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# Retinoic acid-mediated activation of HNF-3 $\alpha$ during EC stem cell differentiation

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## ABSTRACT

**We present evidence demonstrating that the liver-enriched transcription factor HNF-3 $\alpha$  is activated upon retinoic acid-induced differentiation of mouse F9 embryonal carcinoma cells. We have detected increases in the DNA binding activity and mRNA level of HNF-3 $\alpha$ . Both are reflections of the actual activation mechanism at the level of transcriptional initiation, which we showed with the help of HNF-3 $\alpha$  promoter constructs. Time course studies clearly show that HNF-3 $\alpha$  activation is a transient event. Employing Northern blots, HNF-3 $\alpha$  mRNA can be detected between 16 and 24 hours post-differentiation, reaches its zenith at approximately 1 day, and then declines to virtually undetectable levels. F9 cells can give rise to three distinct differentiated cell types; visceral endoderm, parietal endoderm, and primitive endoderm. We have clearly shown that HNF-3 $\alpha$  stimulation occurs upon primitive endoderm formation. In addition, the transcription factor is also activated during the induction of cell lineages that give rise to parietal and visceral endoderm. HNF-3 $\alpha$  stimulation upon visceral endoderm differentiation is accompanied by the activation of HNF-3 target genes such as transthyretin, suggesting that HNF-3 $\alpha$  is involved in the developmental activation of this gene. In contrast, HNF-3 $\alpha$  target genes in parietal and primitive endoderm have yet to be identified. However, the stimulation of HNF-3 $\alpha$  during primitive endoderm formation, which is an extremely early event during murine embryogenesis, points towards a role for the factor in crucial determination processes that occur early during development.**

## INTRODUCTION

Embryonal carcinoma (EC) cells, which are cultured lines of the pluripotent stem cells of teratocarcinomas, provide an attractive system for the study of transcriptional regulation in response to extracellular stimuli [1, 2]. One of the best studied EC cell line

is mouse F9 [3]. Treatment with retinoic acid (RA) converts F9 stem cells into a new cell type that resembles primitive extraembryonic endoderm in the mouse embryo [4]. Like authentic primitive endoderm, mouse F9 cells are bipotential: further treatment with cAMP-elevating agents leads to the formation of parietal endoderm [5]; aggregation, on the other hand, yields visceral endoderm [6]. The recent identification of numerous distinct classes of retinoic acid receptors has facilitated studies that try to characterize the initial steps of RA-induced differentiation [Reviewed in 7]. By analogy to structurally related steroid and thyroid hormone receptors, RA receptors, after binding to RA, function as transcriptional enhancer factors for a specific set of genes. Some genes respond rapidly to the action of RA, indicating that their regulatory sequences are direct targets for retinoic acid receptors [8, 9]. This has been corroborated by the identification of retinoic acid response elements in a number of different promoters [10–12]. It has been suggested that at least some of these primary response (early) genes function as transcriptional regulators that control the expression of secondary (late) genes, implying that a hierarchy of steps is involved in EC cell differentiation [13]. The end result is the differentiated phenotype, which is the consequence of both gene activation and repression.

For example, retinoic acid-induced differentiation of mouse F9 stem cells into visceral endoderm leads to the activation of a set of genes that are also expressed in fetal liver, including  $\alpha$ -fetoprotein and the transthyretin genes [14, 15]. It has been demonstrated that liver-specific transcriptional activation of the transthyretin gene and possibly the  $\alpha$ -fetoprotein gene relies on the transcription factor HNF-3 (Hepatocyte Nuclear Factor 3) [16, 17]. Since  $\alpha$ -fetoprotein and transthyretin are transcriptionally induced upon F9 cell differentiation, we asked whether HNF-3 also participates in their developmental activation and whether the transcription factor is activated in response to retinoic acid.

HNF-3 was originally identified in rat hepatocytes as a liver-enriched protein required for the transcriptional activation of transthyretin and  $\alpha$ 1-antitrypsin [16]. Subsequently, it was found

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that HNF-3 actually constitutes a transcription factor family with at least three members: HNF-3 $\alpha$ , HNF-3 $\beta$ , and HNF-3 $\gamma$  [18, 19]. All three proteins share a high degree of homology in their DNA binding domains and in regions that are involved in transcriptional activation [19, 20]. More recently, additional members of the HNF-3 family were identified, which are expressed in non-hepatic murine tissues [21]. This suggests that HNF-3 proteins are also crucial for transcriptional regulation of genes in tissues other than liver.

Mouse embryonic expression studies with HNF-3 $\alpha$  and HNF-3 $\beta$  clones suggest that these liver transcriptional activators may also participate in cellular specialization during gastrulation [22]. Whole mount *in situ* hybridization of mouse embryos with HNF-3 $\beta$  probe demonstrates expression at early stages of development (primitive streak) and HNF-3 $\beta$  is transcribed in the node, notochord floor plate, and gut. The expression of HNF-3 $\alpha$ , on the other hand, is restricted to definitive endoderm, gut, and the floorplate of the midbrain. Although expression of the HNF-3 $\alpha$  and HNF-3 $\beta$  genes is restricted to liver and lung in the adult, they may also play a more extensive role in early determination events.

The presence of HNF-3 activities is not limited to rats and mice, but HNF-3 homologues were observed in other species. For example, the homeotic *Drosophila* protein forkhead shares a high degree of homology with HNF-3 in the DNA binding and transcriptional activation domain [19, 20, 23]. Other HNF-3 homologues include the *Drosophila melanogaster* sloppy paired proteins [24], the *Xenopus laevis* activin-inducible XFKH1 activity [25], the human ILF and HTLF proteins [26, 27], the *Caenorhabditis elegans* lin-31 regulator [28], and *Saccharomyces cerevisiae* factors [29, 30]. The *Drosophila*, *Caenorhabditis*, and *Xenopus* proteins are clearly involved in early developmental decision processes, confirming that members of the HNF-3 family play a critical role in early development and are not restricted to the regulation of tissue-specific gene expression [31].

