Trans-dominant inhibition of transcription activator LFB1

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ABSTRACT

Liver-enriched factor LFB1 (also named HNF1) is a dimeric transcription activator which is essential for the expression of many hepatocyte-specific genes. Here we demonstrate that LFB1 mutants in the POU A-like or in the homeo domains inhibit wild-type DNA binding by forming inactive heterodimeric complexes. Co-transfection of one of these mutants with wild-type LFB1 in HeLa cells eliminated LFB1 DNA binding and transcriptional activities through a trans-dominant mechanism. Expression of the same dominant negative mutant in human hepatoma HepG2 cells only partially inhibited endogenous LFB1 activity, due to stabilization of LFB1 dimers in these cells. Dimer stabilization in hepatoma cells is mediated by a heat-labile association with an 11kD polypeptide, analogous to the DCoH cofactor identified in rat liver by Mendel et al. (1). The property of stabilizing LFB1 dimers is also shared by HeLa cells which produce a HeLa homolog of DCoH. These results demonstrate that LFB1 dimer stabilization as well as the synthesis of 'stabilizing factors' are not restricted to cells expressing LFB1 or other members of its family.

INTRODUCTION

A set of liver-enriched transcription activators is responsible for the co-ordinated expression of genes in the hepatocyte (2-5). LFB1 is one such factor, and plays a crucial role in cell type-specific transcription of several liver genes (6). LFB1 is composed of two physically separable fragments: the DNA binding domain, consisting of 281 N-terminal amino acids, and the C-terminus, where sequences responsible for transcriptional enhancement are present (7). There are three distinct regions within the DNA binding domain that are required for efficient and specific DNA binding: A (amino acids 1–31), B (amino acids 100–184) and C (amino acids 198–281). The B region is distantly related to the subdomain A of POU proteins (8). The C region is a novel type of homeodomain containing 19 extra amino acids with respect to canonic homeodomains (7, 9, 10). Both B and C regions are involved in DNA recognition (11). The A region mediates LFB1 dimerization through a mechanism similar to that found in classical coiled-coils (12, 13).

LFB1 binds to DNA as a dimer and already exists in solution in dimeric form prior to DNA binding. The recombinant protein,

produced by *in vitro* translation or by the vaccinia virus expression system, exchanges subunits *in vitro* at a rapid rate. Binding to DNA does not lock LFB1 in its dimeric form: monomers are rapidly incorporated into an equilibrium between monomer, dimer and dimer-DNA complexes (7). In contrast, native LFB1 dimers from liver nuclear extracts are stable (14).

Recently, the cDNA for a protein of 11kD which copurifies with rat liver LFB1 has been cloned. This cofactor, named DCoH, cannot bind to the DNA, but forms stable heterotetrameric complexes with LFB1. DCoH alone does not stimulate transcription, but it enhances LFB1 transcriptional activity in co-transfection experiments (1).

Dimerization is a common property of many trans-acting factors. Dimeric transcription activators can be grouped in protein families whose members are able to cross-interact through a common dimerization structure. Heterodimerization often creates new DNA binding or transcriptional activities different from those of the parental homodimers, thus expanding the repertoire of a limited number of regulatory proteins (15).

We wished to investigate whether LFB1 activity could be modulated *in vivo* by heterodimerization. For this purpose we constructed a series of LFB1 mutants that are able to inhibit DNA binding of wild-type molecules *in vitro* by a trans-dominant mechanism. Expression of one of these mutants eliminates the transcriptional activity of a co-transfected wild-type LFB1 in HeLa cells, but has a reduced inhibitory effect on the endogenous protein in human hepatoma HepG2 cells. Our data indicate that LFB1 dimer stability in HepG2 accounts for the lower efficiency of trans-dominant inhibition.

By generating HeLa cells stably expressing LFB1, we demonstrate that the ability to form stable dimers is not restricted to cells of hepatic origin. We also show that HeLa cells contain a small factor which is able to associate with LFB1 and is likely to be a functional homolog of DCoH.

MATERIALS AND METHODS

Plasmid clones

The LFB1 expression plasmid RSV-LFB1 has been constructed by inserting the HincII-BamHI fragment from plasmid pB1.2 (nucleotide position –86 to + 3161 relative to the LFB1 ATG (7) under the control of the Rous Sarcoma Virus LTR sequence in the vector RSV-0 (16). The plasmid RSV-LFB1/SM14 has been generated by inserting the HincII-BamHI fragment from

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plasmid pB1.2/SM14 (7) into the RSV-0 vector. A synthetic double-stranded oligonucleotide harbouring a termination codon in all three possible frames has been inserted at position +844 relative to the ATG (B_{all} site) to generate a C-terminal truncated gene.

