Hepatocyte Nuclear Factor 3 Relieves Chromatin-mediated Repression of the α-Fetoprotein Gene*

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The α-fetoprotein gene (AFP) is tightly regulated at the tissue-specific level, with expression confined to endoderm-derived cells. We have reconstituted AFP transcription on chromatin-assembled DNA templates in vitro. Our studies show that chromatin assembly is essential for hepatic-specific expression of the AFP gene. While nucleosome-free AFP DNA is robustly transcribed in vitro by both cervical (HeLa) and hepatocellular (HepG2) carcinoma extracts, the general transcription factors and transactivators present in HeLa extract cannot relieve chromatin-mediated repression of AFP. In contrast, preincubation with either HepG2 extract or HeLa extract supplemented with recombinant hepatocyte nuclear factor 3 α (HNF3α), a hepatic-enriched factor expressed very early during liver development, is sufficient to confer transcriptional activation on a chromatin-repressed AFP template.Transient transfection studies illustrate that HNF3α can activate AFP expression in a non-liver cellular environment, confirming a pivotal role for HNF3α in establishing hepatic-specific gene expression. Restriction enzyme accessibility assays reveal that HNF3α promotes the assembly of an open chromatin structure at the AFP promoter. Combined, these functional and structural data suggest that chromatin assembly establishes a barrier to block inappropriate expression of AFP in non-hepatic tissues and that tissue-specific factors, such as HNF3α, are required to alleviate the chromatin-mediated repression.

During differentiation, patterns of cell-type specific gene expression are established which must be maintained throughout the life of the cell. It is generally assumed that tissue-specific transcription is achieved through selective synthesis and concentration of cell-type restricted transcription factors. These regulatory proteins are usually not as confined in expression as their downstream targets (1, 2). Additional levels of control in vivo may rely on chromatin structure that restricts access of ubiquitous or widely expressed regulatory factors, as well as facilitates synergy between transcription activators (3, 4). Eukaryotic DNA is highly condensed into chromatin; this compaction results in a general repression of gene expression (see Refs. 5 and 6, and reviewed in Ref. 7). Nuclease sensitivity mapping of a number of genes has indicated a clear pattern of accessible chromatin structure around actively transcribing genes during development (2, 8–13). The chicken β-globin locus, for example, is maintained in a DNase I-sensitive chromatin structure in erythroid cells, whereas this region is inaccessible to enzyme digestion in non-erythroid cells (14). The β2 globin promoter acquires an open chromatin conformation only at the onset of expression in definitive red blood cells (13, 14). Thus, tissue-specific and developmental expression patterns are accompanied by distinct alterations in chromatin structure.

The liver tumor marker gene, α-fetoprotein (AFP), displays strict tissue-specific and developmental regulation in vivo (reviewed in Refs. 15 and 16). AFP is highly expressed during fetal development in endoderm-derived tissues including the yolk sac, liver, and gut. At birth, unlike other liver-specific genes, AFP expression is rapidly repressed (17) and is only reactivated in cases of renewed cellular proliferation, including liver regeneration and hepatocellular carcinoma (17, 18). Differential AFP expression patterns are accompanied by discrete changes in local chromatin structure as measured by DNase I hypersensitivity (reviewed in Refs. 15 and 19–21). AFP thus provides an excellent model to assay the potential contribution of chromatin structure to tissue-specific gene regulation.

Previous studies using transient transfections and transgenic mice have identified multiple regulatory elements that control AFP expression. These include three distinct enhancers and a promoter/repressor region (19, 20, 22, 23). The enhancers were found to activate a heterologous promoter in non-hepatic cells, while a 1-kilobase fragment containing only the distal and proximal promoter regions exhibited absolute tissue specificity (19). The AFP promoter from –1 kb to the start site is therefore a major determinant of liver-specific transcription. Several liver-enriched proteins which direct hepatocyte-specific expression have been shown to bind multiple sites within the AFP promoter (reviewed in Ref. 24), including CAAT/enhancer-binding protein (25), hepatocyte nuclear factor 1 (HNF1; 26), and hepatocyte nuclear factor 3 (HNF3; 27). The HNF3 family is composed of three members, HNF3α, HNF3β, and HNF3γ, which, along with the Drosophila fork head protein, constitute a growing family of winged-helix DNA-binding proteins (28). HNF3/fork head proteins that regulate gene expression in endoderm-derived tissues are required for pattern formation in the embryonic gut (see Ref. 29, reviewed in Ref. 30). HNF3α is of particular interest as it has been shown to position nucleosomes within the enhancer of the liver-specific
Chromatin Structure Confers Tissue-specific Expression

