The Coordinated Regulation of Fibrinogen Gene Transcription by Hepatocyte-stimulating Factor and Dexamethasone

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Abstract. Glucocorticoids and hepatocyte-stimulating factor (HSF; a monocyte/macrophage-derived polypeptide) are potent regulators of fibrinogen biosynthesis. Using primary rat hepatocytes and a rat hepatoma cell line (FAZA) we have determined, more precisely, the interaction between these two molecules in the control of fibrinogen production. When dexamethasone (DEX) or HSF is added to the cells, there is a substantial increase in fibrinogen production (1.5–3-fold). However, if both agents are administered simultaneously the response is much greater with a 15–20-fold rise in synthesis. Quantitative RNA analysis demonstrates that when the factors are present individually only HSF elevates fibrinogen mRNA levels, but the effect is much enhanced in the presence of DEX. This pattern is also seen in the results of the in vitro transcription assays which allow quantitation of mRNA synthesis in isolated nuclei. Cycloheximide does not significantly interfere with the increased transcription brought about by HSF in either cell type. However, the DEX enhancement is blocked by cycloheximide in FAZA cells, thus indicating that in the transformed cell protein synthesis is required for maximal transcription to occur. Data presented here demonstrates the requirement for two types of regulator molecules in the control of fibrinogen gene expression; a polypeptide hormone (HSF) that increases transcription and a steroid (DEX) that enhances the action of the polypeptide.

O VER the past few years considerable interest has focused on the biosynthesis of fibrinogen. In part this has been due to the unique molecular arrangement of the protein. The functional molecule is composed of three pairs of nonidentical polypeptides bound together through complex sets of disulfide bridges. Each fibrinogen subunit (Aα, Bβ, and γ) is encoded by separate, but closely linked, genes (located in chromosome 4 in man and 2 in rat) that are regulated as a stringently coordinated family (12, 13, 19).

Recently we reported that all three fibrinogen mRNAs increase simultaneously and to the same extent when hepatocytes are incubated in the presence of hepatocyte-stimulating factor (HSF),[1] a polypeptide which is derived from activated peripheral blood monocytes and certain myelogenous tumor lines (9, 24). Certain glucocorticoids have also been shown to stimulate fibrinogen synthesis in hepatocyte cultures (23). During an acute inflammatory challenge the concentration of adrenal glucocorticoids are markedly increased in the intact animal (20). The levels of several hepatically derived plasma proteins are substantially altered during acute inflammation ostensibly in response to greater concentrations of glucocorticoids (14, 15). Glucocorticoids have also been implicated in elevating fibrinogen mRNA levels in the liver (5, 22), but little information has been published detailing the mechanism(s) through which they elicit this response.

In this study, we partitioned out the role that is played by HSF and glucocorticoids in the control of fibrinogen biosynthesis. We provide evidence that although HSF and glucocorticoids by themselves increase fibrinogen secretion (and cytoplasmic mRNA levels in the case of HSF), a much greater elevation in fibrinogen production occurs when both are present. Nuclear run-off experiments reveal that HSF stimulates transcription whereas glucocorticoids themselves did not. However, both factors are required for maximum transcription. These findings provide strong evidence that HSF is a transcriptional regulator of the fibrinogen genes and that the glucocorticoid enhancement of transcription is exerted through a mechanism which involves protein synthesis.

Materials and Methods

Cell Culture

Primary hepatocytes were prepared from 200–300-g Sprague-Dawley rats using the collagenase perfusion procedure described by Seglen (28) with several modifications (23). Confluent monolayers were prepared by plating 0.5 ml of a washed hepatocyte suspension (6.0 × 10⁶ cells/ml) on 35-mm tissue culture plates precoated with fibronectin (100 μg/ml) in sterile PBS. The medium was changed after 1 h and the attached cells were allowed to spread overnight. Hepatocytes were cultured in Williams' Medium E lacking arginine, but supplemented with ornithine (100 μM), heparin (3.5 U/ml), insulin (20 mU/ml), penicillin G (100 U/ml), streptomycin sulfate (200 μg/ml), gentamycin (60 μg/ml), and FBS (5% vol/vol). To remove glucocorticoids, the serum was extracted with activated charcoal as described by Dobner et al. (7). FAZA cells, from a Ruber-derived rat hepatoma cell line, were grown to confluency on Falcon 35-mm tissue culture plates (17). The

