

Regulation of Fibronectin Biosynthesis by Dexamethasone, Transforming Growth Factor β , and cAMP in Human Cell Lines

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Abstract. The regulation of fibronectin (FN) biosynthesis by dexamethasone (a synthetic glucocorticoid), forskolin (an activator of adenylate cyclase), and transforming growth factor β (TGF- β) was examined in six human cell lines. Dexamethasone treatment produced the largest increase in FN biosynthesis in the fibrosarcoma cell line, HT-1080 (\sim 45-fold). This seems to result from a dexamethasone-mediated increase in FN mRNA stability which increases the message half-life from \sim 11 to 26 h. The relative instability of FN mRNA in the fibrosarcoma ($t_{1/2}$ 11 h) compared to normal fibroblasts (70 h) appears to result from the particular transformed phenotype of the HT-1080 cells. Forskolin and TGF- β increase the rate of FN gene transcription

in most of the cell lines. These effects (four- to six-fold) occur rapidly and do not require protein synthesis in the responsive cell lines which include normal fibroblasts. However, in the fibrosarcoma (HT-1080), a surprisingly large induction (20–30-fold) is observed and this induction is different from that in the normal fibroblasts and the other cell lines in that both protein synthesis and a lag period are required. Synergism is seen with dexamethasone and either forskolin or TGF- β in HT-1080 cells increasing the rate of FN biosynthesis \sim 200-fold to a level similar to normal fibroblasts. This seems to result from a combination of FN mRNA stabilization (dexamethasone) and increased transcription (forskolin and TGF- β).

FIBRONECTINS (FNs)¹ are a group of large glycoproteins found both as an insoluble form (cellular FN) in extracellular matrices and in a soluble form (plasma FN) in plasma. They contain two subunits of \sim 230 and 250 kD that are cross-linked by disulfide bonding (reviewed by Ruoslahti et al., 1981; Hynes and Yamada, 1982; Yamada, 1983). Up to 20 different FN polypeptides can be generated by differential splicing of the mRNA transcribed from a single gene (Kornblihtt et al., 1985; Gutman and Kornblihtt, 1987; Schwarzbauer et al., 1987). Such differential splicing seems to account for both the difference between the cellular and plasma forms and the difference between the two subunits (Atherton and Hynes, 1981; Kornblihtt et al., 1985; Schwarzbauer et al., 1987).

At the amino acid level, FN is composed of three different types of internal repeats designated I, II, and III which contain \sim 40, 60, and 90 amino acid residues, respectively (Skorstengaard et al., 1982; Petersen et al., 1983; Patel et al., 1987). The FN molecule is arranged in a series of globular domains which can bind independently to a number of different molecules such as heparin, DNA, collagen, actin, and fibrin, as well as to bacteria (Yamada, 1983) and to the

surface of cells through a dimeric membrane-bound FN receptor (Pytela et al., 1985; Hynes, 1987). The primary role of FN appears to be in cellular adhesion which is important in a variety of functions such as cellular differentiation and migration, wound healing, blood coagulation, and tumor metastasis (Yamada, 1983). It is of interest to determine how FN biosynthesis is regulated to better understand its role in each of these processes.

The expression of FN is greatly inhibited by neoplastic transformation in most of the cases examined (Vaheri et al., 1976; Olden and Yamada, 1977; Yamada et al., 1977; Hynes et al., 1978; Vaheri and Mosher, 1978; Fagan et al., 1981). This inhibition can be reproduced in cell culture by transfection of certain oncogenes (Levinson et al., 1975; Setoyama et al., 1985). It has been demonstrated that this inhibition of FN expression results from both a depression of transcription and a decrease in FN half-life (Olden and Yamada, 1977; Tyagi et al., 1983). However, the mechanism of this suppression has not been defined. The loss of FN from the cell surface after transformation results in decreased cellular adhesion which has been correlated with increased metastasis of tumor cells (reviewed in Roos, 1984).

The synthesis of FN is induced by glucocorticoids (Furcht et al., 1979a, b; Oliver et al., 1983; Nimmer et al., 1987) and by transforming growth factor β (TGF- β) (Sporn et al., 1983; Igotz and Massague, 1985; Roberts et al., 1986; Ig-

1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyltransferase; CRE, cAMP response element; IBMX, 3-isobutyl-1-methyl-xanthine; FN, fibronectin; LTR, long terminal repeat; MMTV, mouse mammary tumor virus; TGF- β , transforming growth factor β .

notz and Massague, 1986; Ignatz et al., 1987). Glucocorticoids have a well-known role in response to stress, suggesting that they may provide an early mechanism of inducing FN in processes such as wound healing. TGF- β seems to play a role in cellular differentiation (reviewed in Massague, 1985; Sporn et al., 1986). It is also well known that extracellular matrix plays an important role in determining cellular morphology and behavior. Therefore, the effect of TGF- β on cellular differentiation may be mediated through its regulation of extracellular matrix components.

In an effort to better understand how FN synthesis is controlled, we have recently cloned the promoter region of the human gene (Dean et al., 1987). Studies on the nature of this promoter should provide insight into how the gene is regulated. In this paper, we examine the mechanisms of FN induction by both TGF- β and glucocorticoids in different human cell lines. We also show that FN is responsive to cAMP and analyze the mechanisms involved.

Materials and Methods

Cell Culture and DNA Transfection

All cells were grown in DME in the presence or absence of 10% FCS at 37°C as described previously (Oliver et al., 1983). The HT-1080 cell line (Rasheed et al., 1974) was subcloned and a clone designated HT-1080C was used (Oliver et al., 1983). Drugs were added to cell cultures at the concentrations and for the time periods indicated in the figure legends. Forskolin was from Calbiochem-Behring Corp., La Jolla, CA, and TGF- β was from R and D Systems, Inc., Minneapolis, MN, or was a gift from Michael Sporn, National Cancer Institute, Bethesda, MD. Cells were transfected by the calcium phosphate method as described previously (Knoll et al., 1983). Approximately 15 μ g of plasmid was used to transfect each 10-cm plate which was ~30% confluent. 5 μ g of the plasmid RSV- β -Gal (Edlund et al., 1985) was cotransfected with each plasmid. Assays for β -galactosidase (Edlund et al., 1985) were then used to standardize each of the transfections. Approximately 100 μ g of protein was used for β -galactosidase assays in the HT-1080 and WI38 cell lines, while 20 μ g was used from JEG-3 cells. Under these conditions, 5–6 h were usually required for a color reaction to occur in HT-1080 and WI38 cells but only ~20 min was required in JEG-3 cells. Values for nontransfected cells were used as a control. Drugs were added to the transfected cells as indicated in the figure legends and the cells were harvested after 48 h. Chloramphenicol acetyltransferase (CAT) assays were done as described previously (Gorman, 1985) using 50 μ g of protein and a 1-h reaction time for both JEG-3 and HT-1080 cells. WI38 cells required a 6-h reaction time.

Plasmid Construction

The plasmid pFN-CAT contains a *pst* I fragment of the human FN gene from +69 to ~-1.6 kb (Dean et al., 1987). The 5' end of this fragment contains ~1.1 kb of lambda DNA from the original charon 28 vector. This *pst* I fragment was blunt ended with T4-DNA polymerase (Maniatis et al., 1982a) and subcloned into the *sma* I site of pSVOCAT (*sma* I). Orientation was determined by restriction digestion. The plasmid pRSV- β -Gal was from P. Mellon (The Salk Institute for Biological Studies, San Diego, CA) and the plasmid pMMTV-CAT was from R. Evans (The Salk Institute for Biological Studies).

RNA Isolation and Analysis

Total RNA was isolated from cell monolayers by the guanidinium thiocyanate method (Maniatis et al., 1982b). S-1 analysis was done as described previously with 5'-³²P-labeled probes (Dean et al., 1987) and ~50 μ g of total RNA was used for each analysis. As a control, equal amounts of RNA used for S-1 analysis were slot blotted and probed with a constitutively expressed gene, CHO-B (Harpold et al., 1979). The CHO-B gene was not responsive to any of the drugs tested (unpublished data). The inhibition of protein synthesis by cycloheximide was determined by incubating cells in various concentrations of cycloheximide for 1 h after which time the rate

of incorporation of [³⁵S]methionine into protein was determined. The effect of cycloheximide on protein synthesis was rapid, occurring in 1 h or less. To examine the effect of cycloheximide on dexamethasone induction of the mouse mammary tumor virus promoter (MMTV), the plasmid pMMTV-CAT (20 μ g) was transfected into 10-cm dishes of HT-1080 cells as described above. Dexamethasone and cycloheximide were added after 18 h and incubation was continued for an additional 36 h. Total RNA was isolated from treated or untreated cells and 30 μ g was slot blotted onto hybrid-N membrane (Amersham Corp., Arlington Heights, IL). The membrane was then hybridized, as described previously (Dean et al., 1987), to pMMTV-CAT labeled with ³²P by nick translation (Rigby et al., 1977).

