rates (data not shown).

The abundance of processed nuclear FN mRNA is reduced

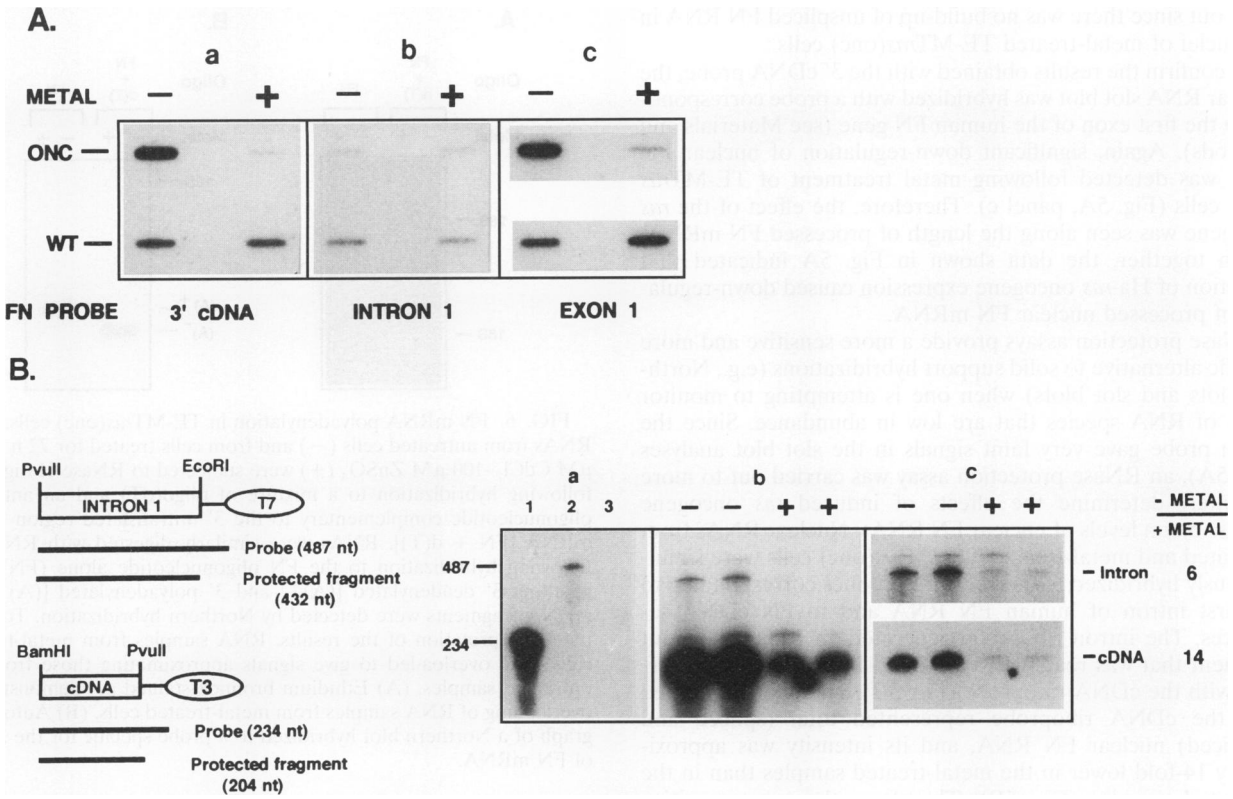

FIG. 5. Effects of induced *ras* expression on levels of nuclear FN RNA. Nuclear RNAs were isolated from TE-MT*ras*(onc) and TE-MT*ras*(wt) cells grown for 72 h in the absence (−) or presence (+) of 4 μM $CdCl_2$–100 μM $ZnSO_4$. (A) Slot blots containing 5 μg of each nuclear RNA sample were hybridized to probes corresponding to the 3′ end of the FN cDNA (panel a), the first intron of the FN gene (panel b), and the first exon of the FN gene (panel c) (see Materials and Methods). Equal sample loading was verified by rehybridizing with a probe for GAPD (data not shown). (B) Plasmids used to make cDNA and intron 1 riboprobes are illustrated on the left (see also Materials and Methods). Following digestion of the cDNA plasmid with *Bam*HI, transcription with T3 RNA polymerase in the presence of [^{32}P]UTP generates a 234-nucleotide (nt) riboprobe. Hybridization of the cDNA riboprobe to nuclear RNA generates a 204-nucleotide RNase-resistant fragment. Similarly, digestion of the intron 1 plasmid with *Pvu*II and transcription with T7 RNA polymerase generates a 487-nucleotide riboprobe that protects a 432-nucleotide fragment when hybridized to nuclear RNA. The autoradiographs show the protected fragments generated by nuclear RNAs from TE-MT*ras*(onc) cells grown for 72 h in the absence (−) or presence (+) of 4 μM $CdCl_2$–100 μM $ZnSO_4$. Panel a shows the migration of the individual riboprobes (lane 1, cDNA; lane 2, intron 1) whose sizes (in nucleotides) are indicated on the left. As a control, a protection assay was also carried out using yeast tRNA instead of cellular RNA (panel a, lane 3). Duplicate samples of nuclear RNAs from untreated (−) and metal-treated (+) cells were hybridized to a mixture of cDNA and intron riboprobes. Panel b shows an exposure of the autoradiograph in which the protected fragments generated by both riboprobes can be detected. In panel c, different exposures of the autoradiograph shown in panel b have been spliced together in order to facilitate quantification and comparison of the protected fragments generated with each riboprobe. The positions of the protected fragments generated with each riboprobe are indicated on the right. The relative signal intensities (−metal/+metal) of the bands generated by each probe are given on the right. These numbers are the means for two experiments in which each sample was analyzed in duplicate, and multiple exposure times were used for quantification.

