The EMBO Journal vol.7 no.8 pp.2485 – 2493, 1988

A variant nuclear protein in dedifferentiated hepatoma cells binds to the same functional sequences in the β fibrinogen gene promoter as HNF-1

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Communicated by A.C.Allison

Normal liver and differentiated hepatoma cell lines contain a nuclear factor, HNF-1, which binds functional sequences within the promoters of the α and β chains of fibrinogen and α_1-antitrypsin. In UV cross-linking studies we find that HNF-1 has an apparent mol. wt of 92 kd in differentiated hepatocytes. Nuclear extracts from a dedifferentiated hepatoma cell line, Fao flC2 (C2), selected on the basis of morphological and biochemical dedifferentiation from Fao contains a protein, vHNF, which binds to the same DNA sequence motif as HNF-1 but has an apparent mol. wt of 72 rather than 92 kd. Mixing experiments indicate that this variant nuclear factor does not arise from HNF-1 by proteolysis. Reversion to the differentiated phenotype in C2-Rev7 (Rev7), selected by growth in glucose-free media, results in the re-expression of many liver-specific functions including the fibrinogen genes. In Rev7, HNF-1 is indistinguishable from that in the original differentiated cell line Fao. Transfection studies and nuclear run-on experiments indicate that reduced expression of fibrinogen RNA in C2 relative to Fao is related to reduced transcription. vHNF but not HNF-1 is present in somatic hybrids between fibroblasts and liver cells which show extinction of liver specific traits and it can also be detected in normal tissue, predominantly in lung nuclear extracts. Since vHNF and HNF-1 are not co-expressed yet correlate with the non-hepatic and hepatic phenotype, respectively, we suggest that the expression of these variant forms reflects determination events in establishing the hepatic phenotype.

Key words: hepatocyte differentiation/gene expression/transcriptional regulation/*trans*-acting factors

Introduction

One of the most challenging problems in modern biology is the elucidation of the molecular mechanisms controlling eucaryotic gene expression in relation to differentiation, development and adaptation to physiologic environmental stimuli. The present view is that regulation is mostly transcriptional and is brought about by the interplay between *cis*-acting DNA elements present in the promoter/enhancer region of a gene and proteins which modulate gene activity in *trans* through specific binding to such DNA sequences (Serfling *et al.*, 1985; Dynan and Tjian, 1985; McKnight and Tjian, 1986; Maniatis *et al.*, 1987). Much emphasis is currently put upon the identification and functional characterization of DNA binding proteins which might be involved in tissue-specific gene expression. There are now several examples of nuclear proteins with restricted tissue distribution and promoter specificity including OTF2, a human B cell-specific factor for immunoglobulin expression (Scheidereit *et al.*, 1987), a pituitary-specific factor, GHF1, involved in the transcriptional regulation of human growth hormone (Bodner and Karin, 1987) and a chicken ovalbumin-specific transcription factor (Bagchi *et al.*, 1987). The identification of these tissue-specific factors now allows an approach to the more fundamental question of the regulation of cell-type-specific gene expression during differentiation.

One possible mechanism by which the coordinated expression of a group of genes characteristic of a developmental stage can be achieved is through the use of a common transcriptional factor by members of a co-expressed group. A prediction of this model is that selection for expression of one member of the group could result in expression of several members of the group. However, few eukaryotic cells provide the opportunity to select for the differentiated phenotype. Hepatocytes are an exception since the hepatocyte-specific gluconeogenic enzymes, phosphoenolpyruvate carboxykinase and fructose diphosphatase, allow these cells to grow in media lacking glucose (Bertollotti, 1977a,b). Since these enzymes are characteristic of highly differentiated hepatocytes but not dedifferentiated cells, growth in glucose-free media places selective pressure upon the factors which control differentiated functions and not simply a single enzyme. Deschatrette *et al.* (1979) selected the dedifferentiated variant Fao flC2 (C2) from the hepatoma cell line Fao on the basis of non-hepatocyte morphology. Detailed studies of the C2 variant line showed that not only the morphology was changed but also many of the functions characteristic for the differentiated liver cell had been lost. These included lack of the two key enzymes of the gluconeogenic pathway and the greatly reduced synthesis of albumin, tyrosine and aminotransferase and other liver-specific proteins. Furthermore Deschatrette *et al.* (1980) isolated a redifferentiated revertant from C2, C2-Rev7 (Rev 7) in glucose-free medium, which had regained many of the functions characteristic for the hepatocyte.

Thus a test is possible of one model of the mechanisms underlying coordinate gene regulation in that selection of variants or mutants having or lacking gluconeogenesis should be correlated with changes in the transcriptional factors controlling other hepatocyte-specific genes.

Recently we have identified a hepatocyte-specific nuclear factor, HNF-1 (Courtois *et al.*, 1987), which is required for the expresson of the fibrinogen α and β chain genes and also interacts with several other promoters of genes expressed in the liver, including α_1-antitrypsin, α fetoprotein, albumin and prealbumin (Courtois *et al.*, 1988, manuscript submitted). These findings suggested to us that HNF-1 was

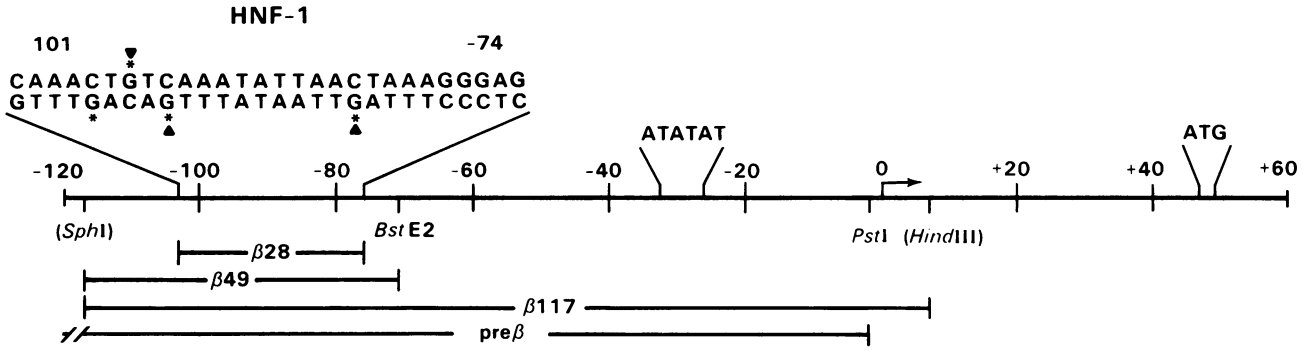

Fig. 1. Promoter region of the β fibrinogen gene. Indicated above the partial restriction enzyme map are the TATA box, the initiation start site and the nucleotide sequence of the binding site of HNF-1. Protein−DNA contact residues (taken from Figure 4B) are marked by asterisks for HNF-1 and triangles for vHNF. Detailed below the map are the probes used for gel retardation assays (β-28 and β-49), DNase I footprint analysis (β-117), methylation interference (β-49) and nuclear run-on experiments (pre β). The two retriction enzyme sites *Sph*I and *Hin*dIII were created in the process of subcloning the β-117 fragment into pJYM-cAT to construct the vector used in the transfection assays.

