Nucleic Acids Research, Vol. 20, No. 8 1983–1990

# Functional characterization of the promoter of pp63, a gene encoding a natural inhibitor of the insulin receptor tyrosine kinase

Laurence Falquerho, Laurent Paquereau, Marie José Vilarem, Simon Galas, Gilles Patey and Alphonse Le Cam*
Centre CNRS-INSERM de Pharmacologie-Endocrinologie, rue de la Cardonille, 34090 Montpellier cedex 5, France

Received December 16, 1991; Revised and Accepted March 25, 1992

## ABSTRACT

**PP63 is a liver specific phosphorylated glycoprotein encoded by a single copy gene, which has the property of inhibiting both autophosphorylation and tyrosine kinase activity of the insulin receptor. In this study, we have analyzed the structure activity relationship of the pp63 gene promoter. Five protein binding sites were found in the proximal 5′ flanking region of the gene (−223 to +4). Using oligonucleotides as competitors and purified recombinant C/EBP in footprinting and gel retardation assays, we identified two typical C/EBP sites (X1 and X3) plus a heterogenous, C/EBP-NF1 like site (X5), separated by two classical NF1 binding sites (X2 and X4). C/EBP or the related proteins were predominantly involved in supporting cell-free transcription. Occupancy of the first high affinity C/EBP site conferred almost maximal promoter efficiency, *in vitro*. However, this pp63 promoter activity remained very low as compared to that in intact hepatocytes. In these cells, occupancy of the first C/EBP (X1) and NF1 (X2) sites was already required for achieving a weak transcriptional activity. The use of the second C/EBP site (X3) strongly enhanced transcription, up to 60–70% of the maximum, whereas occupancy of the two more distal sites (X4 and X5) was necessary to fully activate the promoter. Thus, the strength of the promoter as well as the liver specific expression of pp63 gene appear to result from the interplay of several DNA-protein complexes involving mainly C/EBP and/or related proteins as well as the ubiquitous NF1 factor(s), rather than from the interaction of a more liver specific *trans*-acting factor with the promoter.**

## INTRODUCTION

Many hormones and growth factor receptors possess an intrinsic tyrosine kinase activity which has been shown to play a major role in the control of cell division in both the normal and malignant states (1, 2). Tyrosine kinases can be regulated *in vivo* by different mechanisms, including the action of activators and inhibitors. A protein secreted by normal rat hepatocytes which we have identified and called PP63 (3), is one of the very rare examples of a natural inhibitor of a receptor associated tyrosine kinase which has so far been characterized. This protein has the capacity to inhibit both the autophosphorylation and the tyrosine kinase activity of the insulin receptor and, concomitantly, block the mitogenic effect of the hormone *in vitro* (4). We have recently cloned the pp63 gene (5) which appears to be expressed constitutively at a fairly high level in normal liver (4) and severely down regulated in acutely inflamed animals (6). The protein shares extensive structural features with human α2-HS glycoprotein (7) and bovine fetuin (8), proteins that belong to the cystatin family (9). The physiological function of this potentially important molecule is presently unknown. Elucidating the mechanisms which control constitutive pp63 gene expression is a prerequisite to understand the regulatory mechanisms involved in pathological situations (e.g., inflammation); this may also provide some insights into the role of this protein.

In this study, we have dissected the pp63 gene promoter by measuring its transcriptional activity both in cell-free and in intact cell systems, and by characterizing the *cis* and *trans*-acting elements which are presumably important for its constitutive transcription.

## MATERIALS AND METHODS

### Preparation of nuclear extracts

Crude nuclear extracts (CNE) from liver, spleen or kidney were prepared from male Wistar rats (250–300 g), as described by Gorski *et al.* (10), with the modifications introduced by Sierra (11). Extreme care was taken to minimize protein degradation, specifically by adding low fat milk and a cocktail of protease inhibitors to the homogenization buffer (11). Only nuclear extracts that achieved high levels of transcription were used for footprinting, gel retardation and transcription assays.

* To whom correspondence should be addressed

### DNase I footprinting

Nuclear proteins derived from different organs or recombinant bacterial C/EBP were incubated in 15 μl of 50 mM Nacl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 4 mM spermidine, 17.5% glycerol, 10 mM HEPES (pH 7.9), 250 ng poly(dI.dC), 100 μg/ml bovine serum albumin, for 10 min at 0°C, with end-labeled DNA probes ($2\times10^4$ cpm, approximately 1 ng). The mixtures were then digested with DNase I for 1 min at 20°C in the presence of 2.5 mM $CaCl_2$. 35 μl of a stop solution containing 150 μg/ml of yeast tRNA, 6 mM EDTA, 0.06% SDS and 450 μg/ml of proteinase K were added and incubations were continued for 30 min at 42°C. The DNA was phenol extracted, precipitated with ethanol, suspended in formamide dye and the fragments were separated in a standard 6% sequencing gel. Maxam−Gilbert reaction products (12) were used as size markers. Gels were then dried and autoradiographed at −80°C with an intensifying screen. DNA fragments used as probes were prepared from a plasmid (pUC 18) bearing the pp63 proximal promoter region (−282 to +52), by cutting the vector with appropriate restriction enzymes which allowed labeling of the probes on either strand. Probes were end-labeled by filling in the overhanging 5′ ends, using DNA-polymerase (Klenow fragment) and [α-$^{32}$P] dATP and dCTP, and purified by conventional polyacrylamide gel electrophoresis.

### Gel retardation assays

Oligonucleotides synthesized in an automatic DNA synthesizer (Applied Biosystems), were annealed and end-labeled as described above. The binding conditions were identical to those described for the footprinting experiments, except that 1−2 μg poly (dI.dC) was used, in a 20 μl final volume. Electrophoretic separations were performed at room temperature (20−22°C) on 6% acrylamide gels run for 3 h at 150 V. The following oligonucleotides, which include base pairs (bp) required to generate protruding ends (in small characters), were used: 5′-gtgTCCAGTGATGTAATCAGGC-3′, the CCAAT enhancer binding protein (C/EBP) site derived from the rat hemopexin gene (13); 5′-ttgCTTTTTGGCAAGGATGGTATG-3′, the nuclear factor 1 (NF1) binding site derived from the rat liver pyruvate kinase (L-PK) gene (14); 5′-gatcTCAAACTGTCAAATATTAA-CTAAAGGGAG-3′, the hepatic nuclear factor 1 (HNF1) binding site derived from the rat β fibrinogen gene (15). The binding sites derived from the pp63 promoter (5) were:

X1: 5′-tcgaCGCCTTTACGCAATTCCTTCG-3′ (−64 to −44)