In Vitro Transcription in Isolated Nuclei

In vitro transcription assays in isolated nuclei were done essentially as described elsewhere (Greenberg and Ziff, 1984). Cells were incubated for 48 h in the presence or absence of drugs and $\sim 1 \times 10^7$ cells were used for each assay. Cells were washed three times with ice cold PBS and lysed in a buffer containing 0.5% NP-40 (Sigma Chemical Co., St. Louis, MO). Nuclei were collected after centrifugation at 600 g for 5 min and gently resuspended in 100 μ l of 50 mM Tris-HCl, pH 8.3, 5 mM MgCl₂, 0.1 mM EDTA with 40% glycerol. The nuclei were then frozen at -100°C for later use. For the transcription experiments, nuclei were thawed and mixed with 100 μ l of reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 300 mM KCl, 1 mM each of ATP, CTP, and GTP, and 200 μ Ci of [α -³²P]UTP, >600 Ci/mmol; Amersham Corp.) and incubated at 30°C for 30 min. The mixture was then treated with 75 U of RNase-free DNase (Promega Biotechnologies, Madison, WI) for 10 min at room temperature and phenol/chloroform extracted two times. RNA was then ethanol precipitated three times in the presence of 3.75 M ammonium acetate. Incorporation of ³²P into RNA was measured by TCA precipitation. Approximately 2–5 $\times 10^7$ cpm was incorporated in 1×10^7 cell nuclei. The [³²P]RNA was then hybridized to 2 μ g of the plasmid pgHF 3.7 (Dean et al., 1987) which contains a 1-kb piece of the human FN gene or to 2 μ g of the control vector pGEM-4 (Promega Biotechnologies) immobilized to nitrocellulose by slot blotting. This fragment does not contain any repeated sequences as analyzed by Southern blot hybridization to human genomic DNA (data not shown). Equal amounts of cpm (see figure legend) were hybridized to the filters in 1 ml of solution as described (Dean et al., 1987). The in vitro transcription assay was repeated four times for each drug.

Metabolic Labeling and Immunoprecipitation

For metabolic labeling, each 60-mm dish of cells (near confluency) was incubated for 90 min in 0.4 ml of DME (minus methionine) containing 150 μ Ci/ml of [³⁵S]methionine (600–1,400 Ci/mmol; New England Nuclear, Boston, MA). An equal volume of 2 \times cell lysing buffer (40 mM Tris-HCl, pH 8.8, 4% deoxycholate, 4 mM phenylmethylsulfonyl fluoride [PMSF], 4 mM EDTA, 4 mM iodoacetic acid, 2 mM *N*-ethyl maleimide, and 1% NP-40) was added, the lysate was sheared through a syringe needle and centrifuged at 10,000 g for 10 min at 4°C. Equal cpm, determined by TCA precipitation, were incubated with an excess of goat anti-human FN IgG followed by rabbit anti-goat IgG (both antisera were from Cappel Laboratories, Cochranville, PA). Precipitated proteins were washed three times in 20 mM Tris-HCl, pH 8.8, 0.5% deoxycholate, 50 mM NaCl, 2 mM PMSF, 2 mM EDTA, 0.5% NP-40, and subjected to electrophoresis on SDS-polyacrylamide gels as described previously (Oliver et al., 1983).

RNA Pulse-Chase

FN mRNA decay experiments were done as described elsewhere (Paek and Axel, 1987). HT-1080 cells were treated with 2×10^{-7} M dexamethasone for 48 h before pulse labeling. Cells were treated with 200 μ Ci/ml of [5-³H]uridine (16 Ci/mmol) for 5 h in DME and 10% FCS. After this time, the labeling media was removed and the cells were rinsed with DME three times and incubated for various times in the presence of 5 mM uridine. RNA was isolated as described above and ethanol precipitated three times in the presence of 3.75 M ammonium acetate. Incorporation of [³H]uridine into RNA was quantitated by TCA precipitation. [³H]RNA was hybridized to the plasmid, pgHF3.7 (Dean et al., 1987) or to the vector pGEM-4 bound to nitrocellulose filters. Hybridization was done in 200 μ l as described previously (Dean et al., 1987). Filters were treated with 40 mM NaOH for 2 h and the eluate was counted in a scintillation counter. The background cpm bound to the pGEM-4 filters was subtracted from the cpm on the pgHF3.2 filters at each time point. Background ranged from $\sim 10^{-5}$ to 10^{-6} of input

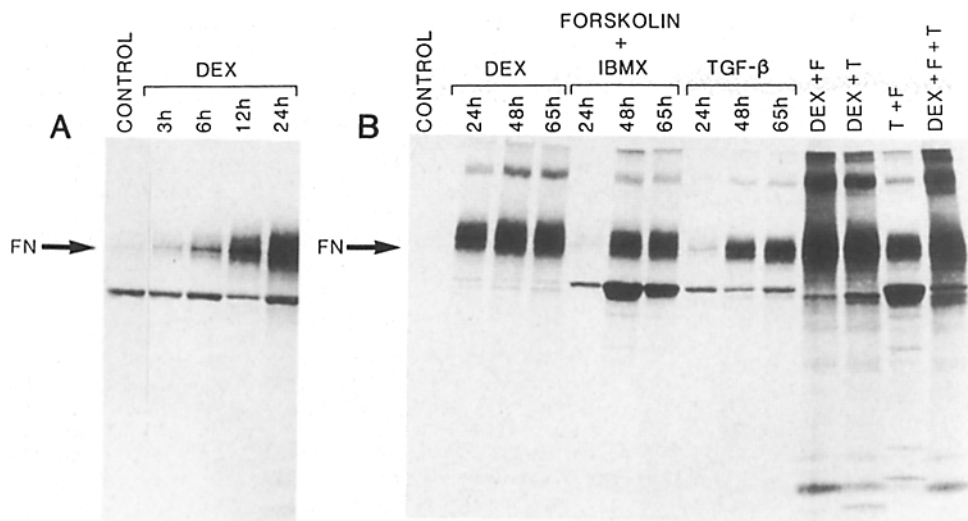


Figure 1. Effect of dexamethasone, forskolin, and TGF- β on the rate of FN biosynthesis in the human fibrosarcoma cell line, HT-1080. Cells were treated with drugs for the indicated times. Incubation in the presence of drug combinations was for 48 h. After drug treatment, cells were labeled with [35 S]methionine and FN was immunoprecipitated and subjected to electrophoresis on an SDS-polyacrylamide gel (see Materials and Methods). *Dex*, dexamethasone (2×10^{-7} M); *IBMX*, 3-isobutyl-1-methyl-xanthine (2×10^{-4} M); *T*, TGF- β (500 pM); *F*, forskolin (1.8×10^{-5} M).

cpm. The points shown in Fig. 6 B are the average of two independent experiments each being representative of the average.

Results

Regulation of FN by Glucocorticoids

Six human cell lines have been used in this study: the fibrosarcoma, HT-1080 (Rasheed et al., 1974; Oliver et al., 1983); JEG-3, a choriocarcinoma (Kohler and Bridson, 1971); TE671, a medulloblastoma (McAllister et al., 1977); HeLa Bu25, a cervical carcinoma (Kit et al., 1966); and two normal fibroblasts, from lung (WI38) and from foreskin (FS-1). All of the cells in the study exhibit a basal level of FN expression although this level varies greatly. Normal fibroblasts exhibit high basal levels of FN (0.3% of total protein) while the transformed cell lines all had much lower levels: JEG-3 (0.01%), TE671 (0.002%), HeLa Bu25 (0.008%), and HT-1080 (0.004%). It is of particular interest that the level of FN is so low in the fibrosarcoma cell line compared to normal fibroblasts.