Reporter plasmid pB1-CRP42CAT is described in Toniatti et al. (7xAlbCRP-CAT; 17). The construction of recombinant vaccinia viruses expressing LFB1 derivatives (vLFB1 and vLFB1 Δ C281) and the relative nuclear extract preparation from infected cells has been described (7). In the same paper a detailed description of the LFB1 mutants used in dimerization interference assays is reported.

The plasmid pEBO-LFB1 has been constructed by inserting an Asp718/blunted-NotI fragment from plasmid pB1.1 (containing the cDNA sequences from nucleotide position -45 to +2273 relative to the LFB1 ATG) (7) under the control of the SV40 promoter/enhancer between the sites HindIII/blunted-NotI of the vector pEBOpLPP (18).

In vitro transcription and translation

In vitro transcription and translation experiments were carried out as previously described (7). Co-translation of DCoH cRNA (0.5 μ l) in the presence of heat treated rat liver nuclear extract (6 μ l) was performed in the same conditions. The DCoH-LFB1 heterocomplex was partially purified on heparin-Sepharose as described in Nicosia et al. (7), prior to heat treatment.

Binding assays

Dimerization interference experiments were carried out by incubating increasing amounts of *in vitro* translated LFB1 mutants or synthetic peptides with 0.5 l of *in vitro* translated LFB1 Δ C281. Binding reactions were assembled as previously described (7).

Oxidized bovine insulin chain B (INS-b) was purchased from Sigma.

In heterodimerization experiments, increasing amounts of the vLFB1 Δ C281 nuclear extract (0.8 μ g and 1.6 μ g) were incubated with the indicated nuclear extract for 45 min on ice. The radiolabelled probe was then added to the mixture and incubation prolonged for further 30 min.

Heat treatment of nuclear extracts was performed as follows: 30–100 μ l of extract were treated for 5 min at 80°C in a water bath, chilled 5 min on ice and spun for 5 min at 13,000 rpm in eppendorf microfuge. Soluble proteins were saved and used for bandshift experiments.

Quantification by densitometric scanning was performed with a Macintosh IIfx computer using the program Image, version 1.22.

Cell transfections and immunological techniques

All cells were grown in DMEM supplemented with 10% fetal calf serum and 25 mM Hepes pH 7.3 in 5% CO₂.

HepG2, Hep3B and HeLa cells were transfected with the indicated amounts of reporter and expression plasmids together with 0.5 μ g of RSV-luciferase plasmid (19) as internal control, by the calcium-phosphate co-precipitation procedure.

LFB1 expression was obtained by transfecting 5 μ g (Hep3B) or 50 μ g (HeLa and Hep3B) of plasmid RSV-LFB1. In control transfections 50 μ g of the vector RSV-0 were used.

Twenty-four to seventy-two hours after transfection, cells were harvested and whole cell extracts were prepared by freeze and thaw. Chloramphenicol acetyltransferase (CAT) activities, determined according to Crabb and Dixon (20), were normalized to luciferase activity, according to de Wet et al. (21).

HeLa cells stably expressing LFB1 were produced by transfecting 10, 20 or 40 μ g of pEBO-LFB1 by the calcium phosphate procedure. Control transfections were performed with the vector pEBOpLPP. Seventy-two hours after transfection, cells were split 1:5 into fresh DMEM containing 150 mg/ml of hygromycin. Cells were kept under hygromycin selection for 20 days. Nuclear cell extracts were prepared according to Lee et al. (22). Rat liver nuclear extract was prepared according to Lichtsteiner and Schibler (23).

Anti-LFB1 rabbit polyclonal antibodies and western blotting techniques have been previously described (24). Metabolic labelling of cultured cells and LFB1 immunoprecipitation were performed as described (25). LFB1 from metabolically labelled cells was affinity purified as described (23).

RESULTS

LFB1 mutants in B and C regions interfere with wild-type DNA binding

Mutations in each region of the tripartite LFB1 DNA binding domain abolish protein-DNA interaction (7). We tested whether any of these mutants would inhibit DNA binding of wild-type LFB1 in bandshift experiments. The results obtained allowed us to group the tested mutants into two classes: mutants in the A region (Δ 1 and IM1) do not affect binding of the indicator homodimer to DNA; in contrast, protein derivatives which are not altered in the dimerization domain (Δ C211, SM14, SM13, SM11, SM8, SM6) interfere with the formation of indicator-DNA complexes (Fig. 1).

