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Independent regulation of HNF-1α and HNF-1β by retinoic acid in F9 teratocarcinoma cells

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Hepatocyte Nuclear Factor-1α (HNF-1α) and HNF-1β are homeodomain-containing transcription factors which interact with the GTTAATNATTAAC motif essential to the function of more than 15 promoters selectively expressed in the liver. These homeoproteins can form homo- and heterodimers in solution and share identical DNA-binding domains but have different transcriptional activation properties. During retinoic acid (RA) induced differentiation of F9 embryonal carcinoma (EC) cells, which simulates aspects of pre-implantation embryogenesis, both HNF-1β mRNA and immunoreactive DNA-binding activity are strongly induced ~24 h post RA-treatment. In contrast, HNF-1α mRNA increases ~4-fold after 5 days, concomitant with elevation of HNF-1α DNA-binding activity and expression of the HNF-1 target gene α-fetoprotein. These results indicate that HNF-1α and -1β expression can be controlled by regulatory hierarchies downstream of primary RA-response genes, and suggest that independent regulatory mechanisms for these factors can confer distinct and interactive developmental functions.

Key words: F9 cells/HNF-1α/HNF-1β/retinoic acid

Introduction

The homeodomain-containing transcription factor Hepatocyte Nuclear Factor-1α (HNF-1α, also LF-B1, APF) recognizes a 13 bp consensus sequence present in over 15 promoters of genes whose expression is highly enriched in liver, including α- and β- fibrinogen, albumin, α-fetoprotein and α-1-antitrypsin (Courtois *et al.*, 1987, 1988; Hardon *et al.*, 1988; Monaci *et al.*, 1988; Herbomel *et al.*, 1989; Lichtsteiner and Schibler, 1989; Maire *et al.*, 1989). Several lines of evidence indicate that HNF-1α is essential for maximum and tissue-specific expression of its target genes, since (i) HNF-1α DNA-binding activity is greatly enriched in hepatocytes versus other cell types, but is absent in dedifferentiated hepatomas or somatic cell hybrids which have extinguished liver functions (Courtois *et al.*, 1987; Baumhueter *et al.*, 1988; Cereghini *et al.*, 1988, 1990); (ii) addition of an HNF-1 binding site to heterologous promoters can confer liver-specific expression (Courtois *et al.*, 1987; Maire *et al.*, 1989; Monaci *et al.*, 1988); (iii) deletion or mutation of HNF-1 sites in target promoters greatly impairs their expression in transient transfection or *in vitro* transcription analysis (Hardon *et al.*, 1988; Courtois *et al.*, 1987; Feuerman *et al.*, 1989; Maire *et al.*, 1989); (iv) transfection of HNF-1α cDNA or addition of recombinant or native HNF-1α protein to *in vitro* transcription extracts produces marked transactivation of reporter constructs (Lichtensteiner and Schibler, 1989; Frain *et al.*, 1989; Kuo *et al.*, 1990). These studies indicate that HNF-1α participates in specification of the hepatocyte phenotype by activating, in a characteristically liver-enriched fashion, the transcription of numerous target genes.

HNF-1α is remarkable amongst characterized homeoproteins in its ability to homodimerize in the presence or absence of DNA via an N-terminal domain (Frain *et al.*, 1989; Chourard *et al.*, 1990; Nicosia *et al.*, 1990). To discern the existence of combinational control of HNF-1α, we have cloned a gene encoding a highly related protein, HNF-1β, which shares the HNF-1α dimerization domain and homeodomain, binds avidly to HNF-1α target sites, and can heterodimerize with HNF-1α upon co-translation or co-transfection (Mendel *et al.*, submitted). Consistent with its weak transactivation properties, HNF-1β is identical to vHNF-1 and vAPF which appear in dedifferentiated hepatocyte cell lines and in somatic hybrids between fibroblasts and hepatocytes which exhibit partial extinction of the liver phenotype (Mendel *et al.*, 1991; Baumhueter *et al.*, 1988; Cereghini *et al.*, 1988). Intriguingly, only HNF-1α DNA-binding activity is detectable in adult mouse liver, although abundant amounts of both HNF-1α and -1β mRNA are expressed, suggesting an extremely effective post-transcriptional repression of HNF-1β in the terminally differentiated hepatocyte (Mendel *et al.*, submitted).

