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Analysis of proteins binding to the proximal promoter region of two rat serine protease inhibitor genes

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ABSTRACT

The three serine protease inhibitor (SPI) rat genes expressed preferentially in liver share considerable structural features and, nonetheless, are transcriptionally regulated in completely different manners, more particularly after hypophysectomy or upon acute inflammation. DNase I footprinting and gel mobility shift analyses of the SPI 2.1 and 2.3 proximal promoter regions reveal the presence of three common protein binding sites (1 to 3, 3′ to 5′) located immediately upstream from the transcription start site. C/EBP, the liver-enriched factor, specifically interacts with site 1 whereas its related proteins (e.g; DBP, LAP/NFIL6) most likely recognize sites 2 and 3. Another ubiquitous unidentified factor also binds to site 2. A liver-specific protein dependent on growth hormone, whose binding is competed out by an oligonucleotide reproducing an HNF3 motif, interacts exclusively with site 3. The 42 bp sequence which is found only within the SPI 2.3 promoter interacts with two ubiquitous factors, one of which is related to NFκB. Acute inflammation does not significantly affect the protein binding patterns observed with the SPI 2.1 or 2.3 proximal promoter sequences. Our results show an apparent discrepancy between the large magnitude of *in vivo* changes in SPI gene transcription mediated by hormones and the small alterations detected *in vitro*, in the DNA-protein interactions on the promoters.

INTRODUCTION

The serine protease inhibitor (serpin) family includes genes in both plants and animals (see 1 for a compilation) which display a large diversity in their gene structure and regulation as well as the function of their products. In rat liver, three members of this family, the SPI 2.1, SPI 2.2 and SPI 2.3 genes (formerly referred to as SPI 1, SPI 2 and SPI 3) (2) have been cloned and their nucleotide sequences determined (3). Despite the very large sequence similarity in the proteins, mRNAs and structural part of the SPI genes (2–4), they are differentially expressed to extremely variable extents, depending on the physiological status of the animal. Thus, SPI 2.1 and SPI 2.2 genes, which are the most tightly related (2, 3) and appear to be co-regulated in all the situations so far examined (2, 5), are maximally expressed in normal rats (2). Inversely, they are totally or partially repressed in hypophysectomized (2, 4, 6) and acutely inflamed (2, 5) animals, respectively. The third gene, SPI 2.3, is virtually silent in normal rats (2) and becomes transiently active during the acute-phase reaction (5, 7) or after a glucocorticoid treatment (7). Several hormones play a major role in controlling SPI gene expression. Thus, growth hormone (GH) together with glucocorticoids (GC) acting as potentiating factors, is the major transcriptional regulator of the SPI 2.1 and SPI 2.2 genes (6). The effectors which down (SPI 2.1 and 2.2) or up-regulate (SPI 2.3) the genes during inflammation have not yet been characterized but are likely to belong to the class of acute-phase mediators acting in liver (9). Comparison of the 5′ flanking SPI gene sequences reveals several specific features which might provide some structural basis for their differential regulation. Thus, unlike the more distal regions, the proximal promoter sequences (i.e., the first 145 bp) are strongly conserved in all three SPI genes (3). Interestingly, the SPI 2.3 gene which escapes GH regulation (2) and is induced during inflammation (2, 5), contains an additional specific 42 bp element (−187 to −146). It has recently been shown that a short sequence contained within a 200 bp 5′ flanking fragment of the SPI 2.1 gene is transcriptionally active, binds GH inducible factor(s) and can confer some hormone responsiveness on a heterologous promoter in adult rat hepatocytes in primary cultures (8), three observations that we have now confirmed (Paquereau *et al.*, in preparation). This strongly suggests that proximal 5′ flanking sequences are important for basal and hormonally-controlled SPI genes expression. We therefore decided to restrict the present investigation to the analysis of proximal promoter regions. The aim of this study was to characterize the *cis*-acting elements and the cognate *trans*-acting factors interacting with the proximal region of the SPI 2.1 and 2.3 gene promoters, and analyze the variations occurring in DNA-binding protein patterns after hypophysectomy and acute inflammation. We show here that

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C/EBP (10) and/or its related liver-enriched proteins, DBP (11), LAP/NFIL6 (12, 13) or others (14), are the major but not the only *trans*-acting factors interacting with the SPI promoters, and that very minor changes can be detected in the DNA-protein complexes formed *in vitro*, in the pathophysiological situations linked to extensive transcriptional variations *in vivo*.

MATERIALS AND METHODS

Preparation of nuclear extracts

Nuclei were purified from livers of control, hypophysectomized (hypox) and inflamed adult male Wistar rats (250 to 300 g), or from the spleen or kidney of control animals (15). Crude nuclear extracts (CNE), prepared according to Gorski *et al.* (16) as modified by Sierra (17), were systematically tested for their capacity to support a high *in vitro* transcription level (17) before use. Inflammation was induced by injecting turpentine to the rat 20 h prior to sacrifice (5), and hypophysectomy was performed 10 days before CNE preparation.

