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An alternative splice variant of the tissue specific transcription factor HNF4α predominates in undifferentiated murine cell types

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ABSTRACT

The transcription factor hepatocyte nuclear factor 4α (HNF4α) is a tissue specific transcription factor mainly expressed in the liver, kidney, intestine and the endocrine pancreas, but is also an essential regulator for early embryonic events. Based on its protein structure HNF4α is classified as an orphan member of the nuclear receptor superfamily. Comparing HNF4α transcription factors in the differentiated and dedifferentiated murine hepatocyte cell line MHSV-12 we identified in dedifferentiated cells the novel splice variant HNF4α7. This variant is characterized by an alternative first exon and has a lower transactivation potential in transient transfection assays using HNF4 dependent reporter genes. HNF4α7 mRNA and the corresponding protein are expressed in the undifferentiated pluripotent embryonal carcinoma cell line F9, whereas HNF4α1 only appears after differentiation of F9 cells to visceral endoderm. HNF4α7 mRNA is also found in totipotent embryonic stem cells. However, the function of HNF4α7 seems not to be restricted to embryonic cells as the HNF4α7 mRNA is also present in adult tissues, most notably the stomach. All these features suggest that the presence of distinct splice variants of HNF4α modulates the activity of HNF4α in a cell type specific way.

INTRODUCTION

Hepatocyte nuclear factor 4 (HNF4) constitutes a transcription factor family (1,2) whose first member HNF4α has been identified as a factor interacting with promoter elements mediating liver specific transcription (3). Based on the zinc finger motif of the DNA binding domain and on a potential ligand binding domain HNF4 is classified as a member of the nuclear orphan receptor superfamily (4). HNF4α turned out to be present also in non-hepatic cells such as kidney, intestine, stomach and pancreas (5–7). The importance of HNF4α in gene control in tissues distinct from the liver has been documented by the fact that a human inherited disease is based on the expression of a mutated HNF4α gene in the endocrine pancreas leading to maturity-onset diabetes of the young (MODY1; 8). In addition to the role of HNF4α as a tissue specific transcription factor it functions also in early embryogenesis: in the *Xenopus* egg we identified the HNF4α protein as a maternal transcription factor that contributes to the zygotic activation of the gene encoding HNF1α (9). In mouse embryos HNF4α transcripts have been identified in the primary endoderm as early as day 4.5 and later in the liver diverticulum, the gut and nephrogenic tissues starting from day 8.5 (6,10). The early embryonic requirement of HNF4α is well established in the mouse, as the knock-out of the HNF4α gene leads to a disturbance of gastrulation and thus to early embryonic lethality (11). The embryonic role of HNF4 seems to be evolutionary conserved, as maternal HNF4 mRNA has been identified in the *Drosophila* egg. Furthermore, a large chromosomal deletion spanning the HNF4 locus results in defects in the gut and malpighian tubules of the developing *Drosophila* and these malformations are interpreted that HNF4 plays similar functions in invertebrates and vertebrates (7).

In addition to HNF4α we have recently identified two other members of the HNF4 family, i.e. HNF4β in *Xenopus* (2) and HNF4γ in humans (1). Both these new members of the HNF4 family seem to have distinct but overlapping functions compared to HNF4α, since both gene products show a differential expression pattern and a distinct transactivation potential of HNF4 dependent reporter genes.

Further complexity of gene control by HNF4α transcription factors is apparently given by the differential splicing of the 10 initially identified exons of the HNF4α gene (6,8). Thus far, six distinct splice variants of HNF4α have been identified in human and murine cDNA samples. We use the nomenclature most recently used for the human splice variants (12): HNF4α1 represents the initially identified transcript (3) and HNF4α2 through HNF4α6 are the splice variants identified subsequently. HNF4α2 is a splice variant that contains a larger exon 9 resulting in an insertion of 10 additional amino acids in the Pro-rich C-terminal part and has been identified in the rat (13), the mouse (14) and human (1,15). HNF4α3 initially refered to as HNF-4C (16) has been identified in human liver cDNA and is characterized by a shorter and distinct C-terminus, whereas HNF4α4 found in human kidney and liver RNA contains in the N-terminal region the two additional exons 1B and 1C (1,12). The fifth splice variant HNF4α5 carries the exon 1B and 1C found in

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HNF4α4 together with the larger exon 9 found in HNF4α2, whereas HNF4α6 combines exon 1B and exon 1C with the C-terminal truncation found in HNF4α3 (12). We speculate that all these splice variants have unique properties allowing a modulation of the HNF4α activity. Indeed, we have shown in transient transfection assays using HNF4 dependent reporter genes, that HNF4α4 has a lower transactivation potential compared to HNF4α2 (1). However, it should be stressed that all these HNF4α variants have only been identified as mRNAs and the existence of the corresponding proteins has not been proven.

The broad spectrum of tissues expressing HNF4α argues for a function of HNF4α in the establishment of the differentiation of various cell types, but most data concerning the role of HNF4α have been investigated in hepatic cells. Most notably, the presence of HNF4α expression has been correlated with the differentiated phenotype of hepatocytes (reviewed in 17) . In fact, it has been shown that the introduction of HNF4α in dedifferentiated hepatocytes can lead to a redifferentiation as shown by the activation of several liver specific functions (18–20), whereas the forced expression of a dominant negative mutant down-regulates several liver specific genes in differentiated hepatoma cells (21,22).

In the present report we analyzed in the murine cell line MHSV-12 the highly differentiated hepatic phenotype that is reversibly decreased in serum-containing culture medium (23). We reasoned that this change in hepatic differentiation might correlate with an altered expression of HNF4α. Using these cells we succeeded to define a novel HNF4α7 splice variant that is expressed in a tissue specific manner and most notably found as the major component in embryonic stem cells.

MATERIALS AND METHODS

Nucleotide sequence accession number

The sequence of the splice variant HNF4α7 was deposited in GenBank (accession number AF015275).

Cell cultures and transfections

All culture media contained penicillin (100 U/ml) and streptomycin (100 U/ml). The MHSV-12 cell clone was maintained in the chemically defined MX83 medium supplemented with 1 µg/ml transferrin, 10 µg/ml insulin, 1 µg/ml hydrocortisone and 100 ng/ml EGF as described (23). For splitting, the cells were trypsinized and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated calf serum for one day before adding the MX83 medium. F9 cells were propagated on gelatine coated cell culture dishes in DMEM with 10% heat-inactivated calf serum. To induce visceral endoderm differentiation the F9 cells were seeded into bacterial dishes in the presence of 2×10^{-7} M retinoic acid (24,25). Embryonic stem cells (ES cells) (E14-1) were cultured on mitomycin-treated feeder cells in DMEM containing 15% heat inactivated fetal calf serum and supplemented with MEM non-essential amino acids (Sigma), 2 mM glutamine and 100 µM β-mercaptoethanol.