In Vitro Transcription Reactions—Prior to nucleosome assembly, immobilized AFP templates were preincubated for 20 min at room temperature with the indicated extracts or NDB. Xenopus egg cytoplasmic fraction (HSF) in an amount previously determined to fully repress transcription was added to assemble the bead-DNA into chromatin for 1 h at 22 °C. Prior to transcription, the assembled templates were washed 3 times in NDB (unless otherwise indicated). Washed templates were then in vitro transcribed upon addition of an RNA polymerase II-containing nuclear HeLa extract (37) and an NTP/salts/energy-generating mixture to give final concentrations of 0.6 mM CTP, UTP, GTP, and ATP, 1.5 mM MgCl2, 50 mM KCl, 50 mM NaCl, 5 mM creatine phosphate, 10 units/ml of creatine kinase, 0.02% Nonidet P-40 (Sigma), and 12.6 mM HEPES, pH 7.9. After a 60-min incubation at 30 °C, RNA products were purified and analyzed by primer extension and gel electrophoresis (41).

Micrococcal Nuclease Analysis—Nucleosome assembly on the AFP bead DNA was assessed by micrococcal nuclease (Roche Molecular Biochemicals) digestion after a 2-h incubation at 22 °C with fractionated Xenopus egg extract HSS containing 3 mM ATP and 5 mM MgCl2. Samples were digested and analyzed exactly as described previously.

Chromatin Structure Analysis—AFP bead/DNA was assembled into chromatin under conditions exactly as described for in vitro transcription analysis and then subjected to restriction enzyme digestion. Chromatin-assembled bead/DNA was washed once in 1× React 2 restriction enzyme buffer (50 mM Tris-Cl, pH 8.0, 10 mM MgCl2, 50 mM NaCl; Life Technologies, Inc.) prior to resuspension in the same buffer containing HincII (Life Technologies, Inc.) at 25 units/μg of DNA. Following a 30-min incubation at 37 °C, samples were digested with 1 mg/ml protease K (Life Technologies, Inc.) in 0.25% SDS, 12.5 mM EDTA, pH 8.0, for 1 h at 37 °C. For the HincII fragment release assay (Fig. 5A), the purified DNA was resuspended in agarose gel loading dye and samples were analyzed by Southern blot as described previously (42). A 25-bp probe (+5 to +28) was used to detect the 84-bp released fragment (−55 to +29). To measure accessibility at the distal HincII site (Fig. 5B), the purified DNA was resuspended in 1× React 1 restriction enzyme buffer (50 mM Tris-Cl, pH 8.0, 10 mM MgCl2, Life Technologies, Inc.) and digested with 1 unit of AcI (Life Technologies, Inc.). Digested samples were analyzed by Southern blot using a 24-bp probe (−3333 to −3356) to detect a 4.0-kb Eco/HincII fragment. Autoradiograms were scanned and quantified using ImageQuanNT (Molecular Dynamics, version 4.2) software. For the HincII fragment release assay, activation over a buffer preincubated control was determined for three independent experiments and the average fold activation (± S.E.) was obtained. The buffer normalization was determined by the percent accessibility at the distal HincII site, we divided the intensity of the 4.0 kb released fragment by the sum of the 6.4-kb parent band and the released fragment. The fold activation was determined after normalizing to the buffer control.

Analysis of Start Complex Stability—AFP bead/DNA was mock chromatin-assembled by preincubation with protein extracts in the presence or absence of HNF3 (50 μg) for 20 min prior to a 60-min incubation in egg extract buffer (100 mM KCl, 4 mM MgCl2, 10 mM HEPES, pH 7.2, 100 mM sucrose, 0.1 mM EDTA) ± 3 mM ATP. Assembled bead/DNA was washed 3 times in NDB containing 0.5% Nonidet P-40, and then incubated in an NTP/salts/energy-generating mixture to give final concentrations of 0.6 mM CTP, UTP, GTP, ATP, 5 mM MgCl2, 50 mM KCl, 5 mM creatine phosphate, 10 units/ml of creatine kinase, 0.02% Nonidet P-40 (Sigma) and 12.6 mM HEPES, pH 7.9. After a 60-min incubation at 30 °C, RNA products were purified and analyzed by primer extension and gel electrophoresis (41).

RESULTS

In Vitro Transcription of AFP Does Not Recapitulate Tissue-specific Expression Patterns in the Absence of Chromatin Structure—To explore the basis for tissue-specific AFP regulation, we have employed an in vitro transcription system for AFP. The well characterized human hepatoma cell line HepG2 (43) was used as a source of AFP trans-activating factors. Human cervical carcinoma HeLa cells were used as a source of liver transcription factors. HepG2 cells actively express both endogenous and transiently introduced AFP, whereas HeLa cells do not (19). To establish an in vitro chromatin transcription assay for the AFP gene, we have attached AFP DNA to streptavidin-coated paramagnetic beads. This system, based on a design for transcriptional analysis of the Drosophila hsp70