Figure 1. Dimerization interference analysis. *In vitro* translated LFB1 derivative defective for DNA binding or synthetic peptides were incubated with a truncated LFB1 molecule (C-terminal deletion Δ C281, indicated as wt), which retains wild-type dimerization and binding ability, as an indicator. After a period of time sufficient to reach the equilibrium, a radiolabelled oligonucleotide reproducing the LFB1 binding site in the human α 1-antitrypsin promoter (oligo B1) was added to the mixture, and protein-DNA complexes were fractionated by polyacrylamide gel electrophoresis (PAGE). Bands corresponding to protein-DNA complexes formed in the absence (upper left) and in the presence of mutant derivatives or peptides are shown. The molar excess of mutant versus wild-type, determined by SDS-PAGE of labelled proteins, is indicated above the panels. The amounts of peptides used are also indicated. The name and a schematic diagram of the LFB1 mutant or synthetic peptide used is shown beside each panel. Detailed descriptions of mutant sequences have been previously published (7). A, B and C domain boundaries are indicated at the top. Numbers refer to amino acid positions relative to the starting methionine. Stippled boxes represent substituted regions. The wedge represents inserted amino acids.

Since the A domain is necessary and sufficient to promote dimerization (7), we concluded that inhibition of wild-type binding to DNA is due to the formation of heterodimeric complexes between mutant and indicator molecules. In accordance with this interpretation, a synthetic peptide corresponding to the A domain (PEP1, from aa 1 to aa 31) interferes with binding of wild-type. In a control experiment, a

Figure 2. Trans-dominant inhibition of LFB1 activity in HeLa and HepG2 cells. Transfected plasmids are indicated below the figure. In all experiments the total amount of transfected plasmids was kept constant by adding the vector RSV-0. Cells were harvested forty-eight hours after transfection. Transcription from the pB1-CRP42CAT responsive promoter was determined by measuring CAT activity in whole-cell extracts from transfected cells. All results represent an average of four independent experiments. A)–HeLa cells were transfected with 5 μ g of reporter plasmid, 5 μ g of RSV-LFB1 plasmid and 65 μ g of RSV-LFB1/SM14 plasmid. Black columns represent the levels of CAT activity obtained. Stippled columns represent the levels of LFB1 DNA binding activity. Values have been normalized over transfection efficiency and expressed as a percentage of the activity measured in extracts from cells transfected with the reporter and the activator expressing plasmids. DNA binding levels have been determined by densitometric scanning of the autoradiographs. B)–HepG2 cells were transfected with 5 μ g of reporter plasmid and 65 μ g of RSV-LFB1/SM14 plasmid. CAT activities have been normalized over transfection efficiency. Columns represent the fold-inhibition of pB1-CRP42 promoter activity relative to control transfections without the RSV-LFB1/SM14 plasmid.

peptide which corresponds to a different region of LFB1 (PEP 2, from aa 113 to aa 128), or a peptide of unrelated sequence (INS-b, the 30 amino acid long insulin chain B), does not affect indicator binding (Fig. 1 and data not shown).

Besides confirming that the A domain is the portion of the protein responsible for dimer formation, these results indicate that in order to bind DNA with wild-type efficiency, each component of a dimer must have functional B and C domains. Derivatives that are able to dimerize (i.e. with an intact A domain), but unable to bind DNA (i.e. defective in the B or C domain) behave as *trans*-dominant negative mutants.

Trans-dominant inactivation of transfected LFB1 in HeLa cells

We wanted to exploit the properties of dominant negative mutants to block the activity of the transcription factor LFB1 *in vivo*. As a model system we used human cervical carcinoma HeLa cells which do not synthesize LFB1 from the endogenous gene. Transfection of the expression plasmid RSV-LFB1 (17) in HeLa cells led to strong trans-activation of a co-transfected LFB1-responsive construct (plasmid pB1-CRP42CAT, see Materials and Methods; Fig. 2A). No measurable CAT activity was present in extracts from cells transfected with the parental expression plasmid lacking the LFB1 sequence (RSV-0; Fig. 2A).

As a further step in this experiment, we over-expressed the SM14 protein mutant (plasmid RSV-LFB1/SM14) in the same context. This LFB1 derivative bears a substitution of the two most conserved amino acids of the homeodomain recognition helix, Trp-205 and Phe-206, both changed into Ala. In addition, SM14 lacks the transcription activation domain in order to avoid squelching interfering effects. This protein mutant, which can still dimerize but no longer bind DNA, is a powerful inhibitor of wild-type LFB1 DNA binding (Fig. 1).