The expression of the HNF-1α and HNF-1β homeoproteins and the process of liver organogenesis are apparently associated intricately. To identify developmental regulators of these homeoproteins, and thus to gain access to antecedent events in hepatocyte differentiation, we have searched for cell lines permitting induction of HNF-1α and HNF-1β. In an inducible system, regulatory events must occur during the lag period preceding induction; moreover, the molecules involved in such *de novo* induction should be responsible for initiation, and not merely maintenance, of HNF-1 expression. Within the present work, we have utilized the F9 embryonal carcinoma (EC) cell line, which upon retinoic acid (RA) treatment in suspension culture differentiates along a visceral endoderm lineage, and expresses the HNF-1 target gene α-fetoprotein (αFP) (Hogan and Taylor, 1981; Scott *et al.*, 1984; Young and Tilghman, 1984; Feuerman *et al.*, 1989). We demonstrate RA-dependent induction of both HNF-1α and HNF-1β mRNA and DNA-binding activity by temporally distinct mechanisms, and correlate these proteins' appearance with expression of the endogenous F9 cell αFP gene. Given the ability of these proteins to heterodimerize, such independent regulation could confer upon HNF-1α and HNF-1β distinct developmental functions in tissues expressing one form, with combinatorial, interactive functions in co-expressing tissues.

Results

RA-dependent induction of HNF-1 DNA-binding activity in F9 cells

Retinoic acid (RA, 7.5×10^{-7} M) treatment of suspension culture F9 cells produced characteristic embryoid aggregates, accompanied by a >20-fold increase in HNF-1 DNA-binding activity over a low basal level in the gel mobility shift assay. As monitored with a rat β-fibrinogen promoter (βFg) binding site oligomer (Courtois *et al.*, 1987), stimulation of HNF-1 DNA-binding activity began at 16 h post-RA treatment, remaining elevated over an 11-day time course (Figure 1a). This stimulation was RA-dependent, as neither embryoid body formation nor induction of HNF-1 DNA-binding activity was apparent in F9 cells cultured without RA, or with the RA solvent DMSO (data not shown). We noticed a reproducible increase in mobility of the HNF-1 gel shift complex beginning at 16 h which remained present throughout the time course (see Discussion). In contrast, the ubiquitous transcription factor Oct-1 exhibited a constant level of DNA-binding activity during F9 differentiation (Figure 1b), acting as a control for equal protein loading and attesting to the integrity of the nuclear extracts.

The F9 cell HNF-1 DNA-binding activity possessed footprint and methylation interference characteristics indistinguishable from affinity purified or crude HNF-1α, protecting positions −76 to −103 of the βg promoter from DNase I digestion, and contacting the βFg promoter at identical G residues (−93 and −82) in accordance with previous results (Figure 2a and b) (Courtois *et al.*, 1988; Baumhueter *et al.*, 1988). We also characterized the F9 cell HNF-1 and hepatocyte HNF-1 in a variant of one-dimensional peptide mapping, the protease clipping band-shift assay (PCBA, Schrieber *et al.*, 1988). In this assay, protein−DNA complexes are subjected to partial protease digestion, revealing a pattern of DNA-binding proteolytic products upon gel shift electrophoresis. We found that F9 cell HNF-1 and hepatocyte HNF-1 produced similar peptide fragments upon association with a ^{32}P-end-labeled βFg HNF-1 site, partial digestion with V8 protease, and non-denaturing gel electrophoresis, suggesting a high degree of relatedness (Figure 2c). These common biochemical properties indicated that the RA-induced protein(s) were structurally similar to HNF-1.

Distinct temporal expression patterns of HNF-1α and HNF-1β mRNA in F9 cells

We prepared RNA in parallel with the nuclear extracts analyzed in Figure 1 to investigate the relationship between the time kinetics of HNF-1α mRNA induction and of HNF-1 DNA-binding activity. Surprisingly, as assayed by an RNase protection assay, HNF-1α mRNA was present even in uninduced F9 EC cells, and exhibited biphasic time kinetics with a reproducible decrease at 36−48 h, thereafter rising 4-fold at 5−11 days (Figure 3). This temporal variation agreed poorly with the time kinetics of HNF-1 DNA-binding induction (Figures 1a and 3). In contrast, RNase protection of the same samples with a mouse HNF-1β riboprobe revealed a strong induction first detectable 16−24 h post-RA stimulation, peaking at 5 days and gradually declining thereafter, in excellent agreement with the temporal pattern of HNF-1 DNA-binding activity (Figure 3). We estimate the peak levels of HNF-1β mRNA to be ~10 fold greater than peak levels of HNF-1α mRNA; the upper panel in Figure 3 (HNF-1α) was exposed eight times longer than the middle panel (HNF-1β).