X2: 5′-attGATGATTTGGAACCAGAACAAAAATCAG-3′ (−96 to −69)

X3: 5′-tcgaGATAGATGATGTCCTAACTTATTTGCTTTCC-CAGAG-3′ (−145 to −110)

X5: 5′-tcgacTATCGCCATGTTGCAAGCAGACTTTGGAAT-ATCTTCCCCC-3′ (−219 to −179)

### *In vitro* transcription analysis

Transcription reactions were performed as previously described (10, 11), using CNE from liver, spleen or kidney, with templates prepared as follows. Fragments of the promoter, starting from different 5′ end positions chosen as a function of the footprint borders and ending at position + 4 (3′), were synthesized using the polymerase chain reaction (PCR) method. Two cloning sites, Pst I and Sma I were added at the 5′ and 3′ ends, respectively; they allowed oriented cloning of PCR fragments at the Pst I (5′) and the blunt-ended Sac I (3′) sites, in pUC13 containing a G-free cassette (16) which was modified as follows. Part of the polylinker region, containing the Sph I, Pst I, Sal I, Xba I and BamH I sites located downstream of the G-free sequence was moved in front of this sequence, to allow for the use of several different sites for directional cloning. All constructs were verified by sequencing of the relevant portions (17). A plasmid, pAdML, bearing the strong promiscuous major late adenovirus promoter inserted in front of a shortened G-free sequence (180 bp) was transcribed simultaneously with the test template and used as an internal standard to normalize the data. Quantification of the transcripts generated from each promoter was achieved by cutting out the bands corresponding to the 380 bp and the 180 bp species, and counting in a scintillation counter. Plasmids were amplified and prepared according to standard methods (18), by using polyethylene glycol precipitation and column chromatography (Ultrogel, Pharmacia) as final purification steps. Transcription products were extracted with phenol and analyzed on 6% polyacrylamide gels.

### Cell preparation and transfection of CAT plasmids

Highly purified supercoiled DNA (6 μg) was transfected into exponentially growing ($5-7\times10^5$ cells/ 60 mm diameter plastic dish) HepG2 human hepatoma cells (19) or NIH 3T3 fibroblasts grown in Dulbecco and Vogt-modified Eagle's medium (DMEM) containing 10% fetal calf serum, 50 μg gentamycin and 10 μg amphotericin B per ml, as previously described (20). The DNA-calcium phosphate precipitate was left overnight in contact with the cells, and CAT activities were measured 48 h later (21). To account for the variations in transfection efficiency, 2 μg of a plasmid bearing the firefly luciferase gene driven by the Rous sarcoma virus promoter (pRSV LUC), were co-transfected with the test plasmid. Luciferase activity present in detergent lysed cells was determined as previously described (22).

Normal rat hepatocytes prepared from adult male Wistar rats (23) were transfected in suspension, using an electroporation procedure (Paquereau and Le Cam, submitted). Cells were then cultivated as monolayers for 24 h, in a mixture of DMEM and Ham F12 (1/1) supplemented with 10% (v/v) fetal calf serum, 50 μg/ml gentamycin and 10 μg/ml amphotericin B. CAT (chloramphenicol acetyl transferase) activities were measured after 24 h culture, a time that was found to be sufficient for maximal expression of the cat gene. For reasons that are not presently obvious, the co-transfection of a control plasmid (i e., pRSV LUC) with the test plasmid in freshly isolated hepatocytes yielded irreproducible data. We therefore decided not to use this procedure and instead, we repeated the experiments (at least 5 times) to ensure reliability of the results.

CAT activities present in triton cell extracts (22) were measured using the mixed-phase assay described by Nielsen *et al.* (24). Fragments of the pp63 promoter were generated by using the PCR technique or pre-existing restriction sites, and cloned upstream from the cat gene in a pEMBL vector (25). All the plasmids used for transfection were purified by cesium banding (18) and each construct was verified by sequencing (17).

### Nuclear run on assays

Nuclei isolated from normal rat liver (10) were used to evaluate the *in vivo* rate of pp63 gene transcription. Incubation conditions (with [α-$^{32}$P] UTP) and the procedure used to analyze the run on transcripts were as previously described (26). The following plasmids were used as hybridization templates: pUC19 bearing 0.52 kb of the serine protease inhibitor 2 cDNA (27); pBR322 harboring 0.65 kb of the rat α2-macroglobulin cDNA (28); pBR322 bearing 1.6 kb of the mouse β-actin cDNA (29); pBR322 containing 1.2 kb of the rat albumin cDNA (30); Bluescript

SK$^+$ bearing 0.7 kb of the rat $\alpha$1-acid glycoprotein cDNA (31); pUC 19 bearing 1.25 kb of the pp63 cDNA (4). The amount of newly synthesized transcripts hybridized to the templates immobilized on a nylon membrane (Hybond N$^+$, Amersham) corresponded to $20\times10^6$ trichloroacetic acid precipitable counts.

### Materials

[$\alpha$-$^{32}$P] UTP, [$\alpha$-$^{32}$P] dATP and dCTP, [$\alpha$-$^{35}$S] dATP were obtained from New England Nuclear. Restriction endonucleases and DNA modification enzymes were purchased from New England Biolabs or Boehringer Mannheim. All other chemicals used in these studies were of molecular biology grade. Recombinant C/EBP protein (32) containing the DNA-binding site was a gift from Dr. S. L. McKnight. The following constructs were kindly provided to us. pC2AT, the wild type G-free cassette plasmid (Drs Sawadogo and Roeder); a G-free cassette plasmid bearing the L-PK (Drs. A. Kahn and M. Raymondjean); pAdML, a plasmid bearing the adenovirus major late promoter placed in front of a shortened (180 bp) G-free cassette and a plasmid harboring the $\alpha$1-acid glycoprotein cDNA (Dr. F. Sierra); a plasmid containing the rat $\alpha$2-macroglobulin cDNA (Dr. P.C. Heinrich); plasmids containing the rat albumin (Dr. J. Salat-Trépat) and the mouse $\beta$-actin cDNAs (Dr.S. Alonso).

## RESULTS

### Proteins binding to the pp63 gene promoter

As a first step to identify the nature of the transcription factors recognizing the pp63 promoter, we have analyzed the binding of nuclear proteins to the proximal 5′ flanking region of the gene (−282 to +52). We first performed DNase I footprinting analyses in the presence of increasing amounts of liver nuclear proteins. Five regions referred to as boxes X1 to X5, were strongly protected against DNase I digestion (Fig. 1 A). Although some differences could be detected, the extent of the footprints appeared quite similar on both strands. On the other hand, both the extent of the footprints as well as the appearance and intensity of DNase I hypersensitive sites changed upon addition of increasing concentrations of proteins. Thus, X1 and, to a lesser extent X2, were observed with a very low amount of proteins (5 $\mu$g), whereas a higher protein input was required to observe the other ones. Such changes in footprint patterns, depending on the amount of nuclear proteins, are consistent with the notion that multiple factors interact with pp63 promoter.