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1. *Abbreviations used in this paper:* CHX, cycloheximide; DEX, dexamethasone; HSF, hepatocyte-stimulating factor.

cells were cultured in DME supplemented with Ham's F-12 (50%), penicillin G (100 U/ml), streptomycin sulfate (200 µg/ml), gentamycin (60 µg/ml), and FBS (10% vol/vol). The cells were changed to medium containing glucocorticoid-free serum 48 h before treatment.

Monocyte-conditioned Medium

Human buffy coats were obtained from the American Red Cross through anonymous donors. The monocytes were purified as described previously (9). Cells were plated on 60-mm tissue culture plates at a density of 3×10^7 cells/ml. The monocytes were incubated for 24 h in serum-free McCoy's 5A medium in 5% $CO_2/95\%$ air. The conditioned medium was harvested from the cells after 24 h and served as the source of HSF.

Immunoassays

Rat fibrinogen antibody was produced in goats and made monospecific by affinity chromatography using fibrinogen covalently coupled to Sepharose (6). Fibrinogen concentrations in hepatocyte and hepatoma media were determined using a quantitative ELISA as previously described (16).

Quantitation of mRNA

After the removal of media from confluent monolayers of either primary hepatocytes or hepatoma cells, they were washed three times with ice cold PBS. Subsequently the washed cells were scraped and homogenized into 4 M guanidine thiocynate lysis buffer as described by Chirwin et al. (4). The homogenate was then mixed 1:1 vol/vol with a solution of 20× SSC (1× SSC contains 0.15 M NaCl, 15 mM trisodium citrate) and 37% formaldehyde in a 3:2 ratio, and heated to 65°C for 15 min. This mixture was centrifuged to remove insoluble cell debris, and the supernatants were then diluted with 20× SSC and blotted onto nitrocellulose using the slot blot apparatus (Schleicher & Schuell, Inc., Keene, NH). 100 ng of cytoplasmic RNA was applied to each slot. A standard dilution of each sample was carried out for each of six different experiments performed. After baking, the filters were prehybridized in 5× Denhardt's solution, 4× SSC, 0.1% SDS, and 100 µg/ml sonicated salmon sperm DNA for 2–4 h at 42°C. Hybridization was carried out using rat fibrinogen cDNA labeled with ^{32}P by nick translation using [^{32}P]dCTP (3,000 Ci/mM; Amersham Corp., Arlington Heights, IL) (18). The specific activity of each probe was 10^8 cpm/µg. The filter was washed extensively before autoradiography.

RNA Analysis by Northern Blots

RNA extracted by hot phenol method (26) was subjected to electrophoresis on a 1.5% agarose gel in the presence of 6% formaldehyde, then transferred to nitrocellulose filters. The immobilized RNA was prehybridized and hybridized as described above.

Blocking of Protein Synthesis by Cycloheximide (CHX)

2.0 µg/ml (final concentration) CHX (Sigma Chemical Co., St. Louis, MO) was added to confluent monolayers of either primary hepatocytes or hepatoma cells 2 h before the addition of HSF and/or dexamethasone (DEX). Pilot studies revealed that when [^{35}S]methionine (20 µCi/ml) was added to the cells after a 2-h incubation with CHX greater than 90% of the incorporation of the labeled amino acid into TCA-precipitable counts were inhibited in both cell types (data not shown).

