Overexpression of Any Fibrinogen Chain by Hep G2 Cells Specifically Elevates the Expression of the Other Two Chains*

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Earlier studies showed that overexpression of Bβ fibrinogen chains, by transfection of Hep G2 cells with Bβ cDNA, specifically enhanced the synthesis of all three fibrinogen chains (Roy, S. N., Mukhopadhyay, G., and Redman, C. M. (1990) J. Biol. Chem. 265, 6389–6393). To determine whether overexpression of any of the three component chains of fibrinogen affects the synthesis of the other two chains, we developed stable Hep G2 cell lines transfected with individual fibrinogen chain cDNAs. As a control, cells were also transfected with expression vector, which did not contain fibrinogen cDNA. Transfection with any fibrinogen cDNA increased the synthesis of all three fibrinogen chains but not of other plasma proteins. Hep G2 cells transfected with Bβ cDNA produced 3–4-fold more fibrinogen than control cells, and cells transfected with Aα or γ cDNA made about 2-fold more fibrinogen. Northern blot analyses showed that levels of all 3 fibrinogen mRNAs were increased and were highest in Hep G2-Bβ cells. Nuclear run-on transcription assays demonstrated that increased expression of the chains was due to increased transcriptional activity. These studies show that transcription of the three fibrinogen chains is tightly linked, and increased expression of any chain specifically leads to increased synthesis of the other two chains.

Human fibrinogen is composed of equimolar amounts of three different polypeptides (Aα, Bβ, and γ) arranged as a dimer, with each half-molecule containing a set of the three chains (for reviews, see Refs. 1–3). Each of the component chains is the product of a separate gene (4–7), which are clustered together on the long arm of chromosome 4, with the Bβ gene in opposite transcriptional orientation to the Aα and γ genes (8, 9). Each gene is separately transcribed and translated (10), and the nascent chains are discharged into the lumen of the endoplasmic reticulum, where chain assembly into dimeric fibrinogen occurs in a stepwise manner (11–13).

Fibrinogen is an acute-phase protein, and in the early stages of inflammation or on treatment of hepatocytes with interleukin-6 and glucocorticoids its synthesis is increased, and this is directly related to enhanced transcription of the three genes (14–16). In these conditions of enhanced fibrinogen production both increased transcription and the half-life of each mRNA are coordinately regulated (17). The mechanisms by which the expression of the three fibrinogen genes is regulated are not fully understood. There is no single sequence in the fibrinogen gene that is involved in the regulation of all three genes, and although several nuclear factors have been described, which bind to the fibrinogen promoters, no single factor has been found that activates all three genes (18–21).

Hep G2 cells have surplus Aα and γ chains, which also occur as an Aα-γ complex. We have suggested that Bβ chain synthesis may be a rate-limiting step in the production of fibrinogen by Hep G2 cells (12, 13). This was supported by the fact that increased Bβ chain synthesis, elicited by transfection of Hep G2 cells with Bβ cDNA, led to increased synthesis and secretion of fibrinogen. However, transfection with Bβ cDNA not only increased the synthesis of Bβ but also that of Aα and γ chains, and the transfected Hep G2 cells maintained surplus amounts of Aα and γ chains. This indicated that hepatocytes stringently regulate expression of the three fibrinogen genes and that over-production of Bβ chains specifically up-regulates expression of the other two genes (22).

In this study we show that enhanced expression of fibrinogen is not only regulated by Bβ in synthesis but that transfection of Hep G2 cells with any fibrinogen chain cDNA elevates the expression of the other two genes, leading to the specific synthesis and secretion of fibrinogen.

**EXPERIMENTAL PROCEDURES**

Materials—The expression vectors containing full length Aα, Bβ, and γ chain cDNA, pRSVNeo-Aα, pRSVNeo-Bβ, and pRSVNeo-γ have been previously described (22, 23). Human β-actin cDNA was purchased from Clontech and RNasin from Promega Corp. Other reagents used have also been previously described (22–25).