To gain further informations, we next performed footprinting analyses with CNE from different tissues and we carried out competition experiments with unlabeled oligonucleotides (Fig. 1 B). With kidney CNE, footprints X2 and X4 were observed and appeared as strong as with liver CNE. In contrast, footprints X1, X3 and X5, although they were detectable, appeared significantly weaker than with liver proteins. An additional footprint, located 5′ to X5, was exclusively observed with kidney extracts. With spleen extracts, only X1 and X2 were detected but were always much weaker than with the two other organs. In liver CNE, addition of a C/EBP oligonucleotide to the binding reaction eliminated totally footprints X1, X3 and only partially (the most 5′ part) X5. A NF1 motif abolished selectively the footprint X4 and strongly affected X2. In contrast, an HNF1 element had no effect. Interestingly, only footprints X1 and X3 (completely) and X5 (partially, the most 5′ part) were preserved with heated liver

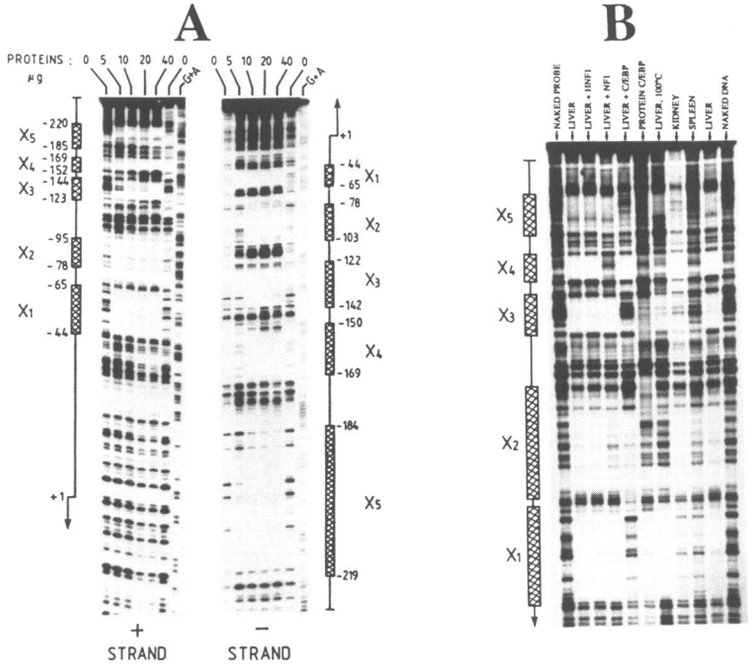


**Figure. 1**. Footprinting analyses of the rat pp63 gene promoter. Panel A. Dependence on protein input. The probe used was a 334 bp fragment from −282 (Pst I site) to + 52 (BstN I site), end-labeled on either the + (5′ to 3′) or the − (3′ to 5′) strand. Various amounts of rat liver nuclear proteins were used and the ratio DNase I / $\mu$g protein was kept constant. The five protected regions are delineated by boxes X1 to X5. The numbers noted alongside correspond to the footprint boundaries relative to the tsp (+1). G+A are the Maxam and Gilbert reaction products. Panel B. Competition and tissue specificity analyses. The 5′ end-labeled + strand probe (−282 to +52), was incubated with either 20 $\mu$g native nuclear proteins from liver, spleen or kidney, or with the same amounts of liver nuclear proteins that were previously heated at 100°C for 5 min, or with 50 ng of pure recombinant C/EBP. When indicated, 20 ng of an unlabeled, double stranded competitor oligonucleotide was added to the binding mixture. Positions of the 5 different footprints are indicated at the left hand side.

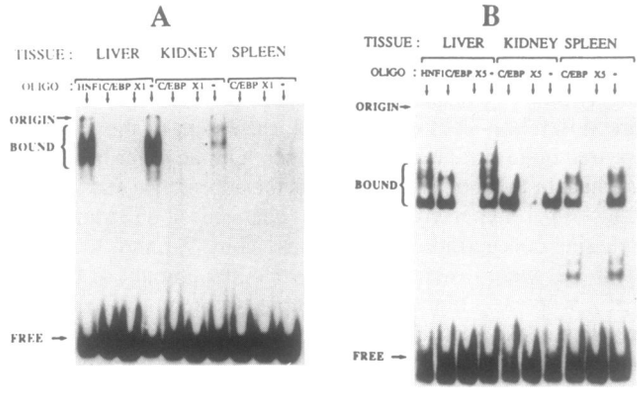


**Figure 2.** Gel mobility shift assays with labeled X1 or X5 oligonucleotides. The X1 (panel A) or X5 (panel B) probes were allowed to bind to CNE (5 μg proteins) from liver, kidney or spleen, in the absence (−) or presence of 50 ng of the unlabeled homologous (X1 or X5), C/EBP or HNF1 oligonucleotides. The smaller complexes seen with spleen extracts (panel B) presumably represent degradation products.

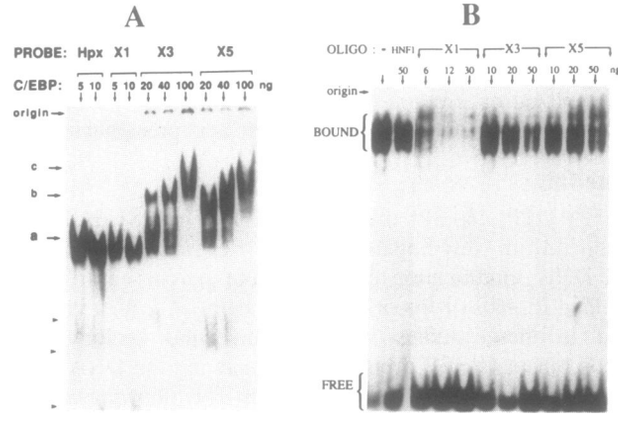


**Figure 3.** Panel A. Binding of purified recombinant C/EBP to a typical C/EBP motif and to the pp63 promoter-derived protein binding sites. Various amounts of recombinant bacterial protein were incubated with 5000 cpm of each of the following probes: the C/EBP binding site from the hemopexin gene (Hpx), or X1, X3 or X5 elements from the pp63 promoter. Migration of free probes is indicated with arrowheads. The larger complexes (b and c) were never seen with the Hpx or X1 probes, even with 100 ng C/EBP; in the presence of smaller amounts of C/EBP (i.e., 5−10 ng), only the smaller complex (a) formed with probes X3 and X5. Panel B. Band shift analysis of the inhibition of liver nuclear proteins binding to a typical C/EBP probe by pp63 promoter elements. A labeled C/EBP oligonucleotide derived from the hemopexin gene was incubated in the absence (−) or presence of various amounts of competitors. HNF1 was used as a non-specific competitor.