We have demonstrated previously that the rate of FN biosynthesis is increased by the synthetic glucocorticoid, dexamethasone, in the human fibrosarcoma cell line HT-1080 (Oliver et al., 1983). The kinetics of this response is shown in Fig. 1. Quantitative estimates of increases in the rate of

FN biosynthesis were obtained by cutting the FN band from SDS-polyacrylamide gels and counting the cpm in the bands (see Table I). The rate of FN biosynthesis is increased ~ 45 -fold, however 48 h is required for full induction. Dexamethasone treatment of other cell lines which synthesize FN results in varying levels of FN induction. After 48 h, no dexamethasone induction is seen in JEG-3 or in TE671 cells and only a small induction is seen in WI38 (less than twofold), HeLa Bu25 cells (threefold), and FS-1 (twofold).

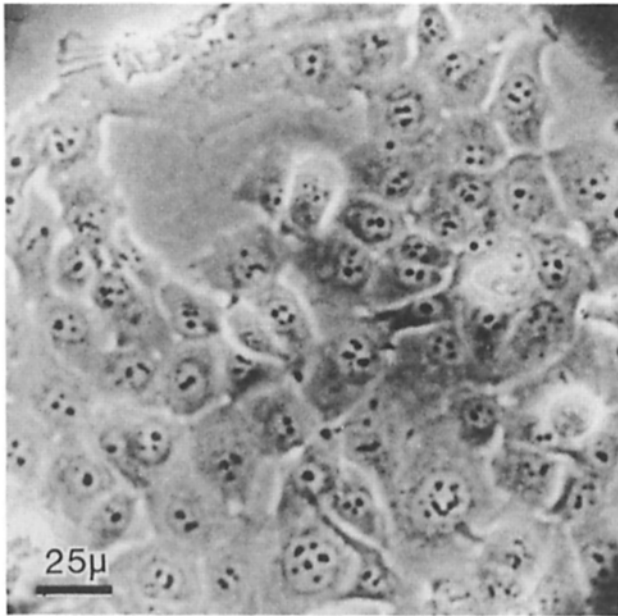
Additional studies to determine the mode of action of dexamethasone on FN biosynthesis were carried out in HT-1080 cells since FN levels in these cells are the most responsive. The morphology of HT-1080 is not drastically changed by dexamethasone (Fig. 2) and this steroid has no detectable effect on the growth of these cells (Fig. 3). There was also no apparent effect of dexamethasone on the morphology or growth rate of the other cell lines (data not shown).

To determine if dexamethasone induction occurs at the level of FN mRNA in HT-1080 cells, an S-1 nuclease protection assay was used. The results demonstrate that dexamethasone increases the level of accumulated FN mRNA transcribed from the FN promoter (Fig. 4). The kinetics of FN induction by dexamethasone (Fig. 1) seem to be relatively slow when compared to induction of the metallothionein gene or the MMTV long terminal repeat (LTR) which are known to be induced directly by glucocorticoids and do not

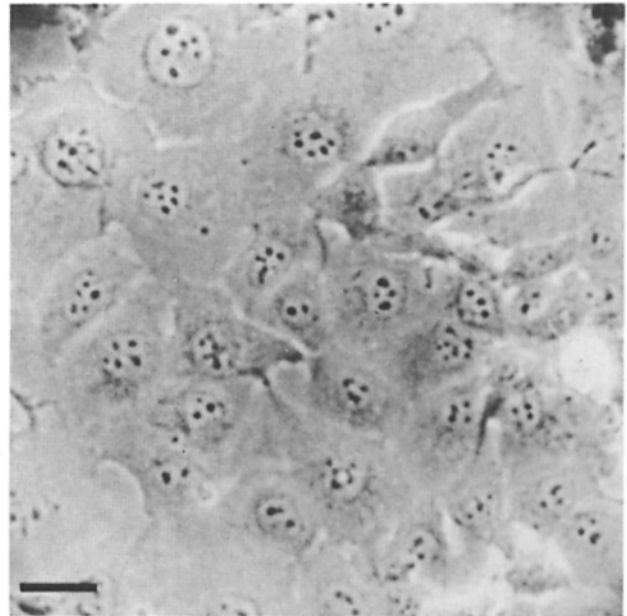
Table I. Induction of Fibronectin in Human Cell Lines

Drugs	HT-1080	JEG-3	TE671	WI38	HeLa	FS-1
Dex	45.0	1.1	0.7	1.6	3.2	1.8
Forskolin	27.5	4.7	0.8	1.1	0.3	0.5
TGF- β	22.0	1.1	2.2	6.7	1.1	3.7
Dex + forskolin	251	3.3	2.9	2.4	2.1	0.6
Dex + TGF- β	171	1.1	1.1	8.5	11.0	3.3
Forskolin + TGF- β	63	5.1	1.7	4.3	0.7	1.7
Dex + forskolin + TGF- β	184	4.9	2.4	5.4	5.1	2.2

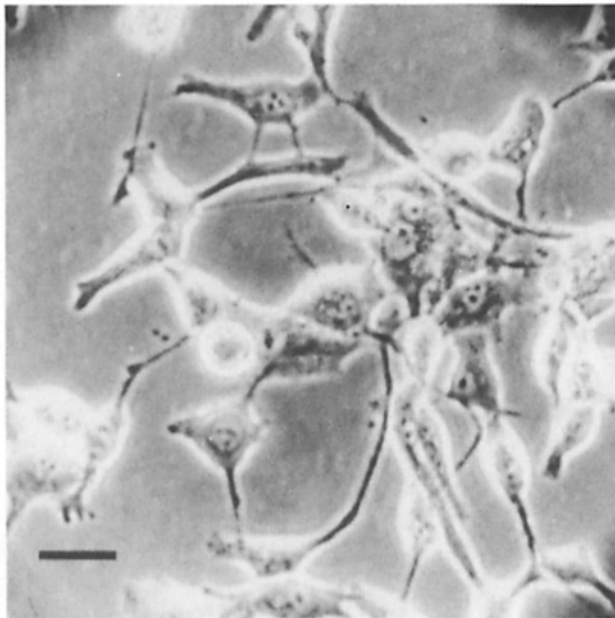
Quantitation of the rate of FN biosynthesis after treatment of various human cells with dexamethasone (*Dex*), forskolin, and TGF- β . The numbers correspond to inductions in the rate of FN biosynthesis and were obtained by cutting the FN-specific band from the gel in Fig. 1 (the 48-h time point) and from similar gels of the other cell lines (photographs of the autoradiograms were submitted to reviewers). Inductions were determined by dividing the cpm in a specific band by the cpm in the band from untreated cells.



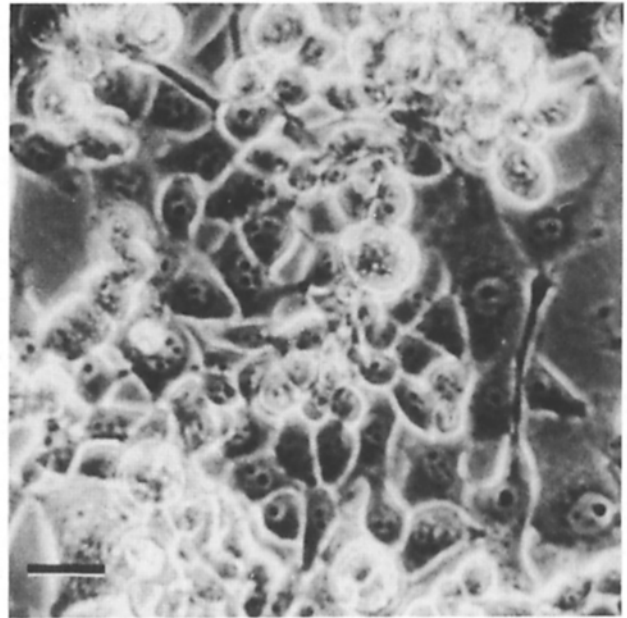
Control



Dexamethasone



Forskolin + IBMX



TGF- β

Figure 2. Phase-contrast photomicrographs of HT-1080 cells in the presence of dexamethasone, forskolin and IBMX, or TGF- β . Cells were treated with the various drugs for 48 h (see Fig. 1 for drug concentrations) and photographed with Kodak plus-X pan film using a Zeiss inverted microscope with phase-contrast optics. Bars, 25 μ m.