In Vitro Nuclear Transcription Assays

Nuclei were prepared from rat hepatocytes and FAZA cells essentially as described by Schibler et al. (27). $1.0–5.0 \times 10^8$ cells were scraped into PBS, washed, homogenized, and the nuclei sedimented through a sucrose cushion. The RNA elongation reaction was carried out according to Gariglio et al. (10). Briefly, 2×10^7 nuclei were incubated for 10 min at 26°C in a 200-ml reaction mixture containing 100 mM Tris-Cl (pH 7.9); 50 mM NaCl; 4 mM $MnCl_2$; 1.2 mM dithiothreitol (DTT); 0.1 mM phenylmethylsulfonyl fluoride (PMSF); 0.4 mM EDTA; 1.0 mM GTP, ATP, and CTP; 29% glycerol; 10 mM phosphocreatine; 5 µM UTP; 150 U/ml RNA-sin; and 0.2 mCi [^{32}P]UTP (650 Ci/mmol from ICN K&K Laboratories Inc., Plainview, NY). The RNA was isolated as described by Groudine et al. (11). The reaction was terminated with DNase I and the protein digested with proteinase K in the presence of SDS. The mixture was phenol/chloroform-extracted and then precipitated with TCA. The DNase I and proteinase digestions were repeated and were followed by two phenol/chloroform ex-

tractions and finally two chloroform extractions. The RNA was precipitated with ethanol and collected by centrifugation. Approximately 3.0×10^7 cpm were recovered in this manner and allowed to hybridize for 3 d at 42°C to the nitrocellulose-bound linearized plasmids containing the fibrinogen and AT-III cDNA inserts. The filters were then washed four times for 15 min at room temperature in 1× SSPE (20× SSPE contains 3.6 M NaCl; 200 mM NaH_2PO_4, pH 7.4, and 20 mM Na_2EDTA), 0.1% SDS, and twice for 30 min at 52°C in 0.1× SSPE, 0.1% SDS and allowed to air dry before autoradiography. The results reported represent the typical finding observed from nuclear runoff experiment using four different preparations of hepatocyte nuclei.

Results

Effect of DEX and HSF on Fibrinogen Secretion

Two different cell types were used to assess the effect of DEX and HSF on fibrinogen production. Primary hepatocytes were used since their response to HSF has been well documented (23). We have previously suggested that a glucocorticoid was a necessary additive for maintaining primary hepatocytes in culture (23). More recent studies, however (data not shown), indicated that glucocorticoids are not absolutely required for cellular maintenance during an incubation period of 24–48 h. It was therefore possible to partition out the role played by the glucocorticoid and the HSF in primary hepatocytes. We have also shown that FAZA cells synthesize and secrete fibrinogen (21). These cells grow indefinitely in the absence of glucocorticoids and therefore they not only serve as a suitable control cell system, but also provide information on the responsiveness of a tumor cell line to the HSF molecule.

A dose–response relationship between the secretion of fibrinogen and the concentration of DEX in the two cell types is shown in Fig. 1. The primary hepatocyte increased its synthesis and secretion 1.5-fold at 10^{-7} M DEX. The FAZA cell response was considerably greater, ~3.5-fold, although the actual amount of fibrinogen being made by the hepatocytes was higher than the transformed cells; 1.0–1.5 µg fibrinogen/mg cell protein/24 h vs. 0.1–0.2 µg fibrinogen/mg cell protein/24 h.

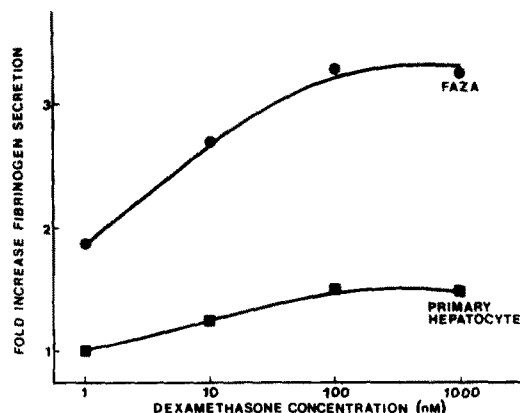The response of primary hepatocytes to increasing concen-