CNE. The same three regions of the promoter were protected to various extents (X1>X3>X5) by purified bacterial recombinant C/EBP, which also bound to another element overlapping site 2 (Fig. 1 B). The latter presumably corresponds to recognition by C/EBP of a sequence (5′-TGTTGTTT-3′) present in the promoter (see Fig. 4), which resembles the viral enhancer core element shown to interact with this protein (33).

To complement the footprint studies, gel retardation assays involving both competition and tissue specificity analyses were performed with oligonucleotide probes corresponding to the footprinted regions. X1 probe bound specifically to liver nuclear proteins, giving rise to several complexes which could barely be resolved, owing to their comparable sizes (Fig. 2 A). Both the C/EBP and X1 unlabeled oligonucleotides totally displaced these complexes, whereas an HNF1 motif had no effect. A weak interaction of nuclear proteins with the same probe was observed with kidney and spleen CNE, giving rise to complexes of slightly different sizes which could also be competed out by the same unlabeled oligonucleotides (Fig. 2 A). Essentially the same binding pattern was obtained using the X3 element as a probe and will therefore not be presented. In contrast, the binding of X5 probe to CNE yielded somewhat different patterns (Fig. 2 B). With liver extracts, at least 4 complexes were observed which could be completely eliminated by the cold homologous oligonucleotide, indicating that they represented specific binding. Two of these complexes which quantitatively predominated and were not displaced by a typical C/EBP oligonucleotide, were observed with spleen CNE. One of them was also detected with kidney extracts. In agreement with the footprinting data, these band shift experiments indicate that, in addition to C/EBP or the related factors, some other ubiquitous nuclear protein(s) binds to site X5.

Both gel retardation and footprinting analyses suggested that C/EBP or liver enriched proteins of the same family such as DBP (34) or LAP (36) bound to the X1, X3 and X5 sites. This was confirmed by two types of an experiment. First, purified recombinant C/EBP was indeed shown to bind to the three sites (Fig. 3 A). However, differences were noted in the binding patterns. A single X1-C/EBP complex similar to that observed with a typical C/EBP probe (i.e., the C/EBP hemopexin site) was detected, whereas several complexes of increasing sizes were obtained with both X3 and X5 probes. This presumably can be accounted for by differences in the number of potential C/EBP binding sites (one for X1, three for X3 and two for X5) contained in these probes (Fig. 4). Second, the three pp63-derived oligonucleotides competed out the binding of the C/EBP consensus probe to liver nuclear proteins with, however, quite different efficiencies (Fig. 3 B). Thus, X1 was at least 10 times more potent than X3, a difference which presumably reflects their various degrees of homology (perfect match for X1 and one or two mismatches for X3) with the core consensus C/EBP element (36). Surprisingly, X5 was even less potent than X3, despite the presence of two potential C/EBP binding sites, one of them (−210 to −202) matches perfectly the consensus sequence, whereas the other one (−196 to −188) displays two mismatches (Fig. 4). This second site includes a sequence (5′-TTGGAA-3′) known to bind to proteins of the NF1 family (37). However, the failure of a typical NF1 oligonucleotide to compete for the formation of footprint X5 (Fig. 1 B), appears to rule out the possibility that NF1 *per se* binds to this element. Nonetheless, the occupancy of this hybrid site (C/EBP/NF1-like) drastically decreased the ability of the other typical C/EBP element to compete for C/EBP binding (Fig. 3 B).

Binding of the X2 probe to liver CNE gave rise to several large complexes of comparable sizes (Fig. 5). Their formation was strongly inhibited by the unlabeled homologous oligonucleotide and, even more efficiently, by a typical NF1 motif, but not an HNF1 site. Smaller amounts of the same DNA-protein complexes were observed with both kidney and spleen CNE. Essentially the same binding pattern was obtained with an X4 probe (unpublished observations). Both footprinting and mobility shift assays suggested that proteins of the NF1 family (37) bound to X2 and X4 sites. This is in agreement with the presence, in the

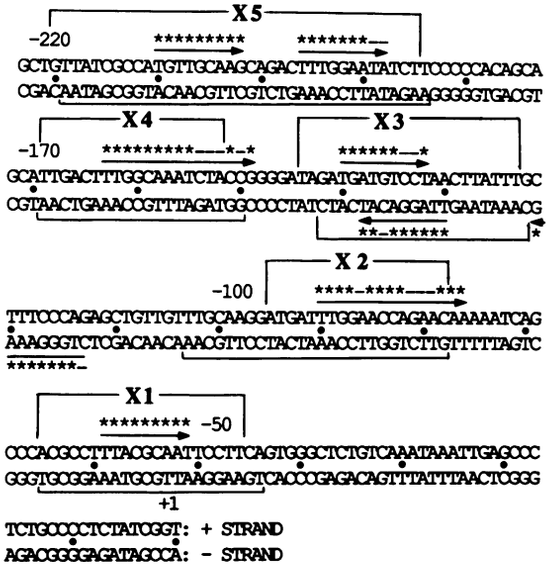


**Figure 4**. Sequence of the pp63 promoter. The footprinted area (X1 to X5) are indicated by brackets. Potential C/EBP and NF1 binding sites, covered with arrows, were identified by comparison with the corresponding core consensus C/EBP (5'-TT/GNNGNAAT/G-3') and NF1 (5'-PyTGGCANNNTGCCAPu-3') sequences. Nucleotides which are conserved (asterisks) or not (dashes) are scored.

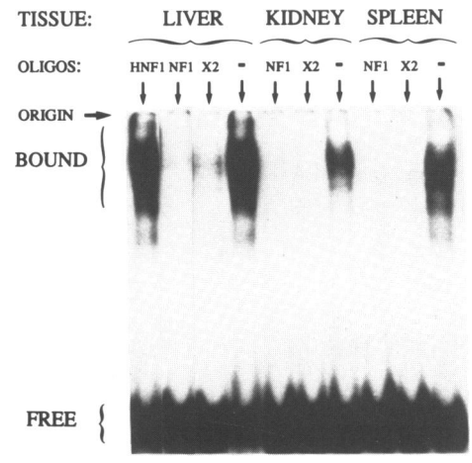


**Figure 5**. Band shift analysis of site 2 DNA-protein complexes. The X2 probe was allowed to bind to nuclear proteins (5 μg) from liver, kidney or spleen, in the absence (−) or presence of 50 ng of the unlabeled homologous X2, NF1 or HNF1 oligonucleotides.

