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Adenovirus-Mediated Increase of HNF-3 Levels Stimulates Expression of Transthyretin and Sonic Hedgehog, Which Is Associated With F9 Cell Differentiation Toward the Visceral Endoderm Lineage

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Retinoic acid-induced differentiation of mouse F9 embryonal carcinoma cells toward the visceral endoderm lineage is accompanied by increased expression of the Forkhead Box (Fox) transcription factors hepatocyte nuclear factor 3α (HNF-3α) and HNF-3β, suggesting that they play a crucial role in visceral endoderm development. Retinoic acid stimulation results in a cascade of HNF-3 induction in which HNF-3α is a primary target for retinoic acid action and its increase is required for subsequent induction of HNF-3β expression. Increased expression of HNF-3β precedes activation of its known target genes, including transthyretin (TTR), Sonic hedgehog (Shh), HNF-1α, HNF-1β, and HNF-4α. In order to examine whether increased HNF-3 expression is sufficient to induce expression of its downstream target genes without retinoic acid stimulation, we have used adenovirus-based expression vectors to increase HNF-3 protein levels in F9 cells. We demonstrate that adenovirus-mediated increase of HNF-3α levels in F9 cells is sufficient to induce activation of endogenous HNF-3β levels followed by increased TTR and Shh expression. Furthermore, we show that elevated HNF-3β levels stimulate expression of endogenous TTR and Shh without retinoic acid stimulation. Moreover, ectopic HNF-3 levels in undifferentiated F9 cells are insufficient to induce HNF-3α, HNF-1α, HNF-1β, and HNF-4α expression, suggesting that their transcriptional activation required other regulatory proteins induced by the retinoic acid differentiation program. Finally, our studies demonstrate the utility of cell infections with adenovirus expressing distinct transcription factors to identify endogenous target genes, which are assembled with the appropriate nucleosome structure.

Hepatocyte nuclear factor 3 Sonic hedgehog Transthyretin Retinoic acid stiumlation

THE hepatocyte nuclear factor 3α (HNF-3α), HNF-3β, and HNF-3γ transcription factors share homology in the winged helix/fork head DNA binding domain (5). The fork head/winged-helix proteins are a growing family of transcription factors that play important roles in cellular proliferation and differentiation during embryonic development (8,20). Recently, the nomenclature of the winged-helix/*fork head* family has

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been revised to *forkhead box* (*Fox*) genes, and HNF-3α and HNF-3β are also known as Foxa1 and Foxa2, respectively (19). The HNF-3 proteins were first identified as mediating transcription of hepatocyte-specific genes (6,23,24). These include the serum proteins transthyretin (TTR), α1-antitrypsin, albumin, α-fetoprotein, Transferrin, and apolipoprotein AI, and the enzymes tyrosine aminotransferase, phosphoenolpyruvate carboxykinase, and glucose 6-phosphatase, and the transcription factors HNF-1α and HNF-4α genes (7). Subsequent studies showed that the HNF-3α and HNF-3β proteins also regulate expression of genes critical in the function of visceral yolk sac endoderm, intestine, stomach, lung, and pancreas (7,8,11,17,33).

Expression of HNF-3α and HNF-3β initiates during gastrulation of mouse embryogenesis in the node, notochord mesoderm, floorplate neuroepithelium, and in visceral, definitive endoderm and gut endoderm (3,27,39,41). Consistent with this expression pattern, homozygous null *Hnf3*β embryos die in utero because of defective formation of the node, notochord, gut endoderm, and visceral endoderm, which are required for development of the primitive streak during gastrulation and for inductive signaling necessary in neurotube formation (2,47). HNF-3β is known to regulate notochord transcription of the sonic hedgehog (Shh) gene, which is required for inductive signaling during the formation of the neurotube (4,12). Moreover, use of *Hnf3*β –/–-deficient embryonic stem (ES) cells to form embryoid bodies (EB) for in vitro differentiation toward visceral (yolk sac) endoderm demonstrates that HNF-3β is involved in cross-regulating expression of the HNF-3α, HNF-1α, and HNF-4α transcription factors (11). These studies also implicated HNF-3β in regulating transcription of apolipoproteins, aldolase B, pyruvate kinase, TTR, and albumin genes in EB-induced visceral endoderm. Moreover, *Hnf3*β –/–-deficient mice fail to thrive, are hypoglycemic, and display reduced pancreatic islet expression and secretion of glucagon, which is required to mobilize hepatic glycogen (18,43).

It is well established that several HNF-3 target genes (e.g., transthyretin and α-fetoprotein) are induced upon retinoic acid-mediated differentiation of mouse F9 embryonal carcinoma cells into visceral endoderm (42,44). Our F9 cell differentiation studies demonstrate that retinoic acid induces a cascade of gene expression in which induction of HNF-3α occurs first, followed by increased HNF-3β levels and then stimulation of TTR levels (15,37). During F9 differentiation, HNF-3α is a primary target of retinoic acid action through a retinoic acid responsive element (RARE) in its promoter region (16). HNF-3α expression is rapidly induced within 6 h, peaks at 1 day postdifferentiation, and then declines to undetectable levels, and its kinetics are consistent with subsequent induction of HNF-3β expression (16,37). Induced HNF-3β expression is consistent with its role in stimulating transcription of downstream target genes TTR, HNF-1α, HNF-1β, and HNF-4α (15,16, 37). Another HNF-3 target gene, Shh, is also expressed in visceral endoderm in mouse embryos (26), but the role of HNF-3 in Shh regulation during F9 cell differentiation remains uncharacterized.

Adenovirus vectors have been used for the overexpression of several different transcription factors (9,13,28,45) and we have therefore generated two adenovirus vectors expressing either HNF-3α or HNF-3β. In this study, we have used adenovirus-based expression vectors to infect F9 cells and increase HNF-3 protein levels without retinoic acid differentiation to examine whether induction of HNF-3 levels alone is sufficient to induce expression of its downstream target genes. We demonstrate that adenovirus-mediated increase of HNF-3α levels in F9 cells is sufficient to induce activation of endogenous HNF-3β, which precedes activation of the presumed target genes: TTR and Shh. We show that elevated HNF-3β levels resulted in activation of endogenous TTR and Shh expression in the absence of retinoic acid stimulation. Moreover, ectopic HNF-3 levels alone were unable to induce a number of other potential target genes, suggesting that their transcriptional activation required other regulatory proteins induced by the retinoic acid differentiation program.