EXPERIMENTAL PROCEDURES

Plasmids and Generation of AFP/Bead DNA Templates—The AFP(1.0)-lucZ vector was constructed by replacing the 177-bp BamHI-HindIII fragment of pA–APΔ44-lucZ (36) with a 1.0-kb BamHI-HindIII fragment containing the AFP promoter/prepressor region. The AFP(3.8)-lucZ vector was generated by inserting a 2.8-kb BamHI fragment containing AFP enhancer element I into the BamHI site of AFP(1.0)-lucZ. The HNF3α and HNF3β Eukaryotic expression vector(s) were a kind gift from Dr. Robert Costa. The HNF3 empty vector was generated by removing the entire HNF3α cDNA insert from the HNF3α vector. The HNF3α bacterial expression vector was kindly provided by Dr. Kenneth Zaret.

To obtain immobilized templates, AFP(1.0)-lucZ and AFP(3.8)-lucZ were digested with EcoRI and ClaI. The resulting fragments were Klenow end-filled with biotin-21-dUTP (CLONTECH) and biotin 14-dATP (Life Technologies, Inc.) to generate uniquely biotin-labeled EcoRI sites. Unincorporated nucleotides and small fragments were removed by gel filtration (Chromaspin 1000, CLONTECH). The largest 9.0-kb fragment encompassing the AFP enhancer I and promoter sequences were coupled to streptavidin-coated paramagnetic beads (Dynal) in Kilobase Binding Buffer (Dynal) on a rotating platform at room temperature overnight exactly as described by the manufacturer. Coupled beads were washed three times with 1× NDB, TE pH 8.0, and stored in phosphate-buffered saline at 4 °C until use.

Transfections—HeLa and HepG2 cells were transfected with an AFP reporter construct (AFP(1.0)-lucZ) and either an HNF3 empty vector or an HNF3 expression vector along with a CAT control vector using the calcium phosphate protocol as described (36). Forty-eight hours after transfection, cells were harvested. β-Galactosidase activity was assayed, expressed as described previously (36) and normalized to CAT activity to control for variations in transfection.

Protein Extracts—Hepatocarcinoma cell extracts were prepared from human HepG2 cells according to the method of Dignam et al. (57) with the following minor modifications. Cells were grown to 70% confluence and harvested by scraping into phosphate-buffered saline. Washed pellets were resuspended in hypotonic buffer (20 mM HEPES, pH 7.9, 10 mM NaCl, 1.5 mM MgCl2, 2 mM dithiothreitol). After swelling 10 min on ice, cells were pelleted and resuspended in hypotonic buffer containing 0.05% Nonidet P-40 prior to Dounce homogenization (Wheaton, type B). Protein extracts were dialyzed against 2 changes of nuclear dialysis buffer (NDB: 20 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride for 2 h). Final protein concentration ranged from 7 to 12 μg/ml. HeLa nuclear extract was prepared exactly as described in Current Protocols in Molecular Biology (38). Final protein concentration ranged from 8 to 10 μg/ml. Xenopus egg chromatin assembly extracts were prepared exactly as described previously (39). Final protein concentrations ranged from 40 to 60 μg/ml. Recombinant HNF3α protein was expressed in Escherichia coli and purified exactly as described previously (40). Final protein concentration was approximately 150 μg/ml.

Transcriptional activation, within the context of chromatin, is achieved by binding of hepatic-enriched factors that mediate the restructuring of chromatin into a transcriptionally competent form. Furthermore, we find that HNF3α, in the absence of other hepatic-specific factors, is capable of activating AFP transcription both in vitro and in vivo, demonstrating a critical role for this fork head homolog in programming liver-specific expression patterns.

In Vitro Transcription of AFP Does Not Recapitulate Tissue-specific Expression Patterns in the Absence of Chromatin Structure—To explore the basis for tissue-specific AFP regulation, we have employed an in vitro transcription system for AFP. The well characterized human hepatoma cell line HepG2 (43) was used as a source of AFP trans-activating factors. Human cervical carcinoma HeLa cells were used as a source of liver transcription factors. HepG2 cells actively express both endogenous and transiently introduced AFP, whereas HeLa cells do not (19). To establish an in vitro chromatin transcription assay for the AFP gene, we have attached AFP DNA to streptavidin-coated paramagnetic beads. This system, based on a design for transcriptional analysis of the Drosophila hsp70

albumin gene (31, 32). A role for HNF3 in organizing chromatin is further supported by the three-dimensional structural similarity of the winged helix conformation to the globular domain of linker histones (33, 34); functional conservation of this domain was confirmed by studies demonstrating the nucleosome-binding properties of HNF3α (35). However, the mechanism by which HNF3α transactivates hepatic-specific genes remains unclear.