Figure 1. Dependence of fibrinogen secretion on the concentration of DEX. Primary hepatocytes and FAZA cells were plated, or grown, to confluency in the absence of glucocorticoids. DEX was added to the medium at the concentrations indicated. After 24 h, the medium was harvested and assayed for fibrinogen using an ELISA.
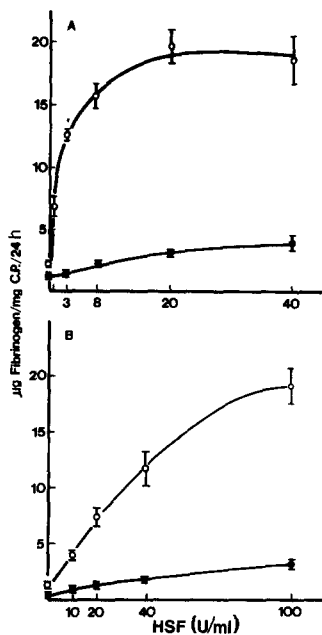
Figure 2. Dependence of fibrinogen secretion on the concentration of HSF in the presence or absence of DEX. (*A*) Primary hepatocytes were prepared in medium lacking glucocorticoids and allowed to spread to form confluent monolayers on 35-mm tissue culture plates. The medium was changed and HSF added at the concentrations indicated either in the presence (○) or absence (●) of 10^{-7} M DEX. The cells were incubated for 24 h, then the medium was assayed for fibrinogen and the total cell protein determined. (*B*) Similar experiment with FAZA cells which were grown to confluency in glucocorticoid-free medium. The values given represent the mean SEM of three replicates from a single experiment.

trations of HSF in the presence and absence of the synthetic glucocorticoid, DEX, is shown in Fig. 2 *A*. Primary rat hepatocytes were prepared as described above in medium lacking glucocorticoids. A maximum response to HSF occurred at 20 U/ml when DEX was present in the medium at a concentration of 100 nM. (A unit of HSF activity is that amount of HSF required for a half-maximal fibrinogen synthesis response in primary hepatocytes; 23, 25). In its absence, there was only a 2.5-fold increase in fibrinogen production over controls during the 24-h incubation period. The FAZA cells showed the same qualitative response to HSF as did the primary hepatocytes (Fig. 2 *b*). One consistent difference between the two cell types is that the FAZA cells require considerably greater concentration of HSF (3–5X) than do the primary hepatocytes to get maximal fibrinogen synthesis.

Effect of Different Steroids on Fibrinogen Production

We tested a number of different steroids on primary hepatocytes in the presence and absence of HSF to determine if steroids belonging to the androgen, estrogen, or mineralocorticoid groups could also interact with HSF to affect fibrinogen production. Fig. 3 shows the results of studies using seven different steroids. Only those steroids in the glucocorticoid family (hydrocortisone, corticosterone, and DEX) enhanced fibrinogen biosynthesis. There was a slight but significant increase with aldosterone reflecting, in part, its known glucocorticoid activity (2).

### Effect of DEX and HSF on Fibrinogen mRNA Levels in the Presence and Absence of CHX

Cytodot Analysis. In a previous study we demonstrated by cytodot hybridization that HSF (in the presence of DEX) elevates the cytoplasmic levels of fibrinogen mRNAs 20-fold (9). Here we performed similar experiments, but under conditions in which each modulator was added individually as well as together. The results shown in Fig. 4 *a* (*A* and *B*) demonstrate that in primary hepatocytes and FAZA cells

maximal mRNA increases occur only when both HSF and DEX were present simultaneously. The fibrinogen mRNA levels in the presence of DEX alone or HSF alone increases in comparison to the control fibrinogen mRNA levels, although to a much less extent ~1.5–3-fold that of control.

Experiments were carried out to determine if protein synthesis was a required step in the increase of fibrinogen cytoplasmic mRNA. It is generally recognized that in certain circumstances there is a requirement for new protein(s) to be made to bring about changes in cytoplasmic mRNA levels (29). Results presented in Fig. 4 *b* (*A* and *B*) are cytodot analyses after each cell type had been incubated for 2 h in the presence of CHX (2 μg/ml) before the addition of DEX or HSF. The cytodot pattern depicting levels of fibrinogen mRNAs for the primary hepatocytes were basically the same as in control, however, the pattern for the FAZA cells were quite different. When protein synthesis was blocked in the FAZA cell the HSF/DEX stimulation of cytoplasmic levels of fibrinogen was inhibited. Tubulin mRNA was also probed under each condition and the results showed that it was unaffected by DEX, HSF, or a combination of the two and thus could serve as a suitable internal control. If the modulators, either individually or together, were acting to stimulate RNA levels in general (either transcriptionally or posttranscriptionally) then tubulin mRNA levels should follow the same increases and/or decreases.