MATERIALS AND METHODS

Adenovirus Vector Preparation

Two 1.6-kb *Eco*RI fragments containing the entire coding sequence for either the rat HNF-3α or rat HNF-3β protein were subcloned into the adenovirus recombination vector, pAdCL-XEB. This vector contained the cytomegalovirus (CMV) promoter, a multiple cloning site (including *Eco*RI), the SV40 poly A site, and a splice donor/acceptor site between human adenovirus type 5 genome sequences 1–355 and 3333–5788 (see also Fig. 1). We used the overlap recombination procedure (14) to construct adenoviruses that overexpressed rat HNF-3α and rat HNF-3β genes. The resulting plasmids, denoted pAdHNF3α or pAdHNF3β, were linearized with *Asc*I and cotransfected with type 5 adenovirus dl324 DNA into HEK (human embryonic kidney) 293 cells. dl324, which contains a deletion in the E1 region (genome positions 1334–3639 are missing) and in the E3 region, was digested with *Cla*I at genome position 918. We transfected 5 μg of each plasmid (HNF-3α or HNF-3β) together with 1 μg of cut virus per 35-mm cell culture plate. After providing sufficient time for

recombination to take place (between 5 and 14 days), 293 cells were lysed and potential recombinant viruses were detected by performing standard plaque assays on 293 cells (35). Following plaque purification, viral DNA was isolated and used for the preparation of Southern blots. HNF-3-containing viruses were detected by probing the blots with probes specific for HNF-3α and HNF-3β, followed by PCR with HNF-3-specific primers. Homologous recombination between the linearized plasmids and dl324 replaced portions of the adenovirus E1 region with the 1.6-kb HNF-3α or HNF-3β gene fragments, and created the AdHNF3α or AdHNF3β, respectively (see Fig. 1). Using the procedure described above, we also generated a control virus (Ad*LacZ*), which contained the prokaryotic *LacZ* (β-galactosidase) gene instead of the HNF-3 cDNAs.

Purification of Recombinant Adenoviruses

For large-scale preparations, 20 tissue culture plates (150 × 25 mm) of HEK 293 cells ($1–2 \times 10^7$ cells/plate) were infected with AdHNF3α, AdHNF3β, or Ad*LacZ* at a multiplicity of infection (MOI) of 1–5 plaque forming units (pfu)/cell. After an additional 2 days of culture, the cells were harvested and washed once with PBS (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 135 mM NaCl, 2.5 mM KCl). The cells were then resuspended in lysis buffer (10 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 0.5% DOC). Freeze thawing lysed the samples and DNase was added to a final concentration of 10 μg/ml. After 30 min at 37°C, an equal volume of fluorocarbon (1,1,2-tricholorotrifluoroethane, Sigma) was added and the samples were incubated on a rocker for 10 min at room temperature. Following a centrifugation (10 min at $1000 \times g$), the supernatant was layered onto a cesium chloride gradient (1.4 g/ml and 1.2 g/ml in 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$) and centrifuged at $90{,}000 \times g$ for 3 h at 4°C (SW 28 rotor). Viral bands were harvested and mixed with an equal volume of loading buffer (50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$). The samples were again layered onto a cesium chloride gradient and centrifuged overnight. Viral bands were removed, mixed with four volumes of freezing solution (0.1% BSA, 50% glycerol, 10 mM Tris-HCl, pH 7.8, 100 mM NaCl), and stored at −20°C.

Cell Culture Experiments

Mouse F9 embryonal carcinoma cells, mouse P19 embryonal carcinoma cells, and HEK 293 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 7.5% ultra calf serum and 2.5% fetal calf serum (Inovar). For viral infections, cultures were grown on tissue culture dishes to 60–70% confluence. Cells were washed once with serum-free DMEM and then exposed to serum-free DMEM containing AdHNF3α, AdHNF3β, or Ad*LacZ* at 20 pfu/cell. After an incubation of 1 h at 37°C, the medium was replaced with DMEM containing 7.5% ultra calf serum and 2.5% fetal calf serum and cells were cultured for various times at 37°C.

To measure infection efficiency, which was represented by the β-galactosidase activity in Ad*LacZ*-infected cells, they were fixed with 0.05% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, for 5 min at room temperature. The cells were then washed with ice-cold PBS (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 135 mM NaCl, 2.5 mM KCl) and stained with X-gal solution [1 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside, 0.1 M phosphate buffer, pH 7.3, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$, 1.3 mM $MgCl_2$] for 4–6 h at 37°C.

RNase Protection Assay

mRNA Isolation. mRNA was isolated from cell cultures using the Fast Track mRNA isolation kit from Invitrogen, following the manufacturer's instructions. Starting with 4×10^8 cells, we routinely obtained 20–50 μg of oligo(dT)-selected mRNA.

Probe Preparation. Antisense RNA probes were generated by transcribing specific cDNA fragments that had been cloned into the Bluescript KS II vector using T7 RNA polymerase as described previously (36). The RNase protection probes for the rat and mouse HNF-3α, HNF-3β, and the mouse TTR, HNF-1α, HNF-1β, and HNF-4α have been described previously (6,36). The following cDNAs were transcribed: mouse GAPDH 318-bp cDNA fragment and Sonic hedgehog (Shh) 249-bp cDNA fragment.

Hybridization reactions were carried out using the RPA II kit distributed by Amicon. For each reaction, 5 μg of oligo(dT)-selected mRNA was hybridized in solution with 1×10^5 cpm of antisense RNA probe (18 h at 45°C), following the recommendations of the kit producer. After RNase treatment, resistant RNA–RNA hybrids were analyzed on 5% polyacrylamide/8 M urea gels (acrylamide/bis 29:1). Autoradiography of gels was performed with Kodak XAR film and Fisher Biotech L-Plus screens at −70°C for 1–4 days. Mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase) control RNA was detected with a specific probe provided by Ambion.