by *ras* oncogene expression. Regulation of cytoplasmic message stability is the most frequently documented means of posttranscriptional regulation. Therefore, Ha-*ras* oncogene-induced cytoplasmic destabilization of FN mRNA seemed a likely explanation for the observed posttranscriptional down-regulation of FN mRNA. FN mRNAs have been reported to exhibit long half-lives (13, 14). When the transcription inhibitor actinomycin D was used to measure the half-life of FN mRNA in TE-MT*ras*(onc) cells, no measurable decay of FN mRNA was observed before significant cell death occurred at 24 h (data not shown). Therefore, as an alternative means of determining if the posttranscriptional down-regulation of FN by the *ras* oncogene was a cytoplasmic or nuclear event, cellular RNAs were fractionated and analyzed by slot blotting. Nuclear RNAs prepared from untreated and metal-treated TE-MT*ras*(onc) and TE-MT*ras*(wt) cells were initially hybridized to a FN cDNA probe (pFH154) (26) spanning the 3′ end of FN mRNA. With this probe, nuclear FN RNA levels were down-regulated to ~20% of control levels following metal treatment of TE-MT*ras*(onc) [but not TE-MT*ras*(wt)] cells (Fig. 5A, panel a). The magnitude of this effect on nuclear FN RNA levels was similar to the ~5× decrease observed when total FN RNA was examined (Fig. 2B). A *ras*-induced mechanism involving inhibition of nuclear export of FN mRNA was ruled out since there was no build-up of FN mRNA in the nuclei of metal-treated TE-MT*ras*(onc) cells.

Several recent investigations have described nuclear posttranscriptional regulation involving changes in the stability and accumulation of primary (unspliced) transcripts (11, 24, 42). To investigate this possibility, nuclear RNA slot blots were rehybridized with a probe corresponding to the first intron of the human FN gene (see Materials and Methods). With the intron 1 probe, there was no detectable effect of induced *ras* oncogene (or proto-oncogene) expression on the accumulated levels of unspliced FN RNA (Fig. 5A, panel b). Furthermore, a mechanism involving inhibition of pre-RNA splicing could be

ruled out since there was no build-up of unspliced FN RNA in the nuclei of metal-treated TE-MT*ras*(onc) cells.