In vitro DNase I footprinting

A 238 bp fragment (−156 to +82) and a 280 bp fragment (−198 to +82) from the SPI 2.1 and SPI 2.3 gene promoters, respectively, were isolated from the corresponding genomic clones (3) and inserted into pUC18. These fragments, used as probes, were end-labeled on either strand with the Klenow fragment of DNA polymerase I and [α-^{32}P] dCTP and dATP. Footprinting experiments were performed as follows. Nuclear proteins (25 μg) or the bacterial recombinant CCAAT/enhancer binding protein (C/EBP) were pre-incubated for 15 min at 4°C in 15 μl of 50 mM NaCl, 50 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 2 mM DTT, 4 mM spermidine, 17.5% glycerol, 10 mM Hepes (pH 7.9), 250 ng poly (dI.dC) and 100 μg/ml bovine serum albumin (BSA). When recombinant C/EBP was employed, poly(dI.dC) was omitted and BSA was increased to 10 mg/ml. The labeled probe (20−40000 cpm, 1 to 2 ng) was then added and incubations were continued for 30 min at 0°C. DNase I digestion, performed for 1 min at 20°C in the presence of 2.5 mM $CaCl_2$, was stopped by adding 35 μl of a solution

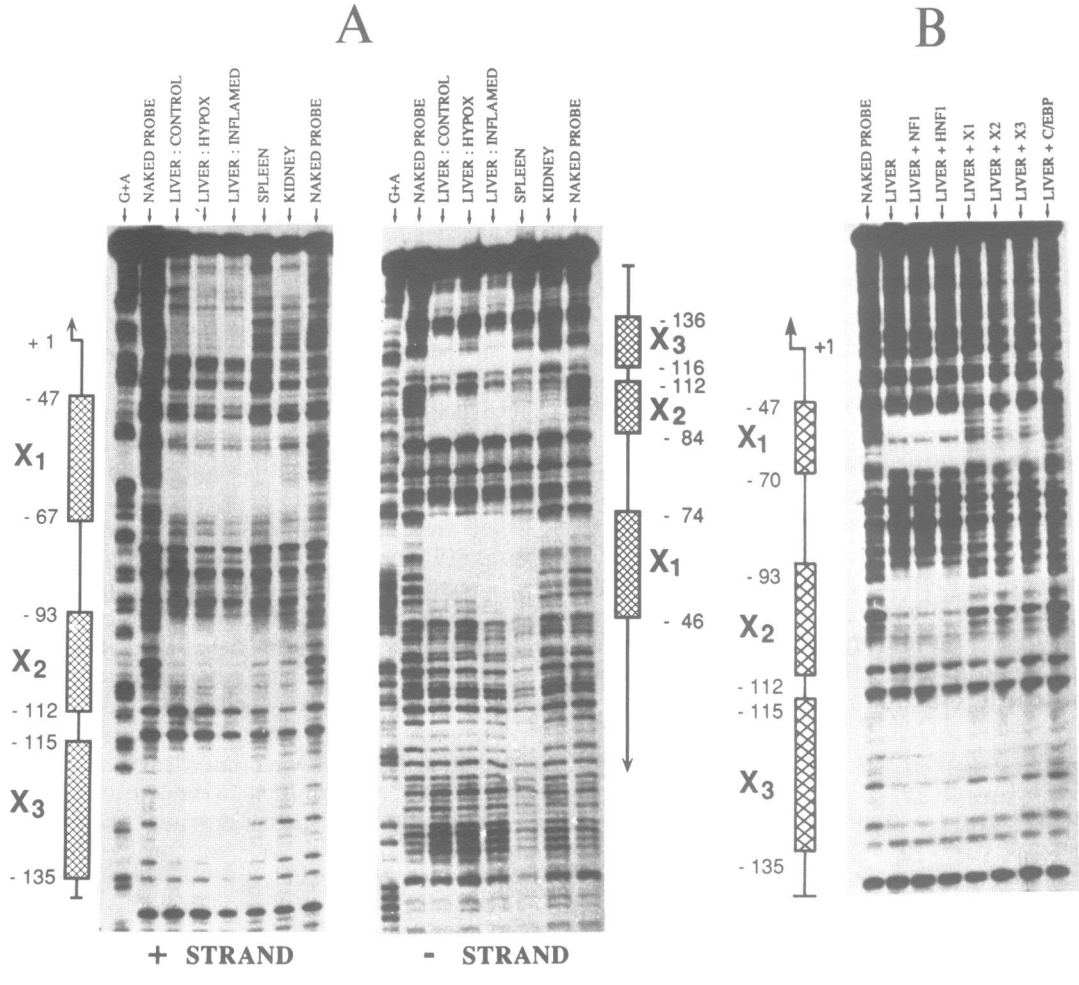

Figure 1. *In vitro* footprinting analysis of the proximal SPI 2.1 promoter region *Panel A*. Tissue specificity. The 238 bp probe, radiolabeled on either the (+) or (−) strand was incubated in the absence (naked probe) or presence of 25 μg of liver CNE from control, hypox or inflamed rats or CNE from spleen or kidney from normal animals, and further digested with DNase I. *Panel B*. Competition studies. The 3′-end labeled (+) strand probe was incubated with 25 μg of control liver nuclear proteins, in the absence or presence of 20 ng of various competitor oligonucleotides reproducing the homologous SPI 2.1-derived binding elements (X1, X2 or X3) or NF1, HNF1, or C/EBP sites. Boxes represent the three protected regions located upstream from the tsp (+1) and the numbers indicate the position of the footprint borders relatively to the tsp. G+A are the Maxam and Gilbert reaction products.

containing 150 μg/ml yeast tRNA, 6 mM EDTA, 0.06% SDS and 450 μg/ml proteinase K, allowing proteins degradation (30 min at 42°C). Labeled DNA species were phenol extracted and separated on 6% (w/v) polyacrylamide/7M urea gels. Maxam-Gilbert sequencing reactions (18) were used as size markers.

Gel mobility shift assays

The binding conditions with CNE (5−10 μg proteins/assay) were as described for the footprinting experiments, except that 1−2 μg poly (dI.dC) were used, in a 20 μl final volume. When recombinant C/EBP (10) or purified NFκB (19) were used, the concentration of BSA was increased to 10 mg/ml and poly (dI.dC) was omitted. Free DNA and DNA-protein complexes were resolved on non-denaturing 6% (w/v) polyacrylamide gels in 0.5×TBE run at 15−18°C for 3 h at 150 V. Oligonucleotides used as probes were end-labeled by filling overhanging 5′ ends with [α-^{32}P] dATP and dCTP using the Klenow fragment.