Nuclear Protein Extraction and Gel Shift Assay

Nuclear proteins were extracted from cell cultures according to the protocol of Andrews and Faller (1). Binding of HNF-3 proteins to DNA oligonucleotides

was initiated by mixing the following components: 1 ng of kinased double-stranded oligonucleotide, binding buffer (20 mM Tris, pH 7.5, 5% glycerol, 40 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT, 1 mM EDTA), 3 μg of poly(dI-dC), and 10 μg of extract. One microliter of HNF-3α-specific or HNF-3β-specific antiserum (15) was added where indicated in a total reaction mixture of 30 μl. After 30 min at room temperature, the reaction mixture was loaded onto a 5% polyacrylamide gel (acrylamide/bis 29:1). Electrophoresis was performed for 120 min at 150 V (room temperature). Subsequently, the gel was dried and subjected to autoradiography as described above. The strong affinity HNF-3 binding site from the TTR promoter (6,29,40) was employed by hybridizing the following double-stranded DNA oligonucleotides: 5′-gttgactaagtcaataatcagaatcagca-3′ and 3′-caactgattcag ttattagtcttagtcgt-5′.

Transfections, Adenovirus Infection, and CAT Assay

Coinfection experiments with an HNF-3-dependent chloramphenicol acetyltransferase (CAT) reporter construct demonstrated that the adenovirus-transduced HNF-3α and HNF-3β proteins were transcriptionally active. We first transfected an HNF-3-dependent CAT reporter plasmid (30) into P19 cells, then infected them with either AdHNF3α, AdHNF3β, or Ad*LacZ* and 24 h later we performed CAT assays with protein extracts prepared from these cells. On the day before transfection, P19 embryonal carcinoma cells were split at a density of $\sim 10^5$ cells per ml in 10-cm tissue culture dishes in 10 ml medium. Transfections of the HNF-3-dependent CAT reporter construct method and SV40 promoter-driven β-galactosidase (β-gal) constructs (pSV-*LacZ*, included as internal control) were done by the calcium phosphate method as described previously (30). The transfected P19 cells were then infected with either AdHNF3α, AdHNFβ, or Ad*LacZ* at a multiplicity of infection (MOI) of 20 pfu/cell. The cells were harvested after another 24 h of culture, lysed by freezing and thawing, and the extract was used for the β-gal assay (38) and the CAT assay. The β-gal assay was performed by reacting a specified amount of protein with substrate ONPG (*O*-nitrophenyl-β-D-galactopyranoside) and measuring the absorbance at 420 nm. Extracts containing equal β-gal activities were used for CAT assay. For this purpose, the extract was incubated at 65°C for 15 min to inactivate the deacetylating enzymes and subsequently incubated with 20 μl 4 mM acetyl CoA and 3 μl [^{14}C]chloramphenicol at 37°C for 2 h with repeated addition of acetyl CoA. CAT activity was monitored by thin-layer chromatography on silica plates in $CHCl_3/CH_3OH$ (95:5) followed by autoradiography.

RESULTS

AdHNF3α and AdHNF3β Give Rise to Functional Protein Products

In order to determine whether the HNF-3 transcription factors alone are sufficient to induce expression of downstream target genes in F9 cells without retinoic acid differentiation, we have used adenovirus-based expression vectors to increase expression of HNF-3α and HNF-3β proteins. We utilized the overlap recombination procedure (14) to construct adenoviruses that overexpress rat HNF-3α and HNF-3β genes, as described in Materials and Methods (Fig. 1). In order to verify that AdHNF3α and AdHNF3β generated functional transcription factors, we first infected mouse P19 embryonal carcinoma cells with AdHNF3α or AdHNF3β at a MOI of 20 pfu/cell. Control adenovirus Ad*LacZ* infections detected by β-gal enzyme staining with the X-gal substrate determined that greater than 90% of the cells were infected using an MOI of 20 pfu/cell. RNA was isolated from P19 cells at different intervals following infection with either AdHNF3α or AdHNF3β and used for RNase protection assays to determine HNF-3α or HNF-3β mRNA levels (see Materials and Methods). Infection of P19 cells with AdHNF3α led to increased HNF-3α mRNA levels between 2 and 12 h postinfection (PI) (Fig. 2A, lanes 6–9). No HNF-3α mRNA was detected in P19 cells that were either infected with control *LacZ*-expressing adenoviruses (Ad*LacZ*) or mock infected cells (Fig. 2A, lanes 1–5). Likewise, infection of P19 cells with AdHNF3β caused induction of HNF-3β mRNA at 2 h PI, with levels gradually increasing through the 12-h PI time point (Fig. 2B, lanes 6–9).

In order to demonstrate that AdHNF3α and AdHNFβ infections led to increased HNF-3α and HNF-3β protein levels, we prepared nuclear extracts from adenovirus-infected cells and gel shift experiments were carried out to visualize the HNF-3 protein–DNA complexes (see Materials and Methods). HNF-3α protein–DNA complex formation was detected between 6 and 72 h PI (Fig. 3A, lanes 4–8), and disruption of this protein–DNA complex by addition of the HNF-3α antibody in the binding reaction confirmed the presence of HNF-3α protein in the complex (Fig. 3A, lane 9). Furthermore, no abundant HNF-3α protein–DNA complex was found in binding reactions using nuclear extracts from either mock-infected or Ad*LacZ*-infected controls (Fig. 3A, lanes 1–3). Likewise, infection of P19 cells with AdHNF3β resulted in the increased HNF-3β protein–DNA complexes by 6 h PI, with continued gradual increase until the 24-h PI time point (Fig. 3A, lanes 1–7). Furthermore, supershifting of the adenovirus-