For transient transfection of C2 cells the DNA-calcium phosphate coprecipitation method was used (26), whereas for Hela and MHSV-12 cells the lipofectin transfection protocol was applied (1).

Extract preparation and gel retardation assays

High salt extracts of crude nuclear pellets of cultured cells were prepared as described (27) and the gel retardation assays (28) were performed using the HNF4 binding site of the human apolipoprotein B promoter (2) or the *Xenopus* HNF1α promoter (26). The monoclonal antibody recognizing the A/B-domain of HNF4α1 has been described (29). The polyclonal antibodies anti-A/B/C and anti-F were raised against the N-terminus of the rat HNF4α1 protein (29) and a peptide of the F-domain of the *Xenopus* HNF4α protein (2), respectively. These polyclonal antisera were produced in rabbits by Eurogentec.

5′ RACE and RT-PCR

For the 5′ RACE, RNA was purified with the RNA-Clean™ System (AGS, Heidelberg) from MHSV-12 cells grown in serum containing DMEM and 5 µg aliquots reverse transcribed with the HN7 primer into cDNA using the Superscript™ II plus kit (GIBCO/BRL). The 5′-end of the cDNA was amplified with the 5′-AmpliFINDER™ RACE kit (CLONTECH) by ligating the oligonucleotide H_2N-TGGGCCTCTAGACTTAAG-5′ to the 3′-end of the cDNA. This tag was used for priming with the oligonucleotide P11 (5′-ACCCGGAGATCTGAATTC-3′) and the PCR product was cloned. For RT-PCR cDNA was synthesized with random primer using 5 µg RNA. To detect HNF4α transcripts the reverse primer HN7 (5′-CCTTAAGGCTTCCTTCTTCATGCCAGCCCGG-3′) and the forward primers HN6 (5′-GGAATTCCGGCATGGATAT-GGCCGACTACAGCGC-′) or HN18 (5′-GGGTACCCTTGGTC-ATGGTCAGTG-3′) were used (see Fig. 3A). The oligonucleotide HN7 and HN6 contain linker sequences (underlined) at the 5′-end not present in the cDNA. To quantitate GAPDH mRNA, the oligonucleotides 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′, were used as forward and reverse primers, respectively. To quantitate the HNF4α transcripts as illustrated in Figures 6 and 7 the PCR reactions contained in addition of the 200 µM dNTP a labeled triphosphate, i.e. 1 µCi/50 µl reaction (α-^{32}P)dCTP. This allows quantification of the PCR fragments with a phosphor imager. In addition this sensitive detection permits a lower cycle number and thus avoids reaching the saturation of the PCR (30). All reactions were done at 62 and 72°C for annealing and extension, respectively.

RESULTS

A novel HNF4α protein in dedifferentiated hepatocytes

It is well established that the composition of the culture medium has a profound effect on the differentiation state of hepatocytes (23). In general the presence of serum leads to substantial dedifferentiation of the hepatic phenotype. To explore whether the cultivation conditions of hepatocytes has any effect on the expression of tissue specific transcription factors known to be responsible for the hepatic phenotype, we determined the presence of liver transcription factors in the immortalized murine hepatic cell line MHSV-12. This cell line has been found to express most abundantly transcripts of liver specific proteins such as albumin, aldolase A and B as well as tyrosine amino transferase, but this liver specific expression pattern is reversibly decreased in serum containing medium (23). Analyzing the expression of HNF1α in the MHSV-12 cell line cultured under various conditions we did not detect any change in this transcription factor (data not shown). However, probing HNF4α

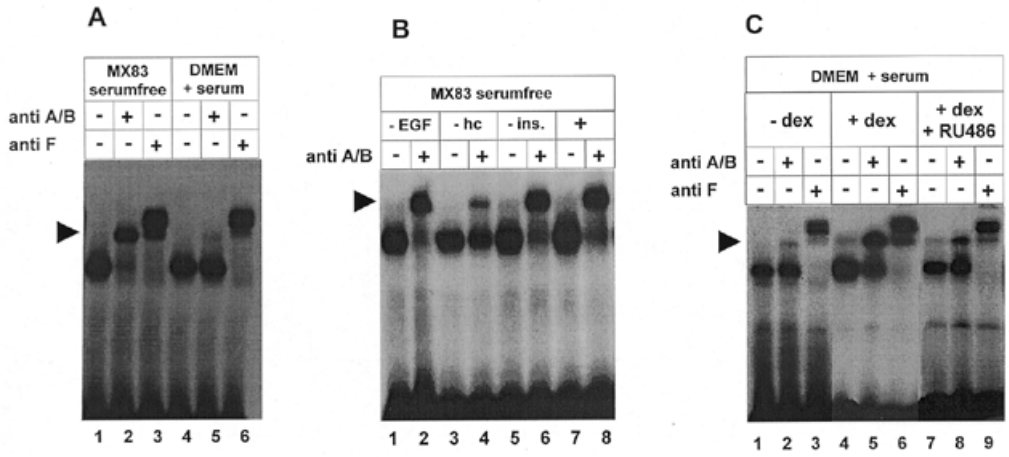

Figure 1. The HNF4α protein of MHSV-12 cells reacts with the A/B-domain specific antibody depending on the conditions used to culture the cells. Nuclear extracts of MHSV-12 cells were used for gel retardation assays with the HNF4 binding site of the apolipoprotein promoter. The addition of specific antibodies is given and the arrow head points to the complex supershifted by the A/B-domain specific antibody. (**A**) Extracts were prepared from cells grown either in the chemically defined MX83 medium lacking serum (lanes 1–3) or in DMEM containing calf serum (lanes 4–6). (**B**) Extracts were derived from cells maintained in complete MX83 medium (lanes 7 and 8) or in MX83 medium lacking either epidermal growth factor (lanes 1 and 2), hydrocortisone (lanes 3 and 4) or insulin (lanes 5 and 6). (**C**) Nuclear proteins were extracted from cells grown in DMEM with 10% serum. Dexamethasone (dex) and its antagonist RU486 were added to the cells at 10^{-7}M and 3×10^{-4}M, respectively, as indicated.

in gel retardation assays using various antibodies we detected some significant differences: as illustrated in Figure 1A, in extracts of MHSV-12 cells grown in serum-free MX83 medium, that maintains a highly differentiated hepatic phenotype (23), HNF4α could readily be detected in gel retardation experiments (lane 1) and this DNA–protein complex could be supershifted by an antibody raised against the A/B-domain or the F-domain of HNF4α (lanes 2 and 3, respectively). In contrast, in extracts derived from MHSV-12 cells cultivated in the presence of serum, that leads to a dramatic dedifferentiation (23), we observed that the HNF4α protein detected in gel retardation assays (lane 4) did not react with the antibody specific for the A/B-domain of HNF4α (lane 5). However, in the same extracts the presence of HNF4α protein could be verified with the antibody specific for the F-domain (lane 6). These results imply that the properties of HNF4α change in MHSV-12 cells depending on the culture conditions.