require protein synthesis for induction (Yamamoto and Alberts, 1976). This suggested that dexamethasone induction of FN might occur through an indirect mechanism. Other proteins that are indirectly induced by glucocorticoids are α_1 -acid glycoprotein (Baumann and Maquat, 1986) and α_{2u} -globulin (Addison and Kurtz, 1986). To determine if this was the case, HT-1080 cells were incubated in the presence of cycloheximide to inhibit protein synthesis and the effect on dexa-

methasone induction of FN mRNA was analyzed (Fig. 5). As a control, a plasmid containing the MMTV LTR linked to the CAT gene was transfected into the HT-1080 cells. The MMTV LTR is directly inducible by dexamethasone and this induction should not require protein synthesis. The results confirm that dexamethasone is able to induce expression of CAT mRNA from the MMTV LTR in the presence of cycloheximide; however, under the same conditions, induction of

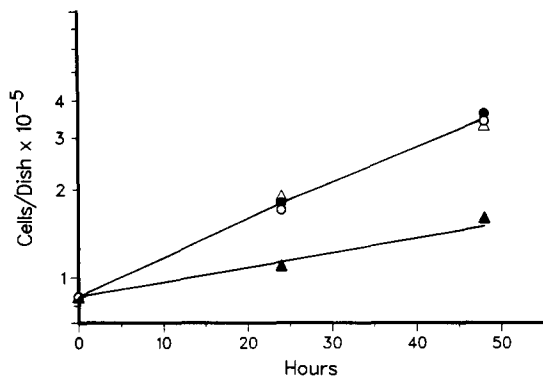


Figure 3. Growth rate of the HT-1080 cell line in the presence of dexamethasone (●), forskolin and IBMX (▲), and TGF- β (Δ). Equal numbers of HT-1080 cells were aliquoted into 60-mm dishes and grown for 3 d in the presence or absence of drugs (see Fig. 1 for concentrations) to near confluency. Cells were removed from dishes with trypsin-EDTA and counted with a Coulter counter. The data shown is an average of two experiments. (○) Control.

FN mRNA was blocked demonstrating that protein synthesis is required for dexamethasone induction of FN (Fig. 5). Similar effects were observed if cycloheximide was added at the same time as dexamethasone or 2 h before or after dexamethasone addition (data not shown).

To determine if dexamethasone induction of FN is due to an increase in the rate of transcription of the FN gene, *in vitro* transcription in isolated nuclei was performed. The results, shown in Fig. 6 A, illustrate that dexamethasone does not stimulate the rate of FN transcription in HT-1080 cells. Since the level of FN mRNA is increased but the rate of transcription is unchanged, dexamethasone must increase the stability of FN mRNA. When the FN promoter is fused to the bacterial CAT gene and transfected into various human cell lines, no dexamethasone induction is observed in any of the cells. The results for HT-1080 and WI38 cells are shown in Fig. 7; the results for the other cell lines are not shown. This supports the hypothesis that the effect of dexamethasone on FN expression is not at the level of transcription.

A pulse-chase experiment was used to measure the effect of dexamethasone on the decay rate of FN mRNA in HT-1080 cells. Cells were incubated for 48 h in the presence or absence of dexamethasone and labeled for 5 h with [3 H]uridine as described previously (Paek and Axel, 1987). The time course of [3 H]FN mRNA decay was measured by hybridization to a plasmid containing a fragment of the FN gene bound to filters. Initially, the levels of pulse-labeled FN mRNA were similar in HT-1080 and WI38 cells and were unaffected by dexamethasone in HT-1080 cells. This observation is in agreement with our measurements of rates of transcription (Fig. 6 A) in which no effect of dexamethasone was observed. However, FN mRNA decayed more rapidly in the untreated HT-1080 cells; this decay suggests that dexamethasone treatment increases the stability of FN mRNA in these cells (Fig. 6 B). The half-life of FN mRNA is estimated to be 11 h in untreated HT-1080 cells and 26 h in dexamethasone-treated cells. The half-life in WI38 cells is \sim 70 h.

TGF- β Can Increase FN Levels by Two Different Mechanisms

The effect of TGF- β on the rate of FN biosynthesis was first

examined in the fibrosarcoma cell line, HT-1080. A concentration of 500 pM was chosen for the experiments. Increasing this concentration to 1 nM did not significantly increase the rate of FN biosynthesis; however, lowering the concentration to 50 pM eliminated most of the FN induction (data not shown).

The effect of TGF- β on FN biosynthesis is shown in Fig. 1 and Table I. In HT-1080 cells, FN is induced by TGF- β \sim 22-fold (see Table I). In WI38 cells the induction is sevenfold, in FS-1 fourfold, and in TE671 about twofold. No significant induction is seen in JEG-3 or HeLa Bu25 cells. Since HT-1080 cells exhibited the largest induction with TGF- β , these cells were used to study the effect of this drug on morphology and growth. Fig. 2 shows that TGF- β causes HT-1080 to grow as spherical cells which form aggregates that are weakly attached to the culture dish. No effect of TGF- β on the growth rate of HT-1080 cells was observed (Fig. 3) and no striking effects on the morphology or growth rate in the other cell lines was apparent.

The kinetics of TGF- β induction in HT-1080 cells appears to be biphasic (Fig. 8). A relatively small induction of approximately sixfold occurs in the first 24 h. After 24 h, the induction increases sharply to \sim 30-fold; however, in WI38 cells induction is apparent by 5 h and increases in a linear fashion with time up to between 24 and 48 h (Fig. 8).

S-1 nuclease protection experiments illustrate that TGF- β treatment increases the amount of FN mRNA in both HT-1080 and WI38 cells rather than increasing the rate of translation (Fig. 5). When protein synthesis is inhibited with cycloheximide, TGF- β induction of FN mRNA is substantially blocked in HT-1080 cells (Fig. 5); however, no effect of cycloheximide is observed in WI38 cells (Fig. 5). This indicates that the mechanism of TGF- β induction differs in the fibrosarcoma (HT-1080) and the normal fibroblast.

When the FN-CAT construct is transfected into both HT-1080 and WI38 cells, a three- to sixfold induction of CAT synthesis is observed with TGF- β in both cells (Fig. 7). This induction is similar in magnitude to that observed for FN and FN mRNA in WI38, but is smaller than the induction observed in HT-1080 (\sim 20-fold). Therefore, the full effect of TGF- β on the level of FN in WI38 cells can be accounted for by increased transcription; however, only a portion of the TGF- β induction of FN and FN mRNA in HT-1080 can be accounted for by an increased transcription rate, suggesting the possibility that the remainder of the induction could result from FN mRNA stabilization as suggested above with dexamethasone.

As mentioned above, the kinetics of TGF- β induction in HT-1080 appears to be biphasic. The early induction between 6 and 24 h is about sixfold and is similar to the level of induction observed in both *in vitro* transcription in isolated nuclei and in transfection experiments. It is, therefore, possible that this early event corresponds to an increase in gene transcription, while the second phase of induction observed after 24 h could involve FN mRNA stabilization; however, more experiments are required to confirm this.

We have also examined the effect of serum on the rate of FN biosynthesis in HT-1080 cells. The basal level of FN expression is stimulated two- to threefold in the presence of 10% serum; however, induction by TGF- β decreases approximately two- to threefold in the presence of serum (Fig. 9).