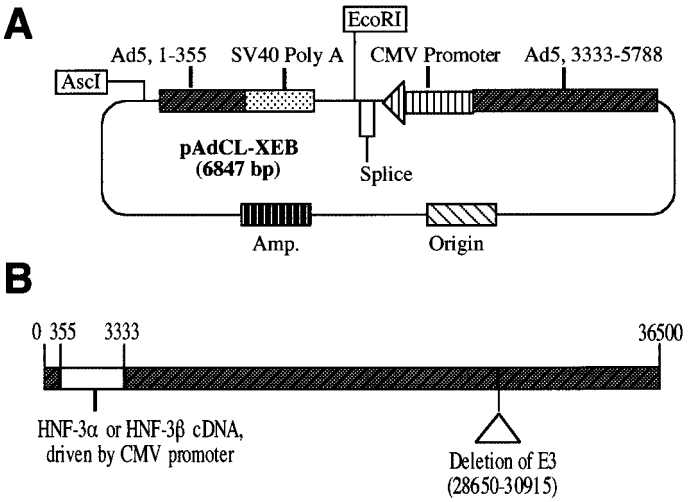

Figure 1. Construction of adenovirus vectors expressing HNF-3α and HNF-3β. (A) Structure of adenovirus recombination pAdCL-XEB. Schematically shown is the adenovirus recombination plasmid containing the expression cassette consisting of the CMV promoter, RNA splice site, *Eco*RI site for insertion of the rat HNF-3 and HNF-3 cDNAs, and the SV-40 polyadenylation sequence. The expression cassette was bounded on both sides by adenovirus sequences 1–355 and 3333–5788, which are required for adenovirus recombination to reconstitute the full-length virus. (B) Schematic representation of the replication-deficient recombinant adenoviruses in which the HNF-3 cDNAs replaces the E1 region. Note that this figure is not drawn to scale.

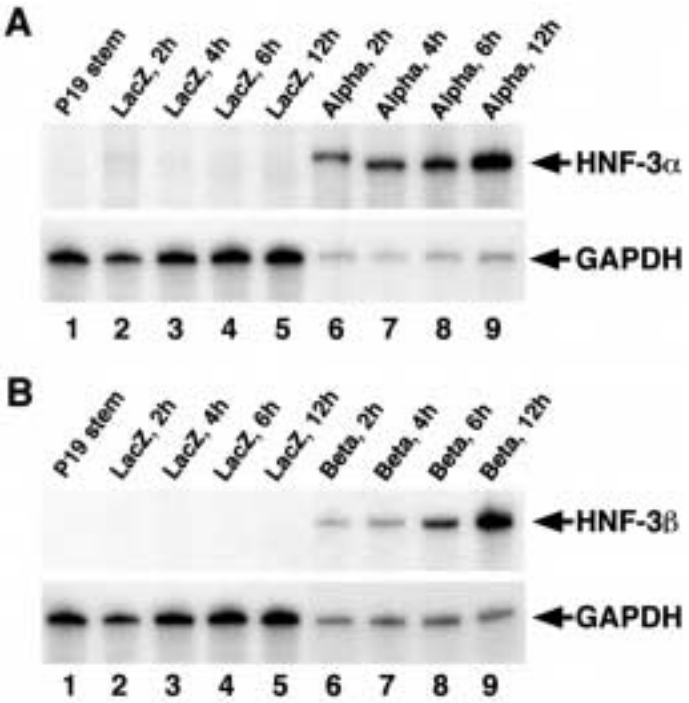

Figure 2. Detection of adenovirus-derived HNF-3 mRNAs. (A) Time course of adenovirus-derived HNF-3α mRNA. To determine HNF-3α mRNA levels, total RNA was prepared from P19 stem cells at various intervals following infection with adenovirus (at a multiplicity of 20 pfu/cell) and RNase protection assays were performed with antisense rat HNF-3α RNA probe as described in Materials and Methods. Shown are RNase protection assays with either P19 stem cell mRNA (lane 1) or Ad*LacZ*-infected P19 cell mRNA isolated at 2 h (lane 2), 4 h (lane 3), 6 h (lane 4), and 12 h (lane 5) postinfection (PI). In addition, RNase protection assays with AdHNF3α-infected P19 cell mRNA isolated at 2 h (lane 6), 4 h (lane 7), 6 h (lane 8), and 12 h (lane 9) PI. For normalization purposes, signals of GAPDH-specific mRNA are shown in the bottom panel. (B) Temporal profile of adenovirus-derived HNF-3β mRNA. To determine HNF-3β mRNA levels, RNase protection assays were performed as described in (A) with antisense rat HNF-3β RNA probe. RNase protection assays with AdHNF3β-infected P19 cell mRNA isolated 2 h (lane 6), 4 h (lane 7), 6 h (lane 8), and 12 h (lane 9) PI and controls are described in (A).