To confirm the results obtained with the 3′ cDNA probe, the nuclear RNA slot blot was hybridized with a probe corresponding to the first exon of the human FN gene (see Materials and Methods). Again, significant down-regulation of nuclear FN RNA was detected following metal treatment of TE-MT*ras* (onc) cells (Fig. 5A, panel c). Therefore, the effect of the *ras* oncogene was seen along the length of processed FN mRNA. Taken together, the data shown in Fig. 5A indicated that induction of Ha-*ras* oncogene expression caused down-regulation of processed nuclear FN mRNA.

RNase protection assays provide a more sensitive and more specific alternative to solid support hybridizations (e.g., Northern blots and slot blots) when one is attempting to monitor levels of RNA species that are low in abundance. Since the intron probe gave very faint signals in the slot blot analyses (Fig. 5A), an RNase protection assay was carried out to more accurately determine the effects of induced *ras* oncogene expression on levels of nuclear FN RNAs. Nuclear RNAs from untreated and metal-treated TE-MT*ras*(onc) cells were simultaneously hybridized to antisense riboprobes corresponding to the first intron of human FN RNA and to FN cDNA sequences. The intron riboprobe generated an RNase-resistant fragment that was readily distinguished from the band generated with the cDNA riboprobe (Fig. 5B). The band generated with the cDNA riboprobe represented total (spliced and unspliced) nuclear FN RNA, and its intensity was approximately 14-fold lower in the metal-treated samples than in the untreated samples (Fig. 5B). Therefore, the more sensitive RNase protection analysis revealed an even greater effect of *ras* oncogene expression on nuclear FN RNA levels than had been detected by Northern analysis of total RNA (Fig. 2) or by slot blot analysis of nuclear RNA (Fig. 5A). The band generated with the intron riboprobe represented only unspliced FN RNA. As expected, and in agreement with Fig. 5A, the band generated with the intron riboprobe was considerably less intense than the band generated with the cDNA riboprobe since unspliced FN RNA is a transient intermediate that does not accumulate in the nucleus (Fig. 5B, panel b). However, the bands generated with the intron riboprobe were of sufficient intensity to be quantified. The data revealed that there was only a 6× decrease in the level of unspliced FN RNA in the oncogene-expressing cells. This *ras* effect on unspliced FN RNA could not, therefore, account for the greater effect seen with the cDNA riboprobe. The observed effect on the intron-containing RNA could reflect altered stability of the unprocessed message or a transcriptional effect that was undetected by the transfection experiments (Fig. 4). However, if down-regulation of FN by the *ras* oncogene were purely a transcriptional effect, the magnitude of the effect observed with the intron riboprobe would be expected to equal that observed with the cDNA riboprobe. Since this was not the case, the RNase protection data confirmed that one component of *ras* action involved posttranscriptional down-regulation of processed nuclear FN mRNA.

FN mRNA polyadenylation is unaffected by *ras* oncogene expression. The results presented above suggested that the Ha-*ras* oncogene exerts a posttranscriptional effect on FN expression in the nucleus at the level of RNA processing and/or stability of the processed message. Several studies have demonstrated posttranscriptional regulation involving changes in poly(A) tail length on fully spliced nuclear RNAs (30, 38). We therefore investigated whether changes in the site or extent of polyadenylation correlated with the decrease in nuclear FN mRNA levels that was induced by *ras* oncogene expression.