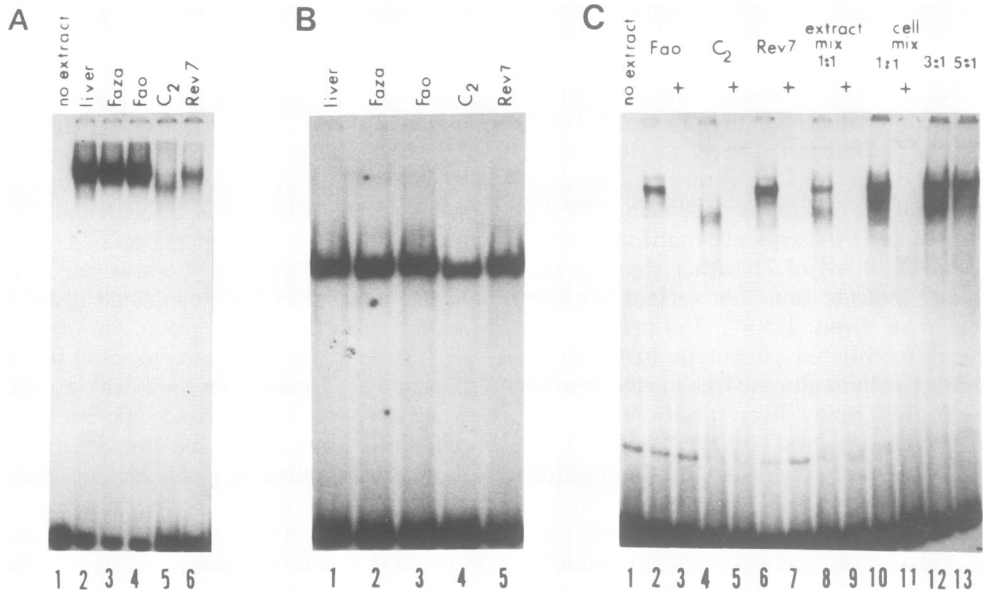

Fig. 2. Detection of two distinct proteins, HNF-1 and vHNF, with specificity for the same sequence of the β fibrinogen gene promoter in differentiated and dedifferentiated hepatoma cells. (**A**) Gel retardation assay comparing complexes formed between nuclear extracts from the cell lines Faza, Fao, C2 and Rev7 with the synthetic oligomer β-28. The ^{32}P-labeled probe (0.2 ng, 5000 c.p.m.) was incubated with 5 μg of crude nuclear extract and bound and free probe separated on a 4% native polyacrylamide gel. (**B**) Comparison of CCAAT binding activity present in the nuclear extracts of the cell lines. Equal amounts of extract (5 μg) were incubated with a labeled human α globin probe and analyzed as above. (**C**) Competition analysis and mixing experiments demonstrating specificity of the binding and absence of proteolytic degradation. Nuclear extracts were incubated with the labeled probe β-49 in the presence (indicated by +) or absence of a 20-fold excess of the synthetic oligomer β-28. The amounts of extract used were 2 μg Fao, 5 μg C2 and Rev7, and in the extract mix 3 μg each of C2 and Rev7 were added to the same binding reaction. In **lanes 10−13** 8 μg of nuclear extract was used which had been prepared from C2 and Rev7 cells mixed at ratios 1:1, 3:1 and 5:1 prior to the preparation of the extract.

regulating the expression of a group of genes and might therefore be involved in the establishment of the differentiated liver phenotype. To test the possible correlation between the presence of HNF-1 and expression of genes characteristic for the differentiated hepatocyte we have studied the well differentiated hepatoma cell line, Fao, the dedifferentiated variant, C2, and the revertant, Rev7.

We have analyzed these cell lines, representing various stages of differentiation, for the presence of HNF-1 and the expression of the fibrinogen genes. Our results show that the dedifferentiated variant C2 contains a nuclear protein, vHNF, of lower mol. wt than HNF-1 but displaying very similar sequence specificity. Reversion to the hepatocyte phenotype in Rev7 is accompanied by the re-expression of HNF-1. The presence of the variant protein in C2 is associated with greatly reduced steady-state levels of fibrinogen mRNA. Furthermore, we demonstrate that hybrids showing extinction of many liver-specific functions as well as certain normal tissues contain a protein, indistinguishable from vHNF in C2, indicating a physiological role for this nuclear factor.

Results

Detection of a variant form of HNF-1 in dedifferentiated cells

The structure of the β fibrinogen promoter and the position of the HNF-1 recognition sequence, the TATA box and