We show here that in vitro reconstitution of AFP expression patterns requires both activating and repressive influences. In order to model the tissue-specific expression of the AFP gene, we have used Xenopus laevis egg extracts as a source of histones and nucleosome assembly factors that can reconstitute physiologically spaced nucleosomes in vitro. Our results indicate that the general repressive nature of nucleosomal DNA is necessary to restrict transcription factor access in a non-hepatic environment. Transcription activation, within the context of chromatin, is achieved by binding of hepatic-enriched factors that mediate the restructuring of chromatin into a transcriptionally competent form. Furthermore, we find that HNF3α, in the absence of other hepatic-specific factors, is capable of activating AFP transcription both in vitro and in vivo, demonstrating a critical role for this fork head homolog in programming liver-specific expression patterns.

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promoter (44), facilitates the rapid purification and concentration of chromatin-assembled templates. A plasmid containing 3.8 kb of mouse AFP regulatory sequence (AFP(3.8)-lacZ), including enhancer I and the distal and proximal promoter elements (36), was restriction enzyme-digested, biotin-end labeled and coupled to streptavidin-coated magnetic beads. The immobilized DNA (AFP/bead DNA) was then transcribed under standard in vitro transcription conditions with either HeLa or HepG2 extract (Fig. 1). Nucleosome-free AFP/bead DNA is efficiently transcribed by both extracts, with peak transcription occurring in the presence of 15 μl of extract, each containing approximately 150 μg of total protein (lanes 3 and 6). These results indicate that restrictions required to direct tissue-specific expression of AFP in vivo cannot be recapitulated on free DNA.

Solid-phase Chromatin Assembly—To recreate in vitro the physiological constraints conferred by chromatin in vivo, AFP/bead DNA was subjected to nucleosome assembly. Chromatin assembly was achieved by incubation with fractionated Xenopus egg extract. The cytoplasmic fraction or high speed supernatant (HSS) of Xenopus eggs efficiently assembles physiologically spaced nucleosomes (45) and has been used in previous chromatin transcription studies (42, 46). Efficiency of chromatin assembly was assessed by micrococcal nuclease digestion. A time course of digestion with micrococcal nuclease indicated the formation of a repetitive array of nucleosomes on the bead/DNA (Fig. 2A). This pattern is identical to that observed on uncoupled AFP DNA incubated in Xenopus HSS (data not shown). We further assessed the extent of chromatin assembly by measuring transcription levels. Assembly of AFP/bead DNA into nucleosomes in the presence of HSS resulted in repression of AFP transcription (Fig. 2B, compare lanes 1 and 2). This transcriptional repression was maintained during washes in a low salt (50 mM KCl) buffer. However, repression was alleviated by washing the chromatin-assembled bead/DNA in a high salt (3 M KCl) wash buffer (lane 3). As 3 M KCl is a sufficiently high salt concentration to disrupt histone/histone and histone/DNA interactions, these data suggest that the repression of AFP transcription observed in lane 2 is mediated by nucleosome assembly. Together, these results indicate that AFP/bead DNA is efficiently assembled into nucleosomal DNA in the presence of Xenopus egg extract.

Hepatoma-specific Derepression of Chromatin-assembled

AFP Templates—As shown above (Fig. 1), HepG2 and HeLa extracts transcribe naked AFP DNA templates in vitro with equal efficiencies. To determine whether these transcriptionally competent extracts were capable of establishing active transcription on a chromatin-assembled template, AFP/bead DNA was incubated with increasing amounts of either HeLa or HepG2 extracts or in nuclear extract buffer alone prior to chromatin assembly (Fig. 3). This “programming” phase allows transactivators and repressors to bind their respective sites on the nucleosome-free template prior to reconstitution into chromatin. Nucleosome-assembled templates, programmed in this way, were washed in low salt buffer prior to transcription to remove any unassociated proteins and nonspecific transcription repressors; this washing step further ensures that only DNA-associated proteins and protein complexes are present during the in vitro transcription analysis.