We co-transfected in HeLa cells the expression plasmids RSV-LFB1/SM14 and RSV-LFB1, in a ratio of 13 to 1, along with the pB1-CRP42CAT construct. Whole-cell extracts from two independent transfections were prepared, and the expression levels of wild-type and mutant LFB1, determined by Western blotting, showed a ratio of about ten-fold mutant versus wild-type LFB1 (data not shown). Measurements of CAT activity from the same extracts revealed a twenty-fold inhibition of LFB1-dependent transcription (Fig. 2A).

Figure 3. Hepatic LFB1 is a stable dimer. The position of bands corresponding to homodimeric and heterodimeric species are indicated on the right of the figure. The asterisk indicates the protein-DNA complex formed by the ubiquitous species LFBu (24). Increasing amounts of the vLFB1 Δ C281 challenging protein were incubated with different extracts for heterodimerization with full-length LFB1. vLFB1: 1 μ g of HeLa nuclear extract containing full-length LFB1 produced by the vaccinia virus expression system. HepG2: 10 μ g (4 μ l) of nuclear extract from HepG2 cells. HT-HepG2: 10 μ l of HepG2 extract treated for 5 min at 80°C, as described in Materials and Methods.

To confirm that inhibiting LFB1-induced transcription in HeLa cells does, in fact, correspond to sequestering the wild-type LFB1 in inactive heterodimeric complexes, we prepared nuclear extracts from transfected cells and quantified the amount of active LFB1 dimers by gel retardation assay. Expression of the SM14 mutant reduced the level of the LFB1-DNA complex to 12% of that obtained with an equivalent amount of nuclear extract from control transfected cells (Fig. 2A).

The low amount of wild-type LFB1 homodimers which still bind to DNA even in the presence of the repressor molecules can explain the residual CAT activity observed.

Trans-dominant inhibition of endogenous LFB1 transcriptional activity in hepatoma cells

The human hepatoma HepG2 cell-line expresses high amounts of LFB1, as well as all known LFB1-dependent genes (26). Transfection of the pB1-CRP42CAT construct in these cells gave rise to high levels of CAT conversion.

We assayed the effect of the SM14 expression on the endogenous LFB1 activity, as measured by inhibition of transcription from the pB1-CRP42CAT template in HepG2 cells. As shown in Fig. 2B, over-expression of the SM14 derivative resulted in a five-fold reduction of the LFB1-induced transcription. Surprisingly, the inhibition observed was significantly lower than that obtained in LFB1-transfected HeLa cells. Similar results were also obtained when using different LFB1-responsive templates and by measuring the inhibition levels by RNase mapping. Higher amounts of transfected mutant-expressing plasmid did not increase inhibition. Furthermore, changing incubation time after transfection (36 and 72 hours) did not alter the level of repression (data not shown).

By measuring the amount of SM14 produced in transfected HepG2 and normalizing it over the number of transfected cells, we can estimate that each transfected cell contains at least 10 molecules of repressor for every wild-type LFB1 molecule (data not shown). Therefore, the partial inhibition obtained in HepG2 cells cannot be ascribed to the ratio between SM14 and wild-type LFB1 being lower than that obtained in HeLa cells.

LFB1 from hepatoma cells is stabilized in its dimeric form

We interpret the discrepancy between the levels of repression obtained in HeLa and HepG2 cells as indication that an additional mechanism exists which operates on the LFB1 synthesized in hepatoma cells from the resident gene and partially prevents inhibition by the transfected mutant.

Since native LFB1 from rat liver is a stable dimer (14), we hypothesized that also the protein from hepatoma cells is not completely available for heterodimer formation. To provide experimental evidence in support of this hypothesis, we tested native LFB1 extracted from hepatoma cells of different origin for its ability to exchange subunits *in vitro*. In this experiment we detected heterodimer formation by using a truncated LFB1 derivative produced in HeLa cells through recombinant vaccinia virus infection (vLFB1ΔC281) as a challenging molecule. This protein freely exchanges subunits *in vitro* (7).

Bandshift assays using vLFB1ΔC281 and nuclear extracts from HepG2 cells did not show significant levels of heterodimerization (Fig. 3, lanes 5 and 6). Longer incubation times (three hours at room temperature or overnight at 0°C) did not alter the pattern of retarded bands. The same results were obtained using nuclear extracts from human hepatoma Hep3B or rat hepatoma H4 cells (Fig. 6A, lanes 13, 14 and data not shown). We concluded that

Figure 4. Half-life of LFB1 in HepG2 cells. HepG2 cells were [³⁵S]Met-labelled as described in Materials and Methods and collected at increasing times after chasing with fresh medium containing cold methionine. Nuclear extracts were prepared and LFB1 was immunoprecipitated with either αB1 or αB2 as described in Materials and Methods, resolved by SDS-PAGE and autoradiographed. The amount of labelled LFB1 immunoprecipitated from each extract was quantified by densitometric scanning. The values obtained with αB1 (filled triangles) and αB2 (open triangles) are reported as a percentage of the labelled LFB1 present before chasing.

dimeric LFB1 synthesized by the endogenous gene in hepatoma cells cannot exchange subunits.