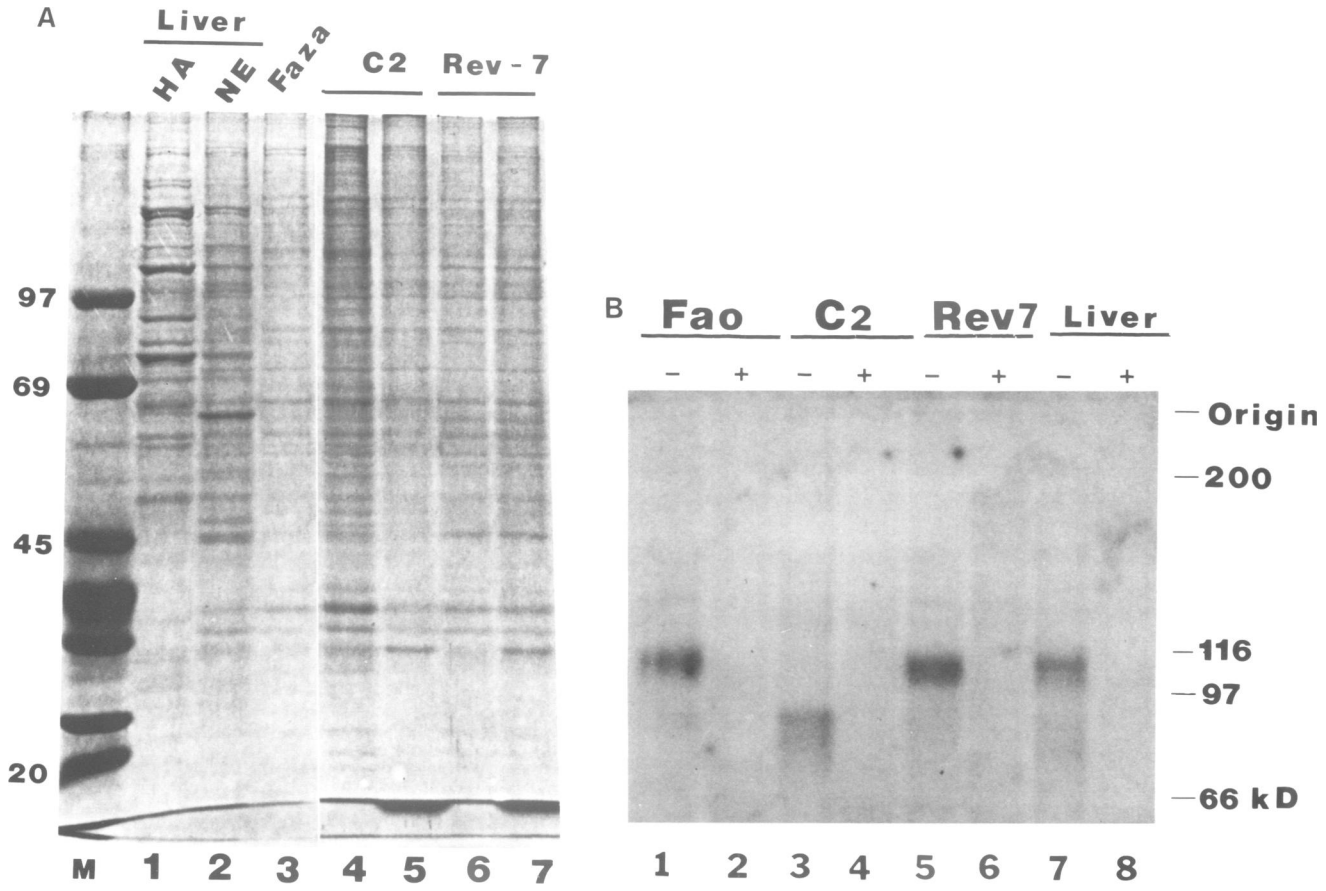

Fig. 3. Determination of the mol. wt of HNF-1 and vHNF by photoaffinity labeling and SDS−polyacrylamide gel electrophoresis. (**A**) Coomassie blue staining of nuclear proteins in liver, Faza, C2 and Rev7 resolved on a 10% SDS−polyacrylamide gel under reducing conditions. HA and NE refer to heparin−sepharose purified material and unfractionated liver nuclear extracts, respectively. (**B**) Autoradiographic detection of proteins specifically bound to the probe β-28. Nuclear extracts from Fao (10 μg), C2 (20 μg), Rev7 (20 μg) and liver (10 μg) were incubated with 1 ng (50 000 c.p.m.) of the body-labeled, BrdU-substituted probe β-28 for 45 min at room temperature in the presence of 10 μg poly(dI-dC), with (indicated by + above the lane) or without a 20-fold excess of unlabeled β-28. After cross-linking proteins were resolved on a 10% SDS−polyacrylamide gel under reducing conditions. Specific DNA−protein complexes were detected by autoradiography. Positions of the mol. wt markers are indicated on the right.

transcription initiation site are shown in Figure 1. The probes used for DNase I footprinting analysis (Galas and Schmitz, 1987) and gel retardation assays (Fried and Crothers, 1981) are also delimited. Nuclear extracts were prepared from the cell lines Faza, Fao, C2 and Rev7 as well as from rat liver and were analyzed for the presence of HNF-1 using the gel retardation assay. Equal amounts of nuclear proteins were incubated with the 3′ end-labeled synthetic oligomer, β-28 (Figure 1) which spans the recognition sequence of HNF-1. After electrophoresis on a native 4% polyacrylamide gel, protein−DNA complexes showing identical migration were detected in extracts from liver, the hepatoma cell lines Faza and Fao and the redifferentiated cell line Rev7, indicating that each of the extracts contained HNF-1 (Figure 2A lanes 2−4, 6). Interestingly, a faster migrating complex was observed when an extract from the dedifferentiated variant C2 was used in the assay (Figure 2A, lane 5). It thus seemed that a protein distinct from HNF-1 but specific for the same DNA sequence was present in extracts from this cell line. We wanted to be sure that we were not dealing, in the case of C2, with a degraded form of HNF-1 and performed several control experiments. First each extract was tested for the intactness of the CCAAT transcription factor (CTF) using a probe prepared from the human α globin promoter which contains a CTF binding site (Jones *et al.*, 1987). Similar amounts of CTF were present in all cell lines tested with no sign of degradation (Figure 2B). To rule out the possibility that selective degradation of the C2 nuclear proteins occurred either during the preparation of the extract or the incubation with the probe we performed mixing experiments. Extracts prepared from C2 and Rev7 were mixed at a 1:1 protein ratio and incubated with the 3′ end-labeled probe β-49 (Figure 1). The gel retardation assay showed that the Rev7 as well as the C2 protein−DNA complex could be distinguished with no indication of degradation of HNF-1 in the Rev7 nuclear extract (Figure 2C, lane 7). Furthermore, by mixing the cells prior to the preparation of a nuclear extract at ratios ranging from 1:1 to 5:1 of C2 and Rev7 we obtained the same result. The specificity of the interaction between β-49 and HNF-1 was confirmed by competition experiments. In the presence of a 20-fold excess of the unlabeled synthetic oligomer β-28, complex formation with each of the extracts was efficiently abolished (Figure 2C, lanes 3, 5, 7, 9, 11). Thus the smaller complex observed with the C2 cell line does not represent a degradation product and yet has the same sequence specificity as HNF-1. We therefore denoted it variant hepatocyte nuclear factor, vHNF.

Determination of the mol. wt of HNF-1 and vHNF

Since the gel mobility shift assay is done under non-denaturing conditions, it was not possible to conclude with certainty whether there was a true difference in the mol. wt of HNF-1 and vHNF in C2. We therefore performed a cross-linking experiment in which nuclear extracts from rat liver, Fao, C2 and Rev7 were incubated with the bromodeoxyuridine-substituted, body-labeled synthetic oligomer β-28 in the absence or presence of a 100-fold excess of cold β-28 competitor DNA. After UV irradiation the protein mixtures were resolved on a 10% SDS−polyacrylamide gel (Figure 3A). Proteins which specifically bound to the radiolabeled probe were detected in each of the three extracts (Figure 3B). In the case of rat liver, Fao and Rev7, the protein−DNA complexes migrated with an identical apparent mol. wt of 110 kd. In contrast, the protein observed with the C2 nuclear extract migrated with an apparent mol. wt of only 90 kd (Figure 3B, lane 3). Assuming a contribution of ~18 kd by the oligomer to the observed mol. wt, the corrected values would be 92 and 72 kd for HNF-1 and vHNF, respectively. The results obtained in the gel mobility shift assay and in the cross-linking experiment show that dedifferentiation in C2 is accompanied by the loss of HNF-1 and the expression of vHNF. Reversion to the hepatocyte phenotype in Rev7 is accompanied by the reappearance of HNF-1.

Sequence specificity of HNF-1 and vHNF

We wished to determine more clearly the sequence specificity of vHNF and relate it to the binding properties of HNF-1. To this end, we did a footprint analysis of the β fibrinogen gene promoter and methylation interference experiments. For the DNase I protection studies we prepared a probe spanning 117 bp of the β fibrinogen promoter (Figure 1, β-117), labeled on the coding strand, and incubated it with nuclear extract from Faza, C2 and Rev7 in the absence or presence of either specific or non-specific cold competitor DNA fragments. With each extract the region from position −76 to −103 of β-117 was protected from DNase I digestion. The footprint was efficiently competed in the presence of a 20-fold excess of cold synthetic oligomer β-28 but not with a DNA fragment prepared from the γ fibrinogen promoter