Cell Culture, Transfection, and Selection of Stable Cell Lines—Hep G2 cells were transfected with pRSVNeo-Aα, pRSVNeo-Bβ, pRSVNeo-γ, or as a control with pRSVNeo, which did not contain a fibrinogen cDNA insert, by the calcium phosphate method (26). Stable cell lines were selected by resistance to 0.4 mg/ml Geneticin (Life Technologies, Inc.) for 2 months. The cells transfected with vectors containing fibrinogen chain cDNAs were named Hep G2-Aα, Hep G2-Bβ, and Hep G2-γ. The control cells transfected with vector only were named Hep G2-Neo.

Metabolic Labeling and Immunoprecipitation of Fibrinogen and Other Plasma Proteins—Incubation of transfected cells with [35S]methionine and immunoprecipitation of nascent fibrinogen, albumin, transferrin, α1-antichymotrypsin, and C-reactive protein were performed as previously described (11, 12, 23). Protein radioactivity of individual proteins was determined by cutting out the radioactive areas from the SDS-PAGE1 gels and counting by liquid scintillation spectrometry (12) or by measuring the optical density of the fluorograms using a scanning densitometer.

Northern Blot Analyses—Poly(A)+ RNA from Hep G2-Neo, Hep...
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G2-Aα, Hep G2-Bβ, and Hep G2-γ were separated by electrophoresis on 12.5% agarose-formaldehyde gels, transferred to nitrocellulose membranes, and hybridized separately with nick-translated 32P-labeled Aα, Bβ, or γ cDNA or with human actin cDNA (27).

Nuclear Run-on Transcription Assay—Nuclear transcription assays were performed according to Groudine et al. (28). The nuclei from about 1 × 10⁷ Hep G2-Neo, Hep G2-Aα, Hep G2-Bβ, or Hep G2-γ cells were isolated and resuspended in reaction buffer (150 mM KCl, 10 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 1 mM MnCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 10% glycerol, 80 units of RNasin, 5 mM dithiothreitol, 1 mM each ATP, GTP, and CTP, and 250 μCi of [³²P]UTP (800 Ci/mmol, 10 μCi/ml) and incubated at 25 °C for 45 min. Transcripts were deproteinized, DNase I-treated, and further purified by chromatography on a G-50 Sephadex spin column. Hybridizations were performed in slots on nitrocellulose membranes containing 0.5 or 5.0 μg of alkaline-denatured Aα, Bβ, or γ cDNA at 42 °C for 3 days. Radioactivity was detected by fluorography and quantitated by measuring the density of the film in a scanning densitometer.

RESULTS

Synthesis and Secretion of Fibrinogen by Hep G2 Cells Transfected with Individual Chain cDNAs—Transfection with any fibrinogen chain cDNA increased the synthesis of fibrinogen, as measured intracellularly in a 5-min pulse period. As previously described (22) Hep G2-Bβ cells synthesized 2.5-3-fold more fibrinogen than the control Hep G2-Neo cells. Hep G2-Aα and Hep G2-γ, however, also synthesized more fibrinogen (40-80%) than control Hep G2-Neo cells (Fig. 1).

Although the rate of secretion of all cells was similar, Hep G2-Aα, Hep G2-Bβ, and Hep G2-γ secreted more fibrinogen than Hep G2-Neo cells. Most of the fibrinogen synthesized by Hep G2-Aα, Hep G2-Bβ, and Hep G2-γ cells were secreted into the medium during the first 45 min of the chase period. Only a small amount, less than 20%, of the pulse-labeled fibrinogen was retained intracellularly after 2 h of chase incubation (Fig. 1). About 80% of the pulse-labeled fibrinogen was recovered.

Increased Synthesis of Individual Fibrinogen Chains—To determine the initial rate of synthesis of individual fibrinogen chains, the intracellular radioactive fibrinogen chains synthesized at the end of a 5-min pulse incubation with L-[³⁵S]methionine were immunoprecipitated, separated in reducing conditions on SDS-PAGE, and the radioactivities of the individual chains measured. The amount of radioactivity in each of the chains was corrected to account for different amounts of methionine in each of the chains.

Transfection of Hep G2 cells with cDNA for individual fibrinogen chains caused an increase in synthesis not only of a single chain but also of the other two component chains of fibrinogen (Fig. 2). Hep G2-Aα, Hep G2-Bβ, and Hep G2-γ synthesized increased amounts of all three chains as compared with Hep G2-Neo. The increases varied from 1.7-4.2-fold (Table I). Hep G2-Bβ cells synthesized more of each of the fibrinogen chains than Hep G2-Aα or Hep G2-γ (Fig. 2 and Table I).