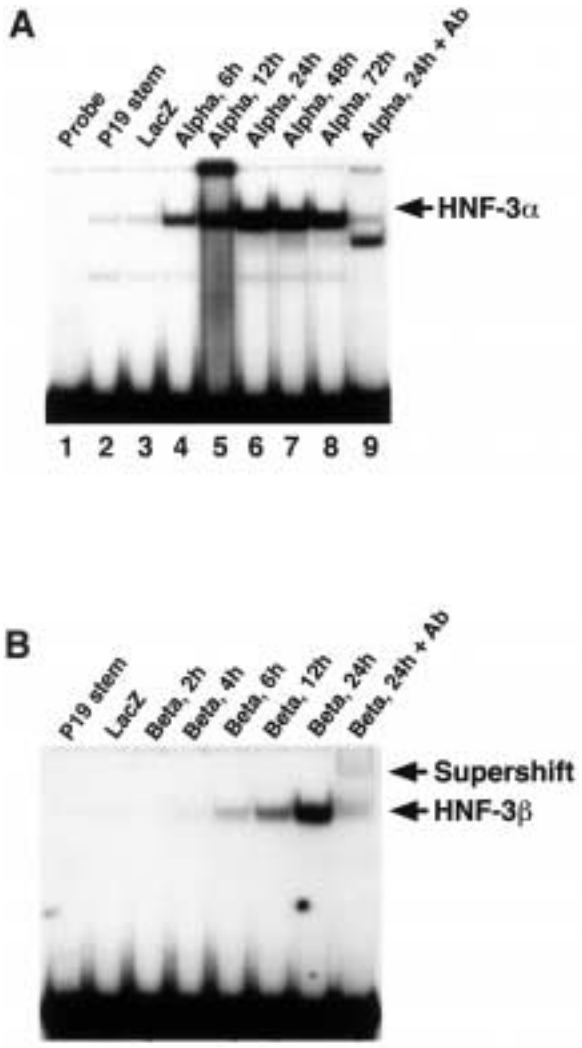

Figure 3. DNA binding properties of adenovirus-derived HNF-3 proteins. (A) Time course of adenovirus-derived HNF-3α protein. To determine HNF-3α mRNA levels, nuclear extracts were prepared from P19 stem cells at various intervals following infection with adenovirus (at a multiplicity of 20 pfu/cell) and used for gel shift assays with an HNF-3 binding site from the TTR promoter as described in Materials and Methods. Shown are gel shift assays using nuclear extracts isolated from either P19 stem cells (lane 2) or Ad*LacZ*-infected P19 cells isolated at 24 h PI (lane 3) or AdH NF3α-infected P19 cells isolated at 6 h (lane 4), 12 h (lane 5), 24 h (lane 6), 48 h (lane 7), and 72 h (lane 8) PI. Lane 1 contains probe only. Lane 9 depicts a gel shift reaction carried out with extract derived from AdHNF3α-infected P19 cells (prepared 24 h PI) and HNF-3α-specific antiserum, which disrupted HNF-3α protein–DNA complex formation. (B) Time course of adenovirus-derived HNF-3β protein. To determine HNF-3β protein levels, gel shift assays were performed as described in (A). Shown are gel shift assays using nuclear extracts isolated from either P19 stem cells (lane 1), Ad*LacZ*-infected P19 cells isolated at 24 h PI (lane 2) or AdHNF3β-infected P19 cells isolated at 2 h (lane 3), 4 h (lane 4), 6 h (lane 5), 12 h (lane 6), and 24 h (lane 7) PI. Lane 8 depicts a gel shift assay performed with nuclear extracts prepared from P19 cells infected with AdHNF3β at 24 h PI and HNF-3β-specific antiserum, which supershifted the HNF-3β protein–DNA complex.

derived HNF-3β protein–DNA complex by addition of the HNF-3β-specific antibody confirmed the presence of HNF-3β protein there (37).

Finally, coinfection experiments with an HNF-3-dependent reporter construct demonstrated that the adenovirus-transduced HNF-3α and HNF-3β proteins were transcriptionally active. We first transfected an HNF-3-dependent CAT reporter plasmid (30) into P19 cells, then infected them with either AdHNF3α, AdHNF3β, or Ad*LacZ*, and then 24 h later we performed CAT assays with protein extracts prepared from these cells (see Materials and Methods). Both AdHNF3α and AdHNF3β were potent activators of HNF-3-dependent CAT reporter gene expression, whereas no transcriptional activation was found with Ad*LacZ* infection, indicating that this stimulation was specific (Fig. 4). In summary, these studies demonstrated that adenovirus infections could increase levels of transcriptionally active HNF-3α and HNF-3β proteins.

HNF-3α Induces Expression of HNF-3β, Shh, and TTR in F9 Cells

We have previously shown that retinoic acid-mediated differentiation of F9 cells causes a cascade of gene expression in which increased HNF-3α levels

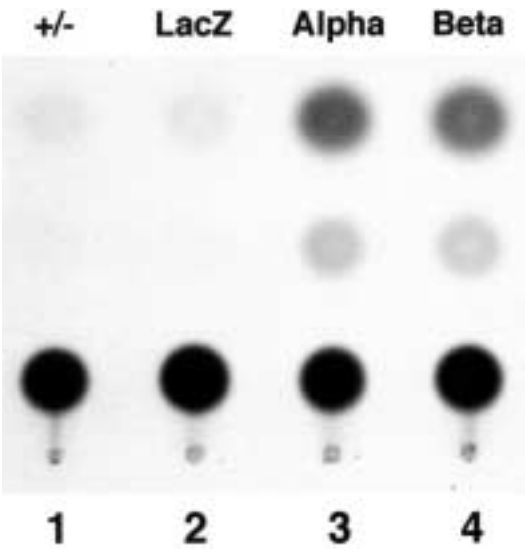

Figure 4. Activation of HNF-3 binding site-containing promoter by AdHNF3α and AdHNF3β. A CAT reporter construct that contained four contiguous HNF-3 binding sites fused to the minimal TATA sequence from the CMV promoter was transfected into P19 cells. Sixteen hours later, the transfected cells were infected with Ad*LacZ* (lane 2), AdHNF3α (lane 3), or AdHNF3β (lane 4) at a multiplicity of 20 pfu/cell. Twenty-four hours after the viral infections, cell extracts were prepared and CAT assays were performed as detailed in Materials and Methods. Lane 1 depicts a CAT assay carried out with cell extract prepared from transfected cells that had not been infected.

are observed first, followed by elevated HNF-3β, which precedes activation of TTR (15,37). Because HNF-3β Shh and TTR promoter regions contain HNF-3 binding sites (4,6,12,23,31), we next examined whether HNF-3α could mediate activation of these genes in the absence of retinoic acid stimulation. Infections of F9 cells with AdHNF3α (20 pfu/cell) caused a large increase in rat HNF-3α levels between 12 h and 3 days PI, with subsequent, gradual decrease of these levels between 3 and 5 days PI (Fig. 5, HNF-3α panel). HNF-3α-mediated induction of endogenous mouse HNF-3β, however, was not observed until 4 days PI and elevated HNF-3β levels were sustained throughout the time points examined (Fig. 5A, HNF-3β lanes 7–9). These studies demonstrate that although HNF-3α is involved in stimulating endogenous HNF-3β expression in F9 cells, sustained HNF-3α levels are not necessary to maintain HNF-3β expression. Furthermore, HNF-3α mediated a later increase of TTR expression at 6 days PI, which is 2 days after the induction of endogenous HNF-3β levels (Fig. 5A, TTR lane 9). Control reactions performed with either F9 stem cell mRNA or Ad*LacZ*-infected F9 cell mRNA (Fig. 5A lanes 1–2) demonstrated that induction of HNF-3β and transthyretin were specific for AdHNF3α infection. Moreover, AdHNF3α infection of F9 cells stimulated a transient increase in Shh expression between 12 h and