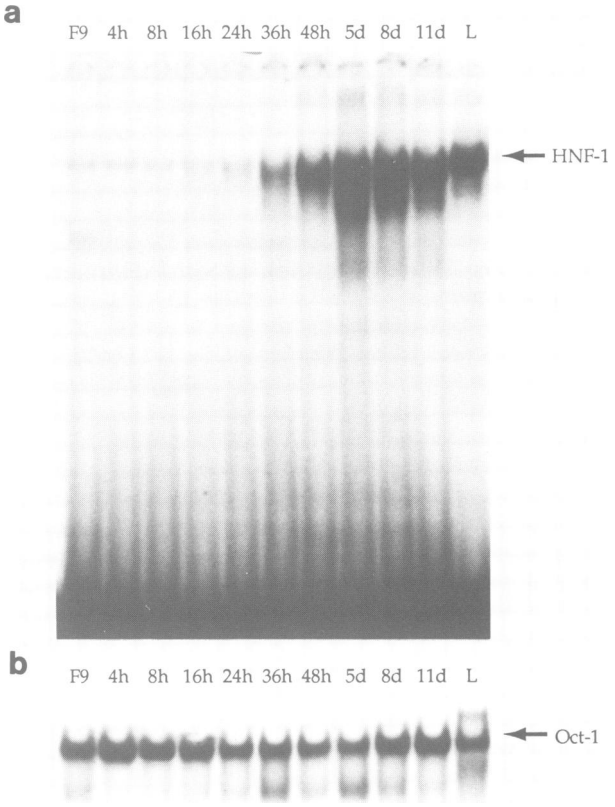

Fig. 1. Induction of HNF-1 DNA-binding activity by RA in F9 cells. (**a**) RA-induced F9 cells express HNF-1 DNA-binding activity with delayed time kinetics. Five micrograms of F9 cell nuclear extract or 1 μg mouse liver extract were bound to a ^{32}P-end-labeled HNF-1 site from the rat β-fibrinogen promoter (Courtois *et al.*, 1987) and gel mobility shift assay performed as in Materials and methods. (**b**) Oct-1 DNA-binding activity is unaffected by RA. Five micrograms of F9 cell nuclear extract or 1 μg mouse liver extract were bound to a ^{32}P-end-labeled Oct-1 site from the human IL-2 enhancer (Durand *et al.*, 1988), and gel mobility shift assay was performed as described above. Similar time kinetics have been observed in four independent experiments.

Suspension culture of RA-treated F9 cells produces vigorous transcriptional activation of the α-fetoprotein (αFP) gene, whose promoter is dependent on an HNF-1 binding site for maximal and tissue-specific expression (Scott *et al.*, 1984; Young and Tilghman, 1984; Feuerman *et al.*, 1989). We observed an approximately 30-fold increase in αFP mRNA after 5−11 days of differentiation (Figure 3), in agreement with previous results in which αFP mRNA is induced at 5−7 days and persists over 17 days at high levels (Scott *et al.*, 1984; Young and Tilghman, 1984; Vogt *et al.*, 1988).

Transient induction of HNF-1β DNA-binding activity in RA-treated F9 cells

Because the time course of HNF-1β mRNA induction (Figure 3) closely paralleled the temporal pattern of total HNF-1 DNA-binding activity (Figure 1a), we characterized the induced DNA-binding activity with polyclonal antisera monospecific for HNF-1β (Mendel *et al.*, submitted). In gel shift analysis of the RA-induced nuclear extracts examined in Figure 1, anti-HNF-1β serum formed a ternary