Synthesis of Variant Aα Chain—In addition to the normal Aα chain (M, = 65,000) commonly present in fibrinogen, a variant Aα chain, containing a C-terminal extension, due to the additional splicing of a sixth exon, is also present in fibrinogen. This variant Aα chain that contains 847 amino acids is termed Aαε and is synthesized and secreted by Hep G2 cells (29).

At the end of a 5-min pulse incubation with L-[³⁵S]methionine Hep G2-Aα, Hep G2-Bβ, and Hep G2-γ cells synthesized more radioactive Aαε than Hep G2-Neo cells (Fig. 3). To detect Aαε the autoradiogram was over-exposed. Aαε appears as a doublet, possibly due to incomplete glycosylation. Aαε contains two possible N-linked glycosylation sites (29).

Transfection with Fibrinogen cDNA Does Not Affect the Synthesis of Other Plasma Proteins—To determine whether transfection with the expression vectors containing fibrinogen cDNAs specifically enhanced the synthesis and secretion of fibrinogen, other plasma proteins, secreted by Hep G2 cells, were also measured. Hep G2-Neo, Hep G2-Aα, Hep G2-Bβ, and Hep G2-γ were pulse-labeled for 5 min with L-[³⁵S]methionine and chase-incubated for 2 h. The radioactivities of secreted fibrinogen, albumin, transferrin, a1-antichymo-

![Graph](https://example.com/graph.png)

**Fig. 1.** Synthesis and secretion of fibrinogen by transfected Hep G2 cells. Hep G2-Neo, Hep G2-Aα, Hep G2-Bβ, and Hep G2-γ cells were pulse-labeled for 5 min with L-[³⁵S]methionine and chase incubated for 2 h with 20 μM L-methionine for 2 h. At the chase periods indicated, the incubation medium was collected and replaced with fresh medium. The amount of intracellular radioactive fibrinogen and fibrinogen-related proteins present at the end of the 5-min pulse or retained after a 2-h chase is shown as bar graphs. Radioactive fibrinogen, secreted into the medium during the chase period, is shown by line drawings.
Expression of Fibrinogen

MW kDa

200 -
100 -
50 -

I2 34
2.0 . =

An
x
Chain
y
Chain,

EBg
Chain

HepCZ-  HepCZ-  HepGZ-  HepCZ-
Neo

Aa
BB
7

FIG. 2. Synthesis of individual fibrinogen chains. Transfected Hep G2 cells were pulse-labeled for 5 min with L-[35S]methionine, and the intracellular fibrinogen chains were isolated by immunoprecipitation, separated by SDS-PAGE in reducing conditions, and detected by fluorography. An autoradiogram is shown in the upper panel. The autoradiogram was scanned, and the intensity of each band, which reflects radioactivity, is presented in the bottom panel. The values in the bottom panel are corrected for different amounts of methionine in the individual fibrinogen chains.

| Transcription | mRNA | Protein |
|---------------|------|---------|
| Aα            | Aα   | 4.2     |
| Bβ            | Bβ   | 7.2     |
| γ             | γ    | 3.9     |
| Aα            | Aα   | 4.1     |
| Bβ            | Bβ   | 6.7     |
| γ             | γ    | 3.7     |
| Aα            | Aα   | 4.3     |
| Bβ            | Bβ   | 7.1     |
| γ             | γ    | 3.9     |
| Aα            | Aα   | 2.1     |
| Bβ            | Bβ   | 2.1     |
| γ             | γ    | 2.2     |
| Aα            | Aα   | 2.8     |
| Bβ            | Bβ   | 4.7     |
| γ             | γ    | 2.3     |
| Aα            | Aα   | 2.4     |
| Bβ            | Bβ   | 2.1     |
| γ             | γ    | 2.5     |
| Aα            | Aα   | 2.3     |
| Bβ            | Bβ   | 3.5     |
| γ             | γ    | 1.8     |
| Aα            | Aα   | 3.0     |
| Bβ            | Bβ   | 4.2     |
| γ             | γ    | 1.7     |
| Aα            | Aα   | 3.0     |
| Bβ            | Bβ   | 4.0     |
| γ             | γ    | 2.4     |

FIG. 3. Increased synthesis of variant αE chain. Hep G2-Neo, Hep G2-Aα, Hep G2-Bβ, and Hep G2-γ cells were labeled for 5 min with L-[35S]methionine and intracellular radioactive fibrinogen chains isolated as described in Fig. 3. An autoradiogram was overexposed to detect αE, which migrated as a doublet, with an apparent molecular mass of approximately 100 kDa. Lane 1, Hep G2-Neo; lane 2, Hep G2-Aα; lane 3, Hep G2-Bβ; lane 4, Hep G2-γ cells.

trypsin, and C-reactive protein were determined.