X2 and X4 elements, of sequences (see Fig. 4) that closely match that of the consensus NF1 motif (38) . Such sequences have previously been found in other typical liver promoters such as the albumin (39) and the L-PK (40) promoters, and identified as NF1 binding sites.

### Transcriptional activity of pp63 gene promoter in cell-free systems

The *in vitro* transcription assay developed by Sawadogo and Roeder (16) allows to directly investigate, in a very simple and rapid way, the role of the various DNA-binding protein complexes in constitutive transcription. In the absence of competitors, a significant amount of the 380 bp transcript representing the activity of the pp63 promoter was detected (Fig. 6 A). To evaluate the contribution of liver factors which belong to the C/EBP and NF1 families, to the transcriptional activation of pp63 promoter *in vitro*, we added a large molar excess (300−600 fold) of the corresponding binding sites to the transcription mixtures. A C/EBP oligonucleotide was able to strongly inhibit transcription (>80% with 0.3 μg), whereas a NF1 site, although it was active, was significantly less efficient (inhibition < 50% with 0.3 μg) (Fig. 6 A). These effects appeared to be largely specific since an HNF1 element did not affect transcription from the pp63 promoter, *in vitro*. Transcriptional activity of this promoter was also measured in spleen and kidney CNE (Fig. 6 B). The promoter appeared to work, at least partly, in a tissue specific manner since it was active in kidney (approximately 50% of liver) but almost completely silent in spleen. For the sake of comparison, the activity of the L-PK promoter was analyzed under the same conditions. The latter which was active both in liver and, to a lesser extent, in kidney extracts, was at least 50 times more potent than pp63 promoter in liver CNE (Fig. 6 B). It should be noted that, in all instances, the activity of pp63 promoter remained low *in vitro*, since it reached at best 6−7% that of the strong adenovirus promoter and 2−3% that of the L-PK promoter.

To evaluate the relative importance of the various binding elements (X1 to X5) identified in the pp63 promoter for its overall transcriptional activity, deletion mutants bearing fragments of different lengths of the 5' flanking gene region were used as templates. A construct containing the transcription start point (tsp) and a region that encompasses the potential TATA box (−35 to +4) had only a very low level of activity (about 5% of the maximum) (Fig. 6 C). When the first footprinted region was included (construct −67 to +4), transcription increased by about 20 fold. Addition of the four other footprinted regions, up to position −223, caused only a small additional increase (20%) in transcriptional activity of the promoter (Fig. 6 C).

### Transcriptional activity of pp63 gene promoter in intact cells

Because it appeared to represent the only cell type in which pp63 gene is expressed (4), we decided to use the normal hepatocyte as a recipient cell in transfection experiments. When a fragment of the promoter encompassing 67 bp upstream from the tsp (thus bearing the first footprinted element) was used (construct −67 to +4), virtually no CAT activity could be measured in cell extracts (Fig. 7 A). Adding the second footprinted region (construct −99 to +4) increased transcription level up to about 20% of the maximum. This value was further enhanced by about three-fold with a construct bearing the first three footprinted elements (−143 to +4) and, an additional 30−40% increase was obtained when the promoter contained the five footprinted regions (construct −282 to +52). This seems to represent the highest transcriptional activity that could be achieved since increasing the length of the promoter up to about 700 bp had no further effect (Fig. 7 A). It is interesting to note that the most efficient pp63 construct always yielded a higher CAT value than the one bearing the simian virus promoter.

To assess the liver specificity of pp63 promoter, a plasmid harboring 692 bp of the 5' flanking gene region was transfected into both hepatic and non-hepatic cell types. As shown in Fig. 7 B, the promoter was active in the HepG2 hepatoma cell line

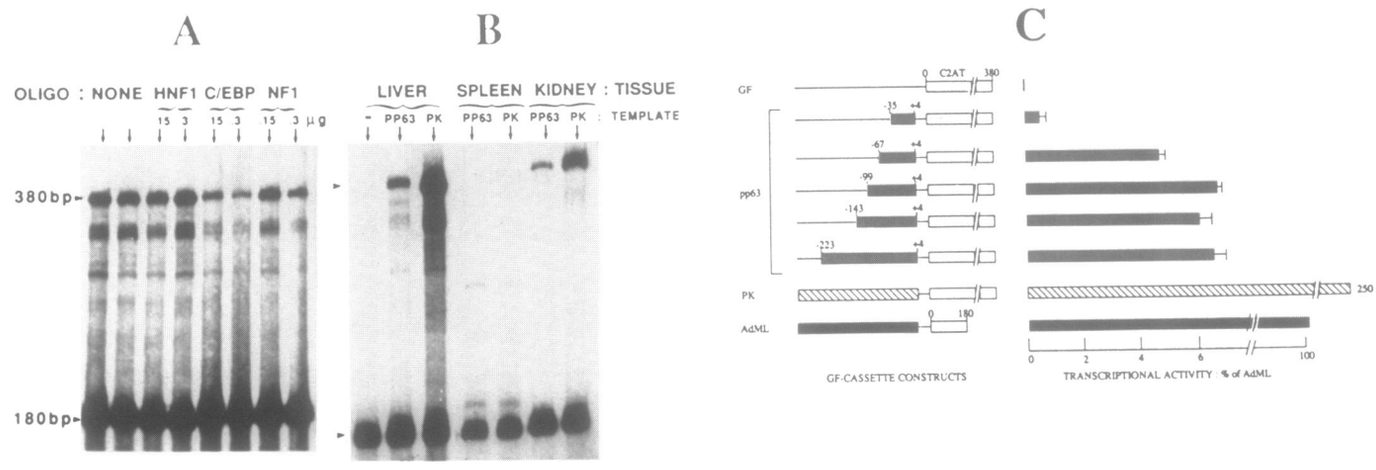


**Figure 6**. Cell-free transcription from the pp63 promoter. Panel A. Competition analysis. *In vitro* transcription was performed with a mixture of pp63 (−223 to +4) (900 ng) and AdML (100 ng) templates, with 36 μg of liver nuclear proteins, in the absence (none) or presence of various competitor oligonucleotides. Panel B. Tissue specificity analysis. Transcription assays were performed with nuclear proteins from liver (36 μg), spleen (50 μg) and kidney (54 μg), using various mixtures of templates containing the AdML plasmid (100 ng) together with a G-free cassette vector (900 ng) without promoter (−) or containing the pp63 (−223 to +4) or L-PK promoters. Transcripts from the pp63 or L-PK (380 bp), and AdML (180 bp) promoters were separated on a sequencing gel. Panel C. Analysis of the transcriptional activity of various pp63 promoter deletion mutants in liver CNE. Transcription assays were performed as described above (panel A), with various pp63 or the L-PK constructs, together with the AdML template. The radioactivity contained in the 380 bp (test promoter) and 180 bp (adenovirus promoter) bands was quantified by counting, and activities of the pp63 and L-PK promoters are compared to that of the viral promoter. Values are the mean +/− SEM of 3 separate experiments performed with different extracts.