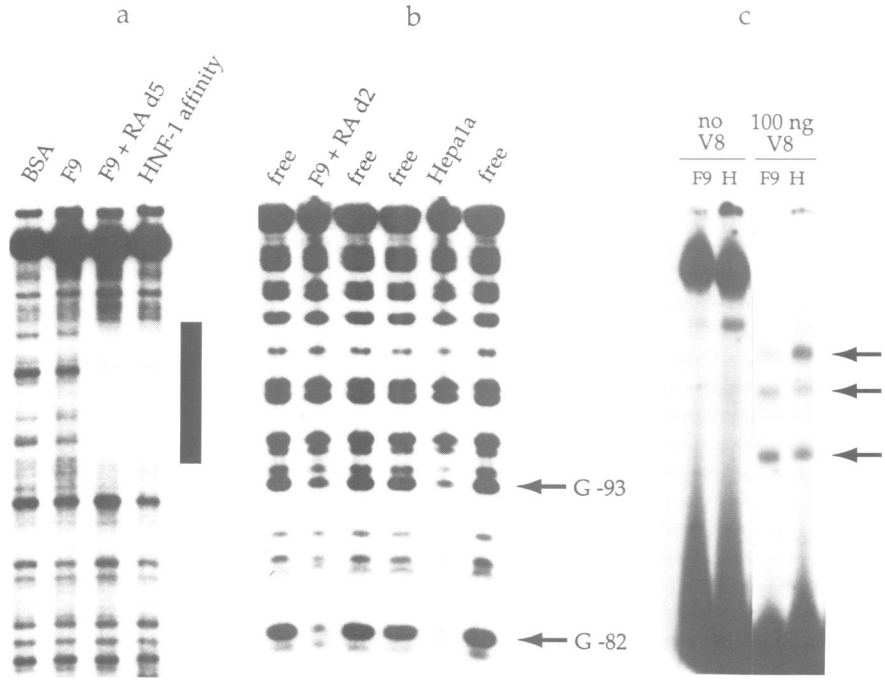

Fig. 2. Biochemical similarities between the RA-induced DNA-binding activity and HNF-1α. (**a**) DNase I footprinting assay. Nuclear extract from 5 day RA-treated F9 cells (25 μg), BSA (25 μg) or affinity purified HNF-1α were incubated with a 117 bp *Sph*I−*Hin*dIII fragment from the rat β-fibrinogen promoter containing an HNF-1 binding site and subsequently subjected to DNase I digestion. See the text for further details. (**b**) Methylation interference assay. Nuclear extract from 48 h RA-induced F9 cells (10 μg) or the Hepa1a cell line (10 μg) was incubated with a DMS-methylated, ^{32}P-end-labeled 49 bp *Sph*I−*Bst*EII rat β-fibrinogen promoter fragment prior to piperidine cleavage. See the text for further details. G (−93) and G(−82) refer to sites of interference relative to the rat βFg transcription start site. (**c**) Partial V8 proteolysis of protein−DNA complexes from 48 h RA-induced F9 cells ('F9') or the Hepa1a cell line ('H'). V8 protease (100 ng) was added to the gel shift incubation for 10 min at room temperature prior to electrophoresis.

Ab−HNF-1β−DNA 'supershift' complex of slower mobility than the binary HNF-1−DNA complex formed without antiserum. These HNF-1β supershift complexes (Figure 4a) peaked at 5 days and declined thereafter, exhibiting temporal variation identical to HNF-1 DNA-binding activity (Figure 1a) and HNF-1β mRNA (Figure 3).

Intriguingly, in later (5−11 day) extracts, not all the HNF-1 DNA-binding activity could be supershifted with HNF-1β antiserum, indicating the presence of an additional activity, possibly HNF-1α (Figure 4a). Consistent with this possibility, the antibody-resistant population exhibited slightly slower mobility than the unshifted complex; it is well established that HNF-1α migrates more slowly than HNF-1β in gel retardation assays (Baumhueter *et al.*, 1988; Cereghini *et al.*, 1988). These experiments were performed under conditions of antibody excess, as (i) an equivalent DNA-binding activity from HNF-1β-transfected Jurkat cells was quantitatively supershifted by the same amount of HNF-1β antiserum, and (ii) increase in the amount of antisera, or up to 10-fold antibody dilution did not alter the quantity of supershift complex (data not shown).