This finding suggests that the reduced efficiency of inhibition in HepG2 cells is caused by the pre-existing endogenous pool of stable LFB1 dimers which cannot form heterodimers with the transfected repressor. In order to estimate the contribution of this pool to the induction of the pB1-CRP42CAT promoter activity, we determined the half-life of LFB1 in HepG2 cells.

Nuclear extracts from [³⁵S]-methionine-labelled cells were prepared, LFB1 was immunoprecipitated with two different polyclonal antibodies (αB1 and αB2; 24) and resolved by SDS-PAGE. Both antisera gave similar results. Densitometric scanning indicated that the LFB1 half-life in HepG2 cells is about five hours, and that more than 15% of the protein is still present after 23 hours (Fig. 4).

These data demonstrate that even several hours after transfection a significant portion of endogenous LFB1 is still present and can activate transcription from the pB1-CRP42CAT template until the physiological turn over of the cell has completely substituted wild-type LFB1 dimers with inactive wild-type/SM14 heterodimers. In this scenario only newly synthesized molecules are repressed, as these interact with the SM14 derivative before the stabilizing process takes place.

A DCoH-like cofactor stabilizes LFB1 dimers in hepatoma cells

We have shown that, similarly to liver LFB1, the protein extracted from different hepatoma cells forms stable dimers. To further characterize this property we tested whether it would be possible to dissociate the monomers without impairing dimerization and DNA binding.

Incubation of nuclear extracts from rat liver or from hepatoma cells with different detergents (Nonidet P40, Chaps, deoxycholate) in critical micelle concentrations did not affect

Figure 5. Heat-labile association between DCoH and LFB1. The position of bands corresponding to homodimeric and heterodimeric species are shown. The asterisk indicates the protein-DNA complex formed by the ubiquitous species LFBu (24). Increasing amounts of the vLFB1 Δ C281 challenging protein were incubated with different extracts for heterodimerization with full-length LFB1. RRL: 10 μ l of heat treated rat liver extract incubated with non programmed rabbit reticulocyte lysate. RRL+DCoH: 4 μ l of heat-treated rat liver extract incubated with DCoH cRNA-programmed rabbit reticulocyte lysate. Where indicated, samples were heat treated as described in Materials and Methods.

dimer stability. Similarly, addition of dithiotreitol from 0.1 mM to 100 mM did not dissociate LFB1 dimers (data not shown).

We have previously shown that LFB1 is partially resistant to heat (27, 28). As shown in Fig. 3 (lane 7), incubation of HepG2 nuclear extract at 80°C did not eliminate LFB1-DNA binding. However, it irreversibly abolished dimer stability and rendered LFB1 molecules completely available for heterodimerization with the indicator protein vLFB1 Δ C281 (Fig. 3, lanes 8 and 9). Interestingly, a lower incubation temperature (65°C) failed to destabilize the dimeric complex. The same results were obtained using rat liver nuclear extract (Fig. 5 and data not shown). Heat treatment of nuclear extracts from hepatoma cells or from rat liver always resulted in a faster migration of the LFB1-DNA complex in gel retardation assay (Fig. 3, lanes 4 and 7 and data not shown). These observations suggest that a common mechanism involving a heat-labile association of LFB1 with an ancillary factor is responsible for LFB1 dimer stabilization in liver and in hepatoma cells.

Mendel et al. (1) have recently reported the isolation of an 11kD protein named DCoH, which is associated with LFB1 in rat liver nuclei. This cofactor associates with LFB1 in cotranslation experiments *in vitro* and stabilizes the LFB1 dimers. To prove that heat treatment eliminates the association between LFB1 and DCoH, we tested whether it was possible to reverse the changes produced by heat treatment of the LFB1 dimers by adding recombinant DCoH. DCoH cRNA was translated *in vitro* in the presence of heat treated rat liver nuclear extract. The resulting LFB1-DNA complex displayed a slower rate of migration in native PAGE than the original heat treated complex (compare lanes 2 and 4 in Fig. 5), reflecting the association between the LFB1 present in the liver extract and the *in vitro* translated DCoH. Concomitantly, DCoH association with the heat treated LFB1 rendered the dimers incapable of exchanging subunits with the Δ C281 derivative (Fig. 5, lane 5). In a control experiment, heat treated LFB1 dimers incubated with non programmed rabbit reticulocyte lysate did not show any change either in electrophoretic mobility or in their ability to heterodimerize with the Δ C281 derivative (Fig. 5, lanes 6 and 7). Further treatment of the *in vitro* produced LFB1-DCoH