Northern Gel Analysis

The effects of CHX on fibrinogen mRNA levels in the two cell types was further assessed by Northern gel analysis (Fig. 5). Results shown in *A* indicate the typical response of the two modulators on fibrinogen mRNAs. The effects of a 2-h preincubation of CHX before adding DEX, HSF, and the combination of the two does not alter the fibrinogen response in primary hepatocytes, but it dramatically reduces the stimulating effect in the FAZA cell. The RNAs shown in *B* were taken from cells that had been in the presence of CHX for a total of 8 h (2 h preincubation before the addition of modulators, then an additional 6 h in their presence). *C* shows the
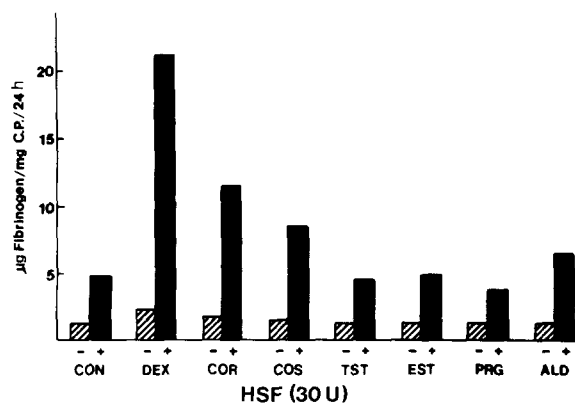
Figure 3. Effects of various steroids and HSF on fibrinogen production. Primary hepatocytes were prepared as in Fig. 1. Maximum stimulating concentrations of the steroids were determined in previous studies and found to be near 10^{-7} M (data not shown). Cells were treated with steroids either in the absence (▨) or presence (■) of 30 U/ml HSF. Control, *CON*; dexamethasone, *DEX*; hydrocortisone, *COR*; corticosterone, *COS*; testosterone, *TST*; 17-β-estradiol, *EST*; 17-α-hydroxyprogesterone, *PRG*; aldosterone, *ALD*.
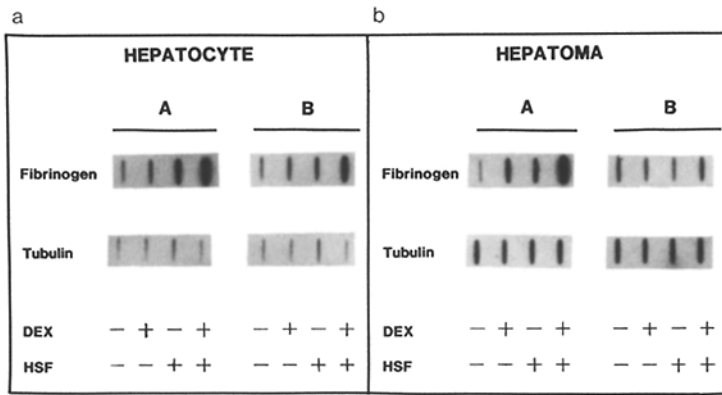
Figure 4. The effects of DEX and HSF on fibrinogen mRNA levels in primary hepatocytes and hepatoma cells (FAZA). (a) Slot blots of cytoplasmic RNA taken from primary hepatocytes and FAZA cells after 12 h exposure to DEX (10^{-7} M) and/or HSF (20 U/ml for hepatocytes and 100 U/ml for FAZA). Fibrinogen and tubulin mRNAs were probed with nick-translated cDNAs for fibrinogen subunits and for tubulin, respectively. (b) Slot blots as described in a except the cells had been treated with 2 μg/ml CHX for 2 h before the addition of DEX or HSF. 0.1 μg of RNA for slot blots was taken at the end of a 12-h incubation.

results of a Northern gel after the cells had seen CHX for 8 h. They were washed twice with media removing the antibiotic and then were incubated for an additional 6 h in the presence of the modulators. These findings demonstrate that the FAZA cells are capable of responding to the modulator once the block in protein synthesis has been removed. These data provide additional information that protein synthesis is required to get the full stimulating effect of HSF in FAZA cells.