In this article, we have focused on the developmental regulation of the  $\alpha$  form of the HNF-3 family. According to our studies, HNF-3 $\alpha$  activation upon retinoic acid-induced F9 cell differentiation occurs at the level of transcriptional initiation. Surprisingly, we observed that HNF-3 $\alpha$  is induced upon differentiation into primitive endoderm, a tissue for which HNF-3 target genes have not been identified yet. Finally, our data suggest that retinoic acid-dependent induction of HNF-3 $\alpha$  and maintenance of HNF-3 $\alpha$  in hepatocytes are two distinct events mediated by different *cis*-acting elements.

## MATERIALS AND METHODS

### Cell culture and differentiation

Mouse F9 embryonic carcinoma cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) + 7.5% Ultra calf serum/2.5% Fetal calf serum (Inovar). Differentiation of F9 cells with all-*trans* retinoic acid (Sigma) and dibutyl cAMP (Boehringer) was initiated as described elsewhere [32]. Differentiated phenotypes were confirmed by Northern blot analysis using appropriate markers. Human HepG2 cells were maintained as described previously [20].

### Preparation of nuclear extract

F9 cells were removed from tissue culture plates by scraping. Subsequently, the cells were washed with PBS (8mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5mM KH<sub>2</sub>PO<sub>4</sub>, 135mM NaCl, 2.5mM KCl) and pelleted by

centrifugation (1,000×g, 4°C, 5 min, Beckman AccuSpin table top centrifuge). The cell pellet was resuspended in an equal volume of lysis buffer (10mM Tris-HCl (8.0), 5mM KCl, 1.5mM MgCl<sub>2</sub>, 1mM DTT [Dithiothreitol], 0.5mM PMSF [Phenylmethanesulfonyl fluoride]) and left on ice for 15min. Lysis was initiated by douncing with a tight fitting pestle (20–25 strokes). Subsequently, cell nuclei were isolated by centrifugation (1,000×g, 4°C, 5 min, Beckman AccuSpin table top centrifuge) and extracted with 4 volumes of 0.4M NaCl buffer (20mM Tris-HCl (7.5), 0.4M NaCl, 1mM EDTA, 10% glycerol, 1mM DTT [Dithiothreitol], 0.5mM PMSF [Phenylmethanesulfonyl fluoride]) for 45 min at 4°C. The nuclear fraction was sedimented (1,000×g, 4°C, 5 min, Beckman AccuSpin table top centrifuge) and the supernatant was dialysed overnight at 4°C against an excess of dialysis buffer (20mM Tris-HCl (7.5), 50mM NaCl, 1mM EDTA, 10% glycerol, 1mM DTT [Dithiothreitol], 0.5mM PMSF [Phenylmethanesulfonyl fluoride]). Insoluble materials were removed by a centrifugation step (1,000×g 4°C, 5 min, Beckman AccuSpin table top centrifuge) and the dialysed nuclear extract was stored in aliquots at –70°C. Liver nuclear extracts were prepared as described previously [16].

### Gel shift assay and oligonucleotides

Binding of HNF-3 $\alpha$  to DNA oligonucleotides was initiated by mixing the following components: 1ng of kinased double-stranded oligonucleotide, binding buffer (20mM Tris-HCl (7.5), 5% glycerol, 40mM KCl, 1mM MgCl<sub>2</sub>, 0.5mM DTT [Dithiothreitol], 1mM EDTA), 3 $\mu$ g of poly [d(I-C)] (Boehringer), and 10–30 $\mu$ g of extract. The total reaction mixture was 30ml. After 30min at room temperature, the samples were loaded onto a 4% Polyacrylamide gel (Acrylamide/Bisacrylamide 29:1). Electrophoresis was performed for 60–90 min at 150V (room temperature). Subsequently, the gel was dried and subjected to autoradiography. The following double-stranded DNA oligonucleotide was employed. HNF-3 DNA oligonucleotide (strong HNF-3 binding site of the transthyretin promoter):

5' GTTGACTAAGTCAATAATCAGAATCAGCA 3'  
3' CAACTGATTCAGTTATTAGTCTTAGTCGT 5'

### Antiserum

To raise the anti-HNF-3 $\alpha$  antibodies, cDNA sequences encoding amino acids 7–103 of HNF-3 $\alpha$  were cloned in frame with the TrpE protein using the pATH2 expression vector [33]. TrpE-HNF-3 $\alpha$  fusion protein was expressed in *E. coli* from the induction of trp promoter by indole-3-acrylic acid (IAA), purified by preparative SDS-acrylamide gel electrophoresis, and used as an antigen to immunize New Zealand White rabbits by standard protocols [34]. The rabbit polyclonal anti-HNF-3 $\beta$  antisera was prepared by using affinity-purified glutathione-S-transferase (GST-HNF-3 $\beta$ ) (amino acid 7–86) fusion protein as an antigen, which was expressed in bacteria via the pGEX2T plasmid (Pharmacia). The production of HNF-3 antibodies was monitored during the immunization process by immunoprecipitation of the corresponding <sup>35</sup>S labeled HNF-3 protein synthesized through an *in vitro* transcription/translation system (Promega). Affinity purification involved two sequential columns containing different proteins coupled to BioRad Affi-gel 10. To remove anti-TrpE antibodies, the anti-HNF-3 $\alpha$  antisera was applied to a trpE protein column and then the flow through was affinity purified on a column containing GST-HNF-3 $\alpha$  (amino acid 7–103). The antibodies specific to HNF-3 $\alpha$  were eluted from the second

column with 0.1M glycine, pH 2.5 and were subsequently neutralized with 20 mM Tris base. The antibodies specific for HNF-3 $\beta$  were affinity purified in a similar manner except that a GST column was used as the first step to remove the anti-GST antibodies and GST-HNF-3 $\beta$  (amino acid 7–86) fusion protein was used for affinity purification.

#### Northern blot analysis

**RNA isolation.** mRNA was extracted from F9 stem and differentiated F9 cells using the Fast Track mRNA isolation kit from Invitrogen, following the instructions of the company. Starting with  $4 \times 10^8$  cells, we routinely obtained 20–50 $\mu$ g of oligo (dT)-selected mRNA.