Chromatin-mediated repression could not be alleviated by providing HeLa factors prior to nucleosome assembly (Fig. 3A, compare lanes 1 and 2–4). Thus, general transcription factors and non-hepatic transactivators provided by the HeLa extract are insufficient to mediate assembly of a chromatin template which supports transcription. In contrast, incubating AFP/bead DNA with HepG2 extract during the programming stage, prior to nucleosome assembly, resulted in transcriptionally active templates (lanes 5–7). Comparison with a nucleosome-free AFP template control transcription (data not shown) revealed that the HepG2 preincubation rescued 90% (±3.9% S.E.) of free DNA transcription levels, indicating almost complete derepression of the chromatin template. An equivalent level of derepression was obtained when hepatoma factors were introduced concomitant with egg extract addition (Fig. 3B, A).
FIG. 3. Hepatoma-specific derepression of nucleosome assembled AFP DNA. A, hepatoma-specific factors establish transcriptionally active chromatin templates. Immobilized AFP templates were incubated with NDB (lane 1), or increasing amounts of either HeLa nuclear extract (lanes 2–4) or HepG2 extract (lanes 5–7) prior to a 1-h chromatin assembly in fractionated Xenopus egg extract (Xl HSS). Amounts of nuclear extract added during preincubation were as follows: 5 µl (approximately 50 µg of total protein; lanes 2 and 5), 10 µl (approximately 100 µg of total protein; lanes 3 and 6), and 20 µl (approximately 200 µg of total protein; lanes 4 and 7). Chromatin-assembled templates were washed in nuclear extract buffer and transcribed in vitro in a RNA polymerase II-containing HeLa nuclear extract. B, AFP enhancer element 1 is not required for hepatoma-mediated derepression. Immobilized AFP templates which contained (lanes 1–5) or lacked enhancer I (lanes 6–9) were incubated with NDB (lanes 1, 3, 6, and 9), HeLa (lane 5), or hepatoma extract (lanes 2, 4, 7, and 9), either prior to (lanes 1, 2, 5, 6, and 7) or during (lanes 3, 4, 8, and 9) chromatin assembly. Chromatin-assembled templates were processed as in A. The AFP primer extension product is indicated by an arrow. C, preincubation with cellular extracts does not disrupt nucleosome assembly. Immobilized AFP DNA was incubated in NDB (lanes 1–3 and 10–12), HepG2 extract (lanes 4–6 and 13–15), or HeLa extract (lanes 7–9 and 16–18) prior to chromatin assembly in fractionated Xl HSS under transcription conditions. Nucleosome-assembled templates were subjected to digestion with micrococcal nuclease for 60 min (lanes 2, 5, 8, 11, 14, and 17), 120 min (lanes 3, 6, 9, 12, 15, and 18) or left undigested (lanes 1, 4, 7, 10, 13, and 16). Samples were analyzed by Southern analysis using either a transcriptional start site probe (lanes 1–9) or a full-length AFP(3.8)-lacZ probe (lanes 10–18). Mononucleosomes are indicated by an arrow. A 123-bp ladder (Life Technologies, Inc.) was used as a molecular weight marker (horizontal lines).

compare lanes 2 and 4, 7 and 9). Hepatoma factors can, therefore, successfully compete with histones to bind their respective sites during on-going chromatin assembly and maturation. Addition of a low salt wash step immediately after pre-binding of the hepatoma extract did not significantly alter hepatoma-mediated assembly into an active chromatin template, indicating the stability of the protein/DNA interactions (data not shown). To confirm that preincubation with cellular extract under transcription conditions did not disrupt nucleosome assembly, chromatin templates preincubated with buffer, HepG2, or HeLa extract were subjected to a limit digest with micrococcal nuclease (Fig. 3C). Southern analysis of the digested DNA was performed using either a transcriptional start site oligo (+5 to +28) or the full-length AFP(3.8)-lacZ plasmid as a probe. Equivalent amounts of mononucleosomes were observed under all three transcription conditions, indicating that HepG2-mediated transcription activation was not due to gross inhibition of nucleosome assembly.

Nucleosome-free AFP templates which lack the enhancer element but retain 1.0 kb of upstream regulatory sequence (AFP(1.0)-lacZ), are transcribed as efficiently as the full-length enhancer-containing construct (AFP(3.8)-lacZ) in in vitro transcription assays with nuclear HeLa extract (data not shown). Transcription analysis of chromatin-assembled templates indicates that the enhancer is not required to establish hepatoma-activated AFP expression in vitro (Fig. 3B, lanes 6–9). These results indicate that 1.0 kb of AFP upstream regulatory sequence is sufficient to confer liver-specific expression, consistent with previous transfection studies by Tilghman and colleagues (19).

These data demonstrate that HepG2 extract contains factors which are capable of stably associating with AFP regulatory sequences and directing the assembly of an accessible chromatin structure. HeLa extract is unable to program transcriptionally competent nucleosome-assembled DNA, indicating either a lack of essential activators or the presence of repressors which only function within the context of chromatin. Thus, our in vitro chromatin transcription assay system successfully recapitulates the restricted AFP expression pattern observed in vivo, indicating the importance of chromatin structure for tissue-specific regulation of AFP. These results along with the robust transcription of nucleosome-free AFP DNA observed in HeLa extract (Fig. 1), suggest that hepatic-specific factors may enhance the formation of functional preinitiation complexes on
the nucleosome-assembled AFP template.