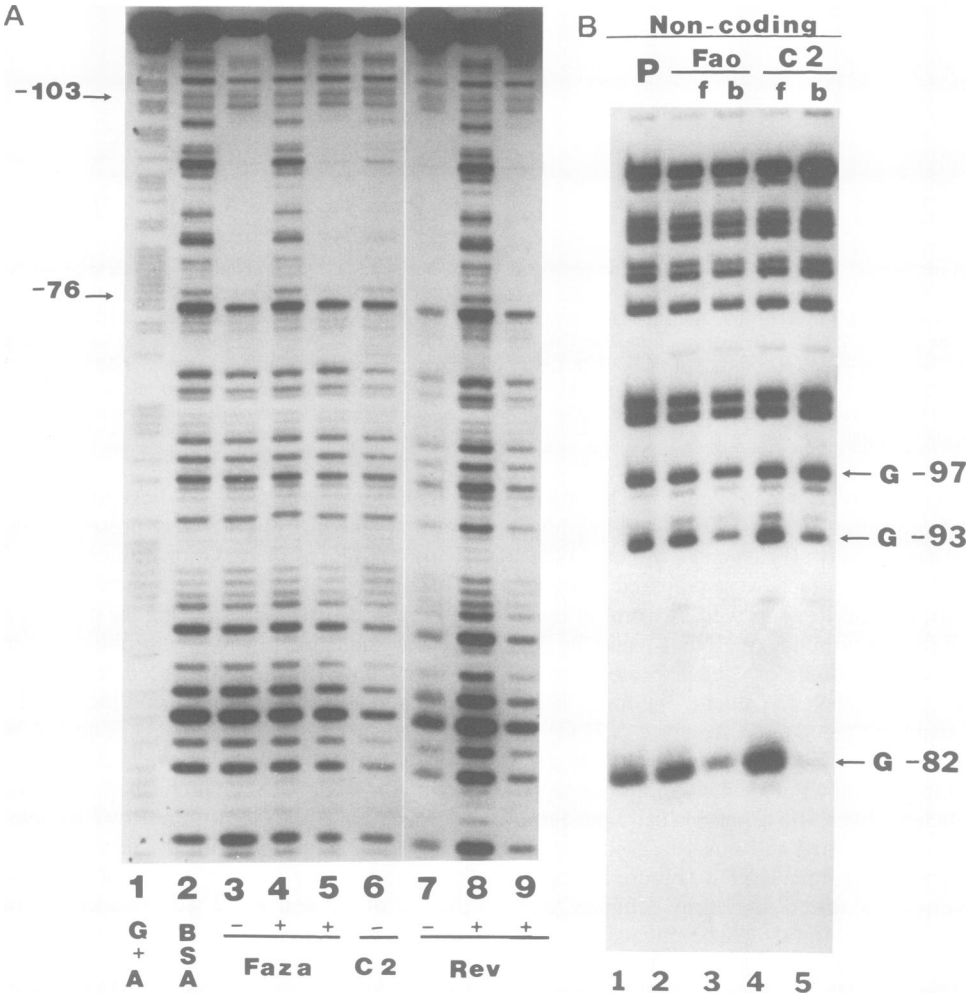

Fig. 4. Comparison of the DNA binding properties of HNF-1 and vHNF. (**A**) DNase I footprint analysis of the interaction of HNF-1 and vHNF with the β fibrinogen gene promoter. Binding reactions were carried out with 0.6 ng (15 000 c.p.m.) of the 3′ end-labeled probe β-117 (Figure 1) and either BSA (25 μg) or nuclear extract from Faza (10 μg), C2 (20 μg) and Rev7 (20 μg). A '+' below the lane indicates the presence of a 20-fold excess of β-28 competitor DNA (**lanes 4** and **8**) or a 100-fold excess of an unrelated DNA fragment derived from the γ fibrinogen gene promoter (**lanes 5** and **9**). In **lane 1** a G + A sequencing reaction of the probe (Maxam and Gilbert) was loaded. The margins of the protected region are indicated on the left (−76 to −103). (**B**) Determination of essential contact residues by methylation interference experiments. Binding reactions were carried out with the partly methylated probe β-49 (Figure 1) in the presence of Fao or C2 nuclear extracts (50 μg) as detailed in Materials and methods. Protected G residues and their position in the recognition sequence are indicated and the results summarized in Figure 1.

containing binding sites for SpI, MLTF and CTF (Morgan *et al.*, submitted; Chodosh *et al.*, 1986). There was no detectable qualitative difference between the footprinted region obtained with nuclear extracts Faza, Rev7 and C2. However, 2-fold more extract of C2 and Rev7 was necessary to produce similar protection as the Faza nuclear extract. Although the footprint studies indicated identical sequence specificity of HNF-1 and vHNF, this technique is not sensitive enough to detect minor differences in the binding properties.

To obtain a more accurate measure of the contact sites important for the binding of the two distinct proteins, we performed methylation interference experiments. This allows determination of which G residues in the recognition sequence are critically involved in the protein–DNA interaction. Nuclear extracts from Fao and C2 were incubated with the partially methylated end-labeled probe β-49 and protein-bound and free probe were resolved on a native polyacrylamide gel. After excision of the bands the DNA was chemically cleaved at the G residues and analyzed on 8% sequencing gels. The results obtained in these experiments showed that methylation at G residues at positions −82 and −93 on the non-coding strand (Figure 4B) and at position −95 on the coding strand (data not shown) interfere with binding of both HNF-1 and vHNF (summarized in Figure 1). However, interference by methylation of the G residue at position −97 was only observed when the Fao extract was used and thus seemed to affect binding of HNF-1 but not vHNF. On the other hand, a methylated G residue at position −82 more severely interfered with the binding of vHNF than that of HNF-1. This interpretation was confirmed by densitometric scanning of the autoradiograph and correction for the slightly different amounts of radioactivity loaded on the sequencing gel. Hence, the two proteins exhibit minor but detectable differences in their binding properties.