**Blotting.** 10  $\mu$ g of F9 cell mRNA was denatured at 65°C for 15 min in the presence of 50% formamide, 1 $\times$ MOPS/EDTA (20mM MOPS [3-[N-morpholino] propanesulfonic acid], 5mM sodium acetate, 1mM EDTA, pH 7.0), 6% formaldehyde, 6% glycerol, and 0.5% bromophenol blue. After addition of 40 $\mu$ g/ml ethidium bromide, the mRNA was loaded onto a gel containing 1.2% agarose, 1 $\times$ MOPS/EDTA, and 2% formaldehyde. Following electrophoresis at 150V for 60min, the mRNA was blotted onto Zeta-Probe nylon membrane (Bio-Rad) in the presence of 10 $\times$ SET (100mM Tris-HCl, 10mM EDTA, and 1.5M NaCl, pH 7.5). 15–20 h later, the blot was baked at 80°C for 45 min.

**Probe preparation.** 20–50ng of HNF-3 $\alpha$  1.6kb cDNA insert [19] was radioactively labeled using the random primer labeling kit of Stratagene. We routinely obtained probes with specific activities of 10<sup>9</sup>cpm/ $\mu$ g. In order to detect glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA, we utilized a 1.6kb PstI GAPDH fragment as described previously [35].

**Hybridization.** Northern blots were prehybridized at 42°C for 4–8 h in 50% Formamide, 100mM Tris-HCl (pH 8.0), 6mM EDTA, 0.9M NaCl, 5 $\times$ Denhardt's (0.1% BSA, 0.1% polyvinylpyrrolidone, 0.1% polyethylene glycol 6000), 1% SDS (Sodium dodecyl sulfate), and 0.05mg/ml denatured, sonicated salmon sperm DNA. Hybridization was initiated by adding radioactively labeled HNF-3 $\alpha$ -specific probe to the hybridization solution ( $2-3 \times 10^6$  cpm/ml). After 18–24 h at 42°C, the blot was washed at 65°C with 2 $\times$ SET/0.1% SDS (60min), 0.5 $\times$ SET/0.1% SDS (60min), and 0.2 $\times$ SET/0.1% SDS (60min). Subsequent autoradiography with Kodak XAR film and Fisher Biotech L-Plus screens at –70°C was performed for 1–7 days.

#### Transfections and CAT assay

F9 stem cells were split on the day before transfection at a density of  $\sim 10^5$  cells per ml into 10cm tissue culture dishes in 10ml medium. Transfections were done by the calcium phosphate method [36, 37]. The DNA–Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> coprecipitate was formed by mixing 120mM calcium chloride with 20 $\mu$ g plasmid in 1ml HEPES buffered saline (140mM NaCl, 5mM KCl, 0.75mM Na<sub>2</sub>HPO<sub>4</sub>, 6mM glucose, 25mM HEPES [N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid], pH 7.05) and leaving at room temperature for 25 min. After the addition of fresh medium the plates were incubated at 37°C for 16 h. Cells were split 1:2 and one of the plates was differentiated into primitive, parietal, or visceral endoderm for the required

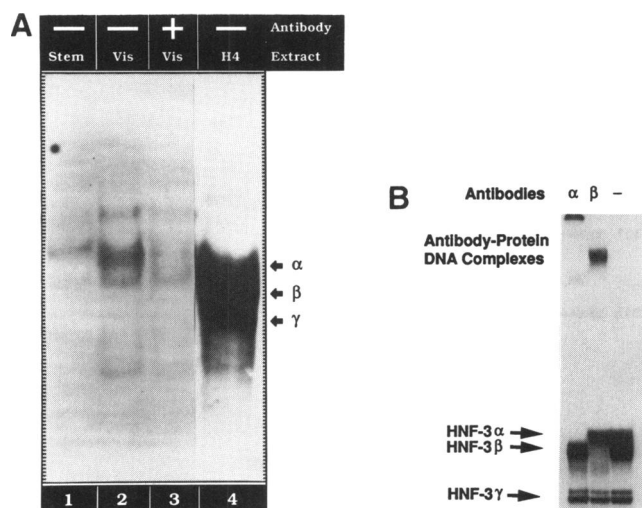
period of time while the other plate served as the stem cell control [32]. Cells were harvested after the required period of time, lysed by freezing and thawing and the extract was used for the chloramphenicol acetyl transferase (CAT) assay [36, 37]. A specified amount of protein was incubated at 65°C for 15min to inactivate the deacetylating enzymes and subsequently incubated with 20 $\mu$ l 4mM Acetyl CoA and 3 $\mu$ l [<sup>14</sup>C]Chloramphenicol at 37°C for 2 h with repeated addition of Acetyl CoA. CAT activity was monitored by thin layer chromatography on silica gel plates in CHCl<sub>3</sub>:CH<sub>3</sub>OH (95:5) followed by autoradiography. Two different constructs were used for the transfection experiments. The first construct contained approximately 5kb of 5' upstream HNF-3 $\alpha$  promoter sequences plus 42 nucleotides of the first HNF-3 $\alpha$  exon fused to the CAT gene. The second construct contained 623 nucleotides of 5' upstream HNF-3 $\alpha$  promoter sequences plus 42 nucleotides of the first HNF-3 $\alpha$  exon fused to the CAT gene. Both HNF-3 $\alpha$  promoter/CAT fusions were inserted into the pGEM2 vector (Promega). The SV40 promoter/enhancer CAT vector was obtained from Promega. Transfections into HepG2 cells were performed as outlined earlier, with CMV  $\beta$ -galactosidase constructs cotransfected as internal control [20]. HepG2 extracts used for CAT assays contained equal amounts of  $\beta$ -galactosidase activity.