The type of HNF4α protein present in MHSV-12 cells is dependent on glucocorticoids

To identify the components in the culture medium that change the properties of the HNF4α protein, we cultivated the MHSV-12 cells in MX83 medium lacking defined components and analyzed the extracts of these cells in the gel retardation assay using the A/B-domain specific antibody. As Figure 1B illustrates, the omission of hydrocortisone in the culture medium results in cell extracts with HNF4α protein that mostly cannot be supershifted by the A/B-domain specific antibody (lanes 3 and 4). In contrast extracts derived from cultures grown in the absence of EGF (lanes 1 and 2) or insulin (lanes 5 and 6) contain HNF4α that is predominantly supershifted by the A/B-domain specific antibody. Consequently, the presence of hydrocortisone in the serum-free medium leads to the expression of the HNF4α form that is recognized by the A/B-domain specific antibody.

In a complementary approach we investigated the effect of the glucocorticoid hormone on HNF4α expression in MHSV-12 cells grown with serum. As shown in the gel retardation assay of Figure 1C, a 24 h incubation with dexamethasone leads to a substantial amount of HNF4α protein that is recognized by the A/B-domain specific antibody (lane 5). Since this dexamethasone mediated change can be inhibited by RU486, a well known antagonist binding to the glucocorticoid receptor as a competitor of the glucocorticoid (lane 8), we assume that the effect is mediated by the nuclear hormone receptor.

The findings with the cells grown without serum as well as the data with the cultures maintained in serum, both imply that a glucocorticoid mediated event alters the properties of the HNF4α protein in such a way that it is recognized by the A/B specific antibody.

The novel HNF4α7 protein is encoded by a differential splice variant of the HNF4α gene

The differential recognition of HNF4α by the A/B-domain specific antibody could either reflect a protein modification or a change in the amino acid sequence in the N-terminus of the HNF4α protein. To distinguish between these two alternatives we transfected an expression vector encoding the originally described rat HNF4α protein (3) into MHSV-12 cells grown in the presence of serum, i.e. in the absence of hydrocortisone, that abolishes the supershift by the A/B-domain specific antibody. Analyzing increasing amounts of extracts derived from these transfected cell lines by gel retardation assays we observed a concentration dependent appearance of a HNF4α protein that can be supershifted by the A/B-domain specific antibody (marked by an arrow head in lanes 2 and 5 in Fig. 2), whereas in extracts of mock transfected cells no HNF4α protein could be supershifted by the A/B specific antibody (lanes 8 and 11). Using the F-domain specific antibody no qualitative difference could be seen between the HNF4α transfected cells and the control, but the supershifted band was stronger in the extract of the transfected cells (compare lanes 3 and 6 with lanes 9 and 12). These results rule out the possibility that protein modification plays a major role but rather

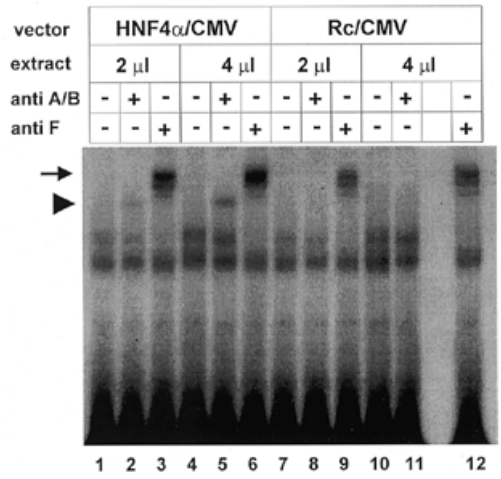

Figure 2. The transfected HNF4α protein is recognized by the A/B specific antibody in MHSV-12 cells grown in serum containing DMEM. An expression vector encoding the rat HNF4α1 protein (HNF4α/CMV) or alternatively the Rc/CMV vector alone were transfected into MHSV-12 cells grown in DMEM with 10% calf serum. Aliquots (2 or 4 µl) of nuclear extracts prepared from the transfected cells were analyzed in gel retardation assays with the HNF4 binding site of the apolipoprotein promoter. The arrow marks the DNA-HNF4α complex supershifted by the antibody specific for the F-domain. The arrow head points to the transfected HNF4α protein detected by the monoclonal anti A/B-antibody.

imply that the transfected HNF4α encodes a protein that is distinct in its amino acid sequence from the protein expressed in undifferentiated MHSV-12 cells.

To prove the assumption that in MHSV-12 cells the endogenous HNF4α protein differs in its amino acid sequence from the cloned sequence, we analyzed the HNF4α mRNA derived from cells cultivated in the presence of serum. As the difference in the HNF4α protein is only detected with the monoclonal antibody raised against the N-terminal A/B-domain, we suspected a distinct 5′-end of the HNF4α mRNA. Therefore, we generated by reverse transcription a cDNA starting in the D-domain (primer HN7 in Fig. 3A) and ligated this DNA at the 5′-end with an oligonucleotide that could be used as a second primer start site. Using this 5′ RACE strategy we succeeded in cloning two independent cDNA fragments, that were analyzed by sequencing. The sequence of the longer cDNA clone, shown in Figure 3B, revealed that the 5′-end from –107 to +47 is completely different from the published nucleotide sequence of mouse HNF4α, whereas the 3′-end (nucleotide +48 to +274) is highly identical to the known sequence. The loss of identity is exactly at the same position in the shorter cDNA clone (data not shown), thus excluding a cloning artefact. As the loss of identity corresponds in both cases to the position of the exon 1-exon 2 boundary (6), we anticipate that the new cDNA encodes a novel splice variant of the HNF4α gene we refer to as HNF4α7.

The 5′-end of the HNF4α7 cDNA encodes an in frame open reading frame preceeded by an in frame stop codon (Fig. 3B). Comparing the amino acid sequence between the HNF4α1 and HNF4α7 proteins, reveals that their N-termini are completely different and that the HNF4α7 splice variant is, by 23 amino acids, shorter than the previously described HNF4α1 protein (Fig. 3C). The novel N-terminal sequence of HNF4α7 shows no significant identity to any sequence in the data bank.