Biphasic RA induction of HNF-1α DNA-binding activity in F9 cells

The presence of HNF-1α DNA-binding activity was directly indicated by HNF-1α supershift complexes apparent throughout the F9 differentiation time course. Initially (0−48 h), a faint, slowly migrating supershift was detected (Figure 4b, upper dark arrow) which co-migrated with the HNF-1α supershift from mouse liver (containing exclusively

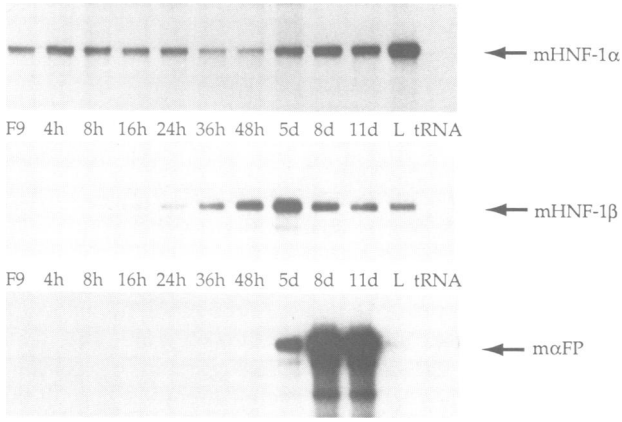

Fig. 3. Comparative time kinetics of appearance of HNF-1α, HNF-1β and αFP mRNA in RA-induced F9 cells. 'L' refers to mouse liver RNA. All time courses have demonstrated identical temporal variation in two (HNF-1β, αFP) or three (HNF-1α) independent RNA isolations. Autoradiography was performed at −70°C with intensifying screen; exposure times were: HNF-1α, 96 h; HNF-1β, 12 h; αFP, 3 h.

HNF-1α; Mendel *et al.*, submitted). After 5 days of RA-induction, we observed prominent ~5-fold stimulation of a faster migrating supershift (Figure 4b, lower dark arrow). No DNA-binding was observed with HNF-1α antiserum alone (data not shown). By 11 days of treatment, HNF-1α supershift complexes rose to ~50% of total DNA-binding