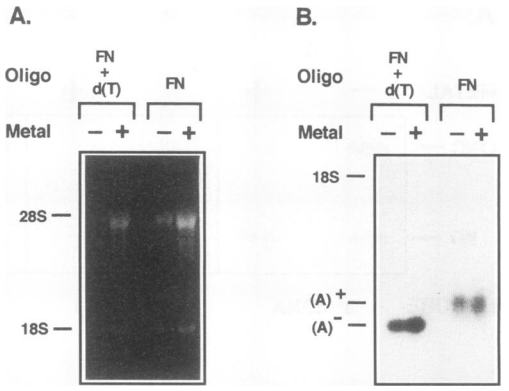

FIG. 6. FN mRNA polyadenylation in TE-MT*ras*(onc) cells. Total RNAs from untreated cells (–) and from cells treated for 72 h with 4 μM $CdCl_2$–100 μM $ZnSO_4$ (+) were subjected to RNase H digestion following hybridization to a mixture of oligo(dT) and an antisense oligonucleotide complementary to the 3′ untranslated region of FN mRNA [FN + d(T)]. RNAs were similarly digested with RNase H following hybridization to the FN oligonucleotide alone (FN). The resulting 3′ deadenylated [$(A)^-$] and 3′ polyadenylated [$(A)^+$] FN mRNA fragments were detected by Northern hybridization. To facilitate interpretation of the results, RNA samples from metal-treated cells were overloaded to give signals approximating those from the untreated samples. (A) Ethidium bromide-stained gel demonstrating overloading of RNA samples from metal-treated cells. (B) Autoradiograph of a Northern blot hybridized to a probe specific for the 3′ end of FN mRNA.

For these experiments, we employed the H-blot method of Carrazana et al. (5). To investigate the site of polyadenylation in FN mRNA, samples of total RNA from untreated and metal-treated TE-MT*ras*(onc) cells were hybridized simultaneously to oligo(dT) and to an antisense FN oligonucleotide (18-mer) complementary to the 3′ untranslated region of FN mRNA (see Materials and Methods). Following digestion with RNase H, the 3′ deadenylated fragments were detected by Northern blotting and hybridization to a probe spanning the 3′ untranslated region of FN mRNA. The data revealed that the size of the deadenylated fragment was unaffected by induction of *ras* oncogene expression (Fig. 6B). Therefore, the site of polyadenylation of FN mRNA was not altered by expression of the Ha-*ras* oncogene. To investigate effects of *ras* expression on the length of the FN mRNA poly(A) tail, cellular RNAs were hybridized to the FN oligonucleotide alone prior to digestion with RNase H. The size distribution of the resulting 3′ polyadenylated fragments was also unaffected by induction of *ras* oncogene expression (Fig. 6B). Therefore, there were no detectable changes in the site of polyadenylation or in the length of the poly(A) tail of FN mRNA that correlated with *ras*-induced changes in nuclear FN mRNA levels.

Alternative splicing at the ED-B domain of FN mRNA is unaffected by *ras* oncogene expression. Alternative mRNA splicing is another potential nuclear posttranscriptional regulatory mechanism (41). Numerous distinct FN mRNAs are generated by alternative splicing of the primary transcript (21). The three best-characterized sites of alternative splicing in human cells are the so-called ED-A, ED-B, and IIICS regions. It has been demonstrated that splicing at these regions is frequently altered in transformed cells (21). In particular, there have been several reports of transformed human cells containing a significantly higher percentage of $ED\text{-}B^+$ FN mRNA than their normal counterparts (4, 33, 48). However, there has been no link made between these qualitative changes in FN