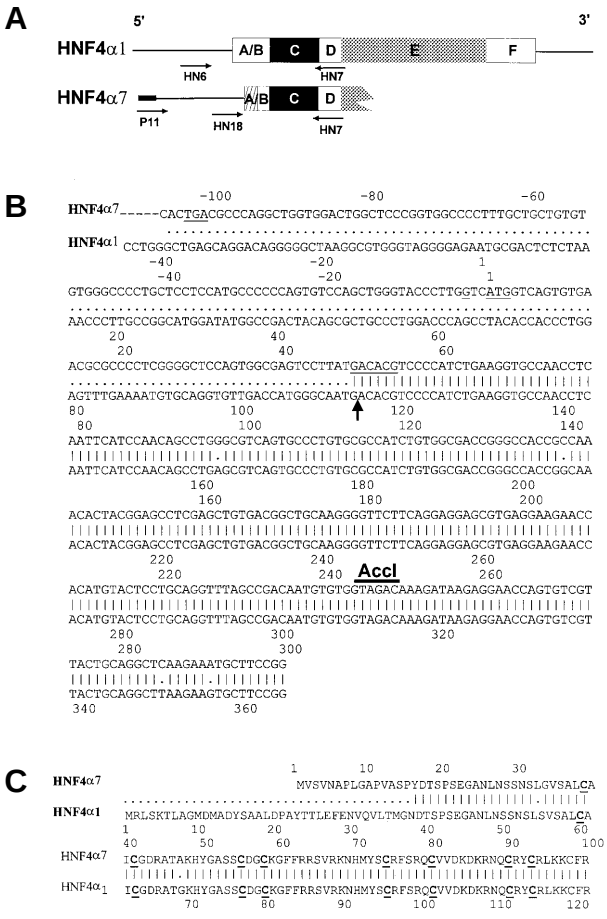

Figure 3. Comparison of the HNF4α1 and HNF4α7 splice variants. (**A**) Schematic drawings of the α1 and α7 splice variants of the mouse HNF4α gene. The boxes designate the domains of the protein as defined (3). The position of the primers used is indicated. The drawing is out of scale. (**B**) Alignment of the nucleotide sequence of the α1 and α7 splice variants. The in frame stop codon TGA preceeding the open reading frame and the initiation codon ATG of HNF4α7 are underlined. The nucleotide sequence of HNF4α1 is as published (14). The initiation codon of HNF4α1 is based on our sequence comparisons of the HNF4α proteins of various species (1). The position of the first intron (6) is marked by an arrow. (**C**) The N-terminal part of the amino acid sequence of the α1 and α7 splice variants are aligned. The cystein residues forming the zinc finger domain are underlined.

The balance of the HNF4α1 and HNF4α7 splice variants changes with the MHSV-12 differentiation status

Comparing extracts derived from MHSV-12 cells grown without serum or in the presence of serum, we observed in western blots that HNF4α is slightly shorter in the presence of serum and thus has the size of the predicted HNF4α7 splice variant (data not shown). The presence of this splice variant was confirmed by RT-PCR using primers specific for the α7 splice variant. Figure 4 reveals the expression of the HNF4α7 splice variant in MHSV-12 cells cultured in the presence of serum (lane 1). Apparently significant amounts of the HNF4α7 variant are also found in cells grown in serum-free medium (lane 2). However, these RT-PCR reactions were performed close to saturation and, therefore, heavily overestimate the amount of HNF4α7 transcripts. In contrast the previously described

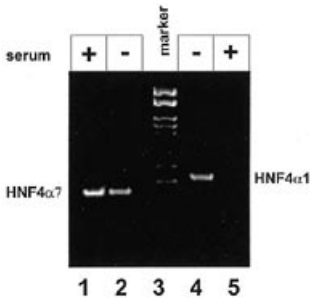

Figure 4. The presence of the HNF4α1 and HNF4α7 splice variants in MHSV-12 cells. By RT-PCR using the primer pair HN18/HN7 (lanes 1 and 2) or HN6/HN7 (lanes 4 and 5) the presence of the α7 and α1 splice variant was determined, respectively. The RNA used was either prepared from cells grown in serumfree MX83 medium (lanes 2 and 4) or from cells maintained in DMEM with 10% serum (lanes 1 and 5). The PCR products were separated on a 1.8% agarose gel and stained with ethidium bromide. pBR322 plasmid DNA restricted with *Alw*441 and *Mva*1 was loaded in lane 3 as size marker.The marker fragment migrating between the HNF4α1 and HNF4α7 PCR product is 383bp in size. The position of the primers is given in Figure 3A.

HNF4α1 splice variant is exclusively detected in MHSV-12 cells grown in serum-free medium (compare lanes 4 and 5). These data confirm that the serum induced change in the differentiation state of the hepatic cells is accompanied by an alteration in the HNF4α splice variants. In differentiated cells grown under serum-free conditions HNF4α1, and to a small extent HNF4α7 transcripts, are present, whereas dedifferentiated cells grown with serum contain almost exclusively the HNF4α7 splice variant.

HNF4α1 and HNF4α7 have distinct transactivation potential

To address the question whether the novel splice variant has a differential transactivation potential we compared its transactivation potential in transfection experiments with the splice variant HNF4α1. Using a luciferase reporter construct containing four HNF4 binding sites in front of the tk promoter (31) we observed in the dedifferentiated hepatoma cell line C2 that lacks HNF4α, (31) a 2-fold lower transactivation potential of HNF4α7 compared to HNF4α1 (Fig. 5A). In Hela cells an even greater difference in the transactivation potential between the two splice variants was observed (Fig. 5B). In fact using high concentrations of the HNF4α7 expression vector no transactivation of the luciferase reporter was seen. Taken together these results establish that MHSV-12 cells grown in serum, that leads to dramatic dedifferentiation of the hepatic phenotype, contain the HNF4α7 variant that has a significantly lower transactivation potential.