## RESULTS

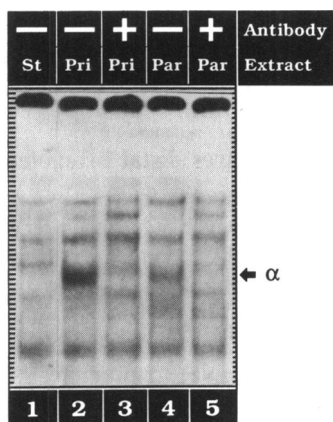
### Increase in HNF-3 $\alpha$ DNA binding activity during F9 cell differentiation

The reported induction of HNF-3-dependent genes upon differentiation of F9 cells into visceral endoderm [14–17] prompted us to examine whether members of the HNF-3 family are activated during the differentiation process. We therefore prepared nuclear extracts from F9 stem cells and F9 cells that were differentiated along the visceral endoderm cell lineage and assayed for HNF-3 binding activity by gel shift analysis. As can be seen in figure 1A, a 24 h retinoic acid treatment results in the induction of a DNA binding protein that specifically recognizes an HNF-3 binding site (lane 2) and co-migrates with HNF-3 $\alpha$  present in rat H4 hepatoma cells (lane 4). The induced band can be competed out with an excess of unlabeled HNF-3 binding site but is not affected by unrelated oligonucleotides (not shown). The binding specificity combined with the relative mobility suggested that the induced protein is indeed mouse HNF-3 $\alpha$ . In order to corroborate this assumption, we preincubated gel shift samples with HNF-3 $\alpha$ -specific antibodies. This results in specific elimination of the induced band, strongly suggesting that the visceral endoderm band is due to binding of HNF-3 $\alpha$  to the labeled oligonucleotide (lane 3). The antibody does not affect the gel shift pattern obtained with stem cell extract (not shown). To illustrate the specificity of the HNF-3 $\alpha$  antisera in gel shift assays, HNF-3 protein-DNA complexes were formed with liver nuclear extracts and then reacted with HNF-3 $\alpha$  antibody prior to gel electrophoresis. The specificity of the HNF-3 $\alpha$  antisera is demonstrated by the fact that the antibody only affected the HNF-3 $\alpha$  complex without altering the migration of the other HNF-3 complexes on the gel (Figure 1B.).

The presence of HNF-3 $\alpha$  in cells that give rise to visceral endoderm is also consistent with the reported detection of HNF-3 protein in yolk sac, which contains visceral endoderm tissue [38]. As outlined earlier, F9 cells, besides leading to visceral endoderm, also have the capacity to differentiate into parietal and

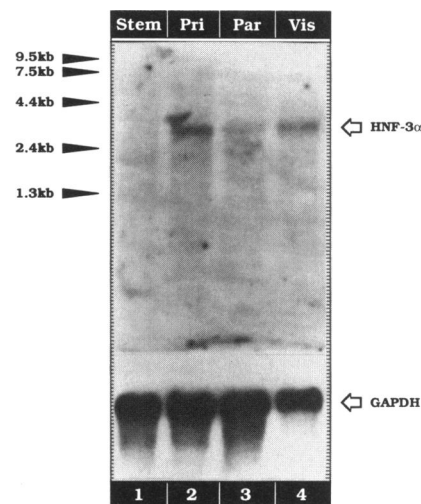


**Figure 1.** Induction of HNF-3 $\alpha$  DNA binding activity upon visceral endoderm differentiation. Panel A: Nuclear extracts were prepared from F9 stem cells (lane 1), visceral endoderm 24 h post-differentiation (lanes 2 and 3), and rat hepatoma H4 cells (lane 4). Gel shift reactions were initiated with 30 $\mu$ g of extract and 1ng of transthyretin oligonucleotide containing HNF-3 binding site in the presence (+) or absence (–) of HNF-3 $\alpha$ -specific antibody. The positions of the three HNF-3 forms are indicated on the right. In order to improve resolution of various HNF-3-DNA complexes, the free probe was electrophoresed out of the gel. Panel B: The HNF-3 oligonucleotide was used for complex formation with liver nuclear extract (6mg) and incubated in the presence ( $\alpha$ ) or absence (–) of HNF-3 $\alpha$  antiserum prior to electrophoresis on a native polyacrylamide gel. As a guide for the position of the HNF-3 $\beta$  complex on the gel, HNF-3 liver complexes were reacted with HNF-3 $\beta$  antiserum (lane b).



**Figure 2.** Induction of HNF-3 $\alpha$  DNA binding activity upon differentiation into primitive and parietal endoderm. Gel retardation reactions were performed as described in materials and methods with HNF-3 DNA oligonucleotides and nuclear extracts derived from F9 stem cells (lane 1), primitive endoderm cells (lanes 2 and 3), and parietal endoderm cells (lanes 4 and 5). (+) indicates the presence of HNF-3 $\alpha$  antibody, (–) its absence. Primitive and parietal endoderm cells were harvested 24 h after induction of differentiation by retinoic acid.

primitive endoderm [4, 5]. We therefore investigated whether HNF-3 $\alpha$  is also induced upon differentiation into these two tissue types. Using gel shift assays, we detected bands in 24h old primitive and 24h old parietal endoderm that exhibit the same electrophoretic mobility as HNF-3 $\alpha$  (Figure 2, lanes 2 and 4).