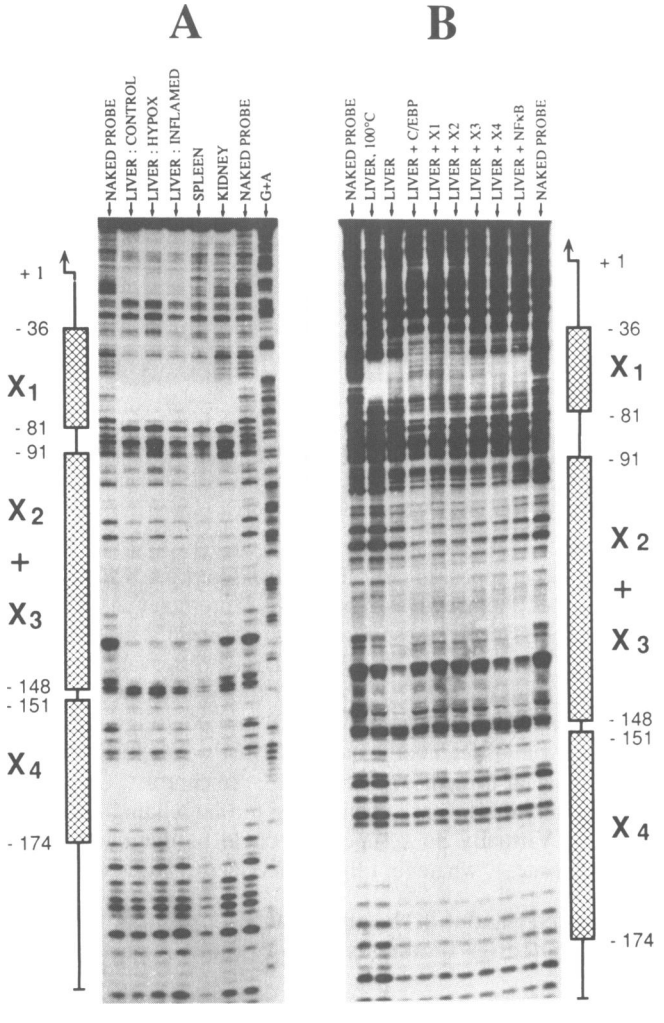

Figure 2. *In vitro* footprinting analysis of the proximal SPI 2.3 promoter region. *Panel A.* Tissue specificity. The 280 bp, SPI 2.3 (+) strand radiolabeled probe was incubated with various nuclear extracts as described in fig. 1 A. *Panel B.* Competition studies. The probe was incubated with 25 μg of control liver nuclear proteins that had been heated at 100°C for 5 min, or with the same amount of native material in the absence or presence of the homologous (elements X1 , X2 or X3 from the SPI 2.1, or X4 from the SPI 2.3 promoters, respectively), or the heterologous (the C/EBP or NFκB elements) competitor oligonucleotides.

Materials

[α-^{32}P] dATP and dCTP were from New England Nuclear. Restriction endonucleases and DNA modification enzymes were purchased from New England Biolabs or from Boehringer Mannheim, except for the DNase I that was from Worthington. Recombinant C/EBP was a gift from Dr. S.L. McKnight and purified NFκB and the NFκB oligonucleotides were kindly provided by Dr. P. Baeuerle. The oligonucleotides used as probes or competitors in the footprinting and gel retardation assays are: 5′-gtgTCCAGTGATGTAATCAGGC-3′, the C/EBP site derived from the rat hemopexin gene (20); 5′-ttgCTTTTTGGCAAGG-ATGGTATG-3′, the nuclear factor 1 (NF1) binding element from the rat liver pyruvate kinase gene (21); 5′-gatcTCAAAC-TGTCAAATATTAACTAAAGGGAG-3′, the hepatocyte nuclear factor 1 (HNF1) binding site from the rat β fibrinogen gene (22); 5′-gttAAATATTGACTTTGCTT-3′, the hepatocyte nuclear factor 3 (HNF3) binding site from a rat α1-antitrypsin gene (23); 5′-gatcCAGAGGGGACTTTCCGAGA-3′, the nuclear factor of the mouse Ig kappa light chain enhancer (NFκB) binding site (24). The binding sites from the SPI 2.1 (X1, X2 and X3) and SPI 2.3 (X4) promoters (3) are: X1 : 5′-tcgacTCC-GAGGCAACATTTCCTAAGAGGAGGGAGGA-3′ (−77 to −46); X2 :5′-ctagATCCAGTCTGCCCATATGTAATCTGA-ACACAAAGCAC-3′ (−120 to −84); X3: 5′-tcgacTACTAA-TCCATGTTCTGAGAAATCATCCAGTC-3′ (−144 to −113) and X4: 5′-tcgacGATGGGAATTTTCCCATC-3′ (−175 to −158).

RESULTS

Footprinting analysis of the proximal 5′ flanking SPI 2.1 and SPI 2.3 gene regions

The SPI 2.1 promoter. Three regions located upstream from the transcriptional start point (tsp) and designated as boxes X1 to X3 were strongly protected against DNase I digestion with liver CNE from control rats (Fig. 1). The extent of the footprints were quite similar on both strands, except for a 3′ end extension of