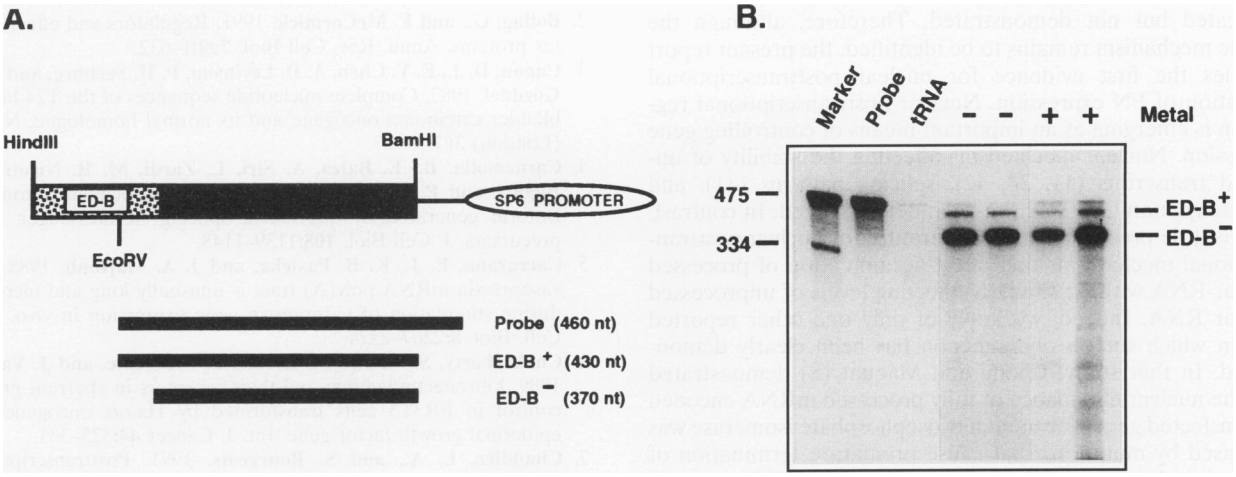

FIG. 7. Alternative splicing of the ED-B exon in TE-MT*ras*(onc) cells. (A) Construction of the plasmid used to make the ED-B riboprobe is described in Materials and Methods. Following digestion of the plasmid with *Eco*RV, transcription with SP6 RNA polymerase in the presence of [^{32}P]UTP generates a 460-nucleotide (nt) riboprobe. Hybridization to ED-B$^+$ cellular RNA generates a 430-nucleotide RNase-resistant fragment, whereas ED-B$^-$ RNA generates a 370-nucleotide protected fragment. (B) Autoradiograph of the protected fragments generated by RNA from TE-MT*ras*(onc) cells grown for 72 h in the absence (−) or presence (+) of 4 μM $CdCl_2$–100 μM $ZnSO_4$. The sizes (in nucleotides) of radiolabeled RNA standards are on the left, and the positions of the ED-B$^+$ (430 nucleotides) and ED-B$^-$ (370 nucleotides) protected fragments are indicated to the right. As a control, a protection assay was also carried out using yeast tRNA instead of cellular RNA. All samples shown were derived from the same experiment, but the *ras*-induced (+) lanes are overexposed to facilitate comparison with the control (−) lanes.

and transformation-associated reductions in FN mRNA levels. RNase protection analysis was used to determine if there were any alterations in the abundance of ED-B$^+$ FN mRNA following induction of *ras* expression in TE-MT*ras*(onc) cells. ED-B$^-$ mRNA was clearly the most abundant isoform present in both the control and the *ras*-expressing cells (Fig. 7B). Quantification of the data by densitometry revealed that ED-B$^+$ mRNA represented approximately 20% of the total in both untreated and metal-treated cells. Therefore, no change in the relative abundance of ED-B$^+$ mRNA accompanied the *ras* oncogene-induced decrease in FN mRNA levels.

DISCUSSION

The disappearance of FN from the surface of oncogenically transformed cells has been well documented and appears to be closely involved in the development of the malignant phenotype (21). However, mechanistically this phenomenon remains poorly understood. In a previous study we obtained evidence supporting posttranscriptional down-regulation of FN in N-*ras*-transformed HT1080 cells compared with a revertant cell line in which the N-*ras* oncogene is underrepresented (7). Since that study compared independently grown, stable cell lines, it could not be determined that the observed differences in FN expression were due to expression of the N-*ras* oncogene. We have therefore established human cell lines containing inducible Ha-*ras* genes in order to further and more directly investigate the effects of *ras* on FN biosynthesis. The nontumorigenic osteosarcoma cell line TE-85 is a valuable and unique experimental tool for oncogene transfection studies in human cells. Tainsky et al. (43) demonstrated that these cells become tumorigenic when transfected with cloned oncogenes. This property of TE-85 cells was exploited in the present study to investigate the effects of manipulated *ras* expression on FN biosynthesis in human cells.