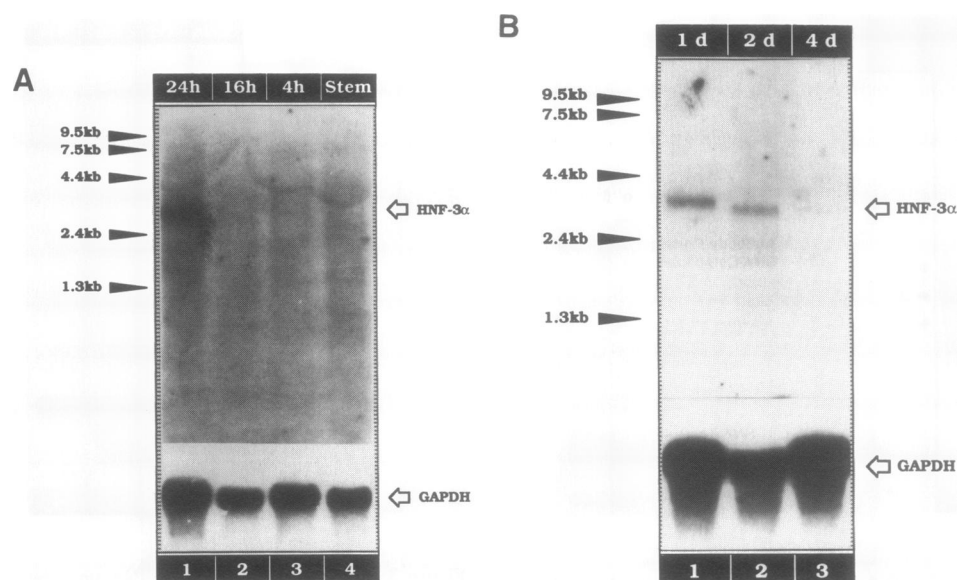


**Figure 3.** Activation of HNF-3 $\alpha$  mRNA upon retinoic acid-induced F9 cell differentiation. mRNA was isolated from F9 stem cells (lane 1), differentiated primitive endoderm (lane 2), differentiated parietal endoderm (lane 3), and differentiated visceral endoderm (lane 4). In each case, cells were allowed to differentiate for 24 h in the presence of retinoic acid. 10 $\mu$ g each of mRNA was analyzed by denaturing agarose gel electrophoresis, blotted onto nylon membrane, and hybridized with a HNF-3 $\alpha$ -specific probe. Following autoradiography, the blot was stripped and rehybridized with a GAPDH probe to determine the loading efficiency. The positions of HNF-3 $\alpha$  mRNA (3.2 kb) and mRNA markers are indicated.

These bands were specifically eliminated by preincubation with HNF-3 $\alpha$  antibodies (lanes 3 and 5). Compared to primitive endoderm, HNF-3 $\alpha$  activity in parietal endoderm is diminished, indicating that dibutyl cAMP (which is used in combination with retinoic acid to induce this phenotype) exerts a negative effect on HNF-3 $\alpha$  induction. Taking this into consideration, we conclude that murine HNF-3 $\alpha$  is also induced upon retinoic acid-mediated differentiation into primitive endoderm and that cAMP suppresses HNF-3 $\alpha$  activation.

### Retinoic acid treatment activates HNF-3 $\alpha$ mRNA

The experiments described so far were performed by means of gel shift assay, a technique that is sensitive to both the level and DNA binding affinity of transcription factors. It is therefore conceivable that the observed induction of HNF-3 $\alpha$  is due to increased amounts or posttranslational modifications which alter the DNA binding affinity of the (pre-existing) factor. Alternatively, stimulation of HNF-3 $\alpha$  binding activity may be the result of increased mRNA levels. In order to discriminate between these mechanisms, we have compared HNF-3 $\alpha$  mRNA concentrations from F9 stem and differentiated F9 cells by Northern blot analysis. The results depicted in figure 3 clearly show that F9 stem cells do not contain any HNF-3 $\alpha$ -specific mRNA molecules (lane 1). In contrast, we were able to detect a ~3.2kb long HNF-3 $\alpha$  mRNA in cells differentiated along the primitive, parietal, and visceral endoderm lineage (lanes 2–4). Consistent with the gel shift data, induction of HNF-3 $\alpha$  mRNA is lowest upon differentiation into parietal endoderm and HNF-3 $\alpha$  mRNA is barely visible (lane 3). This is not due to differential loading since the signal for the GAPDH (Glyceraldehyde-3-phosphate-dehydrogenase) control mRNA is virtually identical in all four lanes (lane 3 actually contains slightly more mRNA than the other lanes). In addition, we have confirmed



**Figure 4.** Temporal profile of HNF-3 $\alpha$  mRNA levels upon visceral endoderm formation. Following the protocol in materials and methods, mRNA was extracted from F9 stem cells, and visceral endoderm cells that had been differentiated with  $10^{-7}$ M of retinoic acid for various times. Northern blots, containing 10 $\mu$ g of mRNA in each lane, were hybridized for 40 h at 42°C with a HNF-3 $\alpha$ -specific probe. In order to control the loading efficiency, the blots were rehybridized with GAPDH probe. The positions of HNF-3 $\alpha$  mRNA (3.2 kb) and mRNA markers are shown. Panel A: F9 stem mRNA (lane 4) and visceral endoderm mRNA prepared 4 h (lane 3), 16 h (lane 2), and 24 h (lane 1) post-differentiation. Panel B: Visceral endoderm mRNA extracted from cells 1d (lane 1), 2d (lane 2), and 4d (lane 3) after addition of retinoic acid.

that differentiation into parietal endoderm did take place by performing Northern blots with a laminin probe, a marker for this cellular lineage ([5], not shown). In addition, we have ensured that F9 cells differentiate into primitive and visceral endoderm by using the following markers: transthyretin [15] (visceral endoderm), tissue plasminogen activator [39] (primitive endoderm). Parietal endoderm formation, in contrast to primitive and visceral endoderm differentiation, requires elevated levels of cAMP [5]. Therefore, it cannot be ruled out that cAMP exerts a negative effect on HNF-3 $\alpha$  expression. In summary, we have observed activation of HNF-3 $\alpha$  upon retinoic acid-induced F9 cell differentiation by performing gel shifts and Northern blots. Both techniques yield similar (if not identical) temporal activation profiles. That is, HNF-3 $\alpha$  activation occurs upon the induction of pathways that lead to primitive, parietal, and visceral endoderm within 24 h post-differentiation. In light of these data, it is highly likely that developmental activation of HNF-3 $\alpha$  transcription factor is mainly due to activation of its corresponding mRNA.