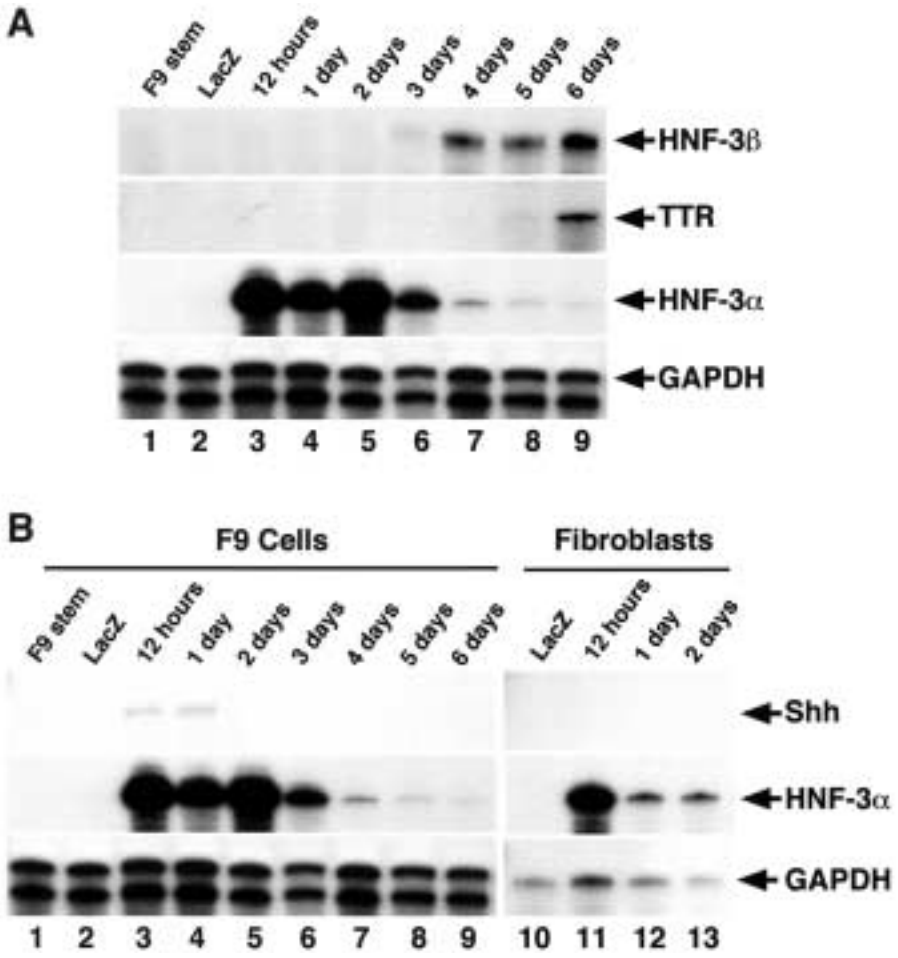

Figure 5. HNF-3α induces HNF-3β Shh and TTR levels in F9 cells. F9 stem cells were infected with Ad*LacZ* or AdHNF3α at a multiplicity of 20 pfu/cell and total RNA was isolated at various intervals PI. RNase protection assays were performed by hybridizing 5 μg of RNA with antisense RNA probes specific for either mouse HNF-3β, mouse transthyretin (TTR), mouse sonic hedgehog (Shh,) or rat HNF-3α as described in Materials and Methods. (A) HNF-3α induces HNF-3β and TTR expression in F9 cells. RNase protection assays with indicated probes and AdHNF3α-infected F9 cell mRNA isolated at 12 h (lane 3), 1 day (lane 4), 2 days (lane 5), 3 days (lane 6), 4 days (lane 7), 5 days (lane 8), and 6 days (lane 9) PI. Control lanes include RNase protection assays from F9 stem cells (lane 1) or Ad*LacZ*-infected F9 cells isolated 3 days PI (lane 2). For normalization purposes, GAPDH mRNA was detected by RNase protection (bottom panel). (B) HNF-3β induces Shh expression in F9 cells but not in fibroblasts. RNase protection assays with indicated probes and AdHNF3α-infected F9 cell mRNA isolated at 12 h (lane 3), 1 day (lane 4), 2 days (lane 5), 3 days (lane 6), 4 days (lane 7), 5 days (lane 8), and 6 days (lane 9) PI. RNase protection assays with indicated probes and AdHNF3α-infected fibroblast cell mRNA isolated at 12 h (lane 11), 1 day (lane 12) and 2 days (lane 13) PI. Controls lanes are identical to those described in (A).

1 day PI during a period when HNF-3α levels are maximal (Fig. 5B, Shh, lanes 3–4). By comparison, AdHNF3α infection of fibroblasts caused elevated HNF-3α levels, but it was unable to stimulate Shh expression in this cell type (Fig. 5B, lanes 11–13). Taken together, these results suggest that HNF-3α mediates the induction of the endogenous mouse HNF-3β, Shh, and TTR genes in F9 cells without retinoic acid stimulation. Moreover, our studies indicate that ectopic expression of HNF-3α stimulated HNF-3β expression followed by TTR, which indicates that the viral system recapitulates the expression pattern observed during retinoic acid-mediated F9 cell differentiation (15,37).

The HNF-1α, HNF-1β, and HNF-4α promoters contain HNF-3 binding sites and are therefore putative targets for HNF-3α in F9 cells during retinoic acid-mediated F9 cell differentiation (10,21,22,46, 49). Furthermore, expression of these transcription factors is activated after the appearance of HNF-3α following retinoic acid stimulation. We therefore examined whether expression of HNF-1α, HNF-1β, or HNF-4α could be affected by the HNF-3α transcription factors in F9 cells. F9 cells were infected with AdHNF3α to elevate HNF-3α protein levels and RNase protection assays indicated that HNF-3α alone was insufficient to induce the expression of these genes (data not shown). These studies demonstrate that transcriptional activation of the HNF-1α, HNF-1β, and HNF-4α genes requires other regulatory proteins induced by the retinoic acid differentiation program.