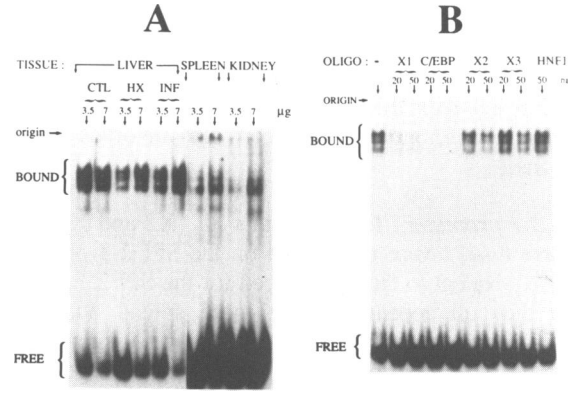

Figure 3. Band shift analysis of site 1 DNA-protein complexes. *Panel A.* Identification and tissue distribution. Gel retardation assays were performed using the labeled X1 oligonucleotide as a probe, with nuclear proteins (3.5 or 7 μg) from control (CTL), hypox (HX) or inflamed (INF) rat livers, or from spleen or kidney from normal rats. Bound and free probes were separated on native polyacrylamide gels and detected by autoradiography after 24 h (liver) or 100 h (spleen and kidney). *Panel B.* Competition analysis. The probe was allowed to bind to control liver nuclear proteins in the absence (−) or presence of the SPI 2.1- derived, the C/EBP or HNF1 unlabeled oligonucleotides.

Table I. Potential binding sites for known proteins found in the proximal SPI promoter regions.

Site	SPI 2.1 Position	Sequence	Strand	SPI 2.3 Position	Sequence	Strand
C/EBP:	+37/+45	GTCAGCAAA	+	+37/+45	GTCGGCAAT	+
C/EBP:	− 58/−66	TTAGGAAAT	−	− 58/−66	TTAGGAAAT	−
C/EBP:	−107/−99	ATATGTAAT	+	−99/−107	ACTGGAAAT	−
C/EBP:	−130/−122	CTGAGAAAT	+	−130/−122	CCCAGAAAT	+
HNF5:	+47/+41	TGTTTGC	−	+47/+41	TGATTGC	−
HNF3:	−117/−127	GGATGATTTCT	−	−117/−127	GGATGATTTCT	−
NFxB:				−172/−163	GGGAATTTTC	+
IL6RE:				−174/−161	ATGGGA	+
IL6RE:				−159/−164	ATGGGA	−
GRE:	−134/−129	TGTTCT	+	−134/−129	TGTTCC	+

DNA motifs present within the SPI 2.1 and 2.3 gene promoters (i.e., the proximal 5′ flanking regions adjacent to the tsp) were compared to the core consensus binding sequences for C/EBP (5′-TT/GNNGNAAT/G-3′), HNF5 (5′-TG/ATTTGC/T-3′), HNF3 (5′-TATTGAT/CTTA/TG-3′), NFxB (5′-GGGAA/CTTTCC-3′), interleukin 6 inducible factor(s) (5′-CTGGGA-3′) and the glucocorticoid receptor (5′-TGTACANNNTGTTCT-3′). The position of the sites on each strand (+ or −) are indicated relatively to the tsp and mismatched nucleotides are underlined.

site 2 observed with the (−) strand (Fig. 1 A). The same three footprints were also observed with liver CNE from hypox or inflamed animals. However, a minor difference was detected in the 5′ portion of the third footprint (X3) which appeared less protected with hypox than control proteins on the (−) strand (panel A, right). Spleen and kidney nuclear proteins also interacted with sites 1 and 2 but the protections were less pronounced that with liver proteins, especially with kidney extracts (Fig. 1 A). In contrast, footprint X3 was either undetectable or very weak with CNE from kidney and spleen, respectively. Besides these 3 upstream sites, a wide protected region encompassing the cap site and the first untranslated exon (approx. from −10 to +82) was observed predominantly with liver CNE (Fig. 1 A).

Competition analysis was performed with oligonucleotides reproducing the SPI 2.1 promoter footprinted upstream regions (X1 to X3) or binding sites for well-characterized DNA-binding proteins (Fig. 1 B). A typical C/EBP oligonucleotide totally eliminated all three footprints, whereas the NF1 or HNF1 oligonucleotides had essentially no effect. The homologous X1 oligonucleotide was as efficient as C/EBP in displacing the binding of liver nuclear proteins to the three sites. The X2 and X3 oligonucleotides almost completely abolished footprints 2 and 3 but only partially the first one (Fig.1 B). Such a cross competition between the different sites suggests that proteins recognizing homologous sequences are involved in these interactions.

The SPI 2.3 promoter. The footprints X1, X2 and X3, the last two almost fused, were also found on the SPI 2.3 promoter at positions equivalent to those described for the SPI 2.1 promoter, although they all appeared to be more spread out (Fig. 2). Footprint X2 was always much weaker than footprints X1 and X3. As observed with the SPI 2.1 promoter, a large footprint covering a partially protected region comprising the tsp and the 3′ adjacent sequence was also detected (Fig. 2 A). The patterns obtained with the three liver CNE (i.e., control, hypox and inflamed) were almost indistinguishable, and the tissue distribution of the binding proteins interacting with these sites was analogous to that observed with the SPI 2.1 promoter. An additional footprint, X4, corresponding to a very weakly protected region (−174 to −151), was observed with liver but also spleen and kidney nuclear extracts (Fig 2 A). Neither hypophysectomy nor acute

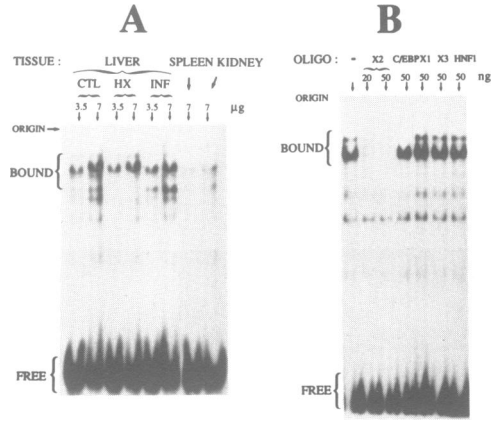

Figure 4. Band shift analysis of site 2 complexes. Tissue distribution (panel A) and competion analysis (panel B) were performed as described in figure 3, using the labeled X2 oligonucleotide as a probe.

inflammation appeared to alter this footprint which partly encompassed the specific 42 bp SPI 2.3 promoter insertion (3).