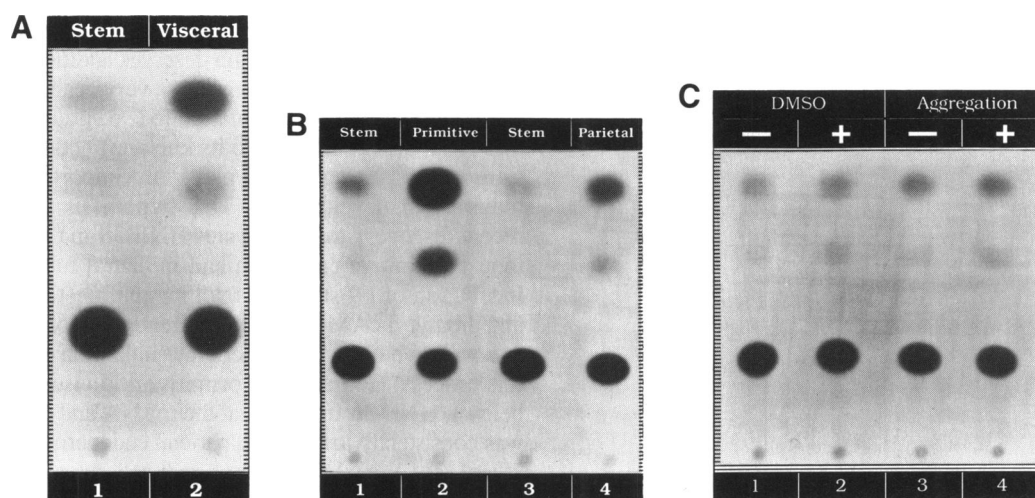
#### Temporal pattern of HNF-3 $\alpha$ induction

According to our results, HNF-3 $\alpha$  is activated upon F9 cell differentiation within a 24 h period, following addition of retinoic acid to the cells (Figures 1–3). In order to obtain more accurate information about the induction profile of HNF-3 $\alpha$ , we have measured the amount of endogenous HNF-3 $\alpha$  mRNA with the help of the Northern blot technique. As depicted in figure 4A, HNF-3 $\alpha$  activation upon visceral endoderm formation takes place between 16 and 24 h post-differentiation. Again, the level of GAPDH (Glyceraldehyde-3-phosphate-dehydrogenase) control mRNA does not fluctuate significantly, indicating that the observed activation pattern is not due to a loading artifact. Furthermore, we also determined HNF-3 $\alpha$  mRNA levels in cells differentiated along the visceral endoderm pathway for more than

24 h. The data in figure 4b clearly show that HNF-3 $\alpha$  stimulation is transient, reaching a peak between 1 and 2d post-differentiation, and followed by a decline of HNF-3 $\alpha$  mRNA to almost undetectable levels. We are still able to detect HNF-3 $\alpha$  mRNA 3 days after induction of differentiation and overexposure of figure 4A reveals low, but detectable, levels of HNF-3 $\alpha$  in the 4 day time point (not shown).

#### HNF-3 $\alpha$ induction requires distal promoter elements

Increase in mRNA abundance is frequently a result of induction of gene transcription. However, it has been reported that changes in the level of certain mRNAs upon F9 cell differentiation involve posttranscriptional mechanisms, such as alterations in transcriptional elongation [40, 41]. In an attempt to determine the mechanism of retinoic acid-induced HNF-3 $\alpha$  activation, we have asked whether the HNF-3 $\alpha$  promoter is able to mediate HNF-3 $\alpha$  stimulation upon F9 cell differentiation. For this purpose, we prepared a construct that contains approximately 5kb of HNF-3 $\alpha$  5' upstream sequences fused to the CAT (Chloramphenicol acetyltransferase) gene. The amount of CAT enzyme activity serves as an indicator of HNF-3 $\alpha$  promoter strength. In order to eliminate the well-documented problem of differential DNA uptake between stem and differentiated cells, we adhered to the following protocol for all transfections: Approximately 24 h after plasmid transfection, the F9 cells were split and one half was left untreated while the other half was differentiated along pathways that led to primitive, parietal, or visceral endoderm. 24 h post-differentiation, all cells were harvested and CAT assays were performed. Using this approach, the 5kb HNF-3 $\alpha$  promoter construct was initially transfected into F9 stem cells and CAT activity was determined upon differentiation along the visceral endoderm cell lineage (figure 5A). While HNF-3 $\alpha$  promoter is only marginally active in stem



**Figure 5.** Induction of a 5kb HNF-3 $\alpha$  promoter upon F9 cell differentiation. Panel A: F9 stem cells were transfected with a HNF-3 $\alpha$  promoter/CAT fusion construct containing approximately 5kb of promoter sequences. 20 h later, the cells were divided. One half was left untreated, the other half was transferred to bacterial petri dishes and visceral endoderm formation was induced by adding  $10^{-7}$ M of retinoic acid. 24 h post-differentiation, cells were harvested, extracts were prepared, and CAT assays were performed as outlined in materials and methods. Lane 1: Promoter activity in F9 stem cells. Lane 2: Promoter activity in visceral endoderm cells. Panel B: Transfections and CAT assays with the 5kb HNF-3 $\alpha$  promoter/CAT construct were done as described under A. However, primitive endoderm differentiation (lane 2) was initiated by adding  $10^{-7}$ M of retinoic acid to regular tissue culture plates and parietal endoderm differentiation required the addition of  $10^{-7}$ M retinoic acid/ $10^{-3}$ M dibutyryl cAMP to regular tissue culture dishes (lane 4). The activity of the construct in F9 stem cells is depicted in lanes 1 and 3. Panel C: Following the transfection of the 5kb HNF-3 $\alpha$  promoter/CAT vector, F9 stem cells were treated for 24 h with  $10^{-3}$ % of DMSO (lane 2) or aggregated for 24 h in bacterial petri dishes (lane 4). Lanes 1 and 3 are untreated controls.