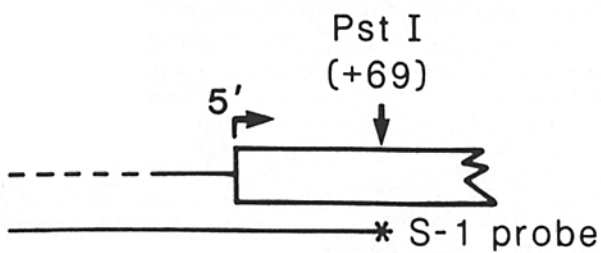
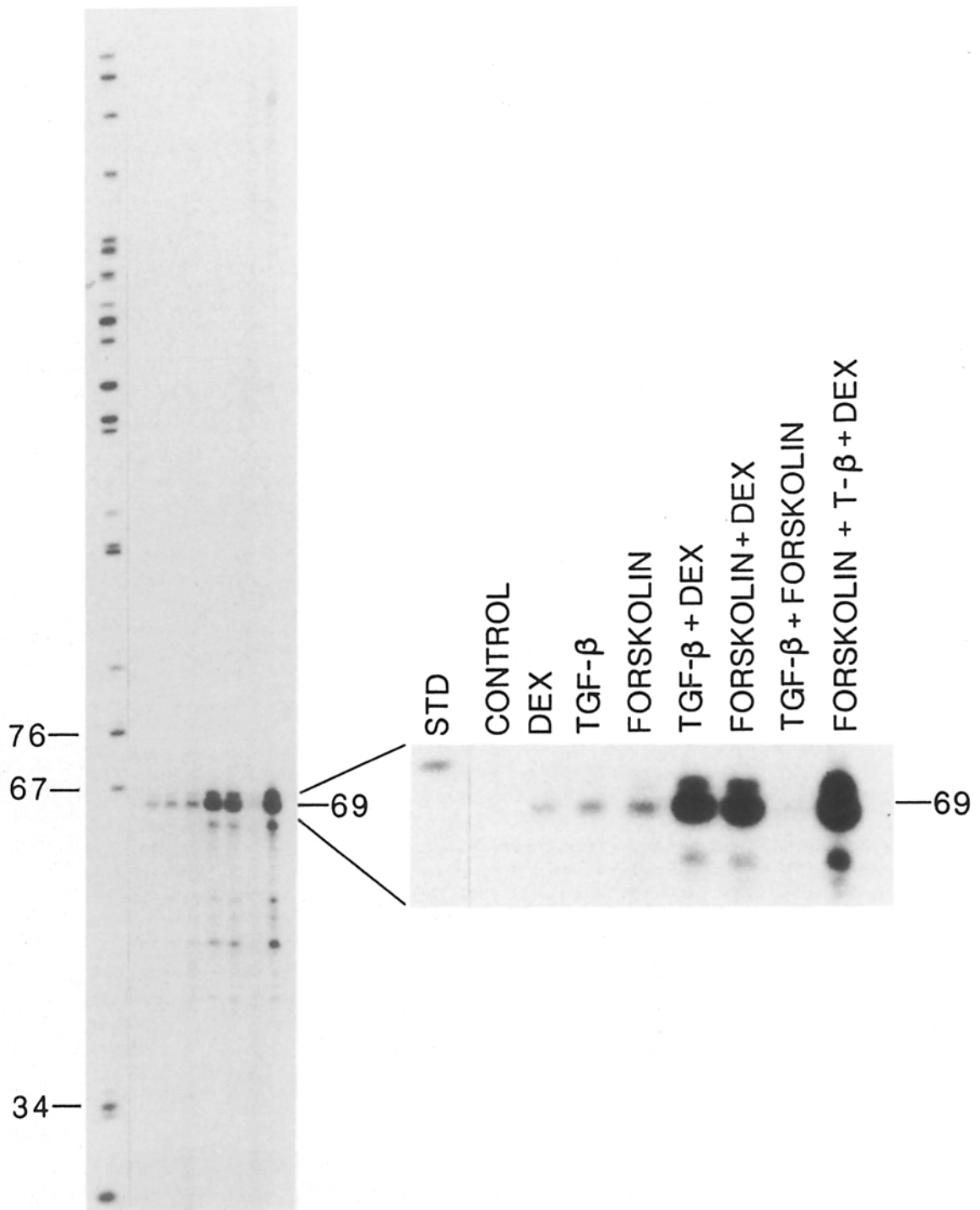


Figure 4. Effect of dexamethasone (*Dex*), forskolin, and TGF- β on FN mRNA levels in HT-1080 cells. An S-1 nuclease assay was used to quantitate the amount of FN mRNA. A $5'$ - ^{32}P -labeled DNA probe was used to map the $5'$ end of the FN mRNA as described previously (Dean et al., 1987). Equal amounts of total RNA ($\sim 50 \mu\text{g}$) were used for each reaction. A schematic diagram of the $5'$ end of the FN gene and S-1 probe is shown at the bottom of the figure. The left-hand lane is a size standard of ^{32}P -labeled pBR322 cut with *Msp* I.

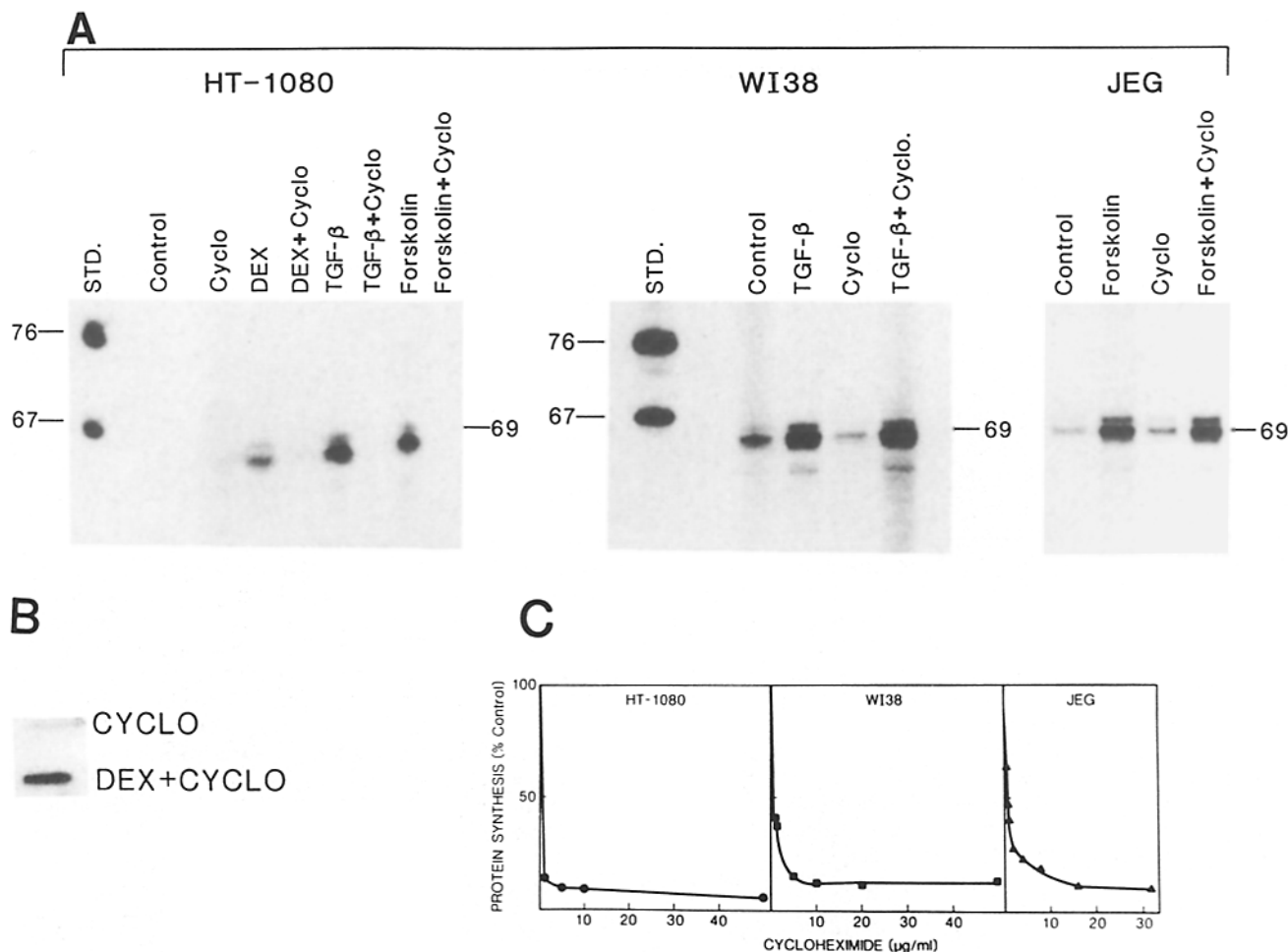


Figure 5. (A) S1 analyses. The effect of cycloheximide on the induction of FN mRNA by dexamethasone (*Dex*), forskolin, and TGF- β in HT-1080, WI38, and JEG-3 (*JEG*) cells. An S-1 nuclease analysis was used to quantitate FN mRNA levels (see Fig. 4). Equal amounts of total RNA ($\sim 50 \mu\text{g}$) were used in each experiment. Cells were incubated for 40 h in the presence or absence of cycloheximide (*Cyclo*) (20 $\mu\text{g/ml}$, HT-1080; 16 $\mu\text{g/ml}$, JEG-3; 4 $\mu\text{g/ml}$, WI38) and the various drugs (see Fig. 1 for concentrations). (B) Slot blots of MMTV-CAT RNA. Effect of cycloheximide on dexamethasone induction of a transfected MMTV promoter. HT-1080 cells were transfected with the plasmid pMMTV-CAT, which contains the MMTV promoter fused to the bacterial CAT gene, and incubated in the presence of cycloheximide (20 $\mu\text{g/ml}$) with or without 2×10^{-7} M dexamethasone for 36 h as described in Materials and Methods. RNA was isolated and 30 μg was immobilized onto a nylon membrane by slot blotting. The membrane was then hybridized to a ^{32}P -labeled pMMTV-CAT probe. (C) Cycloheximide sensitivities. The graphs show the effect of cycloheximide concentration on protein synthesis in the various cells and were used to determine the amount of cycloheximide required to inhibit at least 90% of protein synthesis (see Materials and Methods).