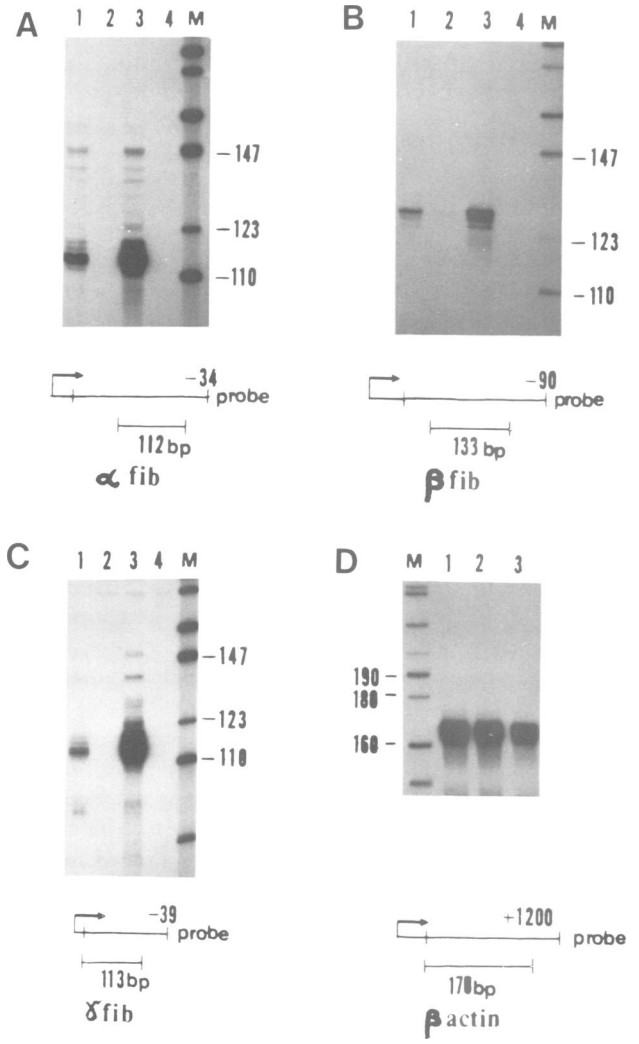

Fig. 5. RNase mapping of the fibrinogen gene transcripts in differentiated, dedifferentiated and revertant cell lines. For each of the cell lines, Fao (A–D, **lanes 1**), C2 (A–D, **lanes 2**), and Rev7 (A–D, **lanes 3**) 10 μg of total cytoplasmic RNA or 10 μg of tRNA as a control (A–D, **lanes 4**) were hybridized to SP-6 antisense riboprobes for the α (**panel A**), β (**panel B**) and γ (**panel C**) fibrinogen genes or cytoplasmic β actin (**panel D**) as described in Materials and methods. The antisense riboprobes generated from the SP-6 promoter (indicated by the arrow) and the expected protected fragment sizes arising from correctly initiated transcripts are indicated below each panel.

vHNF-1 is associated with reduced fibrinogen gene expression

Since vHNF and HNF-1 bound to the same essential DNA element in the β fibrinogen promoter, it was possible that the replacement of HNF-1 would somehow affect the transcriptional activity of this gene. We quantitated correctly initiated transcripts of the α, β and γ fibrinogen genes in the three cell lines Fao, C2 and Rev7 by RNase mapping. Equal amounts of total cytoplasmic RNA were hybridized to ^{32}P-labeled antisense SP-6 probes prepared from the fibrinogen α, β and γ chain genes which include the transcription initiation site. As a control for the intactness of the RNA we used a probe for rat cytoplasmic β actin (P.Gunning, unpublished). In both, Fao and Rev7 correctly initiated transcripts of the three fibrinogen genes were detected (Figure 5A–C, lanes 1 and 3). In Rev7 specific transcripts were about three times more abundant than in Fao. In contrast no α and γ fibrinogen gene transcripts were detected in C2 and mRNA for the β chain gene was present at greatly reduced levels as compared to Fao and Rev7. β Actin transcripts were very similar in Fao, C2 and Rev7 (Figure 4D) indicating that the expression of certain genes is invariant in the three cell lines.

Activity of the transfected β fibrinogen promoter parallels the endogenous fibrinogen genes

Since we were measuring the steady state level of transcripts from each fibrinogen gene, we could not exclude that differential mRNA stability accounted in part for the results obtained by RNase mapping. Furthermore, recent observations (Ott *et al.*, 1984; Clayton *et al.*, 1985; Friedman *et al.*, 1987) indicated that differences in albumin gene expression between C2 and Rev7 are related to both transcriptional and post-transcriptional mechanisms. Thus it was necessary to establish whether the observation made in the RNase mapping was due, in C2, to a reduction in transcriptional activity of fibrinogen genes. To address this question we have taken two different experimental approaches.

In the first instance we used a transient expression assay in which Faza, C2 and Rev7 cells were transfected with a construct in which β fibrinogen promoter sequences up to position −117 were linked to the bacterial chloramphenicol acetyltransferase (CAT) gene at position +7 of the β fibrinogen gene. This promoter fragment contained the DNA

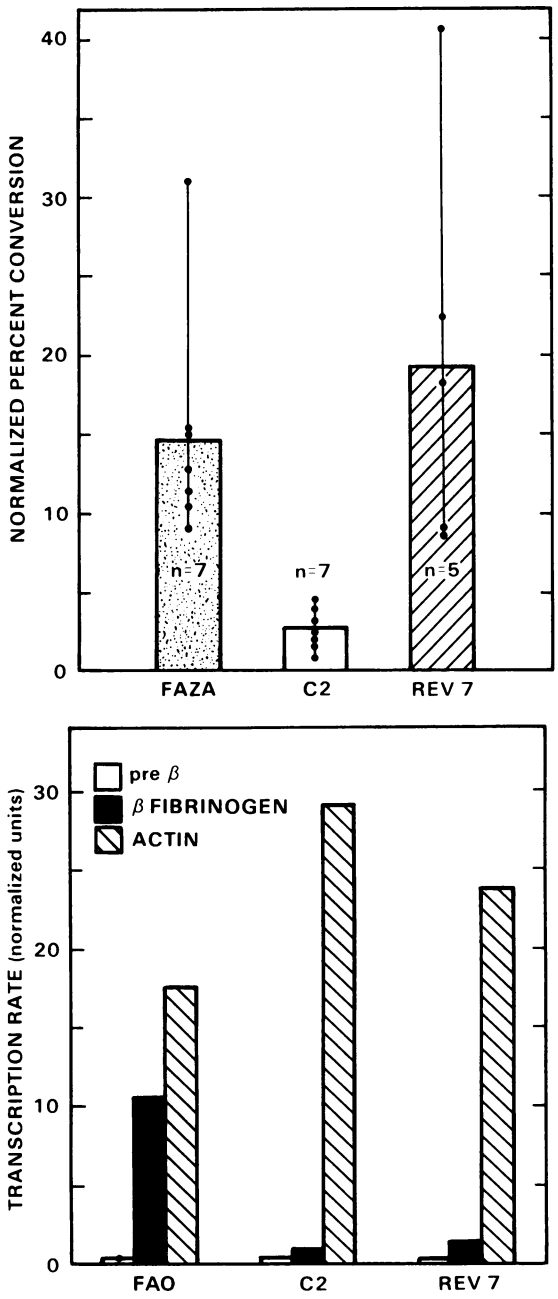

Fig. 6. Function of the β fibrinogen gene promoter in differentiated and dedifferentiated hepatoma cell lines. (**A**) CAT activity was measured in extracts from Faza, C2 and Rev7 transfected with a construct in which transcription of the CAT gene is directed by the β fibrinogen gene promoter (−117 to +7, Courtois *et al.*, 1987). For comparison between the cell lines values were normalized to the co-transfected luciferease gene directed by the RSV-40 promoter. Activity is given in percent conversion of [^{14}C]chloramphenicol to the acetylated forms either as individual experimental values (●) or as the average of all experiments (bars); *n* = number of experiments. (**B**) Nuclear run-on assays to determine the transcriptional activity of the β fibrinogen gene were performed with isolated nuclei from Fao, C2 and Rev7 as described in Materials and methods. Equal amounts of labeled nuclear RNA (2.5 × 10^7 c.p.m.) were hybridized to β fibrinogen gene cDNA, β actin cDNA or plasmid DNA containing 5′ flanking regions of the β fibrinogen gene (pre β). Hybridization signals were quantitated by densitometric scanning of the autoradiographs and liquid scintillation counting of the filters. Transcription rates were normalized to β actin and are given in arbitrary units. In each of three run-on transcription studies the difference between Fao and C2 was at least 5-fold and between C2 and Rev7 at least 2-fold. No hybridization was seen when run-on experiments were performed in the presence of 1 μg/ml α-Amanitin (not shown).

binding site for HNF-1 and had previously been shown to direct transcription of the linked CAT gene in hepatoma cell lines but not in cell lines of non-hepatic origin. Therefore, it was possible to test the influence of the presence of HNF-1 in differentiated hepatoma cell lines or vHNF in dedifferentiated cells on the transcriptional activity of the linked gene. Co-transfection of the luciferase gene driven by the RSV (Rous sarcoma virus) promoter was used as an internal standard to correct for transfection efficiency. A 5- to 6-fold reduction in CAT activity was observed in the C2 variant as compared to either Faza or Rev7 (Figure 6A). This finding parallels the results from the RNase mapping. Thus, the most likely interpretation is that transcription in C2 is impaired due to the absence of HNF-1 and/or the presence of vHNF.