### Fibrinogen Gene Transcription as Measured by Nuclear Run-off Experiments

To determine if HSF is exerting its stimulatory effect on fibrinogen at the level of gene transcription, we carried out gene run-off experiments in isolated nuclei after exposure to HSF. We had previously demonstrated by Northern gel analysis significant increases in fibrinogen mRNA occurred 1.5 h after exposure to HSF (9). We carried out time-course experiments and showed that a maximal radioactive label was incorporated by 2 h (data not shown). Primary hepatocytes were exposed to HSF, DEX, or both for 2 h before isolating the nuclei. When the nuclei were incubated under conditions that permit gene transcription to continue, and then the labeled RNA was isolated and hybridized to immobilized fibrinogen cDNA, the results shown in Fig. 6 were obtained. A clear and dramatic increase in nuclear RNA (representing a 10-fold increase in transcription of the fibrinogen genes) was evident when both DEX and HSF were present. The increases in cytoplasmic mRNA levels therefore can be accounted for entirely by the addition of newly transcribed messages to the cytoplasmic pool. It can also be

seen that the HSF molecule appears to cause an increase (approximately threefold) in transcription when not in the presence of DEX. DEX by itself caused no detectable increase in fibrinogen mRNA synthesis. Taken together these results indicate that HSF is a potent modulator of fibrinogen transcription and DEX appears to enhance the transcriptional activity brought about by the HSF. We also examined the nuclear RNA for alterations in transcription of other genes. The results of probing with a cDNA to antithrombin III (3) (not an acute phase–responding protein) indicate that the condition leading to alteration in fibrinogen gene transcription was specific and not the result of altering cellular transcription in general.

Discussion

In this study we analyzed the regulation of fibrinogen production (synthesis and secretion) after administration of a major acute-phase regulator protein (HSF) and a potent glucocorticoid–hormone analogue (DEX) in an attempt to begin to determine how these two modulators interact. We used both a nontransformed primary cell and a transformed cell line to better evaluate the role that each modulator plays in the regulation of fibrinogen synthesis.

The results obtained from these studies suggest that DEX may regulate fibrinogen synthesis at two levels in the biosynthetic pathway. In the presence of DEX alone the increased secretion of 2–3× above control with a minimal increase in the mRNA levels indicates an enhanced translational or posttranslational process. How this enhancement occurs is not clear from the present study. A second site of DEX action
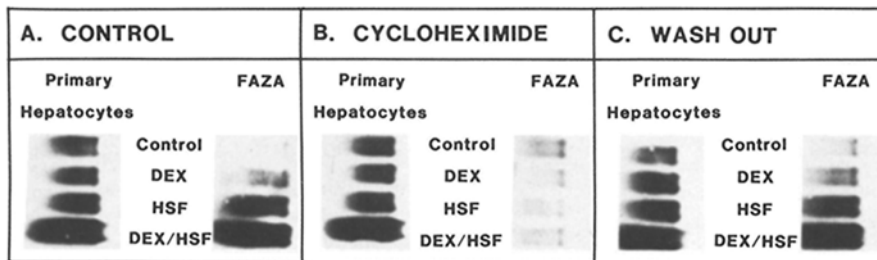
Figure 5. Northern gel analysis. (A) 10 μg of RNA extracted by hot phenol from confluent monolayers of primary hepatocyte and FAZA cells was subjected to electrophoresis in agarose, transferred to nitrocellulose, and probed with nick-translated cDNAs to fibrinogen Bβ chain. DEX concentration was 10^{-7} M; HSF was 20 U/ml for primary or 100 U/ml for FAZA cells. (B) RNA prepared as in A but from cells that had been treated with 2 μg/ml CHX for 2 h before the addition of DEX and/or HSF. RNA was extracted from cells 12 h after the addition of the DEX and/or HSF. (C) RNA extracted as in A. Cells were treated with CHX as in B, but at the end of 6 h, the media was changed to remove CHX; cells were incubated for 1 h, the media was changed again; then HSF and DEX were added as in A. At the end of an additional 6-h incubation the RNA was extracted and analyzed as in A.