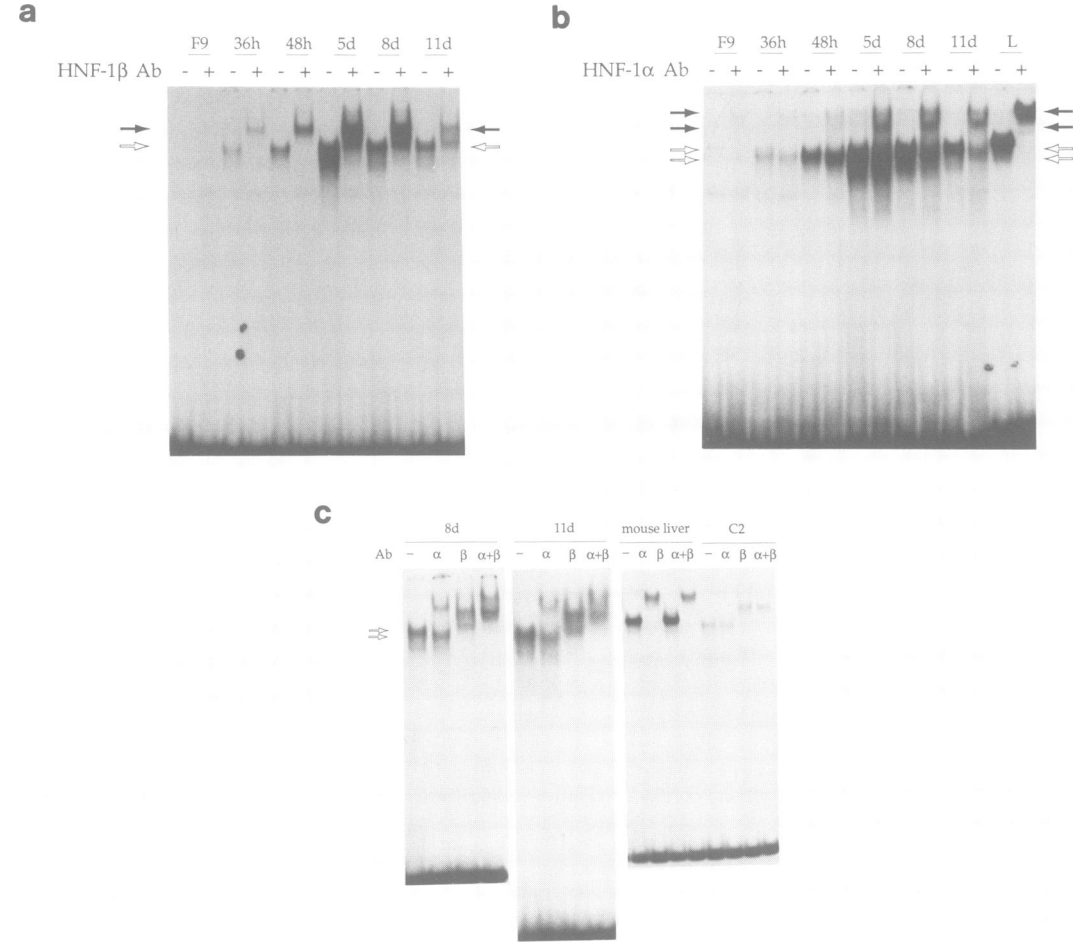

Fig. 4. Identification of the RA-induced DNA-binding activity as HNF-1α and HNF-1β. Five micrograms of nuclear extract or 1 μg mouse liver extract (as in Figure 1) were bound to ^{32}P-end-labeled β28 as previously described. After 30–45 min incubation at room temperature, 1 μl of polyclonal antiserum monospecific for either HNF-1α or HNF-1β was added, and the incubation continued at 4°C for 1 h. Subsequently, the reaction was analyzed in typical gel mobility shift assay. All reactions were performed under conditions of antibody excess (Figure 4b and data not shown). Note antibody-sensitive (closed arrow) and antibody-resistant (open arrow) complexes. (**a**) Time course of appearance of HNF-1β supershift complexes. (**b**) Time course of appearance of HNF-1α supershift complexes. Note co-migration of 0–48 h supershift complexes with mouse liver supershift, but distinct, faster mobility of 5–11 day supershift complexes. A faint, slowly migrating non-specific smear were also observed (see also Figure 1a, to which no antibody has been added) (**c**) Both HNF-1α and -1β antiserum are required to abolish antibody resistant complexes from 8 and 11 day RA-induced F9 nuclear extracts (5 μg) but not from mouse liver (5 μg) or C2 nuclear extracts (10 μg). Similar results were obtained for 5 day extracts (data not shown). Note distinct mobilities of complexes resistant to either HNF-1α or HNF-1β antisera, consistent with HNF-1α homodimers (resistant to anti-β serum) having slower mobility than faster migrating HNF-1β homodimers (resistant to anti-α serum) (Baumheuter, 1988; Cereghini, 1988).

activity, although the magnitude of the HNF-1α supershift remained constant from 5–11 days (Figure 4b). This indicated that HNF-1α DNA-binding remained constant while HNF-1β DNA-binding declined, consistent with the observed decrease in HNF-1β mRNA after 5 days (Figure 3).

The strong induction of HNF-1α DNA-binding activity at 5 days from a low basal level was accompanied by marked stimulation of αFP mRNA (Figure 3; see Discussion). In addition, HNF-1α DNA-binding activity, as measured by supershift (Figure 4b) appeared to parallel the behavior of HNF-1α mRNA over the 36 h–11 day time period (Figure 3), although we are unable to rule out a component of post-transcriptional repression of HNF-1α DNA-binding activity during the 0–48 h time period. This biphasic pattern of HNF-1α DNA-binding activity, mRNA levels and supershift mobility indicated a regulation distinct from HNF-1β, whose mRNA and DNA-binding activity peaked transiently at 5 days (Figures 3 and 4a).

Further corroborating the induction of HNF-1α protein, HNF-1α supershift complexes (Figure 4b) appeared coincidently with gel shift complexes resistant to HNF-1β antisera (Figure 4a), both occurring at 5 days. Moreover, the combination of anti-α and anti-β sera was able to abolish antibody-resistant complexes totally in the 5–11 day extracts (Figure 4c and data not shown), confirming that these DNA-binding activities comprised exclusively HNF-1α and -1β. This is also consistent with the material in 5–11 day extracts resistant to HNF-1α antiserum migrating more quickly than the total DNA-binding activity, as if this resistant sub-population represented HNF-1β, which has faster mobility than HNF-1α (Figure 4b,c) (Baumheuter *et al.*, 1988; Cereghini *et al.*, 1988). Since HNF-1α and HNF-1β antibody-resistant complexes were abolished upon inclusion of the other antibody, these resistant complexes most likely represented HNF-1β and HNF-1α homodimers, respectively. This requirement for both antisera to produce a quantitative supershift in F9 extracts contrasted with the

complete supershift of mouse liver extracts (containing exclusively HNF-1α, Mendel *et al.*, submitted) by HNF-1α antiserum alone, and with the complete supershift of extracts from dedifferentiated C2 hepatoma (containing exclusively HNF-1β, Mendel *et al.*, submitted) by HNF-1β antiserum alone (Figure 4c).