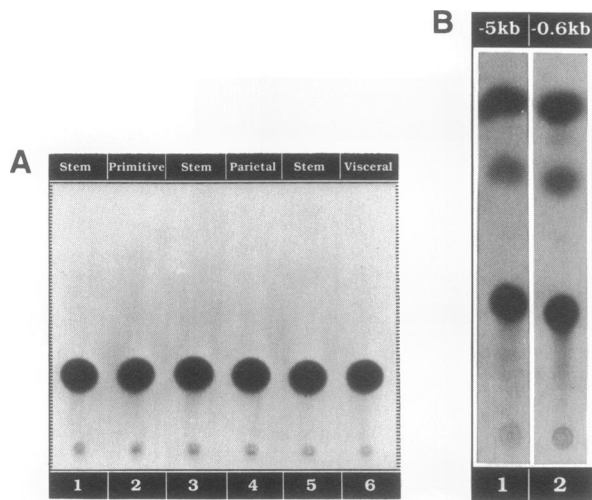
cells (lane 1), it is induced upon differentiation into visceral endoderm (lane 2). Moreover, the promoter is also capable of responding to retinoic acid upon differentiation into parietal and primitive endoderm (figure 5B). We have performed more than six independent transfections for each differentiated pathway. In each case, we detected a strong induction in response to retinoic acid. Occasionally, we observed a weaker response upon formation of parietal endoderm (figure 5B). However, the low level of HNF-3 $\alpha$  induction upon parietal endoderm formation was much more pronounced at the mRNA and DNA binding level, indicating that cAMP, which is needed in addition to retinoic acid for parietal endoderm formation, acts primarily at the posttranscriptional level. To demonstrate the feasibility of our transfection strategy, we have utilized a plasmid construct that contains the SV40 promoter/enhancer fused to the CAT gene. It has been reported that the SV40 promoter/enhancer is only marginally active in F9 stem cells, but is strongly induced upon F9 cell differentiation [42]. Following the above outlined protocol, the viral construct yields the expected result. That is, low activity in F9 stem cells and substantial induction upon retinoic acid-induced differentiation (not shown). In order to show that retinoic acid is responsible for the observed stimulation, we performed an additional control experiments. Our retinoic acid stock solution is made in DMSO (Dimethyl sulfoxide), and we show that the addition of DMSO alone does not effect HNF-3 $\alpha$  induction (figure 5C, lane 2). In addition, we demonstrate that cell aggregation alone, which is needed for visceral endoderm formation, is not sufficient for HNF-3 $\alpha$  promoter activation (figure 5C, lane 4).

In order to delineate the promoter sequences that mediate the response to retinoic acid, we made use of a second chimeric construct that contains 0.6kb of HNF-3 $\alpha$  promoter sequences fused to the CAT gene. As can be seen in figure 6A, this construct

is not able to respond to retinoic acid, irrespective of the differentiation pathway. Again, we have performed five independent transfection studies and never encountered a retinoic acid-dependent induction. Although these results are depicted in a different figure, they were performed in parallel with the transfections that utilize the 5kb HNF-3 $\alpha$  promoter. This clearly shows that the F9 cells were responding to retinoic acid treatment. Furthermore, in order to rule out that the 0.6kb promoter construct is transcriptionally inactive, we transfected the construct into human hepatoma cells (HepG2) which express high levels of endogenous HNF-3 $\alpha$ . Interestingly, both the 5kb and the 0.6kb promoter constructs are equally active in HepG2 cells, as determined by CAT assay (figure 6B). This result suggests that transient expression of HNF-3 $\alpha$  promoter constructs in hepatoma cells is mediated by only 600 nt of upstream sequences, whereas retinoic acid-induced activation requires additional upstream promoter sequences. We therefore conclude that the *cis*-acting elements that confer retinoic acid responsiveness are located between 0.6 and 5kb upstream of the HNF-3 $\alpha$  transcriptional start site.

## DISCUSSION

Given the presented experiments, it is clear that the lipophilic hormone retinoic acid is able to stimulate HNF-3 $\alpha$  expression upon embryonal carcinoma cell differentiation. This is the first report demonstrating that HNF-3 $\alpha$  is able to respond to the vitamin A metabolite. Our data show that the retinoic acid-mediated HNF-3 $\alpha$  induction occurs at the level of transcriptional initiation and is conferred by distal promoter sequences. More recently, we have obtained evidence showing that  $\sim 2.0$  kb of upstream sequences are sufficient to mediate the retinoic acid response [43]. We are now in a position to ask further critical



**Figure 6.** Activity of a 0.6 kb HNF-3 $\alpha$  promoter upon F9 cell differentiation. Panel A: Mouse F9 embryonal carcinoma cells were transfected with plasmid containing approximately 0.6kb of HNF-3 $\alpha$  promoter fused to the CAT gene. 20 h later, cells were divided into equal parts and differentiated into primitive (lane 2), parietal (lane 4), and visceral endoderm (lane 6) as detailed in figure 5. After allowing cells to differentiate for 24 h, extracts were prepared and promoter activity was determined by performing CAT assays. Lanes 1, 3, and 5 depict 0.6kb HNF-3 $\alpha$  promoter strength in F9 stem cells. Panel B: Human HepG2 cells were transfected with HNF-3 $\alpha$  promoter/CAT constructs containing 5kb and 0.6kb of promoter sequences, respectively, as described under Materials and Methods. 36 h later, cells were harvested and promoter activity was measured with the help of CAT assay. Lane 1: Transfection with the 5kb HNF-3 $\alpha$  promoter. Lane 2: Transfection with the 0.6kb HNF-3 $\alpha$  promoter.

questions about the activation mechanism of HNF-3 $\alpha$ . For example, does the HNF-3 $\alpha$  promoter element directly interact with retinoic acid receptor or does it bind to a non-receptor protein? The relatively late induction of HNF-3 $\alpha$  (16–24 h post-differentiation, figure 4A) argues against HNF-3 $\alpha$  being a primary target for activated retinoic acid receptor, since established primary response genes such as Hox 1.3, Hox 1.6, and Hox 4.2 are activated within 2–6 h of retinoic acid treatment [9, 12, 44, 45]. However, it has been demonstrated that the promoter of the laminin B1 gene, whose transcription rate in response to retinoic acid increases after 24–48 h, contains a retinoic acid response element [46]. Further, it is thought that laminin B1 activation is mediated by a direct interaction between retinoic acid receptors and B1 promoter. Therefore, it remains possible that HNF-3 $\alpha$  stimulation is brought about by binding of retinoic acid receptor to the retinoic acid response element in the HNF-3 $\alpha$  promoter. In addition, it has been proposed that primary target genes not only control the expression of tissue-specific genes but also regulate the transcription of secondary transcription factors [7, 13]. The presence of HNF-3 $\alpha$  binding sites in the promoters of transcription factors HNF-3 $\beta$  and HNF-1, both of which are induced after HNF-3 $\alpha$  during F9 cell differentiation, lends further support to the notion that HNF-3 $\alpha$  functions as a primary response gene [47–50]. Future investigations will identify the position of HNF-3 $\alpha$  in the retinoic acid signal transduction pathway.