HNF4α1 and HNF4α7 show a distinct tissue specific expression pattern

To explore the significance of the novel HNF4α7 splice variant we investigated by RT-PCR the presence of HNF4α1 and HNF4α7 transcripts in different mouse tissues. To allow a reliable quantification we made the PCR reaction with ^{32}P-labeled dCTP that permits low cycling numbers and thus ensures quantitative measurements. As shown in Figure 6 both these transcripts can be identified in the liver and the stomach (lanes 1 and 3), whereas in the kidney and to a smaller extent in the intestine only the HNF4α1 transcript can be detected. In the pancreas only small

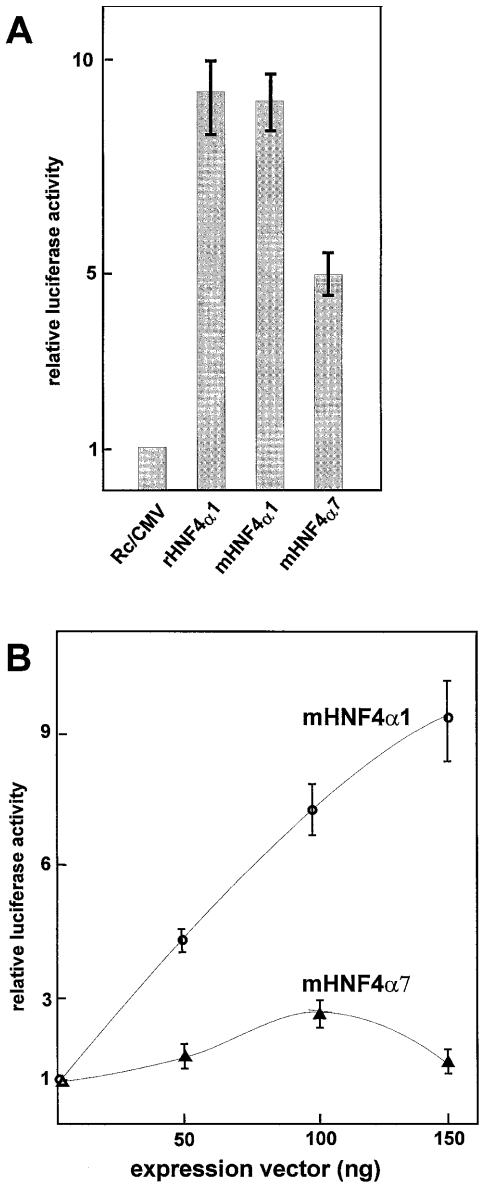

Figure 5. The HNF4α7 transcription factor is a functional transactivator with a reduced activation potential compared to HNF4α1. (**A**) The dedifferentiated hepatoma cell line C2 lacking HNF4α was transiently transfected with 50 ng expression vector encoding HNF4α protein and 2 µg H4-tk-luc as a reporter, that contains four copies of the HNF4 binding site from the human α1-anti-trypsin gene in front of the thymidine kinase promoter as previously described (31). Rc/CMV (InVitrogen) is the expression vector without insert, whereas rHNF4α1 contains the rat transcription factor (3) used to construct the mHNF4α1 and mHNF4α7 constructs. mHNF4α1 is a mouse rat chimeric protein with amino acids 1–102 derived from the mouse α1 protein and amino acids 103 to the C-terminus of the rat protein. mHNF4α7 is the corresponding chimeric protein containing the N-terminus of the α7 splice variant fused to the C-terminus of the rat protein. For both these constructs the *Acc*I site at codon 102 of the HNF4α1 protein was used (see Fig. 3B). (**B**) Increasing amounts of the expression vector encoding mHNF4α1 or mHNF4α7 were transfected into Hela cells with the H4-tk-luc reporter. The amount of plasmid DNA was kept constant by adding Rc/CMV. The error bars given refer to independent transfection experiments.

amounts of HNF4α7 transcripts are present. But the significance of this finding is questionable as the GAPDH mRNA cannot be seen probably because the pancreatic RNase was not sufficiently

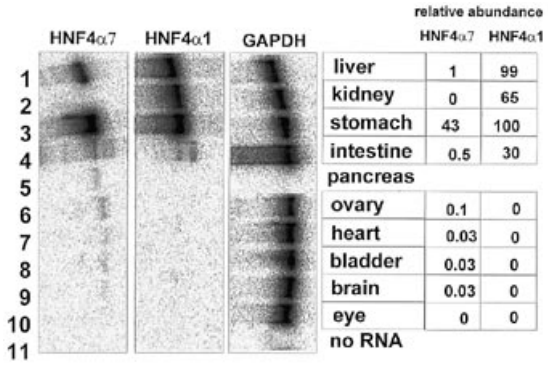

Figure 6. Tissue specific expression pattern of HNF4α1 and HNF4α7. RNA isolated from mouse tissues were analyzed by RT-PCR for the presence of the HNF4α7 and HNF4α1 splice variants using the HN18/HN7 and HN6/HN7 primer pairs, respectively (see Fig 3A). To control the quality of the RNA the presence of GAPDH mRNA was analyzed in aliquots of the same RT reactions. The print-outs of the phosphor imager represent PCR reactions generated by using 35 (HNF4α1 and HNF4α7) or 30 (GAPDH) cycles. To estimate the abundance of the HNF4α1 and HNF4α7 transcripts the signals were counted in the phosphor imager and corrected for the amount of GAPDH mRNA. To ascertain that the PCR reactions were still in the linear range the values given for the high expressing tissues (liver, kidney, stomach and intestine) were calculated from experiments using only 30 cycles of amplification.