Only footprint X1, especially its outmost 5′ portion, was preserved with heated CNE (Fig. 2 B), an observation that was also made with the SPI 2.1 promoter (not shown). Competition experiments showed that the C/EBP and X1 oligonucleotides competed with different efficiencies (C/EBP > X1) for the binding of liver nuclear proteins to sites 1 and 3. In contrast, C/EBP was almost totally inefficient in competing for the binding of proteins to site 2. Virtually no competition could be demonstrated for binding to site 4, whatever oligonucleotide was used (Fig. 2 B).

Footprinted regions in the proximal SPI promoter sequences contain potential binding sites for known transcriptional activators

Comparison of the SPI promoter sequences protected by liver nuclear proteins to that of binding sites for well-characterized *trans*-acting factors revealed that several DNA elements present upstream and downstream in the vicinity of the tsp share significant features with the core C/EBP binding motif (13), both in the SPI 2.1 and SPI 2.3 genes (Table I). A perfect match was observed for an element conserved in all three SPI genes (2) and found in footprint X1, whereas those found in the other upstream

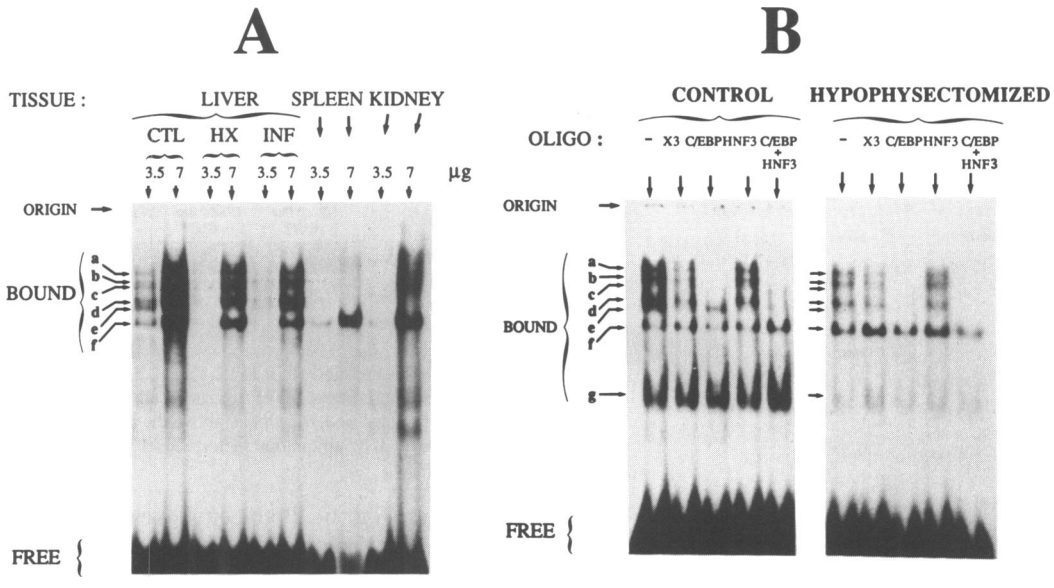

Figure 5. Band shift analysis of site 3 complexes. *Panel A*. Identification and tissue distribution. Binding of nuclear proteins (3.5 or 7 µg) from various origins to the X3 oligonucleotide probe was analyzed as described in fig. 3. *Panel B*. Competition studies. The effect of various oligonucleotides: X3, C/EBP or HNF3, used alone (20 ng) or in combination (10 ng of each), on the binding of liver nuclear proteins (25 µg) from control or hypox rats was studied. Abbreviations are as in Fig. 4.

(X2 and X3) and downstream (i e., 3′ to the tsp) footprints displayed either single or double mismatches with the core C/EBP sequence. A new liver-specific nuclear binding factor called HNF5 has recently been identified (25). Interestingly, HNF5 motifs often appear to overlap C/EBP sites (25), a situation which seems to occur with only one of the potential C/EBP sites in the SPI promoters. Indeed, a heptanucleotide present on the −strand and located 3′ to the tsp (+47 to +41), either totally (SPI 2.1) or partially (SPI 2.3) matches the consensus HNF5 binding sequence. A partial identity (54%) was also found with the core HNF3 sequence (23), for a DNA motif located in footprint X3 which is conserved in all three SPI promoters (3). A short sequence also present in the same X3 region is identical to the second part of a glucocorticoid receptor binding element (GRE) (26) in SPI 2.1 (and SPI 2.2), and displays a single mismatch in SPI 2.3 promoters, respectively. Finally, the palindromic motif present in the SPI 2.3 X4 footprinted element shares structural features with NFκB binding sequences (24) (Table I). This region also contains on each strand a hexanucleotide that closely resembles the interleukin 6 responsive element (IL6RE) found in many acute-phase genes (27).