Ectopic HNF-3β Levels in Mouse F9 Cells Are Sufficient to Induce Expression of TTR and Shh

Retinoic acid differentiation of F9 induces HNF-3α first, followed by delayed stimulation of HNF-3β expression (15,37). Because these HNF-3 proteins are closely related, we next examined whether HNF-3β could substitute for HNF-3α function and stimulate TTR and Shh levels in the absence of retinoic acid stimulation. In order to test our hypothesis, we infected F9 cells with AdHNF3β, which provided maximal HNF-3β levels at 2 days PI and gradually declined until the 5-day time point (Fig. 6A, HNF-3β lanes 3–8). Longer exposure of the HNF-3β signal demonstrated detectable HNF-3β levels throughout the time points examined (Fig. 6B). AdHNF3β infection mediated an increased in TTR expression between 4 and 6 days PI, suggesting that high HNF-3β levels were not required to maintain TTR transcription (Fig. 6, lanes 7–9). Moreover, elevated HNF-3β levels provided greater stimulation of Shh expression in F9 cells than had been observed with the HNF-3α protein (compare Fig. 6B with Fig. 5B). This result suggests that the HNF-3 proteins are not equivalent in their ability to activate target genes and that HNF-3β is the preferred transcriptional regulator for the *Shh* gene. Overall, this result is consistent with our gene cascade model, in which HNF-3α induces HNF-3β and HNF-3β induces expression of the differentiation-specific *Ttr* and *Shh* genes.

Although HNF-3α is a primary target gene for retinoic acid action (16), its promoter region contained an HNF-3 recognition site (34). We therefore examined whether elevated HNF-3β levels in F9 cells could stimulate expression of HNF-3α. RNA was prepared from F9 cells at different time points following AdHNF3β infection, and HNF-3α activation was assessed by RNase protection. Despite achieving elevated HNF-3β levels, this transcription factor was not able to stimulate expression of the endogenous mouse *Hnf3α* gene (Fig. 6, HNF-3α panel). This result indicates that increased transcription of the *Hnf3α* gene requires additional factors that are stimulated by the retinoic acid differentiation program. Similar to HNF-3α experiments in F9 cells, elevated HNF-3β levels were also insufficient to stimulate expression of HNF-1α, HNF-1β, or HNF-4α (data not shown).

DISCUSSION

Previous F9 cell differentiation studies demonstrate that retinoic acid induces a cascade of gene expression in which induction of HNF-3α occurs first, followed by increased HNF-3β levels and then stimulation of TTR expression (15,37). During F9 differentiation, HNF-3α is a primary target of retinoic acid action through a RARE sequence in its promoter region (16). HNF-3α expression is rapidly induced within 6 h, peaks at 1 day postdifferentiation, and then declines to undetectable levels (16,37). The HNF-3α peak is consistent with subsequent activation of HNF-3β expression, whose kinetics of induction are associated with transcription of downstream target genes, Shh, TTR, HNF-1α HNF-1β, and HNF-4α (15,16,37). The role of HNF-3 in regulating transcription of these target genes is supported by the fact that all of their promoter regions contain functional HNF-3 binding sites (4,6,12,23,31). In this study, we examined whether increased HNF-3 levels are sufficient to induce expression of its downstream target genes without retinoic acid stimulation by using adenovirus-based expression vectors to increase HNF-3 protein levels in F9 cells. We demonstrate that adenovirus-mediated increase of HNF-3α levels in F9 cells is sufficient to induce activation of endogenous HNF-