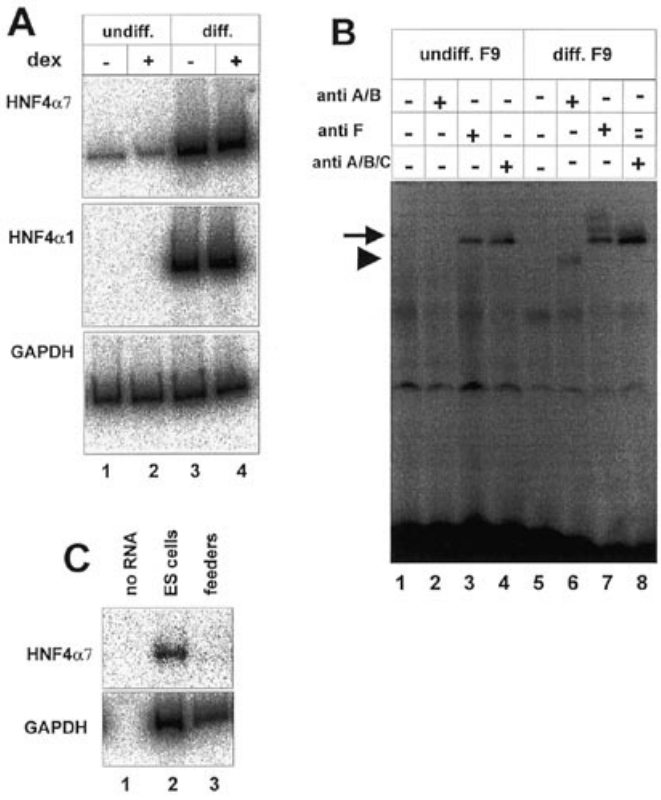

Figure 7. Differential expression of HNF4α1 and HNF4α7 in embryonic cell lines. (**A**) The undifferentiated embryonal carcinoma cell line F9 or F9 cells differentiated during 7 days to visceral endoderm were treated with dexamethasone as indicated. The HNF4α7 and HNF4α1 transcripts were detected by RT-PCR as in Figure 6 using 35 cycles of amplification. The GAPDH RNA was amplified by 25 cycles. (**B**) The HNF4 binding site of the HNF1α promoter was used for gel retardation assays with nuclear extracts of the F9 cells. The addition of the various antibodies is given. The arrow designates the complexes containing HNF4α1 or HNF4α7, whereas the arrow head points to the supershift containing HNF4α1. (**C**) RNA prepared from the embryonic stem cell line E14-1 grown on feeder cells (lane 2) or from the feeder cells alone (lane 3) was analyzed by RT-PCR for the presence of HNF4α7 (35 cycles) and GAPDH (25 cycles) mRNA as in (A). In lane 1 no RNA was added in the RT reaction.

inhibited during RNA preparation. However, traces of HNF4α7 RNA were also detected in the ovary, heart, bladder and brain, where no HNF4α1 transcripts and normal levels of GAPDH RNA were observed. Clearly, the HNF4α1 and HNF4α7 splice variants are expressed differentially in the various tissues analyzed. By quantification of the PCR products in a phosphor imager we estimate that in the stomach about a third of the HNF4α transcripts correspond to the HNF4α7 splice variant (lane 3). Furthermore, the abundance of the HNF4α7 transcripts differs by a factor of ~1000-fold between high and low expressing tissues.

HNF4α7 is expressed in embryonic stem cells of the mouse

Our initial experiments leading to the identification of the HNF4α7 splice variant implied some correlation of the novel splice variant with the undifferentiated phenotype of the hepatocytes. Therefore, we investigated the presence of HNF4α7 in the murine embryonal carcinoma cell line F9 that resembles the pluripotent stem cells of the inner cell mass of the early mouse embryo. This cell line can can be differentiated into distinct endodermal cell types upon retinoic acid treatment. Most interestingly these stem cells can be differentiated by retinoic acid treatment into visceral endoderm when they are cultured in suspension as aggregates and these cell types share properties of hepatocytes (24,32) . Based on RT-PCR we succeeded to show that undifferentiated F9 cells contain exclusively the HNF4α7 splice variant (Fig. 7A, lanes 1 and 2), whereas the differentiated cell population contains the HNF4α1 as well as the HNF4α7 splice variants (lanes 3 and 4). From three independent experiments we estimated by quantification in a phosphor imager a 15-fold increase in HNF4α7 mRNA upon differentiation into visceral endoderm, whereas the level of HNF4α1 transcripts increased from undetectable levels to a value ~60% of the amount of the HNF4α7 transcript. Since in the immortalized hepatic MHSV-12 cells grown in the presence of serum, the balance between HNF4α1 and HNF4α7 is altered by the addition of the glucocorticoid dexamethasone (Fig. 1), we wondered whether a similar effect is seen in F9 cells. As Figure 7A demonstrates the expression pattern of HNF4α1 and HNF4α7 is not changed upon dexamethasone treatment in either undifferentiated or differentiated F9 cells.

To determine whether the presence of the HNF4α7 transcript in the embryonal cell line F9 also reflects the expression of the corresponding protein, we prepared nuclear extracts from F9 cells. In gel retardation assays with extracts from undifferentiated F9 cells (Fig. 7B) we could detect a supershift with the F-domain specific antibody (lane 3) and the A/B/C-domain specific antibody (lane 4) but no reaction with the A/B-domain specific antibody (lane 2). In contrast in F9 cells differentiated into visceral endoderm a supershift was seen with each of the three antibodies (lanes 6, 7 and 8). This result is consistent with the RT-PCR analysis and implies that undifferentiated F9 cells contain HNF4α7 protein, whereas HNF4α1 as well as HNF4α7 proteins are present in differentiated F9 cells.

To further investigate the early embryonic expression of HNF4α7 we searched for the corresponding transcript in the totipotent embryonic stem cell line E14-1. The RT-PCR analysis given in Figure 7C demonstrates the presence of HNF4α7 mRNA in this embryonal stem cell (lane 2), whereas the RNA sample derived from the feeder cells used for the ES cell culture lack any HNF4α7 transcripts (lane 3). Probing the same ES cell RNA for the HNF4α1 mRNA we did not detect any transcript (data not shown).

From the analysis of both the F9 cells and the ES cells we conclude that the presence of HNF4α7 mRNA is a characteristic of undifferentiated embryonic cells.

DISCUSSION

The novel splice variant HNF4α7 is expressed as the exclusive HNF4α transcript in the undifferentiated embryonal carcinoma cell line F9 and the ES cell line E14-1. The transcript is also found in the hepatocyte cell line MHSV-12 that has been established by immortalization of embryonic hepatocytes. In this immortalized hepatic cell culture HNF4α7 predominates provided these cells are cultured under conditions that lead to dedifferentiation. In this context it is important to stress that the MHSV-12 cell line dedifferentiated by serum addition has far more hepatic characters than the dedifferentiated hepatoma cell lines C2 and H5 that both lack any HNF4α and HNF1α transcription factors (18,32).

Recently, the expression of HNF4α has been investigated in the ES cell line D3 (33). Under differentiating conditions allowing the formation of embryoid bodies the appearance of HNF4α RNA has been observed, but no signal was detected in the undifferentiated embryonic stem cells. The lack of HNF4α transcripts in undifferentiated ES cells probably reflects the insensitivity of the Northern blots compared to our RT-PCR assay as these authors used a full length cDNA probe that would hybridize also to the HNF4α7 splice variant.

The presence of HNF4α7 in various embryonic cell types suggests that this novel HNF4α splice variant has some function during early embryogenesis. Early embryonic expression of the HNF4α gene starting in the primary endoderm at embryonic day 4.5 of the mouse has been documented by *in situ* hybridization (10). As the probe used in these experiments did not differentiate between the α1 and α7 splice variant, the identity of this signal in respect to the splice variants is not clear. However, another *in situ* hybridization used a genomic fragment of the exon 1 of the original HNF4α transcript (6) and thus, in retrospect, was a HNF4α1 specific probe. With this probe HNF4α transcripts were first localised only on day 7 p.c. in visceral extraembryonic endoderm. This detection at a later time point in embryogenesis could well reflect the fact that this probe could not hybridize to the HNF4α7 splice variant. Similarly, the relatively late appearance of HNF4α binding activity in gel retardation assays starting at 8.5 days p.c. (25) might also be interpreted as a lack of reactivity of the antibody with the HNF4α7 splice variant. Therefore, by indirect evidence the earlier appearance of HNF4α7 compared to HNF4α1, we have seen in the differentiating F9 cells (Fig. 7A and B) matches the available data in the developing embryo.