Analysis of DNA-protein complexes by gel retardation assays

Analysis of site 1 complexes. Incubation of liver CNE from control animals with the X1 probe gave rise to a composite pattern of bands representing at least three DNA-protein complexes (Fig.3). These complexes were also observed with material from hypox and inflamed rats. Spleen and kidney CNE yielded essentially the same pattern of bands with albeit some clear quantitative differences. Indeed, the amounts of proteins bound to the probe was at least 10 fold lower than with liver extracts and, furthermore, the higher molecular weight complexes were underrepresented (Fig. 3 A). A typical C/EBP oligonucleotide totally prevented the formation of these complexes and was an even more efficient competitor than the homologous X1 binding

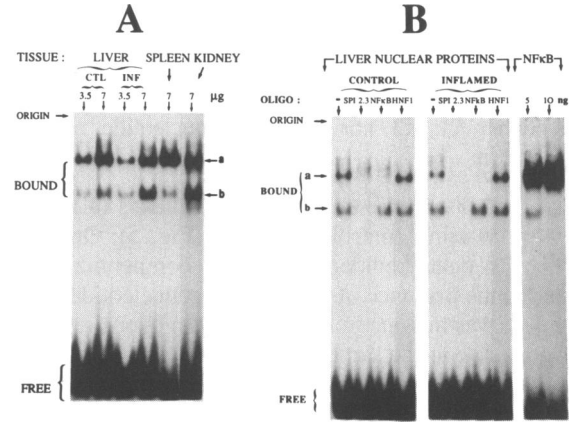

Figure 6. Band shift analysis of site 4 complexes. *Panel A*. Characterization and tissue distribution. The specific SPI 2.3 promoter site 4 oligonucleotide used as a probe was incubated with liver nuclear extracts from control (CTL) or inflamed (INF) rats, or spleen or kidney CNE from normal animals, as described in fig. 3. *Panel B*. Binding of liver nuclear proteins and recombinant NFκB to the X4 probe; competition study. The probe was incubated with 5 µg liver nuclear proteins from control (CTL) or inflamed (INF) rats, in the absence (−) or presence of 20 ng competitor oligonucleotides corresponding to the SPI 2.3-specific X4 element, NFκB or HNF1 sites. Bacterial recombinant NFκB was incubated without competitor, under similar conditions.

site (Fig 3 B). The SPI 2.1 promoter-derived X2 and X3 oligonucleotides were also able to compete, although much less efficiently, for the binding of liver nuclear proteins to this probe, whereas a HNF1 binding site had no effect.

Analysis of site 2 complexes. An even more complex pattern consisting of 5 to 7 bands, of which one predominated, was obtained with all liver CNEs and the X2 probe (Fig. 4). Only small amounts of the same DNA-protein complexes were found

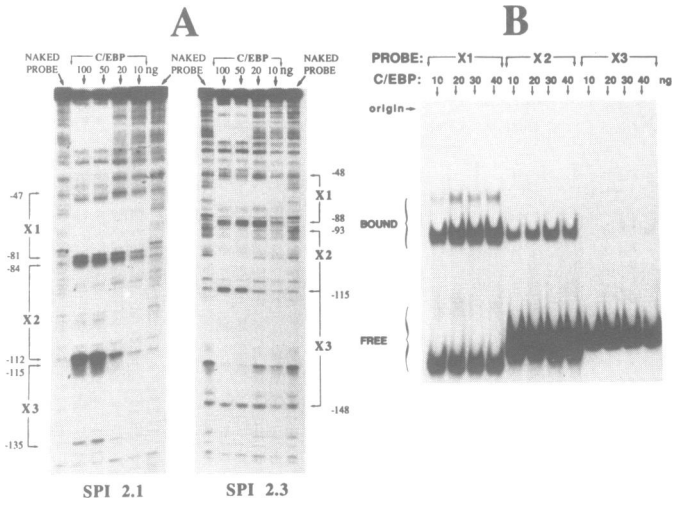

Figure 7. Binding of the recombinant C/EBP to proximal SPI 2.1 and 2.3 promoter elements. *Panel A*: footprinting analysis. Increasing amounts of bacterial C/EBP were incubated with the 238 bp SPI 2.1 or the 280 bp SPI 2.3 (+) strand labeled probes, as described in Materials and Methods. *Panel B*: the interaction of the recombinant C/EBP with the three common SPI promoter binding sites was analyzed using the standard band shift method.

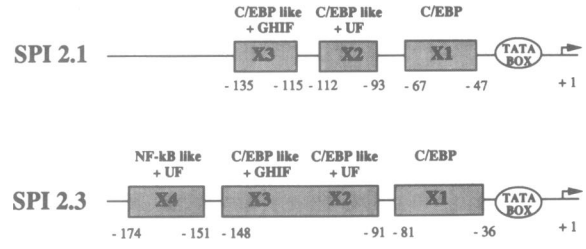

Figure 8. Organization of the SPI 2.1 and 2.3 proximal promoter regions. Positions of the footprint borders on the (+) strand and the putative cognate nuclear binding factors are noted underneath and above the boxes representing the binding sites, respectively. UF: ubiquitous factor; GHIF: growth hormone inducible factor.

when using kidney and spleen extracts. The homologous X2 oligonucleotide totally displaced the two largest complexes but had essentially no effect on the smallest ones, which presumably represent non-specific binding (Fig. 4 B). Interestingly, a C/EBP oligonucleotide eliminated only the largest specific complex, whereas neither X1, X3, nor HNF1 oligonucleotides affected the binding patterns.

Analysis of site 3 complexes. Several complexes (a to g) were observed when using control liver CNE (Fig. 5). One of them (e), which is better evidenced in the competition analysis performed in the presence of a C/EBP oligonucleotide as shown in figure 5 B, was missing in liver CNE from hypox rats. Kidney extracts gave rise to the same array of DNA-protein complexes as control liver CNE with the exception of complex e, whereas only complex f occurred with nuclear proteins derived from spleen. The qualitatively minor difference detected between control and hypox liver CNE in band shift assays (i.e., absence of band e with hypox extracts) might have, from the functional point of view, a major significance because site 3 has recently been shown to be implicated in mediating GH action (8 and Paquereau *et al.*, in preparation). The competition experiments illustrated in figure 5 B provide some interesting pieces of information about the putative nature of nuclear factors interacting with this site. The homologous X3 oligonucleotide reduced to various extents the amounts of the five largest complexes (a to e) but did not affect the other ones (f and g) which presumably represent non-specific binding. A typical C/EBP site completely abolished the formation of 4 complexes (a to d), whereas a HNF3 oligonucleotide specifically eliminated the liver-specific e complex (Fig. 5 B, control). Combining the C/EBP and HNF3 oligonucleotides resulted in additive effects and prevented the formation of the specific (a to e) DNA-protein complexes.