TE-85 cells were stably transfected with plasmids containing a human Ha-*ras* oncogene or proto-oncogene under control of the heavy metal-inducible hMT-II$_A$ promoter. Induction of Ha-*ras* oncogene (but not proto-oncogene) expression was accompanied by decreases in total FN mRNA and protein levels. Transfection experiments to analyze FN promoter activity indicated that the Ha-*ras* oncogene exerts its effect on FN biosynthesis at the posttranscriptional level. The most commonly observed mechanism of posttranscriptional regulation of mRNA accumulation affects mRNA stability in the cytoplasm (1). A cytoplasmic component in the *ras*-induced down-regulation of FN cannot be entirely ruled out. However, the magnitude of the *ras* effect observed when total RNA was analyzed (Fig. 2) could be accounted for by the effect seen when nuclear RNA was analyzed (Fig. 5). The analysis of nuclear RNAs indicated that there may be multiple components in *ras* regulation of FN expression. There was a decrease in levels of intron-containing RNA in the oncogene-expressing cells. This phenomenon could be explained by altered transcription of the FN gene or by altered stability of the unprocessed FN message. However, the decrease in levels of intron-containing FN RNA could not account for the greater *ras* effect seen at the level of total nuclear FN RNA (Fig. 5B). Therefore, one component of the *ras* effect was due to a mechanism affecting accumulation of processed nuclear FN mRNA. Possible mechanisms that could contribute to decreases in processed nuclear RNA levels include altered nuclear RNA processing (splicing or polyadenylation), altered nuclear stability of the processed message, or both. H-blot analyses demonstrated that there was no effect of Ha-*ras* oncogene expression on FN mRNA poly(A) tail length or site of polyadenylation. In addition, there was no evidence for altered splicing at the ED-B ("onco-fetal") domain of FN mRNA.

Posttranscriptional regulation of FN expression has been demonstrated in other experimental systems. For example, transforming growth factor β has been reported to increase the stability of FN mRNA (47). In addition, Dhawan et al. (14) reported that cell adhesion regulates FN expression by posttranscriptional mechanisms that do not involve changes in FN mRNA stability. A mechanism involving RNA processing was

implicated but not demonstrated. Therefore, although the precise mechanism remains to be identified, the present report provides the first evidence for nuclear posttranscriptional regulation of FN expression. Nuclear posttranscriptional regulation is emerging as an important means of controlling gene expression. Nuclear mechanisms affecting the stability of unspliced transcripts (11, 24, 42), splicing patterns (41), and polyadenylation (30, 38) have been demonstrated. In contrast, there is little precedence in the literature for nuclear posttranscriptional mechanisms that affect accumulation of processed nuclear RNA without similarly affecting levels of unprocessed nuclear RNA. Indeed, we know of only one other reported case in which such a phenomenon has been clearly demonstrated. In that study, Cheng and Maquat (8) demonstrated that the nuclear abundance of fully processed mRNA encoded by transfected genes for human triosephosphate isomerase was decreased by mutations that cause premature termination of translation. In contrast, there were no observed differences in levels of intron-containing (unprocessed) RNAs. Our study is different in that the observed nuclear posttranscriptional effect involves regulation of a transcript encoded by an endogenous gene. It remains to be determined if nuclear posttranscriptional down-regulation of FN by the Ha-*ras* oncogene involves nuclear RNA processing, nuclear stability of processed FN mRNA, or both. Unfortunately, because of the stability, size, and complexity of the FN message, experiments designed to address these possibilities may prove technically difficult.

The *ras* oncogenes are activated by point mutations in up to 30% of malignant human tumors (2). Therefore, understanding the mechanisms by which *ras* oncogenes contribute to the transformed phenotype is clearly important. Several studies have identified mechanisms by which *ras* can alter expression of specific genes. *ras*-induced transcriptional activation has been reported (9, 17, 28), and specific promoter elements mediating these effects have been identified for some genes (23, 32). *ras* has also been shown to increase the cytoplasmic stability of specific mRNAs (20, 28). Collagen biosynthesis, like that of FN, is often reduced in transformed cells. Slack et al. (40) recently reported that oncogenic *ras* regulates type I collagen gene expression at both transcriptional and posttranscriptional (cytoplasmic mRNA stability) levels. The present study demonstrates that expression of the Ha-*ras* oncogene causes down-regulation of nuclear FN RNA levels. Furthermore, the activity of *ras* is multifaceted, with effects being exerted on levels of precursor and processed nuclear RNAs. *ras* proteins are associated with the cytoplasmic surface of the plasma membrane. Therefore, a cascade of events is clearly required for *ras* oncoproteins to exert their effects in the nucleus. The nuclear posttranscriptional down-regulation of FN reported here represents a previously unidentified component of this cascade and thus a new level at which *ras* oncoproteins can contribute to development of the malignant phenotype.

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