heterocomplex at 80°C generated LFB1 dimers that had the same electrophoretic mobility of the original heat treated complexes in gel retardation assays and freely heterodimerized with the Δ C281 indicator (Fig. 5, lanes 11-13). These data demonstrate that the LFB1-DCoH heterocomplex is heat labile and that it is possible to reverse the changes caused by heat treatment of LFB1 from liver cells by reconstituting its association with DCoH. Next, we wanted to test whether DCoH or a related factor is responsible for the stability of hepatoma cell-derived LFB1 dimers. LFB1 was immunoprecipitated from H4 nuclear extract with two different rabbit polyclonal antisera (α B1 and α B2; 24). Both antisera co-immunoprecipitate a protein with a molecular weight of about 11kD (Fig. 7, lanes 1 and 3). Since α B1 and α B2 have been raised against distinct LFB1 regions (see Materials and Methods), it is unlikely that immunoprecipitation of the 11kD species is due to a cross-reaction of the two antibodies rather than an LFB1 mediated co-immunoprecipitation. As a negative control we used nuclear extracts prepared from de-differentiated H5 cells. These cells are derived from the H4 lineage and have lost the ability to express LFB1 (29). Both antisera failed to immunoprecipitate the 11kD polypeptide from H5 nuclear extract (Fig. 7, lanes 2 and 4).

Similar experiments were performed using nuclear extracts from other hepatoma cell lines; lane 5 of Fig. 7 shows the products of an immunoprecipitation reaction with α B2 and HepG2 extract. Also in this case, a protein of about 11kD was co-immunoprecipitated with LFB1. LFB1 was purified from HepG2 nuclear extract by DNA-affinity chromatography and immunoprecipitated with α B2. The 11kD polypeptide is still co-immunoprecipitated, confirming its association with LFB1 (Fig. 7, lane 6).

LFB1 dimer stabilization activity is not restricted to hepatic cells

We have shown that all tested hepatoma cells produce stable LFB1 dimers. Moreover, LFB3 (also named vHNF1), another member of the LFB1 family produced by A1251 human kidney tumour cells (30) and by de-differentiated hepatoma C2 cells (31), also forms stable dimeric complexes (32). In contrast, transient

Figure 6. Dimer stability of transfected LFB1 in HeLa and Hep3B cells. The position of bands corresponding to homodimeric and heterodimeric species are shown on the right of each panel. The asterisk indicates the protein-DNA complex formed by the ubiquitous species LFBu (24). Increasing amounts of the vLFB1ΔC281 challenging protein were incubated with different extracts for heterodimerization with full-length LFB1. **A)**—HeLa: 10 μg of nuclear extract from HeLa cells transfected with RSV-0. HeLa/RSV-LFB1: 10 μg of nuclear extract from HeLa cells transfected with RSV-LFB1. vLFB1: 1 μg of vLFB1 HeLa nuclear extract. Hep3B: 10 μg of nuclear extract from Hep3B cells transfected with RSV-0. Hep3B/RSV-LFB1a and Hep3B/RSV-LFB1b: 10 μg of nuclear extract from Hep3B cells transfected with increasing amounts of RSV-LFB1, as described in Materials and Methods. **B)**—HeLa: 10 μg (2 μl) of nuclear extract from a pool of hygromycin resistant cells transfected with the vector pEBOpLPP. HB1.3: 10 μg (2 μl) of nuclear extract from HB1.3 cells stably expressing LFB1. HT-HB1.3: 10 μl of HB1.3 extract treated for 5 min at 80°C as described in Materials and Methods.

transfection of RSV-LFB1 in HeLa cells gives rise to an LFB1 protein which can freely heterodimerize *in vitro* (Fig. 6A, lanes 6 and 7). Similarly, Mendel et al. (14) showed that transient expression of LFB1 in human T-lymphoid Jurkat cells produces unstable LFB1 dimers. These results led to the conclusion that dimer stabilization is a property of cell-types naturally expressing LFB1 or LFB3.