Analysis of site 4 complexes. Two types of nuclear proteins interacted with the oligonucleotide reproducing the sequence of the more distal site (X4) specifically found in the SPI 2.3 promoter (Fig. 6). Both complexes a and b were observed with liver CNE from control or inflamed rats as well as with kidney and spleen extracts, although some variations in the relative amounts in each of them were found. Although differences could occasionally be detected, separate experiments performed with different CNE preparations and similar to that illustrated in figure 6 B, failed to demonstrate significant changes in the binding of liver proteins to site 4 upon inflammation. The two complexes were eliminated in the presence of the homologous X4 binding site, but not with a HNF1 oligonucleotide, suggesting that they represent specific binding (Fig. 6 B). Interestingly, a typical NF$\varkappa$B site displaced only complex a which co-migrates with the one formed between purified NF$\varkappa$B and the X4 probe. Methylation interference experiments have shown that the contact points between purified NF$\varkappa$B and the X4 probe were the nucleotides GGGAA and GAAAA, on the + and − strand, respectively (P. Baeuerle, unpublished observations). A major role for the three guanosine residues which appear fully conserved in all $\varkappa$B sites (28), was previously demonstrated in the interaction of purified NF$\varkappa$B with its most frequent cognate DNA binding motif, 5′-GGGACTTTCC-3′, (29). The nuclear factor forming the ubiquitous b complex has not yet been identified.

Interaction of purified recombinant C/EBP with the SPI promoter proximal regions

Sequences analysis and competition experiments suggested that C/EBP or its related proteins could interact with the three common sites located upstream (X1, X2 and X3), and the one found downstream of the tsp in the SPI promoters. This was directly demonstrated by investigating the binding of bacterial recombinant C/EBP which footprinted both the SPI 2.1 and SPI 2.3 promoters at positions very similar to those found with liver CNE (Fig. 7 A). The purified protein (at high concentrations) protected a wide region encompassing the tsp and covering the 3′ adjacent sequence (approx. from −15 to + 65) (Fig. 7 A). This presumably can be accounted for by the presence in this region of a sequence identical (SPI 2.1) or highly similar to the core consensus C/EBP element (see Table I). In contrast, marked differences were observed for the three upstream footprints. Indeed, the X1 region was protected much more efficiently and with lower amounts of the protein than X2 and X3. Furthermore, interaction of recombinant C/EBP with the SPI 2.1 sequence generated, at the X1/X2 and X2/X3 boundaries, much stronger DNase I hypersensitive sites than that of the protein with the SPI 2.3 promoter. Such strong hypersensitive sites were never

observed with CNE, suggesting that more than a single factor present in CNE interacts with the promoters.

Footprinting data suggested that the affinity of C/EBP for the three upstream sites markedly differed (X1 > X2 > > X3). This was substantiated by a band shift analysis of C/EBP binding to the three oligonucleotides (Fig. 7 B). The purified protein (even at a low concentration) strongly bound to site 1, giving rise to two complexes, the smallest of which predominated (>95%). In contrast, C/EBP bound much less efficiently (5 to 10 fold) to the second site (X2) and, in this type of assay, barely recognized site 3 (X3).

DISCUSSION

In these *in vitro* studies, we found many common features but also some interesting differences with respect to the putative *cis*-acting regulatory elements and the cognate *trans*-acting factors interacting with the proximal region of the SPI promoters which might, at least partly, account for their differential regulation *in vivo*. This obviously does not rule out the possibility that other more distal regulatory regions may exist in these genes. The diagram shown in figure 8 summarizes these characteristics. C/EBP, a protein originally purified from rat liver nuclei (10) and found mainly in terminally differentiated cells such as hepatocytes and adipocytes (30), is most probably the liver factor interacting with the first common protein binding site found upstream from the tsp in SPI gene promoters. Several pieces of experimental evidence support this contention. (i) A typical C/EBP oligonucleotide competed out the binding of liver nuclear protein(s) to this site; (ii) the hepatic protein(s) proved to be thermoresistant which is very specific of C/EBP (31); (iii) bacterial recombinant C/EBP binds with a high affinity to site 1; (iv) the binding appears largely, although not totally, specific of liver proteins. The other common upstream binding sites found in SPI promoters (X2 and X3), which also share significant features with the core C/EBP sequence (Table I), are likely to be recognized by C/EBP-like proteins such as DBP (11) LAP/NFIL6 (12, 13) or possibly others (14), rather than by C/EBP itself. This is suggested by the decreased (X2) or extremely low (X3) affinity of the recombinant protein for these sites as well as by the weak competing effect exerted by the X2 and X3 oligonucleotides toward X1 binding. The almost total lack of inhibitory effect of a C/EBP oligonucleotide on the binding of liver proteins to the SPI 2.3 promoter X2 site in a footprinting assay could result from a very low affinity of the protein(s) for this site or indicate that one or several C/EBP unrelated factors interact with it. Consistent with the latter hypothesis, band shift experiments showed that, besides a protein related to C/EBP, another major unknown ubiquitous factor specifically binds to the X2 site. Nonetheless, a C/EBP oligonucleotide completely eliminates footprint X2. This might mean that the ubiquitous site 2 binding factor either cannot contact the site in the whole promoter due to steric hindrance or that the C/EBP-like protein(s) must first bind to allow the interaction of the second factor with the promoter. C/EBP and its related proteins (11–14) are among the major hepatic transcriptional activators and their cognate binding sites have been found in many promoters specific of the liver (for a review see 32). A typical example of the role of C/EBP is illustrated by the albumin promoter (33, 34) which can be *trans*-activated in a cell-specific manner by this protein (35). However, in most cases, C/EBP alone is not sufficient and appears to act synergistically with other liver-specific proteins such as HNF1, HNF3 and HNF4 (reviewed in 32) or with more ubiquitous factors like NF1 (36), AP1 (37) or NFY (38). In the case of the SPI genes, the situation appears quite different since no conspicuous binding site for any of these factors seems to be present within the proximal promoter regions. Thus, instead of an interplay between heterologous factors, it seems reasonable to assume that all the C/EBP like sites can cooperate to regulate the transcriptional activity of SPI promoters. This is presently under investigation. Comparison of the interactions between the SPI promoters and the C/EBP-related proteins present in liver CNE from control, hypox and inflamed rats failed to reproducibly show significant qualitative or quantitative differences. This might suggest that none of these factors is involved, at least directly, in modulating the dramatic changes occurring in SPI gene expression in these pathophysiological situations (2, 4, 5).