Effect of cAMP on FN Biosynthesis

The effect of increased cAMP levels on the rate of FN biosynthesis was examined initially in the HT-1080 cell line. Induction was observed with dibutyl cAMP, 8-bromo cAMP, forskolin (an activator of adenylate cyclase), and the phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX), as well as with various combinations of these drugs (data not shown). The minimal amount of dibutyl cAMP required for induction was 1×10^{-4} M; 1×10^{-5} M was not effective. The level of forskolin chosen for induction studies was 1.8×10^{-5} M in combination with 2×10^{-4} M IBMX. A three-fold increase or decrease in these concentrations had no effect on induction (data not shown). This combination of forskolin and IBMX was then tested on various cell lines.

The effect of forskolin and IBMX on the growth and morphology of HT-1080 cells was examined since these cells

show the greatest effect of this drug on FN biosynthesis. The growth rate in the presence of forskolin was substantially decreased (Fig. 3) although there was no significant change in the rate of protein biosynthesis (data not shown). The morphology of the cells was markedly altered in the presence of forskolin; the cells became elongated with numerous projections (Fig. 2).

The kinetics of the forskolin effect in HT-1080 cells indicate that a large increase (~ 28 -fold) in the rate of FN biosynthesis occurs between 24 and 48 h of treatment (Figs. 1 and 10). As illustrated with TGF- β , S1 nuclease analysis demonstrates that forskolin increases the amount of FN mRNA in HT-1080 cells rather than increasing the rate of translation (Fig. 4). However, induction of FN mRNA by forskolin in HT-1080 cells is blocked by cycloheximide, thus indicating an indirect effect which requires protein synthesis (Fig. 5).

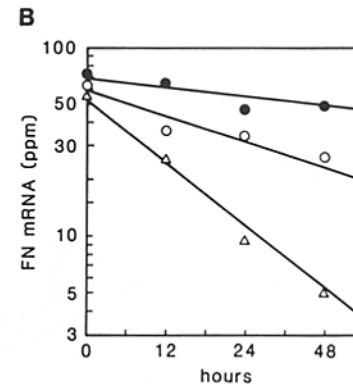
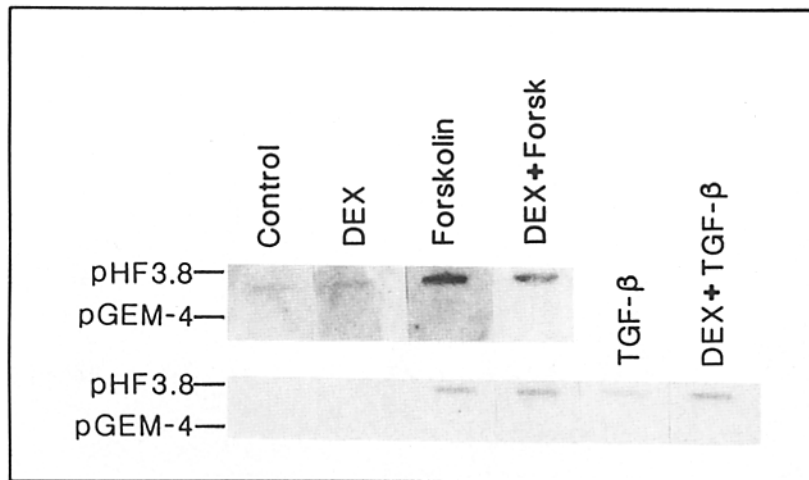
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Figure 6. Measurements of the rate of FN gene transcription and the time course of FN mRNA decay. (A) In vitro transcription in isolated nuclei (see Materials and Methods). Cells were treated with drugs for 48 h (see Fig. 1 for concentrations), nuclei were isolated, and RNA was labeled with [³²P]UTP. The labeled RNA was then hybridized to the plasmid pHF3.7 which contains a genomic DNA fragment of the human FN gene (Dean et al., 1987) or to the control vector pGEM-4 immobilized onto a nitrocellulose filter by slot blotting. 1×10^7 cpm of ³²P-labeled RNA was hybridized to the filters in a 1-ml volume in the bottom experiment. In the top experiment, more ³²P-labeled RNA (5×10^8 cpm/ml) was used in the hybridization in order to better detect transcription in the untreated and dexamethasone-treated cells. *Dex*, dexamethasone; *Forsk*, forskolin. (B) Time course of FN mRNA decay. Cells were labeled for 5 h with [³H]uridine as described in Materials and Methods. Incubation was continued with 5 mM uridine for various times after which [³H]RNA was isolated and [³H]FN mRNA quantitated by hybridization to an excess of an FN gene fragment bound to nitrocellulose filters. Nonspecific hybridization to the vector (pGEM-4) was subtracted from each point. The data are presented as parts per million (ppm). Where indicated, cells were incubated in 2×10^{-7} M dexamethasone for 48 h before labeling which was continued until [³H]RNA isolation. WI38 (●); HT-1080 (△); HT-1080 treated with dexamethasone (○).

The mechanism of this induction appears to be complex. In vitro transcription in isolated nuclei (Fig. 6) and transfection experiments (Fig. 8) indicate a transcriptional effect of only four- to sixfold, which is less than the ~28-fold induction seen at the protein and mRNA levels, suggesting the possibility that the induction also involves FN mRNA stabilization as was suggested for TGF-β in these cells.

In JEG-3, TE671, and WI38 cells, forskolin induces the rate of FN biosynthesis rapidly; the effect is maximal at 24 h and decreases thereafter (see Table I, Fig. 10; other data not shown). The largest response to forskolin is seen in JEG-3 cells (approximately sixfold). In some of the cell lines (TE671, HeLa Bu25, WI38, and FS-1) treatment with forskolin for 48 h results in rates of FN biosynthesis that are even lower than basal levels (Table I). This effect is also seen when the FN-CAT construct is transfected into WI38 (Fig. 7) or HeLa Bu25 (data not shown) cells which are then treated for 48 h with forskolin. The decrease in FN levels after 24 h of forskolin treatment appears to be a feedback mechanism rather than due to degradation of forskolin since addition of more forskolin at 24-h intervals during the incubation had no effect (data not shown). Transfection assays in JEG-3 cells suggest that the forskolin effect in these cells is transcriptional (Fig. 7). The amount of induction of FN (Table I and Fig. 9) and FN mRNA (Fig. 5) by forskolin is similar in magnitude to induction observed in transfection experiments (Fig. 7) suggesting that the forskolin effect in JEG-3 cells can be accounted for entirely by increased transcription. Unlike HT-1080 cells, protein synthesis is not required for forskolin

induction of FN mRNA in JEG-3. The mechanism of forskolin induction of FN is then different in HT-1080 compared to the other cell lines, as was the case with TGF-β induction discussed above.

Synergism between Dexamethasone and TGF-β or Forskolin

In HT-1080 cells, the combination of TGF-β and dexamethasone, or forskolin and dexamethasone, results in a very large increase in the rate of FN biosynthesis (~200-fold) (Fig. 1 and Table I). This synergistic effect is three- to fourfold greater than one would expect from an additive effect. No synergism is observed with the combination of TGF-β and forskolin. Synergism is also observed with TGF-β and dexamethasone in HeLa Bu25 cells (~11-fold induction) but not with forskolin and dexamethasone. No synergism is seen in WI38, FS-1, or JEG-3 cells (Table I). In TE671, induction is observed with the combination of forskolin and dexamethasone (approximately fourfold) even though no induction is observed with dexamethasone or forskolin alone.