As a second approach to assess the *in vivo* role of HNF-1 and vHNF in the transcriptional activation of the β fibrinogen gene, we performed nuclear run-on experiments. In this case the results would reflect not only the action of HNF-1 and vHNF but also other, not yet identified factors contributing to the overall transcriptional control. Equal amounts of radiolabeled RNA produced in isolated nuclei from Fao, C2 and Rev7 were hybridized to β fibrinogen or rat β actin cDNA, to DNA containing the non-transcribed 5′ region of the β fibrinogen gene and to vector (pBR322) DNA. Comparison of the hybridization signals showed that the transcriptional activity of the β fibrinogen gene was reduced by a factor of 10 in C2 as compared to Fao. However, the transcription rate was only slightly increased in Rev7 compared to C2 (Figure 6B). This indicated that reversion to the hepatocyte phenotype in the Rev7 cell line must be accompanied by stabilization of fibrinogen mRNA since the difference in mRNA levels is only partly due to transcriptional reactivation. However, the transfection experiments and the nuclear run-on assay strongly suggest that in C2 the observed reduction in fibrinogen gene expression is primarily due to transcriptional control.

Detection of vHNF in intertypic somatic hybrids and lung

In Rev7 the re-expression of the hepatocyte phenotype is accompanied by the reappearance of HNF-1. It was therefore conceivable that vHNF in C2 was the result of a mutation in HNF-1 which was reversed in Rev7. To test this hypothesis we extended our analysis to intertypic somatic hybrids obtained by fusing the rat hepatoma cell line Fao with mouse embryonic fibroblasts (Killary and Fournier, 1984). These hybrids show extinction of a large number of liver-specific traits (Killary and Fournier, 1984; Chin and Fournier, 1987), including fibrinogen gene expression as seen by Northern blot analysis (K.Fournier, personal communication) and thus display a phenotype very similar to C2. We used the gel retardation assay to detect HNF-1 and vHNF in extracts prepared from the two hybrid cell lines FF5-1 and FF3-3. Surprisingly, in both hybrids a protein was present which bound to the HNF-1 recognition sequence (β-28) and co-migrated with vHNF in C2 (Figure 7A, lanes 1,2 and 3). This result suggested that the presence of vHNF in C2 was not explained by a simple mutational event but rather indicated control at a higher regulatory level. Furthermore vHNF seemed to be more generally associated with a dedifferentiated or non-hepatic phenotype. Consequently we investigated whether a protein with the characteristics of vHNF could be detected in nuclear extracts prepared from rat tissues of other than hepatic origin.

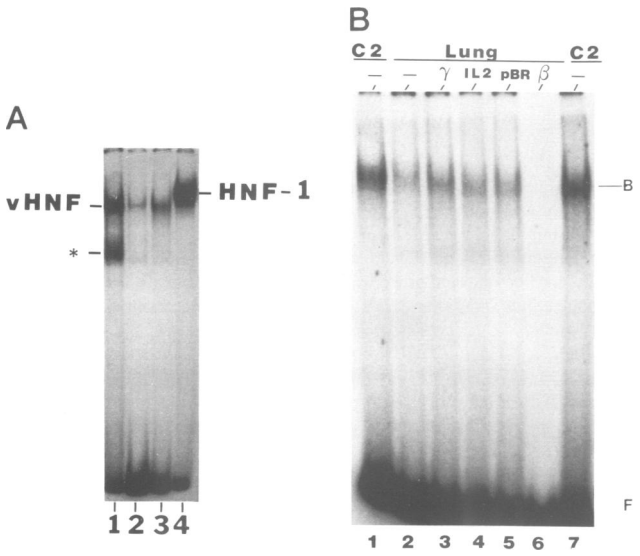

Fig. 7. A protein similar to vHNF is present in nuclear extracts from somatic hybrids displaying extinction of the liver phenotype and in normal lung tissue. (**A**) Gel retardation assay comparing complexes formed between the end-labeled probe β-28 (Figure 1, 5000 c.p.m.) and proteins present in nuclear extracts prepared from the two somatic hybrids FF5-1 (**lane 1**, 15 μg) and FF3-3 (**lane 2**, 12 μg), C2 (**lane 3**, 5 μg) and Fao (**lane 4**, 5 μg). Binding reactions were analyzed on a native polyacrylamide gel. The DNA–protein complex indicated by an asterisk is specifically competed in the presence of an excess of unlabeled β-28 (data not shown) but has not yet been further analyzed. (**B**) Comparison of complexes formed between nuclear extracts from rat lung tissue (**lanes 2–6**) and C2 (**lanes 1,6**) and the probe β-28 in the absence (lanes 1,2,6) or presence of the following competitor DNA fragments: lane 3, 40-fold excess of a γ fibrinogen gene promoter fragment (−137 to −38); lane 4, 60-fold excess of an IL-2 gene promoter fragment (−50 to +45); lane 5, 40-fold excess of pBR322 DNA; lane 6, 10-fold excess of unlabeled β-28.

In thymus, brain and spleen only very low amounts of vHNF seemed to be present (data not shown) but lung nuclear extracts contained a significant amount of a protein showing indistinguishable characteristics from vHNF in the gel retardation assay and competition experiments (Figure 7B). The formation of the complex between β-28 and the lung nuclear protein was not competed with unrelated DNA fragments including the γ fibrinogen gene and IL-2 promoter and pBR322 (Figure 7B, lanes 3,4,5). In contrast effective competition was observed in the presence of a 20-fold excess of cold β-28 (Figure 7B, lane 6).

Discussion

The transcriptional activation of related sets of genes is probably controlled by multiple regulatory mechanisms which determine developmental timing, tissue-specific expression and the levels of transcription. A way to achieve coordinate regulation of a set of cell type-specific genes is through *trans*-acting factors which can interact with a DNA sequence element present in the promoter of each of the genes. HNF-1 is a good candidate for such a regulatory protein in the liver since it binds to essential promoter elements of the genes coding for the α and β chain of fibrinogen, α_1-antitrypsin, albumin, prealbumin, α feto-protein and possibly others containing the HNF-1 consensus sequence, GTTAATNATTAAC (Courtois *et al.*, 1987 and unpublished studies). We have investigated the regulation of HNF-1 in cell types in which liver-specific gene expression is suppressed either as dedifferentiated hepatoma cell variants or in somatic hybrids where liver-specific gene expression is extinguished. Our results demonstrate the absence of HNF-1 in the cell types which do not display the liver phenotype. Instead a variant nuclear protein, vHNF, with similar sequence specificity was detected in extracts from these cells.