Detailed analysis of the proteins interacting with the X3 site reveals that a liver-specific binding factor, different from C/EBP, is either missing or inactivated in nuclear extracts from hypox rats. This protein, which could be re-induced by GH injection to the animal (not shown), is prevented to bind by an oligonucleotide reproducing the binding site for HNF3, a well-characterized liver-specific *trans*-acting factor (23). Nevertheless, it seems unlikely that HNF3 itself is the nuclear factor giving rise to this specific complex because no difference in amounts of this protein(s) could be detected between control and hypox liver CNEs using a specific HNF3 probe (V. Rossi, personal observations). We rather interpret the competition data by the partial identity of a sequence present within site 3 (in all three SPI genes, 2) and the core HNF3 motif (see Table I). Our data on site 3 which also show the binding of a factor specifically found in liver and dependent on GH, appear to differ from those reported by Yoon *et al.* (8). Using a slightly broader oligonucleotide derived from the same region of the SPI 2.1 promoter as a probe (−150 to −106 instead of −144 to −113), these authors described two complexes of small sizes that were dependent on GH, whereas we consistently observed a much more complicated binding pattern with a single large size complex dependent on this hormone. Although we cannot presently explain this discrepancy, we must mention that we have also observed DNA-protein complexes of small sizes in our band shift assays, which we believe to represent proteolytic products, because they occurred only with transcriptionally inactive nuclear extracts.

The specific 42 bp insertion contained in the SPI 2.3 promoter represents a potential regulatory site which might be more particularly involved in the gene induction observed during inflammation (2, 5). Although this remains to be proven by functional studies, both sequence analysis and binding experiments appear to be consistent with this assumption. Indeed, two copies of a hexanucleotide highly resembling the core IL6RE (27), which also overlap a potential NF$\varkappa$B site (24), are present within the site 4 SPI 2.3 promoter region (Table I) and band shift experiments show that two ubiquitous nuclear factors specifically interact with the 42 bp DNA element. One of the complexes is efficiently displaced by a typical $\varkappa$B motif and purified NF$\varkappa$B strongly binds to site 4. This suggests that, as previously demonstrated in the case of the angiotensinogen gene induced by cytokines (39), NF$\varkappa$B or a related factor present in liver interacts with the SPI 2.3 promoter. In this respect, it is interesting that a factor different from NF$\varkappa$B was shown to recognize, in the promoter of the class II major histocompatibility Aα^k gene, a palindromic-like sequence, 5′-GGGAATTTTCCC-3′, identical to that found in the specific SPI 2.3 promoter insertion (40).

However, unlike what has been reported for the angiotensinogen gene (39), acute inflammation did not induce a protein that would specifically bind to this acute-phase responsive element (APRE) in the SPI 2.3 promoter. This might mean that the structurally related APRE SPI 2.3 sequence does not behave, as in the case of the angiotensinogen gene (39), as an enhancer inducible by cytokines for this specific gene. In keeping with this observation, glucocorticoid induction of the SPI 2.3 gene (7) might be related to the presence of a short sequence homologous to a GRE half-site located immediately upstream from the specific SPI 2.3 promoter palindromic region (Table I). It should be noted however, that a similar sequence is also found at the same position in the SPI 2.1 and 2.2 genes which, unlike the SPI 2.3 gene (7), are virtually insensitive to glucocorticoids alone (6). Finally, consistent with the absence of APRE in the proximal 5'-flanking gene region, no detectable change was found in the proteins binding to the SPI 2.1 proximal promoter upon inflammation, despite the fact that expression of this gene is strongly down-regulated in inflamed animals (2, 5). This implies that the regulatory sequences involved in the inflammatory response are located elsewhere in this SPI gene.

In conclusion, we would like to emphasize the following points. The tremendous changes occurring in SPI gene transcription in hypox and inflamed animals are not reflected by large modifications in the DNA-protein complexes formed on the proximal promoter regions, *in vitro*. Mostly proteins of the C/EBP family which have the intrinsic capacity to *trans*-activate hepatic genes both *in vitro* and *in vivo*, either in the basal state or under regulatory conditions (i.e., inflammation) (32), seem to interact with SPI promoters *in vitro*. Nonetheless, the genes are silent in the basal state (SPI 2.3) or in the absence of GH (SPI 2.1 and 2.2), in the whole animal. Although other explanations can be proposed, this might indicate the need for accessory proteins to interact with either the DNA or the C/EBP like factors for transcriptional activation of the SPI genes. Such interactions might have been lost during CNE preparation and only genomic footprinting analyses might overcome this problem. The second point we would like to stress relates to the fact that the protein dependent on GH and binding specifically to site 3 represents, from a quantitative point of view, a minor DNA binding factor. Whether binding of this protein alone can account for the whole transcriptional effect of GH on SPI gene expression remains to be proven.

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