It seems likely that the synergism in FN induction is due, at least in part, to dexamethasone acting to stabilize FN mRNA, while TGF-β and forskolin stimulate transcription, since addition of dexamethasone to TGF-β- or forskolin-treated cells does not further increase the transcription rate of the FN gene (Fig. 6A). The effect may be more complex than this based on the action of dexamethasone and forskolin on TE671 cells. While neither of these drugs alone induces

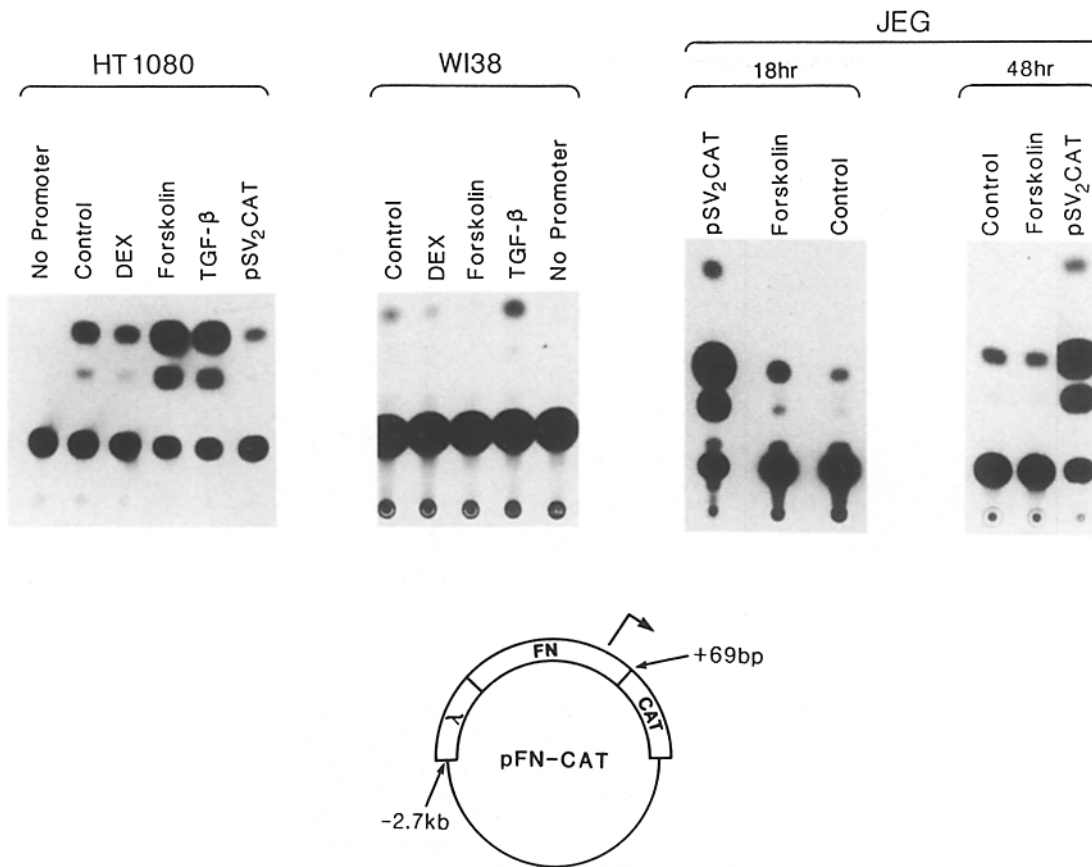


Figure 7. Effect of dexamethasone, forskolin, and TGF- β on expression of CAT from a transfected plasmid (pFN-CAT) containing the FN gene promoter fused to the CAT gene. Transfections were done as described in Materials and Methods. In each transfection, the plasmid pRSV- β -gal was included and β -galactosidase assays were used to normalize the amount of extract used for CAT assays. HT-1080 and WI38 cells were treated with drugs for 40 h after transfection and JEG-3 cells were treated as indicated in the figure. The plasmid pFN-CAT contains \sim 1.6 kb of human FN gene 5' flanking sequence. It also contains \sim 1.1 kb of DNA from the original charon 28 λ -phage vector. This phage DNA does not seem to affect induction of the FN promoter since similar results have been obtained with a vector containing only 520 bp of FN 5' flanking sequence and no phage DNA (data not shown). In addition, when the FN promoter, including the phage DNA is cloned in the opposite orientation, CAT activity is greatly reduced (unpublished data).

FN, the combination of drugs results in induction indicating some potential overlap in the induction pathways of these drugs.

Discussion

In this study we examined how FN is regulated by three different drugs: the synthetic glucocorticoid, dexamethasone; forskolin, which elevates adenylate cyclase levels; and TGF- β . The effect of these drugs on FN biosynthesis varies with cell type.

In cells in which FN biosynthesis is stimulated by glucocorticoids, this effect appears to be mediated through the glucocorticoid receptor (Oliver et al., 1983); however, the presence of the glucocorticoid receptor alone is not sufficient for FN induction since TE671 cells contain glucocorticoid receptors which translocate to the nucleus (Dean, D. C., R. F. Newby, and S. Bourgeois, unpublished results) but FN biosynthesis in these cells does not respond to glucocorticoids. This is not surprising since the effect of

glucocorticoids on FN biosynthesis is indirect and appears to involve selective stabilization of FN mRNA. Protein synthesis is required for this effect, therefore, a protein(s) is being synthesized in response to dexamethasone which is capable of either directly or indirectly stabilizing FN mRNA. The nature of such a protein, as well as the region of FN mRNA required for this effect, may provide insight into the mechanism involved. A similar effect of glucocorticoids on the stability of human growth hormone has been demonstrated (Paek and Axel, 1987). These authors suggested that this increase in growth hormone mRNA half-life is due to a glucocorticoid-induced lengthening of the poly A tail on the mRNA.

The rate of FN biosynthesis is also affected by cAMP and drugs such as forskolin which raise cellular levels of cAMP. In both HT-1080 cells and the choriocarcinoma cell, JEG-3, forskolin produces a significant increase in the rate of FN biosynthesis. The induction in both cell lines seems to result, at least in part, from an increase in the rate of FN gene transcription; however, the mechanisms appear to be different.

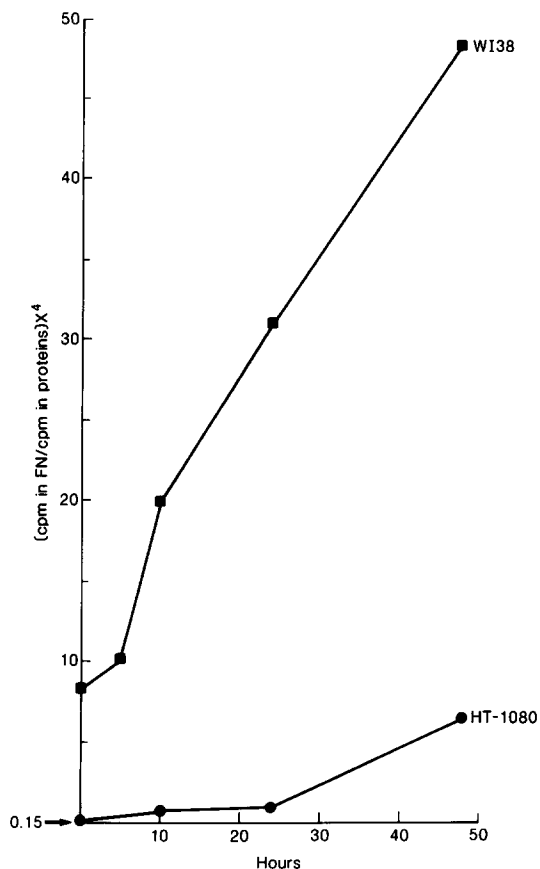


Figure 8. Effect of TGF- β on the kinetics of the rate of FN biosynthesis in HT-1080 and WI38 cells lines. Cells were treated with TGF- β (see Fig. 1) for the indicated times and labeled with [35 S]methionine. FN was then immunoprecipitated and subjected to electrophoresis on an SDS-polyacrylamide gel. The FN band was cut from the gel and the number of cpm in each band was determined.