While the function of vHNF is not clear, present evidence indicates that it is somehow related to reduced transcription of liver-specific genes. vHNF is present in dedifferentiated variants selected from an original differentiated cell line on the basis of a non-hepatic morphology. These cells have reduced rates of transcription of a variety of liver-specific genes (Figure 6A, Clayton *et al.*, 1985). Furthermore a truncated β fibrinogen promoter containing the HNF-1/vHNF binding site functions ~5-fold better in the differentiated cell line and the revertant than in the dedifferentiated cell line. Notably the results obtained in the transfection and nuclear run-on experiment do not correlate regarding the revertant Rev7. In the CAT assay the β fibrinogen promoter–CAT construct was chosen such that one could measure predominantly the contribution of HNF-1 and vHNF to the transcriptional activation of the linked CAT gene. Conversely the nuclear run-on assay is a measure of not only HNF-1 but possibly other activities present in whole nuclei including post-transcriptional influences on β fibrinogen gene expression which are eliminated in the transfection assay. These results indicate that HNF-1 is only one of several regulatory elements necessary for the hepatocyte-specific transcription of the fibrinogen genes. Ultimately *in vitro* transcription comparing the effects of purified vHNF and HNF-1 will be required to assess the role of the two proteins in transcription.

Since vHNF is present in several situations where liver-specific gene expression is repressed or absent, one possibility is that it acts as an inhibitor of liver-specific gene expression. A related observation was made by Cereghini *et al.* (1987) who studied nuclear proteins interacting with the albumin promoter proximal element (PE). In DNase I protection assays, they found that nuclear extracts from a dedifferentiated hepatoma cell line, H5, and from spleen protected a smaller region of the PE than extracts from liver. Furthermore, their experiments seem to indicate a dominance of the binding activity in H5 over that present in liver extracts, suggesting a repressor type mechanism for the regulation of albumin in non-expressing tissues. The fact that vHNF is ~20 kd smaller than HNF-1 suggests that it may lack a domain essential to transcriptional activation. In the bovine papilloma virus alternative splicing of the VPI gene produces a shortened transcript containing a DNA binding domain but lacking a domain required for transcriptional activation (Lambert *et al.*, 1987). This protein acts as a repressor since it lacks the activation domain. By analogy vHNF could represent an alternatively spliced product of the same gene that encodes HNF-1 or the product of a distinct gene lacking a transcriptional activation domain.

A second possible role of vHNF would be that it represents a protein that is capable of activating transcription but has a different promoter specificity by virtue of the context in which the protein is bound. HNF-1 is known to bind to the promoters of several genes which are not exclusively expressed in the liver. For example, α_1-antitrypsin is expressed in the kidney, lung and macrophages. The fact that we find vHNF and not HNF-1 in the lung raises the possibility that vHNF could have an altered promoter

specificity, excluding expression of some genes and activating others. While precedents for this mechanism are unknown, such a possibility could enlarge the repertoire of promoters available to a single transcriptional factor.

The possibility that vHNF represents a mutated form of HNF-1 is effectively disproved by the discovery of vHNF in normal lung as well as somatic hybrids formed between hepatoma cells and embryonic fibroblasts. These later cells have extinguished the expression of liver-specific genes. Thus the protein appears to be characteristic of both extinction and dedifferentiation. From these obervations it seems likely that the change between vHNF and HNF-1 reflects events occurring at a more fundamental level of hepatocyte differentiation.

Materials and methods

Cell lines

The rat hepatoma cell lines Faza, Fao, Fao flC2 and C2-Rev7 (Deschatrette *et al.*, 1980) were obtained from M.Weiss at the Institue Pasteur in Paris, France. The Fao cell line was derived from the Faza cell line and differs only in ouabain resistance. The somatic, intertypic hybrids (FF5-1 and FF3-3) between Fao and mouse embryonic fibroblasts were provided by K.Fournier. All cells were grown in DMEM supplemented with 10% fetal calf serum in 10% CO_2.

Preparation of nuclear extracts

Nuclear extracts were prepared from the cell lines essentially as described (Ohlsson and Edlund, 1986). After extraction of the nuclei with 0.3 M $(NH_4)_2SO_4$, and removal of the DNA, proteins were precipitated by the addition of 0.2 g/ml $(NH_4)_2SO_4$, collected by centrifugation and dialyzed against two changes of 50 mM Hepes, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF. The protein solution was then cleared from insoluble material by centrifugation at 10 000 *g* in a microfuge and the supernatant stored at −70°C in small aliquots. The rat liver nuclear extract was prepared in the same way except for the initial steps. Rats were anesthetized with ether, the liver was perfused with ice-cold 25 mM Hepes, pH 7.4, 150 mM NaCl and then quickly removed The tissue was cut into small pieces, resuspended in cold buffer and homogenized in a Dounce homogenizer with a motor-driven teflon pestle. Large particles were removed by filtration through gauze and the nuclei recovered by centrifugation. Subsequent steps were as described above.

Electrophoretic mobility shift assay

The assay was carried out essentially as described (Fried and Crothers, 1981; Singh *et al.*, 1986) with 0.2 ng (~5000 c.p.m.) of ^{32}P end-labeled probe and the amount of nuclear extracts indicated in the figure legends.

DNase I footprint and methylation interference experiments

Binding reactions were performed in 50 mM KCl, 25 mM Hepes pH 7.9, 0.05 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 5% glycerol in a final volume of 50 μl. Various amounts of nuclear proteins or bovine serum albumin (BSA) were preincubated with 1.5 μg poly(dI-dC); end-labeled probe (~15 000–20 000 c.p.m., 1 ng) was added and the mixture incubated for another 40 min at room temperature. After the addition of 1 μl of 250 mM $MgCl_2$ digestion with freshly diluted DNase I (Worthington, 250 μg/ml in water) was realized for 60 s at 20°C. The amount of DNase I was adjusted empirically for each extract to produce comparable cleavage patterns. Reactions were stopped by the addition of 2 vols 100 mM Tris−HCl pH 8.0, 100 mM NaCl, 10 mM EDTA, 1% SDS, 100 μg/ml proteinase K, 25 μg/ml tRNA followed by a 30-min incubation at 37°C. Proteins were extracted with phenol/chloroform, the DNA precipitated with ethanol and analyzed on 6% polyacrylamide−8 M urea gels.

Methylation interference experiments were performed essentially as described (Siebenlist and Gilbert, 1980) according to L.A.Chodosh (personal communication). First, probe DNA was partly methylated with dimethylsulfate. The methylated DNA was then incubated with nuclear extracts in the presence of poly(dI-dC) and bound and free probe separated on a 4% native polyacrylamide gel. After autoradiographic visualization the bands were cut out and embedded into a 1.5% agarose gel in Tris−borate buffer containing 0.1% SDS. A small piece of NA45 DEAE membrane was placed in front of the gel slice and the DNA electrophoresed onto the membrane in ~15–20 min. The membrane with the bound probe was washed briefly once in Tris−borate buffer and once in 10 mM Tris pH 8.0, 1 mM EDTA, 200 mM NaCl. Bound probe was eluted in 0.4 ml of 10 mM Tris pH 8.0, 1 mM EDTA, 1 mM NaCl at 68°C for 30 min. After clearing the supernatant by centrifugation in a microfuge the probe DNA was precipitated with ethanol and the resulting pellet subjected to chemical cleavage at G residues with piperidine. The DNA corresponding to the bound and free form was then electrophoresed on 8% polyacrylamide−8 M urea gels.