Aα

Bβ

γ

AT-III

| DEX | − | + | − | + |
| HSF | − | − | + | + |
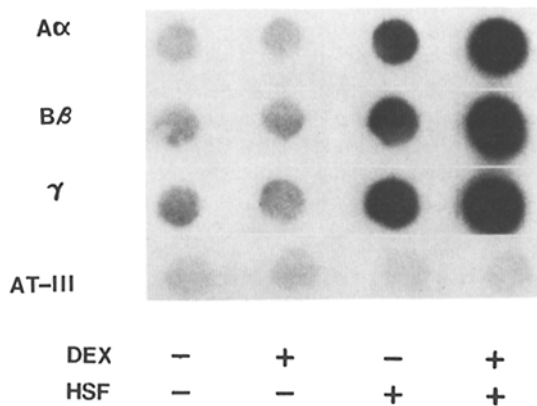
Figure 6. In vitro transcription assay for fibrinogen mRNA synthesis performed on isolated primary hepatocyte nuclei. Approximately 5.0×10^7 cells were incubated for 2 h in the presence of 10^{-7} M DEX or 20 U/ml HSF or both. Nuclei were isolated (2×10^7/assay) and transcription was permitted to continue for 15 min in the presence of $[^{32}P]$ UTP. The labeled RNA (3.0×10^7 cpm) was purified and allowed to hybridize to the nitrocellulose to which Aα, Bβ, and γ fibrinogen cDNAs and antithrombin-III cDNA had been bound. The nitrocellulose was extensively washed and exposed to fibrin as described previously.

is at the gene level since maximal transcription, as measured by nuclear run-off studies, only occurs when DEX is present. The fact that steroids other than those with recognized glucocorticoid activity do not affect the HSF signal reinforces the observation by Fowlkes et al. (8), who demonstrated the presence of putative glucocorticoid receptor–binding sequences in the 5' nontranslational regions of the three fibrinogen genes. These are potential steroid promoter sites where the glucocorticoid receptors may be binding and affecting HSF-stimulated transcription.

Our findings from nuclear run-off studies provide strong evidence that the HSF signal acts exclusively at the level of gene transcription. We believe these are the first and most direct studies demonstrating that HSF controls fibrinogen production by regulating the transcription of fibrinogen mRNAs. Our findings show that all three genes are being transcribed simultaneously since we detect the same amount of radioactive label incorporated in each mRNA. Furthermore, our studies show that DEX is not an essential component of the HSF-stimulated transcription although it does synergistically enhance the HSF effect. When both DEX and HSF are present, a 10-fold increase in transcription occurs. The lack of any significant amplification at later steps in the biosynthetic pathway indicates that HSF operates exclusively at the level of gene transcription.

Baumann et al. (1) showed that in cultured rat hepatocytes the DEX-mediated induction of α_1 acid glycoprotein is dependent on protein synthesis. The findings reported here (Figs. 4 and 5) for the primary hepatocyte indicates that the fibrinogen response to HSF operates independently of protein synthesis. The slight reduction in hybridizable mRNAs can be accounted for by the cytotoxicity that CHX would have on cells in the 12-h exposure time of the experiment. In FAZA cells, however, the HSF induction was completely blocked in the presence of CHX. This finding suggests that the DEX enhancement requires a protein intermediate, a finding not unlike that of α_1 acid glycoprotein (27). 6 h af-

ter CHX was removed from the FAZA cells, a full induction could be achieved indicating that the cells could recover from the metabolic block and thereby reinforcing the finding that protein synthesis is required in the FAZA cells for them to be able to respond to the HSF signal.

In summary, we have provided evidence that maximal stimulation of fibrinogen biosynthesis requires both a glucocorticoid and the polypeptide HSF. We have been able to separate the interaction between these two modulators and show that HSF is exclusively a transcriptional regulator and DEX acts primarily by enhancing the HSF signal. We also show that there are some differences in the way DEX and HSF regulate fibrinogen in normal and transformed cells. For example, the requirement that several times more HSF is required by FAZA cells to bring about a full fibrinogen response as well as some of the requirements for ongoing protein synthesis indicates there are some fundamentally different steps in the HSF signaling. We therefore raise a note of caution in predicting regulatory pathways where only one type of cells is used as the model system. Finally, we predict that several acute-phase genes will contain common sequence elements in their control regions which will be regulated by HSF and DEX in a manner analogous to fibrinogen.

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