**HNF3 Alleviates Chromatin Repression**—One likely candidate for such an hepatic-enriched factor is the winged helix protein, HNF3, which regulates the transcription of numerous liver-specific genes including albumin, transthyretin, and phosphoenolpyruvate carboxykinase (reviewed in Refs. 30 and 47). HNF3α has recently been found to modulate chromatin structure at the albumin enhancer and thereby promote subsequent binding of additional transactivators (31, 35). The AFP proximal and distal promoter regions contain three HNF3-binding sites (27). Transient transfection experiments have demonstrated that HNF3α activates AFP expression (this work and Ref. 27). To directly determine the mechanism of HNF3α-mediated activation, immobilized templates were incubated with increasing amounts of recombinant HNF3α protein in the presence of HeLa extract prior to chromatin assembly (Fig. 4A). Co-incubation with HeLa extract and HNF3α (750 ng) is sufficient to reconstitute 30–40% of the activation achieved on HepG2-assembled chromatin DNA (lanes 4 and 5). As HNF3 expression is restricted to endodermal-derived tissues such as liver, HeLa cells lack this critical hepatic factor (48). Preincubation with HNF3α alone is insufficient to activate transcription of chromatin-assembled DNA (data not shown), indicating that HNF3-mediated relief of chromatin repression requires the presence of HeLa nuclear proteins. The inability of HNF3α to reconstitute 100% of the crude HepG2 extract activity may indicate that other liver-specific factors are required for optimal AFP expression.

**HNF3 Activates AFP Expression in HeLa Cells**—To confirm HNF3-mediated activation of AFP expression in a non-hepatic environment in vitro, transient transfection experiments were performed. Although transiently transfected DNA does not appear to assemble the same higher-order chromatin structure observed with genomic DNA, certain aspects of nucleosome-mediated regulation can be observed on transiently expressed DNA (reviewed in Ref. 49). HeLa cells were co-transfected with an AFP(1.0)-lacZ reporter construct, which lacks AFP enhancer elements, along with either an HNF3α or HNF3β expression vector. The AFP promoter exhibits very low levels of activity upon transfection into HeLa cells (Fig. 4B). The lack of AFP promoter activity in HeLa cells in the absence of hepatic-enriched factors is consistent with previous studies in HeLa cells (19) and supports our assumption that some level of nucleosome assembly is occurring on the introduced plasmid, resulting in transcription repression. In contrast, transfection of the AFP(1.0)-lacZ reporter construct into HepG2 cells results in high levels of β-galactosidase activity, as expected due to the presence of HNF3 and other hepatic activators. Co-transfection of the AFP reporter into HeLa cells with an HNF3α expression vector resulted in strong activation of AFP expression. Comparison of β-galactosidase activity between cell lines revealed that HNF3α-transfected HeLa cells displayed approximately 55% of the activity obtained from transfection of AFP(1.0)-lacZ into HepG2 cells, in close agreement with the level of HNF3 activation observed on in vitro chromatin templates (Fig. 4A). HNF3α and HNF3β were equally capable of activating the AFP promoter, suggesting that there are no differences in the ability of these factors to bind putative sites in this region. We have not yet determined whether HNF3β functions similarly to HNF3α in in vitro chromatin reconstitution experiments. These results illustrate the ability of our in vitro system to identify physiologically relevant regulators of tissue-specific gene expression. Furthermore, the cell culture demonstration of HNF3-induced AFP activation in a non-liver cell confirms the pivotal role HNF3 plays in establishing hepatic-specific expression.

**Stability of Preinitiation Complexes under Chromatin Assembly Conditions**—In order to promote physiological nucleosome spacing, our in vitro chromatin assembly reactions are performed in the presence of 3 mM ATP and 5 mM MgCl2 (50). As high concentrations of ATP have been found to affect the stability of preinitiation complexes (PICs) (51–54), it was possible that the tissue-specific expression patterns achieved upon chromatin assembly reflected a differential sensitivity of PIC formation and/or stability to ATP. To assay PIC response to ATP, we performed mock chromatin assembly reactions in the presence or absence of 3 mM ATP. Normal in vitro transcription conditions were restored by washing and resuspending the DNA templates in transcription reaction mixture lacking additional nuclear extract. Transcription from existing PICs was initiated by addition of 0.6 mM NTPs. As shown in Fig. 5, preincubation with either HeLa or HepG2 extract in the absence of ATP generated stable PICs capable of robust transcrip-
tion (Fig. 5, lanes 3 and 7). Incubation with ATP resulted in destabilization of both HeLa (5.5-fold repressed) and HepG2 (12.3-fold repressed) extract-generated PICs (compare lanes 3 and 4, and lanes 7 and 8), indicating that the tissue-specific expression observed under chromatin assembly conditions was not the result of differential ATP-dependent inhibition. Indeed, transcription complexes assembled by the hepatoma extract were more sensitive to ATP incubation conditions than HeLa PICs.