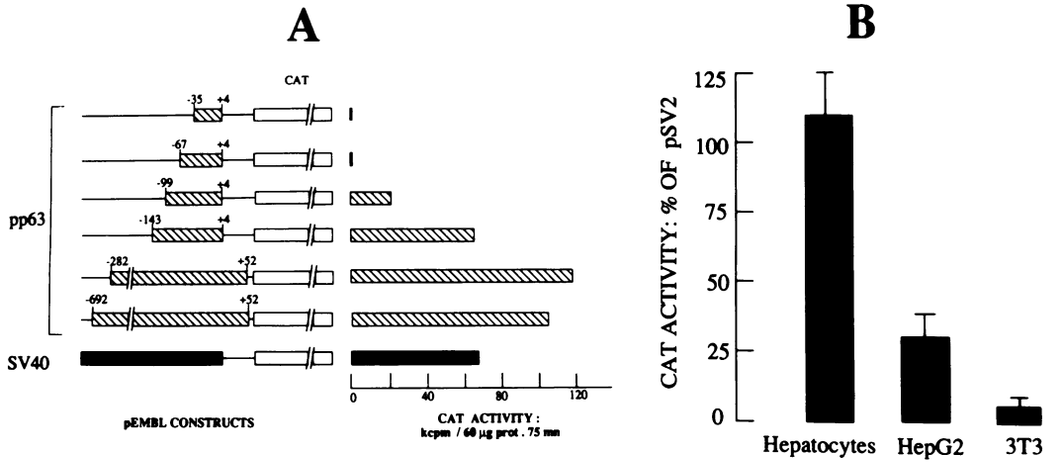


**Figure 7**. Panel A. Analysis of the transcriptional activity of various pp63 promoter deletion mutants in intact hepatocytes. Cells were transfected using an electroporation method, with various pp63-pEMBL constructs or with pSV2CAT (SV40), and cultured as monolayers. CAT activities present in cell lysates were determined 24 h later. Because large variations (3−5 fold) were observed in intrinsic CAT values from one cell preparation to another, the results of a typical experiment are presented. However, the relative efficiency of the different constructs was found to be almost unvariable, in five different experiments. Panel B. Comparison of relative pp63 promoter efficiencies in different cell types. The pp63-pEMBL (−692 to +52) or pSV2CAT constructs were transfected into normal rat hepatocytes, HepG2 or NIH 3T3 cells as described in the Materials and Methods section. CAT activities were measured 24 h (hepatocytes) or 48 h (cell lines) after transfection. kcpm: $10^3$ cpm

(about 25% of the activity measured in hepatocytes), but virtually silent in fibroblastic NIH 3T3 cells.

### Transcriptional activity of pp63 promoter *in vivo*

In an attempt to validate the data obtained *in vitro* with cell-free systems and with intact hepatocytes, the transcriptional activity of pp63 gene promoter *in vivo* was evaluated and compared to that of several other well characterized promoters, by analyzing run on transcripts (Fig. 8). The signal obtained with a pp63 probe was about two-fold stronger than that obtained with the albumin probe. Considering that the sizes of the hybridizing sequences were of the same order of magnitude (cDNA inserts of about 1.2 kb), and that the albumin gene (14.5 kb) (30) is approximately two times larger than the pp63 gene (7 kb) (5), the strength of pp63 promoter appeared to be 3−4 times that of the strong, liver-specific albumin promoter. It also largely exceeded (>10 fold) that of the two other liver specific, α1-acid glycoprotein and α2-macroglobulin gene promoters, and that of the ubiquitous β-actin gene promoter. However, it appeared less than half as strong as the serine protease inhibitor gene promoter.

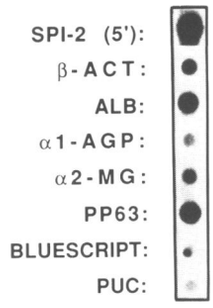


**Figure 8**. Nuclear run on analysis of pp63 promoter activity *in vivo*. Nascent liver RNA transcripts were elongated *in vitro*, in the presence of [$\alpha$-$^{32}$P] UTP, purified and hybridized to various plasmids bearing partial cDNAs encoding the serine protease inhibitor 2 (SPI-2, 5'), $\alpha$2-macroglobulin ($\alpha$2-MG), $\beta$-actin ($\beta$ ACT), albumin (ALB), $\alpha$1-acid glycoprotein ($\alpha$1-AGP) and pp63 (PP63). Bluescript SK$^+$ and pUC 19 are blank controls. After hybridization performed at 42°C for 48 h, the membrane was digested with RNases (A and T1), extensively washed, and autoradiographed at −80°C with an intensifying screen, for 3 days.

## DISCUSSION

These studies show that mainly two major classes of transcription factors bind at five different sites to a proximal 5' flanking region of the pp63 gene which appears to be sufficient for maximal transcription of a reporter gene in intact hepatocytes. These factors probably correspond to transcriptional activators of the C/EBP (41) and NF1 (37, 38) families.

Identification of C/EBP or C/EBP-like proteins as potential binding factors to sites X1, X3 and X5 of pp63 promoter in liver extracts result from the following data. (i) An oligonucleotide corresponding to a well-characterized C/EBP binding element altered, to various extents, all three footprints; furthermore, the corresponding liver nuclear proteins proved to be totally (X1 and X3) or partially (X5) thermoresistant which is a typical feature of C/EBP (33); (ii) the purified recombinant protein footprinted pp63 promoter at the same three sites; (iii) in band shift assays, binding of X1, X3 and X5 elements to liver nuclear proteins was totally (X1 and X3) or partially (X5) competed out by a typical C/EBP motif; (iv) all three pp63 promoter binding elements specifically recognized the bacterial recombinant protein. Consistent with these observations, the sequences of all three binding sites match, either perfectly for X1 and X5 (for one of them) or partially for X3 (Fig. 4), the core consensus C/EBP binding sequence (36). However, these three homologous sites do not seem to be equivalent and might bind to different, although closely related proteins. Several observations suggest that C/EBP is likely to be the binding factor for site 1 and that one or several of the other members of the family bind to sites 3 and 5. First, pure recombinant bacterial C/EBP recognizes very efficiently site 1, but its affinity for site 3 and 5 is much lower. Second, despite the fact that footprints X1, X3 and X5 observed with liver extracts were equally strong, competition analyses showed clear differences in the affinity of the liver binding factors for a typical C/EBP site (X1 > > X3 > X5). C/EBP was originally purified from rat liver (32) and is predominantly found in terminally differentiated cells such as adipocytes and hepatocytes (42, 43), but seems to be absent in tissues like spleen and kidney (41, 44). C/EBP belongs to a continuously growing family of related transcriptional activators which includes DBP (34), proteins recently cloned by Williams *et al.* and called CRP1, CRP2 (equivalent to LAP (35), NF-IL6 (36), IL6-DBP (45) and AGP/DBP (46)), and CRP3 (41), and Ig/EBP-1 (47). These proteins recognize the same or closely resembling DNA sequences and, with the exception of DBP which does not form hetero-dimers (34), have similar leucine zipper dimerization specificities (41). Although most of them are enriched in liver and therefore can interact with the pp63 promoter, further studies will be required to discriminate the various possibilities. The binding of both kidney and spleen nuclear factors to pp63 promoter C/EBP sites presumably represents recognition by proteins with functional homologies, reported to be present in these tissues (41).