Analyzing 200 unfertilized mouse eggs we could not identify HNF4α7 mRNA whereas GAPDH mRNA was readily detectable (data not shown). As unfertilized mouse eggs are about 20 times larger than the ES cells, the input RNA in the RT-PCR corresponds to ~4000 ES cells. Since in such an ES cell aliquot we could identify the HNF4α7 mRNA, we assume that HNF4α7 transcripts are absent in the egg and accumulate by zygotic transcription in early mouse embryogenesis. This is consistent with the appearance of HNF4α transcripts in *in situ* hybridization experiments with a probe detecting HNF4α1 and HNF4α7 only at day 4.5 p.c. (10). Therefore, it seems unlikely that HNF4α mRNA is a maternal component in mammals. This is in contrast to *Drosophila* HNF4 (7) and *Xenopus* HNF4β mRNA (2) that are known to be present as maternal transcripts. However, the HNF4α protein itself might be a maternal component of the mammalian egg, a situation we have found for the HNF4α protein in *Xenopus* whose corresponding mRNA has disappeared at the egg stage (2,34).

An early function of HNF4α gene expression in mammals has been established by the disruption of the gene that results in an impaired gastrulation and early embryonic lethality (11). As the targeting vector used in these knock-out experiments lacked exon 2 and 3, the HNF4α1 as well as the HNF4α7 transcripts are devoid of the DNA binding domain. Therefore, it is not clear at the moment which HNF4α transcripts plays the essential function at this early embryonic stage. It should also be considered that the partial rescue of HNF4α knock-out embryos observed by supplementing the developing embryo with normal visceral endoderm (35) might be an effect relieving only one of the two HNF4α splice variants.

We assume that the presence of HNF4α7 in the undifferentiated embryonal carcinoma cell line F9 and in the embryonic stem cell E14-1 reflects already some first step of embryonic gene regulation. Consistent with this interpretation the presence of transcripts encoding the tissue specific transcription factor HNF1α has been reported in undifferentiated F9 cells (25,36). Since it is well established that HNF1α is a potential target gene of HNF4α (18–20,26,37), we consider the possibility that HNF4α7 triggers HNF1α expression at this early stage. However, the significance of such a potential effect is questionable as the HNF1α transcripts are of aberrant size and no HNF1α protein can be detected in F9 cells (25).

Our analysis demonstrates that HNF4α7 is not restricted to embryonic tissues as it is also found abundantly in the stomach of the adult mouse (Fig. 6). In contrast, in the liver and intestine, HNF4α7 transcripts constitute only a minor fraction as compared to HNF4α1 and in the ovary, heart, bladder and brain only traces can be found. It will be most interesting to investigate whether in these tissues HNF4α7 is restricted to a subpopulation of cells, e.g. the stem cell compartment.

Our finding that the transactivation potential of the HNF4α splice variants differs in transient transfection assays and that the extent of this effect depends on the cell type used (Fig. 5), indicates that the two HNF4α splice variants cooperate differentially with other transcription factors. As the HNF4α1 and HNF4α7 splice variants have distinct N-termini, we expect that this domain determines the differential cooperation. Therefore, we exclude the reported interaction with COUP-TFI and COUP-TFII (38) as well as the binding to HNF1α (39) as the mechanisms mediating the distinct properties of of HNF4α1 and HNF4α7, since these interactions involve the C-terminus common to HNF4α1 and HNF4α7. However, the specific binding observed with the basal transcription factor TFIIB (40) might trigger the distinct properties of the two splice variants, as the formation of this complex requires the N-terminus of HNF4α (38,40). Alternatively, HNF4α1 and HNF4α7 may exert distinct activities due to differential heterodimerization with other members of the HNF4 family (1,2). But we would not expect differential heterodimerization with other

members of the nuclear receptor superfamily, since HNF4α seems usually not to heterodimerize with these components (41,42).

Analyzing the expression of the HNF4α splice variants we revealed that HNF4α7 is under a complex regulatory network. In the hepatic cell line MHSV-12 the glucocorticoid plays a major role as the lack of this hormone seems to be a critical component for the appearance of HNF4α7. Clearly, the glucocorticoid hormone alters the balance between the HNF4α1 and HNF4α7 splice variants. As glucocorticoids are known to trigger the final stage of hepatic differentiation at birth (43), we have investigated the expression of these two HNF4α splice variants. Using liver extracts from 2 days before birth up to 2 days after birth we could not see any change (data not shown). In fact, as all the HNF4 binding activity observed in gel retardation assays could be supershifted by the A/B specific antibody at all stages analyzed, we have no data to support a major change in HNF4α splice variants at birth, although glucocorticoid action is an essential step at this developmental stage. A lack of glucocorticoid control was also seen in F9 cells. Neither in undifferentiated nor in differentiated F9 cells the addition of dexamethasone altered the level of the HNF4α1 and HNF4α7 mRNA (Fig. 7A). On the other hand in these F9 cells the retinoic acid mediated differentiation to visceral endoderm leads to an increase in HNF4α7 mRNA but more significantly also to the appearance of the HNF4α1 splice variant. This change in HNF4α expression can also be seen on the protein level, as HNF4 binding activity reacting with the anti A/B antibody is only detected in the differentiated cells (Fig. 7B).