Discussion

We have demonstrated that RA-induced differentiation of F9 cells along an αFP-expressing visceral endoderm lineage is accompanied by marked increases in both HNF-1α and HNF-1β mRNA and DNA-binding activity. The delayed induction of HNF-1α (5 days) and -1β (24–36 h) protein suggests that these genes are not directly sensitive to nuclear translocation of an RA receptor. Rather, the data suggest an indirect activation mediated by primary RA-response genes such as *Hox 1.3, Hox 2.1* and laminin, which are expressed within 1–3 h of RA treatment (La Rosa *et al.*, 1988; Murphy *et al.*, 1988; Vasios *et al.*, 1989). Further investigation of this cell culture system should enable characterization of developmental regulators of HNF-1α and -1β in F9 cells, as well as definition of downstream targets of primary RA-response genes. It is important to recognize that regulatory molecules identified in differentiated F9 cells, as opposed to regulators present in adult tissues, might participate in *de novo* induction rather than simply maintenance of HNF-1α and -1β expression, thus allowing biochemical analysis of an otherwise inaccessible developmental window.

The distinct time kinetics of HNF-1α and -1β induction in F9 cells, combined with the ability of these factors to heterodimerize, creates great potential variety in the HNF-1 DNA-binding species present at a given point during F9 differentiation. Initially, HNF-1α homodimers are present at very low levels, as (i) HNF-1β mRNA has not yet been induced; and (ii) HNF-1α supershift complexes co-migrate with mouse liver HNF-1α supershift (Figures 1a and 4b). However, with the induction of HNF-1β mRNA and DNA-binding activity (Figures 3 and 4a), the total DNA-binding activity smears towards faster mobility (Figure 1a), consistent with the well-characterized faster mobility of HNF-1β versus HNF-1α (Baumheuter, 1988; Cereghini, 1988). At time points later than 16 h, the possibility therefore exists for simultaneous expression of α–β heterodimers as well as α–α and β–β homodimers. Intriguingly, in 5–11 day extracts, HNF-1α antiserum creates a prominent supershift complex migrating faster than HNF-1α supershift from mouse liver or 0–48 h F9 extracts (Figure 4b). We observed an identical faster supershift in nuclear extracts from cells co-transfected with both HNF-1α and HNF-1β cDNA, but not from cells singly transfected with either HNF-1α or HNF-1β cDNA, indicating that this band may indeed represent an *in vitro* α-β heterodimer (C.J.Kuo and G.R.Crabtree, unpublished observations).

The induction of both HNF-1α and HNF-1β proteins provided an opportunity to correlate their appearance with developmental activation of the endogenous F9 cell, HNF-1-regulated αFP gene. Significantly, endogenous αFP was not expressed with the onset of HNF-1β DNA-binding activity at 24–36 h, but was rather co-induced with high level HNF-1α DNA-binding activity at 5 days (Figures 3 and 4). These data provide a developmental corollary to transient data in which HNF-1α is a potent transactivator while HNF-1β has much weaker activity and is not a trans-dominant repressor of HNF-1α upon co-transfection (Mendel *et al.*, submitted). Moreover, the stimulation of HNF-1α DNA-binding activity at day 5 (Figure 4b), combined with the dependence of the αFP promoter on an intact HNF-1 site for maximal and tissue-specific expression (Feuerman *et al.*, 1989), provides a likely explanation of the absolute requirement for this promoter for αFP induction in RA-treated F9 cells (Vogt *et al.*, 1988).

Independent regulation of HNF-1α and -1β, as described in F9 cells indicates that these proteins possess distinct developmental functions—which can be modified in co-expressing regions by either heterodimerization or competition of α–α and β–β homodimers for common binding sites. An analogous situation of independent regulation producing overlapping tissue distributions is represented by HNF-1α and -1β protein in the adult mouse: HNF-1α is expressed in liver, and HNF-1β in lung, with co-expression in kidney (Baumhueter *et al.*, 1988; Mendel *et al.*, submitted). We suggest that independent regulation of HNF-1α and -1β, such as in F9 cells, could help establish the overlapping expression patterns observed in adult organs. These resulting spatial distributions, creating regions of homo- and heterodimerization, would greatly diversify the ability of the HNF-1 binding site to interpret positional information, enabling its differential participation in the development of a variety of tissues.