An additional ubiquitous, as yet unidentified factor, different from NF1, bound to element X5. Interestingly, the binding of this protein strongly decreased the affinity of the C/EBP site located in its close vicinity, which, based on the sequence, should behave as a high affinity site. Whether such a quenching phenomenon takes place *in vivo* and whether it might have some functional significance remains to be evaluated.

Proteins of the NF1 family (37,38) appear to bind to sites 2 and 4 of the pp63 promoter. This is indicated by the fact that, in addition to the homology with the core consensus NF1 sequence (see Fig. 4), an oligonucleotide corresponding to a typical NF1 binding site eliminated the two heat sensitive footprints and competed out very efficiently the binding of homologous pp63-derived probes (X2 and X4 oligonucleotides) to liver nuclear proteins. One of these proteins might correspond to the NF1-like binding protein recently purified from rat liver (48).

Nuclear run on experiments showed that pp63 promoter works more efficiently than the albumin promoter in liver, *in vivo*. Despite the numerous limitations of the system and more particularly the use of a transient transfection assay, transfection of chimeric genes into intact hepatocytes confirmed that it belongs to the class of high strength liver promoters. Indeed, even a shortened pp63 promoter (i.e., 223 bp) was more efficient than the strong SV40 promoter in driving the cat gene. This strikingly contrasts with the poor efficiency of pp63 promoter in liver nuclear extracts. In the cell-free system, pp63 promoter was at least 20 times less active than the strong adenovirus promoter and 50 times less efficient than the L-PK promoter (14). Analysis of the data obtained with deletion mutants might help understanding the differences observed between the cell-free and the intact cell systems. *In vitro*, the presence of the first high affinity C/EBP binding element was sufficient to ensure almost maximal promoter efficiency. In contrast, this element alone had essentially no activity in intact hepatocytes. In this system, the first C/EBP and NF1 binding sites were both required to activate the promoter and the presence of the second C/EBP element was mandatory to reach a high level of transcription. This strongly suggests that a cooperation between the first three DNA-protein complexes must take place to obtain efficient transcription in intact cells. Disruption of the cellular structure may prevent such a phenomenon to occur, which would explain the very low transcriptional activity of the promoter *in vitro*. The last two distal elements appear to build up moderately the strength of pp63 promoter, but however, might cooperate with the proximal sites. Site directed mutagenesis will allow to more precisely delineate the role of each of these sites in the context of the whole promoter. Competition experiments indicated that C/EBP-like proteins were the most important transcription factors in supporting pp63 promoter activity, *in vitro*. Unlike the L-PK promoter for which

the binding of a single factor (HNF1) is sufficient to achieve almost maximal transcriptional activity (see ref. 14 and Fig. 6 B), the cooperation between several C/EBP and NF1 binding sites which might also involve other accessory factors, appears to be necessary for the activation of pp63 promoter. This might explain the enormous difference observed *in vitro*, in the transcriptional activity of these two largely liver specific promoters.

Four protein factors (or families of proteins): HNF1, C/EBP, HNF3 $\alpha$ and HNF4 have been reported to bind to hepatic genes, and to govern liver specific expression (44). Other binding sites for ubiquitous factors such as AP1 (49), NF1 (37) or NFY/ACF (50) are often found on the same promoters. In most cases, cooperative interactions between different factors were shown to occur, but one of them appeared very often to be functionally dominant (e.g., HNF1 for the L-PK promoter). For the pp63 gene, members of the C/EBP family are clearly the functionally relevant most important factors. However, none of them appears to be totally liver specific. It therefore seems likely that *in vivo*, the liver specific expression of this gene (4) arises from an precise interplay between different DNA-C/EBP-like and DNA-NF1 protein complexes, which form on the promoter. It will be interesting to see whether C/EBP or the related proteins are also involved in the negative regulation of pp63 gene during acute inflammation (6). In this regard, it is worth mentioning that NF-IL6, a transcription factor which is activated during acute inflammation and binds to C/EBP sites, was shown to mediate the transcriptional activation of positive acute phase genes (36).

## ACKNOWLEDGMENTS

We thank S.L.McKnight for kindly providing to us recombinant C/EBP, P.C.Heinrich, J.Salat-Trépat, S.Alonso, A.Kahn and M.Raymondjean for the gift of various plasmids, and J.C.Nicolas for the synthesis of the oligonucleotides. We are indebted to F.Sierra for teaching us the *in vitro* transcription method, his help and continuous support throughout this study and for giving us the $\alpha$1-acid glycoprotein cDNA plasmid and the G-free cassette vector containing the AdML promoter. We deeply thank M.Raymondjean and F.Sierra for many helpful advice and for carefully reading the manuscript. We also acknowledge the contribution of L.Charvet and M.Passama for art work. This work was supported in part by a grant from the 'Association pour la Recherche sur le Cancer', (ARC), Villejuif France.