UV-cross-linking

Photoaffinity labeling of proteins in whole nuclear extracts was performed essentially as described (Ogata and Gilbert, 1977; Chodosh *et al.*, 1986). Briefly, the body-labeled probe was prepared by annealing the 28-bp synthetic oligonucleotide β-28 (Figure 1) with a 14-base complementary primer and filling in with the Klenow fragment in the presence of [α-^{32}P]dATP, dCTP, dGTP and a 1:1 ratio of dTTP:BrdU. Nuclear extracts were incubated with 0.5 ng (50 000 c.p.m.) of the body-labeled probe in the presence of 3 μg poly(dI-dC) for 45 min at room temperature in a final volume of 25 μl. Irradiation of the samples was performed for 30 min under an inverted 302-nm transilluminator. Samples were then reduced with 100 mM DTT and analyzed on 10% SDS−polyacrylamide gels as described (Laemmli, 1970).

Preparation and quantitation of RNA

RNA from cell lines was isolated after lysis in guanidinium thiocyanate as described (Chirgwin *et al.*, 1979). RNase mapping was performed according to published procedures (Melton *et al.*, 1984; Krieg and Melton, 1984). After hybridization of total cytoplasmic RNA to the antisense SP-6 riboprobe digestion was performed for 30 min at 30°C in the presence of 40 μg/ml of RNase A and 125 U/ml of RNase T1 and the samples analyzed on 6% polyacrylamide−8 M urea gels.

SP-6 riboprobes for rat α, β and γ fibrinogen and rat cytoplasmic β actin were prepared as follows: an *Eco*RI−*Hae*III fragment (−34 to +114) spanning the initiation site and part of the first exon of the γ fibrinogen gene (Evans *et al.*, 1987) was cloned into the *Eco*RI−*Sma*I site of SP-64. The β fibrinogen SP-6 construct was prepared by cloning an *Xba*I−*Eco*RV fragment spanning from −504 to +144 and covering the start site and the first exon (Fowlkes *et al.*, 1984) into the *Hin*cII and *Xba*I site of SP-64. Labeled RNA antisense probe was produced from the *Ssp*I-digested vector The α fibrinogen SP-6 construct was prepared by cloning the *Pst*I−*Xho*I fragment (−35 to +153) into the *Sal*I and *Pst*I site of SP-65. An antisense probe for rat cytoplasmic β actin was prepared by cloning the *Ava*I−*Pvu*II fragment (+1030 to +1200) of the β actin cDNA clone pRβ A-1 (P.Gunning, unpublished) into the *Ava*I−*Hin*cII site of SP-65.

Nuclear run-on transcription

For transcription assays 5 × 10^7 cells were washed twice with ice-cold phosphate-buffered saline, the pellet resuspended in 1 ml lysis buffer [10 mM Tris−HCl (pH 7.4), 3 mM $MgCl_2$, 10 mM NaCl, 2 mM DTT, 1 mM PMSF and 0.1% NP-40] and disrupted in a loosely fitted Dounce homogenizer. The nuclei were pelleted in a microfuge for 30 s at 10 000 *g*, and washed once with 1 ml of lysis buffer and once with buffer in which the NP-40 was omitted. The nuclear pellet was resuspended in storage buffer [10 mM Tris−HCl (pH 8.0), 5 mM $MgCl_2$, 0.5 M sucrose, 2.5% Ficoll 400 000, 1 mM DTT, 0.1 mM PMSF and 50% glycerol] and used immediately. Transcription assays were performed for 15 min at 25°C in 40 mM Tris−HCl (pH 8.3), 150 mN NH_4Cl, 7.5 mM $MgCl_2$, 1 mM DTT, 25% glycerol, 0.5 mM ATP, 0.25 mM each of CTP and GTP, 200 μ/ml RNasin, 200 μCi [α-^{32}P]UTP (>760 Ci/mmol; Amersham Corp.) in a final volume of 200 μl. Reactions were terminated by the addition of 7.5 μg DNaseI, 50 μl 10% SDS and 100 μg proteinase K and incubation was continued for 15 min at 25°C. After the addition of 10 μl 0.5 M EDTA (pH 8.0) and 25 μl 3 M NaAc the mixtures were extracted three times with phenol/chloroform. Labeled nuclear RNA was precipitated with 5 ml ice-cold 5% trichloroacetic acid (TCA)−30 mM $Na_4P_2O_7$ in the presence of 100 μg tRNA as a carrier. After 30 min on ice the precipitate was collected on a nitrocellulose filter (type HA, 0.45 μm, Millipore Corp.) the filter washed once with 10 ml of 5% TCA−30 mM $Na_4P_2O_7$, and the RNA eluted with 0.9 ml of 50 mM Tris−HCl (pH 7.5), 600 mM NaCl, 20 mM $MgCl_2$ and 200 μg/ml DNase I. After 30 min the reaction mixture was extracted twice with phenol chloroform and the RNA precipitated with ethanol. Labeled nuclear RNA (10^7–5 × 10^7 c.p.m.) was hybridized to 5 μg of linearized plasmid DNA immobilized on nitrocellulose filters using a dot-blot apparatus. Hybridization was for 48 h at 42°C in 30% formamide, 300 mM NaCl, 10 mM EDTA, 100 mM Tris−HCl (pH 7.5), 0.2% SDS, 0.4% of each Ficoll and polyvinylpyrrolidone, 100 μg/ml tRNA. Filters were washed twice for 15 min at 42°C in 10 mM Tris−HCl (pH 7.5), 300 mM NaCl, 2 mM EDTA, 0.1% SDS and twice for 15 min at 65°C

in 10 mM Tris–HCl (pH 7.5), 60 mM NaCl, 0.1 mM EDTA, 0.1% SDS. Filters were autoradiographed overnight and then counted in a liquid scintillation counter. The following plasmid DNA was used for hybridization: (i) pBR322; (ii) pRβ A-1 (P.Gunning, unpublished) containing the 1.8-kb cDNA of rat cytoplasmic β actin; (iii) pC20, containing a 0.9-kb *Pst*I fragment of the β fibrinogen gene upstream region (Fowlkes *et al.*, 1984) and p β fib, containig the 1.5-kb β fibrinogen gene cDNA (Crabtree and Kant, 1981).

Transfection assay

Transfections were performed essentially as described (Courtois *et al.*, 1987) with the only exception that the calcium phosphate precipitate was removed from the cells after 6 h.

Acknowledgements

We would like to thank K.Fournier for providing the somatic hybrids, P.Gunning for the rat β actin cDNA clone and L.Chodosh for the method of the methylation interference experiment. We are grateful to L.Campbell for performing the transfection assays and to N.Leger for preparation of the manuscript. This research was supported by HL33942 and CA39612 to G.R.Crabtree.

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Received on March 8, 1988; revised on May 20, 1988