Supplementing HeLa extract with recombinant HNF3α prior to mock chromatin assembly had only a negligible effect in the absence of ATP (compare lanes 3 and 5), but stimulated transcription 2.5-fold under ATP conditions (compare lanes 4 and 6). Comparison of ATP inhibition of HeLa transcription in the presence and absence of HNF3 (lanes 4 and 6) reveals an intriguing role for HNF3 in stabilizing start complexes. HNF3 partially protects the established PICs from ATP-dependent disruption, and may therefore contribute to hepatic-specific expression by enhancing the stability of PICs. This level of activation is approximately 30% of that obtained in the presence of chromatin (8.7-fold; see Fig. 4A), indicating that a chromatin context is required to achieve full HNF3α-mediated transactivation.

HNF3α-mediated Changes in Chromatin Structure—Transcriptionally active chromatin templates can frequently be distinguished from their inactive counterparts by analysis of chromatin structure (reviewed in Refs. 55–57). Inactive chromatin generally exists in a “closed” or inaccessible form as measured by both nuclease and restriction enzyme digestion, whereas active chromatin is predominantly in a more accessible state. HNF3α has been shown to position nucleosomes over the albumin gene enhancer (31, 35). Therefore, we examined whether this transactivator could alter chromatin structure at the AFP promoter. Our structural analysis focused on the 1.0-kb sequence immediately upstream of the AFP transcriptional start site as this region has been shown to direct tissue-specific expression of AFP both in vitro and in tissue culture cells (this work and see Ref. 19). HincII restriction enzyme recognition sites flank the AFP transcriptional start site (−55 and +29 bp), resulting in the release of an 84-bp fragment if both sites are accessible. A fragment release assay allows us to determine the relative accessibility of this region in chromatin-assembled templates.

As shown in Fig. 6, recombinant HNF3α significantly increased restriction enzyme accessibility in this region. Unprogrammed chromatin templates (buffer only) are refractory to digestion (Fig. 6A, lane 2). Programming with HeLa extract enhanced accessibility 4-fold over buffer alone (Fig. 6A, lane 4, and C), possibly due to the formation of non-functional preinitiation complexes. Addition of HNF3α protein alone resulted in 6-fold enhanced accessibility (Fig. 6, A, lane 6, and C), suggesting that HNF3α may function on its own at some level to relieve chromatin repression. Chromatin remodeling by HNF3α alone
may be mediated through site-specific binding to one or more of the three putative promoter-binding sites, two of which lie within 200 bp of the transcriptional start site. Co-incubation with HNF3 and HeLa extract resulted in an additive increase (10-fold) in accessibility (Fig. 6A, lane 8, and C), indicating that factors in the HeLa extract enhance HNF3’s ability to establish an “open” promoter structure. To determine whether the HNF3 protein was nonspecifically inhibiting chromatin assembly on the AFP template, we assayed accessibility at a distal HincII site, which lies within vector sequence 4.0 kb upstream of the transcriptional start site. Restriction enzyme analysis of chromatin templates, assembled under identical conditions to those in Fig. 6A, revealed no significant increase in distal site accessibility in the presence of HNF3 and/or HeLa extract when compared with a buffer control (Fig. 6, B and C). Furthermore, a comparison of micrococcal nuclease limit digests performed on chromatin templates preincubated with either HeLa extract (Fig. 3C) or recombinant HNF3α in HeLa extract (data not shown) showed no global disruption of nucleosome assembly in the presence of HNF3. These studies indicate the presence of localized, tissue-specific structural changes that correlate with enhanced transcription activity. Furthermore, these data suggest a critical role for hepatic-specific factors, such as HNF3, in generating discrete alterations in chromatin structure that are required for AFP expression in hepatocytes.

DISCUSSION

The ability to accurately assay transactivator function in vitro depends upon reconstitution of both the constraints and enhancements placed on a gene in vivo. As many activators and repressors display widely varying affinities for nucleosomal versus free DNA, it has become increasingly clear that gene regulation must be analyzed within the context of chromatin. General transcription factors and transactivators present in HeLa extract are capable of efficiently transcribing naked AFP DNA templates, but are unable to interact productively with chromatin-repressed templates and are also insufficient to establish a transcriptionally competent template when provided prior to chromatin assembly. These results are in direct contrast to those obtained using the adenovirus major late promoter as a DNA template (58, 59). HeLa extract efficiently establishes transcriptionally competent adenovirus templates, both on nucleosome-free DNA and when incubated prior to nucleosome reconstitution. The inability of HeLa extract to activate chromatin-reconstituted AFP templates may illustrate the higher level of restriction placed on a tissue-specific cellular gene compared with the relatively promiscuous expression of a viral gene. Tissue-specific activators, such as HNF3, may be required to properly position nucleosomes and recruit RNA polymerase II complexes to the AFP promoter. In contrast, HeLa-induced activation of nucleosome-assembled adenovirus major late promoter may reflect general promoter derepression.