Transfection of Hep G2 cells with expression vectors containing any one fibrinogen chain cDNA caused an increase in synthesis and secretion of fibrinogen but did not affect albumin, transferrin, α1-antichymotrypsin, and C-reactive protein (Fig. 4). The control Hep G2-Neo cells produced more radioactive albumin and transferrin than fibrinogen and less α1-antichymotrypsin and C-reactive protein. However, the cells transfected with the fibrinogen cDNAs (Hep G2-Aα, Hep G2-Bβ, and Hep G2-γ) synthesized and secreted more fibrinogen than the other proteins measured. This demonstrates that overexpression of any one fibrinogen chain specifically increases the synthesis and secretion of fibrinogen but does not affect other plasma proteins, including α1-antichymotrypsin and C-reactive protein, which like fibrinogen are acute-phase proteins.

Fibrinogen mRNA Levels—Hep G2-Aα, Hep G2-Bβ, and Hep G2-γ cells contained elevated levels of mRNA of all three fibrinogen chains when compared with Hep G2-Neo cells. Fig. 6 shows Northern blot analyses. Densitometric readings of the specific hybridized bands showed that in all cases the mRNA levels of the three fibrinogen chains in Hep G2-Aα, Hep G2-Bβ, and Hep G2-γ cells were greater than that of control Hep G2-Neo. The mRNA levels in Hep G2-Aα and Hep G2-γ were nearly double that of Hep G2-Neo and the levels in Hep G2-Bβ were 3–5-fold higher than the control levels. In the three cell lines overproducing fibrinogen, the Bβ mRNA levels were greater than that of Aα and γ (Fig. 5 and Table I). As a control β-actin mRNA levels were also determined and found not to differ.

Nuclear Run-on Transcription—To determine whether the increased synthesis of fibrinogen and the elevated levels of the three fibrinogen chain mRNAs were due to a stimulation of gene expression, nuclear run-on transcription assays were performed on isolated nuclei of Hep G2-Neo, Hep G2-Aα, Hep G2-Bβ, and Hep G2-γ cells. Slots on nitrocellulose membranes containing 0.5 or 5 μg of alkaline-denatured Aα, Bβ, and γ cDNA were hybridized separately with 32P-labeled nuclear RNA from the various stable-transfected Hep G2 cells.
Expression of Fibrinogen

**FIG. 4.** Transfection specifically enhances secretion of fibrinogen and does not affect other plasma proteins. Hep G2-Neo, Hep G2-Aα, Hep G2-Bβ, and Hep G2-γ cells were pulse-labeled for 5 min with L-[35S]methionine and then chase-incubated for 2 h as described in Fig. 1. The radioactivities of secreted fibrinogen, albumin, transferrin, α-antichymotrypsin, and C-reactive protein were determined and shown as bar graphs.

**FIG. 5.** Northern blots. Poly(A+), RNA (5 µg) from Hep G2-Neo, Hep G2-Aα, Hep G2-Bβ, and Hep G2-γ cells was probed separately with 32P-labeled Aα, Bβ, γ fibrinogen, and human β-actin cDNAs. An autoradiogram is shown in the upper panel. The autoradiogram was scanned, and the intensity of the specific hybridized bands is presented in the lower panel. In the upper panel, each autoradiogram has four lanes. Lane A, poly(A+) RNA from control Hep G2-Neo cells; lane Aα, RNA from Hep G2-Aα; lane Bβ, RNA from Hep G2-Bβ; lane γ, RNA from Hep G2-γ cells. Panel A, probed with 32P-labeled Aα cDNA; panel B, probed with Bβ cDNA; panel C, probed with γ cDNA; panel D, probed with β-actin cDNA.