In JEG-3 cells, a rapid induction is observed, which begins to decrease after 24 h suggesting some type of feedback mechanism. Forskolin induction in these cells seems to be entirely due to a transcriptional effect which does not require protein synthesis. This is similar to the mechanism observed for cAMP induction of somatostatin (Montminy et al., 1986), proenkephalin (Comb et al., 1986), phosphoenolpyruvate carboxy-kinase (Short et al., 1986), vasoactive intestinal polypeptide (Tsukada et al., 1987), and the α -subunit of chorionic gonadotropin (Silver et al., 1987). The cAMP effect on these genes is rapid, transcriptional, and insensitive to cycloheximide (Cimbala et al., 1982). It has been demonstrated that the DNA sequence -TGACGTCA- is the core of a cAMP response element (CRE) found in the 5'-flanking region of cAMP-responsive genes (Short et al., 1986; Silver et al., 1987; Montminy and Bilezikjian, 1987) and that this sequence binds to a 43-kD nuclear protein (Montminy and Bilezikjian, 1987). We find two potential CRE sequences in the FN promoter (-170 and -415) that bind nuclear proteins and we have demonstrated that a 25-bp oligonucleotide containing one of these sequences (-170) is sufficient to confer inducibility on an exogenous promoter (Dean et al., 1987; Dean, D. C., M. S. Blakeley, R. F. Newby, and S. Bourgeois, manuscript in preparation). The effect of cAMP could be to

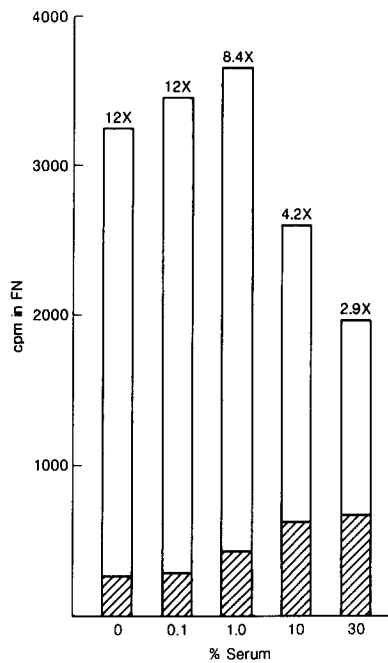


Figure 9. Effect of FCS on the rate of FN biosynthesis in HT-1080 cells. Cells were incubated in the presence or absence of serum and with or without TGF- β (500 pm) as indicated in the figure for 48 h. The amount of FN was quantitated as in Figs. 8 and 10 and Table I. The striped bars represent cells incubated with no added TGF- β , while the open bars represent cells incubated in the presence of TGF- β . The numbers given above the bars are multiples of the induction rate of FN.

induce the synthesis of the 43-kD nuclear protein which binds to the CRE (Montminy and Bilezikjian, 1987); however, we see no apparent difference in the amount of nuclear protein that binds to the FN CRE (as analyzed by DNase I

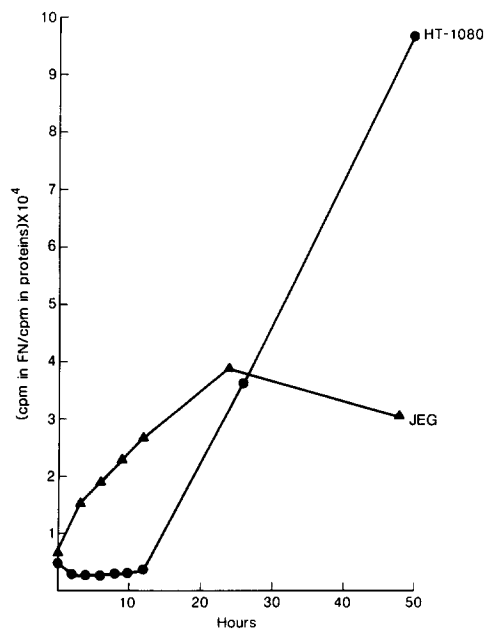


Figure 10. Kinetics of the forskolin-induced increase in the rate of FN biosynthesis in HT-1080 and JEG-3 cell lines. The concentration of forskolin (forskolin and IBMX) is given in Fig. 1 and the experimental procedure is similar to that in Fig. 8.

footprinting) when cells are treated with forskolin (Dean, D. C., R. F. Newby, and S. Bourgeois, unpublished results). There is evidence that cAMP treatment results in phosphorylation of this protein (Montminy and Bilezikjian, 1987) which could stimulate its effect on gene transcription.

The cAMP induction of FN in the fibrosarcoma cell line (HT-1080) occurs through a different mechanism than in the JEG-3 cells. In these cells, protein synthesis is required for cAMP induction and the response time is >24 h. It will be of interest to determine if the transcriptional aspect of cAMP induction in the fibrosarcoma cells is modulated through a CRE or some other DNA sequence.

Induction of FN by TGF- β in fibroblasts seems to be rapid and does not require protein synthesis. This is consistent with previous results (Igotz and Massague, 1985; Igotz and Massague, 1986; Igotz et al., 1987). Transfection experiments done in our study demonstrate that TGF- β induction of FN in fibroblasts is transcriptional and is mediated through FN promoter sequences. TGF- β binds to three distinct types of receptors, however, the detailed mechanism of how induction occurs is unknown (Cheifetz et al., 1986). It will be of interest to determine which FN promoter sequences and nuclear proteins are required for TGF- β induction. This should provide insight into the mechanism of TGF- β induction.

As demonstrated with forskolin, TGF- β induction of FN in the fibrosarcoma cells (HT-1080) occurs through a different mechanism than in fibroblasts. The kinetics of TGF- β induction are similar to those of forskolin induction in these cells and, as with forskolin, protein synthesis is required for the effect. Since the FN promoter mediates induction of FN by TGF- β in both fibrosarcoma (HT-1080) and fibroblast cells, it is of interest to compare the DNA sequences required for this induction in these cell lines.

The synergism between glucocorticoids and either forskolin or TGF- β in the fibrosarcoma cells produces very large increases in the level of FN and FN mRNA which seems to result from the combination of a transcriptional effect (forskolin and TGF- β) and an mRNA stabilization effect (glucocorticoids). A similar mechanism seems to account for the synergistic effect of thyroid hormone (transcriptional) and glucocorticoid (mRNA stabilization and transcriptional) on the growth hormone gene (Paek and Axel, 1987). Synergism between cAMP and glucocorticoids has also been observed with phosphoenolpyruvate carboxykinase (Krone et al., 1976), preproenkephalin (Yoshikawa and Sabol, 1986), tyrosine aminotransferase (Meyer and Morris, 1986), and glyceral-3-phosphate dehydrogenase (Meyer and Morris, 1986).

Results of the induction of FN by all three drugs in lung and foreskin fibroblasts are very similar and are probably representative of the type of FN regulation occurring in normal fibroblasts. In the fibrosarcoma cells, the basal level of FN is greatly decreased (\sim 50-fold), which correlates with a decrease in FN mRNA half-life. Dexamethasone treatment increases FN mRNA half-life in HT-1080 to a level similar to that in fibroblasts. It is also possible that TGF- β and forskolin increase FN mRNA stability in these cells although not to the degree that dexamethasone does. Direct measurements of mRNA half-life will be required to support this hypothesis. It has been demonstrated that HT-1080 cells contain a mutant *N-ras* gene which is active in transformation assays (Brown et al., 1984). Since activated members of the

ras gene family decrease the amount of FN and collagen (I) when transfected into cells (Liau et al., 1985; Sistonen et al., 1987) it is tempting to speculate that this inhibition results, at least in part, from an activated *N-ras*-mediated destabilization of FN mRNA and that each of these drugs causes the synthesis of some protein(s) which directly or indirectly increases FN mRNA half-life. Since the *ras* family of proteins is cytoplasmic, a cascade of events appears to be necessary for *ras* proteins to exert effects in the cell nucleus (reviewed by Marshall, 1987). The action of the drugs in HT-1080 cells could be either on a step in the cascade between the *ras* protein and the nucleus or on steps which occur after an initial set of proteins are induced.

It seems likely that the individual pathways for induction with TGF- β and forskolin are the same in HT-1080 as in other cells. The requirement of protein synthesis for induction with both drugs in HT-1080 may indicate that a component(s) normally present in these pathways is suppressed as a result of this particular transformed phenotype and that synthesis of such a protein(s) is required before a direct transcriptional effect can occur. Inhibition of the cAMP pathway when cells are transfected with an activated *ras* gene has been previously demonstrated (Tarpoly et al., 1986). Identification of any such protein(s) may provide insight into the mechanism involved in transformation by the *ras* gene family as well as assist in determining the pathway of TGF- β induction and clarifying any overlap with the cAMP pathway.

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