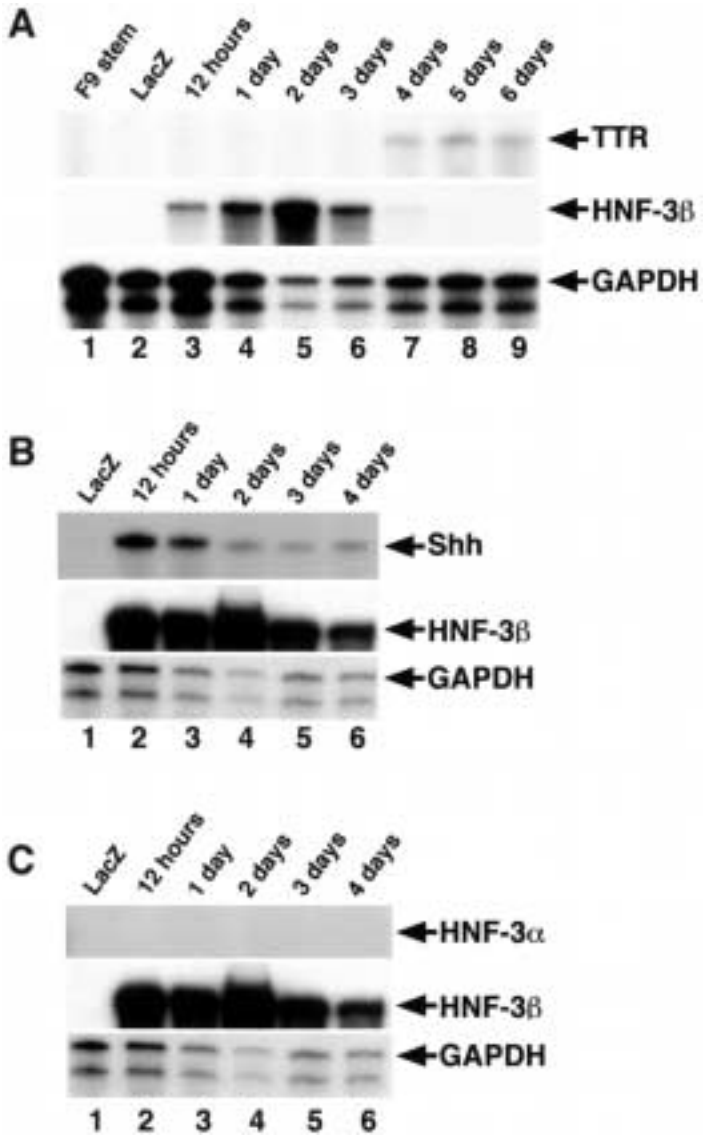

Figure 6. HNF-3β stimulates TTR and Shh expression but not HNF-3α in F9 cells. F9 stem cells were infected with Ad*LacZ* or AdHNF3β at a multiplicity of 20 pfu/cell and RNA was isolated at various intervals postinfection (PI). RNase protection assays were performed by hybridizing 5 μg of RNA with antisense probes for either mouse TTR, Shh, or rat HNF-3β as described in Materials and Methods. (A) HNF-3β stimulates TTR expression in F9 cells. RNase protection assays with indicated probes and AdHNF3β-infected F9 cell mRNA isolated at 12 h (lane 3), 1 day (lane 4), 2 days (lane 5), 3 days (lane 6), 4 days (lane 7), 5 days (lane 8), and 6 days (lane 9) PI. (B) HNF-3β stimulates Shh expression in F9 cells. RNase protection assays with indicated probes and AdHNF3β-infected F9 cell mRNA isolated at 12 h (lane 2), 1 day (lane 3), 2 days (lane 4), 3 days (lane 5), and 4 days (lane 6) PI. (C) Overexpression of HNF-3β in F9 cells does not activate HNF-3α. RNase protection assays with indicated probes and AdHNF3β-infected F9 cell mRNA isolated 12 h (lane 2), 1 day (lane 3), 2 days (lane 4), 3 days (lane 5), and 4 days (lane 6) after adenoviral infection. Controls are described in Figure 5 legend.

3β, TTR, and Shh expression. Furthermore, we show that elevated HNF-3β levels are also sufficient to stimulate endogenous TTR and Shh expression without retinoic acid stimulation. Moreover, ectopic HNF-3 levels were found to be insufficient to induce HNF-3α, HNF-1α, HNF-1β, and HNF-4α expression, suggesting that their transcriptional activation requires other regulatory proteins induced by the retinoic acid differentiation program.

Our studies clearly demonstrate that adenovirus-derived HNF-3α alone is sufficient to induce transcription of endogenous HNF-3β. While HNF-3α transcription was detectable within 1 day postinfection, HNF-3β expression was first seen 4 days postinfection (see Fig. 5). Currently, the mechanism causing delayed induction of HNF-3β expression remains uncharacterized. One possibility is that a certain level of virus-derived HNF-3α protein is required to induce transcription of the HNF-3β gene. Alternatively, HNF-3α may stimulate expression of additional tran-

scription factors that participate, in concert with HNF-3α, in the activation of HNF-3β transcription. Further studies are needed to discriminate between these possibilities.

Overexpression of HNF-3α was sufficient to activate TTR gene expression without retinoic acid stimulation. However, transthyretin induction occurred relatively late, 6 days postinfection (see Fig. 5). Because of this delayed induction of transthyretin, we reasoned that HNF-3α induces transthyretin transcription in an indirect manner. Our model proposes that HNF-3α activates expression of HNF-3β, which in turn induces transcription of the *Ttr* gene. In order to test this hypothesis, we used adenovirus to ectopically express HNF-3β in F9 cells and assayed for TTR expression at various intervals PI. Our F9 cell experiments showed that high HNF-3β levels stimulated TTR expression within 4 days PI whereas HNF-3α required 6 days to induce TTR expression when only elevated HNF-3β levels were observed. This temporal profile is clearly consistent with our hypothesis and suggests that HNF-3β, not HNF-3α, directly participates in the induction of TTR expression. In summary, our studies favor the following model for the participation of HNF-3 factors during F9 cell differentiation. Treatment of F9 cells with retinoic acid leads to the induction of HNF-3α, which is a primary target and directly interacts with retinoic acid receptors (16). HNF-3α then participates in the activation of a second set of transcriptional activators such as HNF-3β. Our model proposes that HNF-3β regulates expression of tissue-specific genes including TTR. This model is further supported by our F9 cell studies demonstrating that ectopic HNF-3β levels resulted in more significant induction of Shh expression compared with only minimal induction by elevated HNF-3α levels. Consistent with these studies, ectopic expression experiments in zebrafish demonstrated that increased levels of HNF-3β (Axial) alone is sufficient to induce endogenous Shh expression (4), which is an important signal transduction pathway in development of numerous organ systems (25,26,32,48).

Besides HNF-3α, HNF-3β, and TTR, retinoic acid-mediated F9 cell differentiation leads to the induction of a set of transcription factor genes (HNF-1α, HNF-1β, and HNF-4) that are presumably stimulated in response to increased HNF-3α levels. Analogous to HNF-3β and TTR, their promoter regions contain HNF-3 binding sites and are induced after HNF-3α during F9 cell differentiation (10,21,22,46,49). Moreover, use of *Hnf3*β-deficient ES cells for EB-mediated in vitro differentiation toward visceral endoderm demonstrates that HNF-3β is essential for the regulation of the HNF-3α, HNF-1α, and HNF-4α transcription factors (11). In our current study, we demonstrated that ectopic expression of neither HNF-3α nor HNF-3β alone resulted in activation of these transcription factors in F9 cells (data not shown). Furthermore, we show that HNF-3β alone is insufficient to activate expression HNF-3α in F9 cells despite the fact that its promoter region contains an HNF-3 recognition site (34). These results suggest that transcriptional activation of the HNF-3α promoter requires binding of other regulatory proteins that are activated by the retinoic acid differentiation pathway (16). Likewise, because retinoic acid induces expression of the HNF-1α, HNF-1β, and HNF-4α transcription factors in F9 cells, our data suggest that retinoic acid-induced transcription factors, in addition to HNF-3 proteins, are required for their transcriptional activation. In combination with visceral endoderm *Hnf3*β –/– genetic data (11), our results indicate that HNF-3 proteins are necessary but not sufficient for induced expression of HNF-3α, HNF-1α, HNF-1β, and HNF-4α in F9 cells.

In summary, we demonstrate that adenovirus mediated increase of HNF-3α levels in F9 cells is sufficient to induce activation of endogenous HNF-3β, which precedes activation of the presumed target genes, TTR and Shh. We show that elevated HNF-3β levels resulted in stimulated expression of endogenous *Ttr* and *Shh* genes without retinoic acid differentiation. Moreover, ectopic HNF-3 levels alone were unable to induce a number of other potential HNF-3 target genes, suggesting that their induced expression required other transcription factors that are stimulated by the retinoic acid differentiation program. Finally, our studies demonstrate the utility of cell infections with adenovirus expressing distinct transcription factors to identify endogenous target genes, which are assembled with the appropriate nucleosome structure.

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