Chromatin structure has been implicated in developmental and tissue-specific regulation of a number of genes (reviewed in Ref. 60). Alterations in chromatin structure of both plant and animal genes have long been known to correlate with changes in gene expression (13, 61–65). Although tissue-specific transactivators and chromatin remodelers (66, 67) have been described, our in vitro analysis of the AFP gene provides the first demonstration of an RNA polymerase II-transcribed animal gene whose tissue-specific expression pattern is imposed by chromatin structure itself. To our knowledge, the only other example of chromatin structure dictating tissue specificity comes from the plant storage protein gene, β-phaseolin, in which rotational positioning of a nucleosome over the TATA region in nonexpressing vegetative tissues blocks β-phaseolin transcription at the level of initiation (68). Thus, tissue-restricted gene expression is likely the result of highly regulated derepression of chromatin by cell type-specific chromatin modulators.

Numerous studies have demonstrated that while increased chromatin accessibility generally precedes transcription activation, structural changes alone are often insufficient to confer gene activation (reviewed in Refs. 69 and 70). Analysis of the Xenopus TRβA promoter (71) and the human immunodeficiency virus-1 promoter (72) reveal that nucleosomal disruption and gene activation are separable events, suggesting that chromatin remodeling is just one in a series of steps which lead to transcription activation. Our data indicate that the structural changes mediated by preincubation with either HeLa extract or HNF3α alone are not sufficient for AFP activation. However, the enhanced accessibility that accompanies co-incubation with HNF3α and HeLa extract correlates with transcription activation. Additionally, HNF3 may perform a role in derepression of HeLa-programmed templates that must precede AFP activation, such as recruitment or stabilizing general transcription factor interaction with the promoter.

The stability of functional PICs on naked DNA templates is greatly inhibited in the presence of ATP. Previous reports have revealed a role for the ATPase Mot1p in Saccharomyces cerevisiae and its closely related mammalian homologue TAFII172/170 in ATP-dependent inhibition of transcription (52, 53, 73). In the presence of ATP, these SNF2 family members inhibit TBP-driven in vitro transcription by dissociating TBP from DNA, but exhibit little or no effect on TFIID complexes (52). In vivo analysis of Mot1p action in yeast has shown that this essential protein can act as both a corepressor (74) and an activator of specific gene expression (75–77), possibly by releasing free TBP from nonpromoter sites. The inhibition of PIC formation in our studies implies either a surprisingly high ratio of TBP versus TFIID-driven transcription in crude nuclear extracts or, alternatively, the presence of a previously uncharacterized ATP-dependent inhibition function. Our results support: 1) a diminished role for general transcription factors in establishing active chromatin on complex promoters; and/or 2) formation of chromatin that stabilizes PIC assembly in the presence of tissue-specific activator(s). Upstream activators, such as HNF3 (this study) or Gal4p-VP16 (52), may act in recruiting general transcription factors, stabilizing PIC formation, and thereby preventing ATP-dependent inhibition. However, we find that the majority of tissue specificity relies on chromatin structure activation: HNF3 plays a 3-fold greater role in transcription activation of chromatin (8.5-fold) than chromatin-free (2.5-fold) DNA templates under the same incubation conditions.

As chromatin structure is required to restrict access of nonhepatic factors, we reasoned that “loosening” of Hepa-assembled chromatin templates through hyperacetylation might be sufficient to relieve repression. To this end, we reconstituted immobilized AFP DNA preincubated with HeLa extract into nucleosomes by supplying Xenopus extract which had been preincubated with increasing amounts of the histone deacetylase inhibitor, trichostatin A (78). Even at micromolar concentrations, this inhibitor had no alleviating effect on chromatin-mediated repression (data not shown), suggesting that hepatoma-specific factors must also be present either to stabilize the accessible chromatin structure or to recruit the general transcription machinery to the chromatin-assembled templates.

A role for HNF3 in establishing a transcriptionally accessible chromatin structure is well supported by structural analysis of the evolutionarily related albumin gene. These studies found that HNF3 binding is required for nucleosome positioning over the albumin enhancer (31, 32), consistent with the hepatic-
specific expression pattern observed for albumin in vivo. HNF3, which structurally resembles linker histone H5 (34), can bind in place of a linker histone molecule to a degenerate HNF3-binding site on the side of a core nucleosome, but lacks the basic amino acids present in linker histones required to mediate compaction of nucleosomal DNA (35). Our in vitro analysis of HNF3 provides the first demonstration that HNF3-directed structural changes functionally mediate derepression of transcription on a chromatin template. Combined, these data suggest a mechanism by which HNF3 potentiates the assembly of a transcriptionally competent chromatin structure poised for expression.

The studies presented here demonstrate that chromatin structure is required to impose tissue-specific regulation on the expression vector and purification protocol and R. Costa for the HNF3 mammalian expression vectors. We are also grateful to I. Cartwright, L. Pile, and J. Ma for helpful discussions and comments on the manuscript. Additionally, we acknowledge reviewer comments that helped in the completion of this work.

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