According to our results, HNF-3 $\alpha$  is induced upon retinoic acid-mediated differentiation into primitive endoderm. This result was somewhat unexpected since no HNF-3 $\alpha$  target genes have

been reported for this tissue yet. The activation of HNF-3 $\alpha$  upon primitive endoderm formation deserves additional attention. That is, primitive endoderm appears very early during murine development, before gastrulation [51, 52], and it is conceivable that HNF-3 $\alpha$ , in addition to its known function as liver-specific transcriptional regulator, plays an important role in early mammalian development. This hypothesis is supported by a recent discovery that implicates HNF-3 $\alpha$  in the establishment of mouse definitive endoderm and in neural tube patterning [22]. Incubation of F9 stem cells with a combination of retinoic acid and dibutyryl cAMP leads to the generation of parietal endoderm and we have observed a moderate induction of HNF-3 $\alpha$  during this process. Compared to primitive and visceral endoderm (cf. below), HNF-3 $\alpha$  induction at the mRNA and DNA binding level was consistently lower upon parietal endoderm formation (figures 2 and 3). We also detected reduced rates of transcriptional initiation (figure 5B). However, this was less consistent and at a lower magnitude. Parietal endoderm is the only tissue that requires cAMP-elevating agents for its induction. This suggests that cAMP is able to modulate HNF-3 $\alpha$  expression in a negative fashion, perhaps at the posttranscriptional level. Finally, HNF-3 $\alpha$  is activated along the visceral endoderm cell lineage and can be detected 3 days after addition of retinoic acid. At this time, the HNF-3 $\alpha$  target genes transthyretin and  $\alpha$ -fetoprotein can be detected in visceral endoderm [14–17], suggesting that HNF-3 $\alpha$  plays a major role in the developmental induction of these two genes. Although HNF-3 $\alpha$  activity disappears around the time of transthyretin and  $\alpha$ -fetoprotein induction (figure 4), it is possible that HNF-3 $\alpha$  is required for the *de novo* induction, but not maintenance, of these genes. The recent identification of HNF-3 $\alpha$  mRNA in mouse yolk sac (not shown) is also consistent with our hypothesis that the transcription factor participates in the activation of visceral endoderm specific genes. On the other hand, it cannot be ruled out that HNF-3 $\alpha$  induction upon F9 cell differentiation occurs only in primitive endoderm which seems to serve as a precursor for both parietal and visceral endoderm [5, 6]. For example, previous data suggest that during the early phase of visceral endoderm formation, primitive and visceral endoderm cells are present at the same time [6]. If HNF-3 $\alpha$  induction occurs only in primitive endoderm, one would detect activation despite differentiation along the visceral endoderm lineage. However, we believe that this is not likely since we have been able to detect HNF-3 $\alpha$  mRNA in mouse visceral yolk sac.

The use of the F9 embryonal carcinoma cell system offers the crucial advantage of identifying regulators that participate in the *de novo* activation of HNF-3 $\alpha$ . Since the transcription factor (and its target genes transthyretin and  $\alpha$ -fetoprotein) are also induced upon hepatogenesis, results obtained with our system will be useful for the elucidation of the mechanism involved in liver-specific gene expression. So far, pertinent investigations have been extremely limited and conclusions about establishment of hepatocyte-specific transcription have been drawn by analyzing the expression pattern of liver-specific promoter factors in various different tissues. Our results show that retinoic acid-induced HNF-3 $\alpha$  stimulation during F9 cell differentiation is mediated by distal promoter elements (figures 5 and 6). We are now in a position to characterize the promoter-binding activities that regulate HNF-3 $\alpha$  induction. In contrast, our results indicate that maintenance of HNF-3 $\alpha$  expression in hepatocytes requires promoter sequences that are positioned closer to the transcriptional start site. This suggests that maintenance and developmental induction of HNF-3 $\alpha$  expression are two distinct

events that are mediated by different *cis*-acting promoter elements.

It is important to realize that visceral endoderm, which is formed upon F9 cell differentiation, is of extraembryonic origin (the same is true for primitive and parietal endoderm) and is not a precursor for fetal liver. However, visceral endoderm formation and hepatogenesis share a number of critical transcriptional regulatory events. That is, both processes are accompanied by the activation of transthyretin,  $\alpha$ -fetoprotein, and HNF-3 $\alpha$  transcription factor. Because of this close resemblance, it is likely that induction of these genes during visceral endoderm differentiation utilizes the same mechanism that is employed during hepatogenesis. Specifically, does HNF-3 $\alpha$  activation during organogenesis require promoter elements that mediate retinoic acid action? Confirmation of a direct role for retinoic acid in the induction of liver-specific transcription would be a major step forward in our understanding of the mechanism that leads to tissue-specific expression. According to the current model, liver-specific transcription is thought to involve a series of consecutive transcriptional regulatory events. That is, liver-specific proteins are transcriptionally controlled by liver-specific transcription factors, which require another set of transcriptional activators for their stimulation, and so on. However, induction of liver-specific gene expression during the early stages of development cannot be performed by yet another transcriptional event but requires a pre-existing activator. Retinoic acid receptor is an ideal candidate for such a latent activator. Our experiments have placed us in a position that will allow us to answer whether this is indeed the case.

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