## REFERENCES

1. Ullrich,A. and Schlessinger,J. (1990) *Cell*, **61**, 203–212.
2. Cross,M. and Dexter,M. (1991) *Cell*, **64**, 271–280.
3. Le Cam,A., Magnaldo,I, Le Cam,G. and Auberger,P. (1985) *J. Biol. Chem.*, **260**, 15965–15971.
4. Auberger,P., Falquerho,L., Contrérès,J.O., Pagès,G., Le Cam,G., Rossi,B. and Le Cam,A. (1989) *Cell*, **58**, 631–640.
5. Falquerho, L., Patey,G., Paquereau,L., Rossi,V., Lahuna,O., Szpirer,J., Szpirer,C., Levan,G. and Le Cam,A. (1991) *Gene*, **98**, 209–216.
6. Le Cam,A. and Le Cam,G. (1985) *Biochem. J.*, **230**, 603–607.
7. Lee,C.C., Bowman,B.H. and Yang,F. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4403–4407.
8. Christie,D.L., Dziegielewski,K.M., Hill,R.M. and Saunders,N.R. (1987) *FEBS Lett.*, **214**, 45–49.
9. Kellerman,J., Haupt,H., Auerswald,E.A. and Müller-Esterl,W. (1989). *J. Biol. Chem.*, **264**, 14121–14128.
10. Gorski,K., Carneiro,M. and Schibler,U. (1986) Cell, 47, 767–776.
11. Sierra,F. (1990). In Azzi,A., Polak,J.M. and Saluz,H.P. (eds) BioMethods-A Laboratory Guide To In Vitro Transcription. Birkhäuser Verlag. Vol. 2.
12. Maxam,A.M. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499–560.
13. Poli,V., L. Silengo,L., Altruda,S. and Cortese,R. (1989) *Nucleic Acids Res.*, **17**, 9351–9365.
14. Vaulont,S., Puzenat,N., Kahn,A. and Raymondjean,M. (1989) Mol. Cell. Biol., 9, 4409–4415.
15. Courtois,G., Baumhueter,S. and Crabtree,G.R. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7937–7941.
16. Sawadogo,M. and Roeder,R.G. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4394–4398.
17. Chen,E.Y. and Seeburg,P.H. (1985) *DNA*, **4**, 165–170.
18. Maniatis,T., Fritsch,E.F. and Sambrook,J. (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. Knowles,B.B., Howe,C.C. and Aden,D.P. (1980) *Science*, **209**, 497–499.
20. Chen,C. and Okayama,H. (1987) *Mol. Cell. Biol.*, **7**, 2745–2752.
21. Gorman,C.M., Moffat,L.M. and Howard,B. (1982) *Mol. Cell. Biol.*, **2**, 1044–1051.
22. Brasier,A.R., Tate,J.E. and Habener,J.F. (1989) *Biotechniques*, **7**, 1116–1122.
23. Le Cam,A., Guillouzo,A. and Freychet,P. (1975) *Exp. Cell Res.*, **98**, 382–396.
24. Nielsen,D.A., Chang,T.C. and Shapiro,D.J. (1989) *Anal. Biochem.*, **179**, 19–23.
25. Dente,L., Cesarini,G. and Cortese,R. (1983) *Nucleic Acids Res.*, **11**, 1475–1489.
26. Iynedjian,P.B., Jotterand,D., Nouspikel,T., Asfari,M. and Pilot,P.R. (1989) J. *Biol. Chem.*, **264**, 21824–21829.
27. Pagès,G., Rouayrenc,J.F., Le Cam,G., Mariller,M. and Le Cam,A. (1990) *Eur. J. Biochem.*, **190**, 385–391.
28. Northemann,W., Heisig,M., Kunz,D. and Heinrich,P.C. (1985) *J. Biol. Chem.*, **260**, 6200–6205.
29. Alonso,S., Minty,A., Bourlet,Y. and Buckingham,M. (1986) *J. Mol. Evol.*, **23**, 11–22.
30. Sargent,T.D., Wu,J.R., Salat-Trepat,J.M., Wallace,R.B., Reyes,A.A. and Bonner,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 3256–3250.
31. Sierra,F., Fey,G.H. and Guigoz,Y. (1989) *Mol. Cell. BioL.*, **9**, 5610–5611.
32. Landschulz,W.H., Johnson,P.F., Adashi,E.Y., Graves,B.J. and McKnight,S.L. (1988) *Genes & Dev.*, **2**, 786–800.
33. Johnson,P.F., Landschulz,W.H., Graves,B.J. and McKnight,S.L. (1987) *Genes & Dev.*, **1**, 133–146.
34. Mueller,C.R., Maire,P. and Schibler,U. (1990) *Cell*, **61**, 279–291.
35. Descombes, P., Chojkier,M., Lichtsteiner,S., Falvey,E. and Schibler,U. (1990) *Genes & Dev.*, **4**, 1541–1551.
36. Akira,S., Isshiki,H., Sugita,T., Tanabe,O., Kinoshita,S., Nishio,Y., Nakajima,T., Hirano,T and Kishimoto,T. (1990) *EMBO J.*, **9**, 1897–1906.
37. Borgmeyer,U., Nowock,J. and Sippel,A.E. (1984) *Nucleic Acids Res.*, **12**, 4295–4311.
38. Nowock,J. , Borgmeyer,U., Püschel,A.W., Rupp,R.A.W. and Sippel,A.E. (1985) *Nucleic Acids Res.*, **13**, 2045–2061.
39. Cereghini,S., Raymondjean, M., Garcia Carranca,A., Herbomel,P. and Yaniv,M. (1987) *Cell*, **50**, 627–638.
40. Vaulont,S., Puzenat,N., Levrat,F., Cognet,M., Kahn,A. and Raymondjean,M. (1989) *J. Mol. Biol.*, **209**, 205–210.
41. Williams,S.C., Cantwell,C.A. and Johnson,P.F. (1991) *Genes & Dev.*, **5**, 1553–15567.
42. Birkenmeier,E. H., Gwynn,B., Howard,S., Jerry,J., Gordon,J.I., Landschulz,W.H. and McKnight,S.L. (1989) *Genes & Dev.*, **3**, 1146–1156.
43. Umek,R.M., Friedman,A.D. and McKnight,S.L. (1991) *Science*, **251**, 288–292.
44. Xanthopoulos,K.G., Prezioso,V.R., Chen,W.S., Sladek,F.M., Cortese,R. and Darnell,J.M. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 3807–3811.
45. Poli,V., Mancini,F.P. and Cortese, R. (1990) *Cell*, **63**, 643–653.
46. Chang,C.J., Chen,T.T., Lei,H.Y., Chen,D.S. and Lee, S.C. (1990) *Mol. Cell. Biol.*, 10, 6642–6653.
47. Roman,C., Platero,J.S., Shuman, J. and Calame, K. (1990) *Genes & Dev.*, 4, 1404–1415.
48. Poanessa,G., Gounari,F., Frank,R. and Cortese,R. (1988) *EMBO J.*, **7**, 3115–3123.
49. Bohmann,D., Bos,T.J., Admon,A., Nishimura,T., Vogt,P.K. and Tjian,R. (1987) *Science*, **238**, 1386–1392.
50. Raymondjean,M., Cereghini,M.S. and Yaniv,M. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 757–761.