**FIG. 6.** Nuclear run-on transcription assays. Slots containing 0.5 and 5.0 µg of alkaline-denatured Aα, Bβ, and γ cDNAs were hybridized separately with 32P-labeled nuclear RNAs from Hep G2-Neo, Hep G2-Aα, Hep G2-Bβ, and Hep G2-γ cells as described under "Experimental Procedures." The upper panel shows an autoradiogram, and the lower panel shows the intensities of the slots containing 5.0 µg of cDNA.

Hep G2-Aα, and Hep G2-γ the increase was nearly 4-fold, and in Hep G2-Bβ it was greatest, about 7-fold (Fig. 6 and Table 1).

**DISCUSSION**

Hepatocytes of different species have unequal amounts of the three fibrinogen chains. Because in Hep G2 cells Bβ chains...
occur in limiting amounts we postulated that synthesis of $\beta$ is a rate-limiting factor in the production of fibrinogen (12, 13). Indeed, increasing $\beta$ chain synthesis by transfection of Hep G2 cells with $\beta$ chain cDNA led to increased synthesis of fibrinogen without using the surplus pools of $\alpha$ and $\gamma$ chains (22). We now show that increased expression of any fibrinogen chain, elicited by transfection with vectors containing individual fibrinogen chain cDNAs, specifically up-regulates the expression of the other two chains. Thus the expression of all three fibrinogen genes is tightly linked, and our results suggest that there are probably feedback mechanisms that maintain a steady intracellular proportion of unequal amounts of fibrinogen chains, which apparently are needed for fibrinogen chain assembly. It is of interest to note that the expression of the variant, C terminus-extended $A\alpha$ chain (29) is also up-regulated indicating that it is under similar control as normal $A\alpha$ chains.

The regulation of fibrinogen chain production occurs at the transcriptional level, as shown by nuclear run-on assays. Increased transcription leads to higher levels of all three fibrinogen mRNAs and to increased initial rates of synthesis for all three chains, irrespective of which fibrinogen cDNA was transfected into Hep G2 cells. However transcription with $\beta$ cDNA had a greater effect than did transfection with $A\alpha$ or $\gamma$ cDNA. This may reflect the fact that $\beta$ does not accumulate in Hep G2 cells, but $A\alpha$ and $\gamma$ chains are maintained in surplus amounts. Therefore fibrinogen expression may be more sensitive to small changes in the intracellular levels of $\beta$.

The mechanism by which transfection with any one fibrinogen chain cDNA up-regulates the expression of the other two is not yet understood. Transfection with any fibrinogen cDNA causes an increase in both fibrinogen mRNA levels and in the amount of fibrinogen chains synthesized. Both fibrinogen mRNA and the individual chains are present in the cell cytoplasm and presumably exert, indirectly, a feedback effect that up-regulates the expression of the other two chains. At present we do not know whether it is the increased levels of a fibrinogen chain or of RNA that triggers the events leading to the up-regulation of the three fibrinogen genes. If individual chains are responsible then events that initially occur within the lumen of the endoplasmic reticulum must trigger increased transcription of fibrinogen genes, which ultimately occur in the nucleus. There are examples of auto-regulation of gene expression by proteins present in the cytoplasm. For example the under- or overexpression of some heat shock proteins regulates the expression of a family of heat shock genes (30, 31).

During the acute-phase reaction the synthesis of fibrinogen increases together with a subset of other plasma proteins. Expression of acute-phase proteins is enhanced by glucocorticoids and by interleukin-6 (32-34). The upstream regions of the $\beta$ fibrinogen gene, which respond to dexamethasone and interleukin-6, have been identified (34-36). However the factor(s) that enhance fibrinogen expression in Hep G2-Aa, Hep G2-\(A\alpha\) and Hep G2-$\gamma$ cells must be different from interleukin-6 or glucocorticoids, because the expression of acute-phase proteins, $\alpha$1-antichymotrypsin and C-reactive protein, was not affected. There may be some unknown factor(s) that specifically regulates the three fibrinogen genes, and over expression of any fibrinogen chain may activate these fibrinogen-specific factors. Although the mechanism is unknown our results show that fibrinogen gene expression is regulated at two different levels. One mechanism enhances fibrinogen gene expression together with that of other acute-phase proteins, and at a second level the fibrinogen genes are independently regulated.

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