However, since transient transfection creates an imbalance of a very high concentration of LFB1 molecules in a small number of cells, it is possible that the mechanism responsible for dimer stabilization cannot efficiently operate under these conditions (i.e. the amount of DCoH would be limiting). According to this hypothesis, over-expression of LFB1 in a hepatic environment should also produce unstable dimers. Human hepatoma Hep3B cells were chosen for this experiment because they contain low levels of LFB1 and allow additional protein produced by the transfected genes to be detected. Various amounts of the RSV-LFB1 construct were transfected into Hep3B cells, giving rise to proportional levels of recombinant LFB1 (compare lanes 12, 15 and 18 in Fig. 6A). Nuclear extracts from transfected cells

were then assayed for their ability to exchange subunits with vLFB1ΔC281. No band corresponding to heterodimeric complexes was detected when using the extract from Hep3B transfected with RSV-0. In contrast, both extracts from cells transfected with the expression plasmid contain LFB1 molecules which form heterodimers with the vLFB1ΔC281 derivative. Maximum heterodimerization levels were observed with the extract from cells expressing higher amounts of recombinant LFB1 (Fig. 6A, lanes 19 and 20).

To verify whether HeLa cells can also synthesize stable LFB1 dimers, we introduced the LFB1 coding region into the expression vector pEBO-pLPP (18) under the control of the SV40 promoter/enhancer regulatory region. The resulting construct (pEBO-LFB1) autonomously replicates as an episome in the nuclei of transfected cells, due to an origin of replication derived from the Epstein-Barr virus which gives rise to a low number of plasmids per cell ($2 \div 8$ copies). Upon transfection of pEBO-LFB1 into HeLa cells, several independent clones were selected and pooled. These cell pools stably express levels of LFB1 that are comparable to those of HepG2 cells. By mixing nuclear

Figure 7. A small cofactor is associated to the LFB1 dimer in hepatoma and in HeLa cells. SDS-PAGE of labelled proteins from H4 (lanes 1 and 3), H5 (lanes 2 and 4), HepG2 (lanes 5, 7 and 8) and HB1.3 (lanes 9 and 10) nuclear extracts or affinity purified LFB1 from HepG2 (lane 6) after immunoprecipitation performed as described in Materials and Methods. Cell-types and antisera used are indicated at the top of the figure ($\alpha 0$ stands for the pre-immune serum). Arrows indicate the bands corresponding to LFB1 and the 11kD species.

extract from one such pool (HB1.3) with vLFB1 Δ C281 only very low amounts of heterodimeric species were detected in gel retardation experiments (Fig. 6B, lanes 7 and 8). Similarly to the hepatic derived LFB1, heat treatment of HB1.3 extract enabled the LFB1 molecules to heterodimerize (Fig. 6B, lanes 12 and 13). Equivalent results were obtained using nuclear extracts from two other different cell pools selected independently (data not shown). We therefore concluded that the capability of forming stable LFB1 dimers is not restricted to those cells which naturally express members of the LFB1 family.

In order to determine whether stable LFB1 complexes synthesized in HB1.3 cells are associated with an HeLa-homolog of DCoH, we performed immunoprecipitation experiments using α B2 antiserum and [35 S]-methionine-labelled HB1.3 nuclear extract. As shown in Fig. 7, lane 10, a polypeptide which comigrates with the 11kD protein from HepG2 was specifically co-immunoprecipitated. Pre-immune serum did not immunoprecipitate this species (Fig. 7, lane 9).

These data demonstrate that a DCoH-like molecule is also present in cells where neither LFB1 nor LFB3 are synthesized from the endogenous genes.

DISCUSSION

Many transcription factors are composed of multiple domains (33). Such a modular structure permits dominant negative mutants to be generated which retain intact some protein modules and their relative activity, but carry a mutated version of the remaining ones so as to be non-functional. Over-production of these inhibitory variants makes the cell deficient in the function of the wild-type protein (34).

The transcription activator LFB1 has a tripartite DNA binding domain whose essential elements perform different functions: the A domain is responsible for dimerization, and the B and C domains mediate DNA recognition (7, 11). LFB1 can bind to its target sequences only as a dimer. If both components of the dimer carry mutated versions of either the Pou A-like or the homeo domain (B and C domain, respectively), DNA recognition is abolished (7).

The experiments reported in this paper show that LFB1

molecules mutated in the B or C domain interfere with the binding activity of wild-type LFB1 *in vitro* by sequestering it in heterodimers defective for DNA recognition. These results imply that each component of the LFB1 dimer must have functional A, B and C domains in order to bind DNA *in vitro*.