Our data show that the splice variant HNF4α7 contains a distinct first exon. As the original exon 1 of the murine HNF4α gene has been shown to contain the initiation site of the promoter (6,44), we assume that the novel exon 1 found in HNF4α7 indicates a second promoter of the HNF4α gene. Therefore, we propose that the differential expression of the two splice variants is mainly due to the differential use of two distinct promoters of the HNF4α gene

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REFERENCES

1 Drewes, T., Senkel, S., Holewa, B. and Ryffel, G.U. (1996) *Mol. Cell. Biol.*, **16**, 925–931.
2 Holewa, B., Zapp, D., Drewes, T., Senkel, S. and Ryffel, G.U. (1997) *Mol. Cell. Biol.*, **17**, 687–694.
3 Sladek, F.M., Zhong, W.M., Lai, E. and Darnell, J.E., Jr. (1990) *Genes Dev.*, **4**, 2353–2365.
4 Sladek, F.M. (1994) In Tronche, F. and Yaniv, M. (eds), *Liver Gene Expression*, R.G. Laundes, Austin, TX, USA.
5 Miquerol, L., Lopez, S., Cartier, N., Tulliez, M., Raymondjean, M. and Kahn, A. (1994) *J. Biol. Chem.*, **269**, 8944–8951.
6 Taraviras, S., Monaghan, A.P., Schütz, G. and Kelsey, G. (1994) *Mech. Dev.*, **48**, 67–79.
7 Zhong, W., Sladek, F.M. and Darnell, J.E., Jr. (1993) *EMBO J.*, **12**, 537–544.
8 Yamagata, K., Furuta, H., Oda, N., Kaisaki, P.J., Menzel, S., Cox, N.J., Fajans, S.S., Signorini, S., Stoffel, M. and Bell, G.I. (1996) *Nature*, **384**, 458–460.
9 Pogge von Strandmann, E., Nastos, A., Holewa, B., Senkel, S., Weber, H. and Ryffel, G.U. (1997) *Mech. Dev.*, **64**, 7–17.
10 Duncan, S.A., Manova, K., Chen, W.S., Hoodless, P., Weinstein, D.C., Bachvarova, R.F. and Darnell, J.E., Jr. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 7598–7602.
11 Chen, W.S., Manova, K., Weinstein, D.C., Duncan, S.A., Plump, A.S., Prezioso, V.R., Bachvarova, R.F. and Darnell, J.E., Jr. (1994) *Genes Dev.*, **8**, 2466–2477.
12 Furuta, H., Iwasaki, N., Oda, N., Hinokio, Y., Horikawa, Y., Yamagata, K., Yano, N., Sugahiro, J., Ogata, M., Ohgawara, H., Omori, Y., *et al* (1997) *Diabetes*, **46**, 1652–1657.
13 Hata, S., Tsukamoto, T. and Osumi, T. (1992) *Biochim. Biophys. Acta*, **1131**, 211–213.
14 Hata, S., Inoue, T., Kosuga, K., Nakashima, T., Tsukamoto, T. and Osumi, T. (1995) *Biochim. Biophys. Acta*, **1260**, 55–61.
15 Chartier, F.L., Bossu, J.P., Laudet, V., Fruchart, J.C. and Laine, B. (1994) *Gene*, **147**, 269–272.
16 Kritis, A.A., Argyrokastritis, A., Moschonas, N.K., Power, S., Katrakili, N., Zannis, V.I., Cereghini, S. and Talianidis, I. (1996) *Gene*, **173**, 275–280.
17 Cereghini, S. (1996) *FASEB J.*, **10**, 267–282.
18 Kuo, C.J., Conley, P.B., Chen, L., Sladek, F.M., Darnell, J.E., Jr. and Crabtree, G.R. (1992) *Nature*, **355**, 457–461.
19 Ktistaki, E., Ktistakis, N.T., Papadogeorgaki, E. and Talianidis, I. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 9876–9880.
20 Spaeth, G.F. and Weiss, M.C. (1997) *Mol. Cell. Biol.*, **17**, 1913–1922.
21 Galson, D.L., Tsuchiya, T., Tendler, D.S., Huang, L.E., Ren, Y., Ogura, T. and Bunn, H.F. (1995) *Mol. Cell. Biol.*, **15**, 2135–2144.
22 Fraser, J.D., Keller, D., Martinez, V., Santisomere, D., Straney, R. and Briggs, M.R. (1997) *J. Biol. Chem.*, **272**, 13892–13898.
23 Stutenkemper, R., Geisse, S., Schwarz, H.J., Look, J., Traub, O., Nicholson, B.J. and Willecke, K. (1992) *Exp. Cell Res.*, **201**, 43–54.
24 Hogan, B.L., Taylor, A. and Adamson, E. (1981) *Nature*, **291**, 235–237.
25 Cereghini, S., Ott, M.O., Power, S. and Maury, M. (1992) *Development*, **116**, 783–797.
26 Zapp, D., Bartkowski, S., Holewa, B., Zoidl, C., Klein-Hitpass, L. and Ryffel, G.U. (1993) *Mol. Cell. Biol.*, **13**, 6416–6426.
27 Dignam, J.D. (1990) *Methods Enzymol.*, **182**, 194–203.
28 Kugler, W., Kaling, M., Ross, K., Wagner, U. and Ryffel, G.U. (1990) *Nucleic. Acids. Res.*, **18**, 6943–6951.
29 Stumpf, H., Senkel, S., Rabes, H.M. and Ryffel, G.U. (1995) *Carcinogenesis*, **16**, 143–145.
30 Rupp, R.A. and Weintraub, H. (1991) *Cell*, **65**, 927–937.
31 Drewes, T., Clairmont, A., Klein-Hitpass, L. and Ryffel, G.U. (1994) *Eur. J. Biochem.*, **225**, 441–448.
32 Cereghini, S., Yaniv, M. and Cortese, R. (1990) *EMBO J.*, **9**, 2257–2263.
33 Abe, K., Niwa, H., Iwase, K., Takiguchi, M., Mori, M., Abe, S.I. and Yamamura, K.I. (1996) *Exp. Cell Res.*, **229**, 27–34.
34 Holewa, B., Strandmann, E.P., Zapp, D., Lorenz, P. and Ryffel, G.U. (1996) *Mech. Dev.*, **54**, 45–57.
35 Duncan, S.A., Nagy, A. and Chan, W. (1997) *Development*, **124**, 279–287.
36 Kuo, C.J., Mendel, D.B., Hansen, L.P. and Crabtree, G.R. (1991) *EMBO J.*, **10**, 2231–2236.
37 Tian, J.M. and Schibler, U. (1991) *Genes Dev.*, **5**, 2225–2234.
38 Ktistaki, E. and Talianidis, I. (1997) *Mol. Cell. Biol.*, **17**, 2790–2797.
39 Ktistaki, E. and Talianidis, I. (1997) *Science*, **277**, 109–112.
40 Malik, S. and Karathanasis, S.K. (1996) *Mol. Cell Biol.*, **16**, 1824–1831.
41 Jiang, G.Q., Nepomuceno, L., Yang, Q. and Sladek, F.M. (1997) *Arch. Biochem. Biophys.*, **340**, 1–9.
42 Jiang, G.Q. and Sladek, F.M. (1997) *J. Biol. Chem.*, **272**, 1218–1225.
43 Montoliu, L., Blendy, J.A., Cole, T.J. and Schütz, G. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 4244–4248.
44 Zhong, W., Mirkovitch, J. and Darnell, J.E., Jr. (1994) *Mol. Cell. Biol.*, **14**, 7276–7284.