Materials and methods

Cell culture, nuclear extract preparation and gel mobility shift assay

Undifferentiated F9 cells were passaged at $<4 \times 10^7$ cells/150 mm tissue culture dish coated with 1% gelatin. For induction, 5×10^6 F9 cells were placed in a 150 mm bacteriological dish (Falcon) with addition of 7.5×10^{-7} M RA (Sigma) previously dissolved as 1000× stock in DMSO. Induced F9 cells aggregated to form embryoid bodies and were subcultured every 2 days. All tissue culture was conducted at 37°C, 10% CO_2, in DMEM with 10% FCS and penicillin streptomycin (Applied Scientific). Nuclear extracts were prepared as previously described (Baumhueter *et al.*, 1988) with a modified buffer A: 10 mM HEPES (pH 7.8), 15 mM KCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.1% NP-40, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 μg/ml antipain, 1 mM spermine and 1 mM spermidine. Extracts were desalted by application to P6DG (Biorad) spun columns and immediately frozen. F9 nuclear protein (5 μg) or adult mouse liver nuclear extract 'L' (1 μg) was incubated with a ^{32}P-end-labeled 28 bp fragment (β28) representing the HNF-1 rat β-fibrinogen promoter binding site (Courtois, 1987) or an Oct-1 site from the human IL-2 enhancer (Durand *et al.*, 1988), and gel mobility shift assay performed as previously described (Baumhueter *et al.*, 1988), except with 1 × TBE as running buffer. Where appropriate, the protein–DNA complex was prebound for 30 min at room temperature; subsequently, 0.75–1 μl of antiserum directed against HNF-1α or HNF-1β was added to the binding reaction and the incubation continued at 4°C for 1 h before gel mobility shift assay.

DNase I footprinting and methylation interference assays

DNase I footprinting assay: Nuclear extract (25 μg) from untreated or 5 day-induced F9 cells, BSA (25 μg) or affinity purified HNF-1α were incubated with a ^{32}P-end-labeled probe containing the proximal 117 bp *Sph*I–*Hin*dIII rat βFg promoter fragment. DNase I footprinting was carried out as previously described (Courtois *et al.*, 1988; Baumhueter *et al.*, 1988). *Methylation interference assay*: Nuclear extract (10 μg) from 48 h RA-induced F9 cells or from the mouse liver cell line Hepala were incubated with a DMS-methylated, ^{32}P-end-labeled 49 bp *Sph*I–*Bst*EII rat βFg promoter fragment, and resolved by gel mobility shift assay. Bound and free probe were electroeluted, and subjected to piperidine cleavage and electrophoresis as described elsewhere (Baumhueter *et al.*, 1988).

Partial proteolysis of DNA–protein complexes

Nuclear extract (10 μg) from 48 h RA-treated F9 cells ('F9') of from Hepala cells ('H') were incubated with βFg probe in gel shift conditions and for

an additional 10 min at room temperature in the presence or absence of 100 ng V8 protease before electrophoresis through 4% non-denaturing gels as in standard gel mobility shift assay.

RNA isolation and RNase protection analysis

Total RNA from induced or non-induced F9 cells was isolated by guanidinium thiocyanate lysis and CsCl gradient centrifugation, and the integrity of ribosomal bands assayed in formaldehyde gels. RNA (15 μg) from each sample or tRNA was hybridized to 2.5×10^5 c.p.m. [^{32}P]UTP-labeled antisense riboprobe, followed by digestion of hybrids with RNase A and T1 under standard conditions (Baumhueter *et al.*, 1988). The digested hybrids were electrophoresed at 90 W through 6% sequencing gels. The mouse HNF-1α (mHNF-1α) riboprobe was prepared by T3 transcription of *Pst*I-linearized pBS KS (−) (Stratagene) bearing a 277 bp insert from the HNF-1α 3′ UTR (Kuo *et al.*, 1990). The mouse HNF-1β (mHNF-1β) riboprobe will be described elsewhere (Mendel *et al.*, submitted). The mouse α-fetoprotein (mαFP) riboprobe was obtained by T3 transcription of *Pst*I-linearized pBS KS (−) containing the 240 bp *Pst*I−*Hin*dIII exon 8 of mαFP (gift of S.Tilghman).

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