Dimerization interference can be reproduced *in vivo* by expressing the dominant negative mutant SM14 in cultured cells. Titration of wild-type LFB1 into inactive heterodimeric complexes with the SM14 derivative ultimately results in repression of its transcriptional activity. At present there is no evidence that LFB1 is regulated *in vivo* by a trans-dominant mechanism in a manner similar to that described for the MyoD/Id pair (35). However, the fact that LFB1 is also present in tissues such as kidney, in which LFB1-dependent promoters are silent, suggests that LFB1 activity in kidney cells is modulated by a trans dominant mechanism.

We and others have cloned cDNAs coding for transcription factors capable of heterodimerizing with LFB1 both *in vitro* and *in vivo* (LFB3/vHNF1-A and vHNF1-B/HNF1b (14, 24, 36). Namely, LFB3 is highly expressed in kidney where it is possible to detect LFB1/LFB3 heterodimers. So far, attempts to assign a functional identity to the LFB1/LFB3 heterodimer by co-transfection experiments in cultured cell lines have failed. However, the possibility that the LFB1/LFB3 heterocomplex would display different biological properties cannot be ruled out at the moment.

LFB1 cis-elements act as 'master' signals for liver-specific promoter activity. In some cases LFB1 has been demonstrated as sufficient for transcription induction *in vitro*, in cultured cells and in transgenic mice (23, 37, 38). Thus constitutive over-expression of a dominant negative mutant is expected to down-regulate all genes controlled by members of the LFB1 family. In this respect it will be interesting to analyze the phenotype of transgenic mice over-expressing the SM14 mutant.

Recombinant LFB1 dimers expressed by vaccinia virus or synthesized *in vitro* freely exchange their subunits. In contrast, dimeric LFB1 derived from rat liver is stable (14). In this work we have shown that the ability of forming LFB1 stable dimers is a common feature of different cells of hepatic origin. Interestingly, also the other members of the LFB1 family form

stable dimeric complexes. We can predict two consequences deriving from this property. The first is that only newly synthesized proteins can be engaged in heterodimeric complexes. Secondly, since multiple loading of ribosomes on the same mRNA molecule during translation favours the formation of homodimers, stabilization of such complexes gives rise to a final ratio of homo-versus hetero-dimers significantly different from that expected on the grounds of the relative expression levels of the genes coding for the two subunits. According to these predictions, the transcription activity of the endogenous LFB1 in HepG2 cells is not completely repressed by the expression of the dominant negative mutant.

Recently Mendel et al. (1) have shown that an 11kD cofactor named DCoH, is responsible for the stabilization of LFB1 dimers in rat liver nuclei. The observation that exposure to heat is able to unlock the dimer subunits and concomitantly increase electrophoretic mobility of the LFB1-DNA complex in native PAGE, led us to identify an 11kD polypeptide associated with LFB1 in human and rat hepatoma cells. This finding, along with the data reported by Mendel et al. (1) showing that murine and rat hepatoma cells synthesize the mRNA coding for DCoH, indicate that stabilization in hepatoma cells is also regulated by DCoH.

An 11kD species is also co-immunoprecipitated with the LFB3/LFB3 stable complex from A1251 cells (32), suggesting that there is a strong correlation in the cell-type distribution between members of the LFB1 family and DCoH. By generating stable transformed HeLa cells expressing physiological amounts of LFB1, we have demonstrated that also in these cells there is a mechanism responsible for LFB1 dimer stabilization. It is likely that such a mechanism is mediated by a HeLa homolog of DCoH. This hypothesis is based on the following observations. Stable HeLa LFB1 dimers display an identical heat sensitivity to those extracted from rat liver or hepatoma cells. Furthermore, a cofactor which shows the same electrophoretic mobility in SDS-PAGE as hepatic DCoH is associated with the LFB1 complex in HB1.3 stable transformants.

Mendel et al. (1), reported that low levels of DCoH mRNA are detectable in tissues such as brain and heart which do not synthesize the mRNA coding for either LFB1 or LFB3, thus indicating that expression of DCoH is not restricted to cells which naturally express members of the LFB1 family. Our experiments in the HB1.3 stable transformant extend this observation to the protein level. On the grounds of these results we hypothesize that DCoH belongs to a class of 'stabilizing factors' whose domain of expression is wider than that of the LFB1 family of transcription activators.

The finding that most of the characterized dimeric transcription factors are not available for reassortment (39–42) suggests that dimer stability is an important requirement for the activity of these proteins *in vivo*. The identification and characterization of 'stabilizing factors' will provide further insight toward clarifying the physiological role played by dimer stability on the activity of regulatory proteins.

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