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Interplay of an original combination of factors: C/EBP, NFY, HNF3, and HNF1 in the rat aldolase B gene promoter

M.Raymondjean, A.-L.Pichard, C.Gregori, F.Ginot and A.Kahn
ICGM, Laboratoire de Recherches en Génétique et Pathologie Moléculaires, INSERM U. 129, 24 rue du Faubourg Saint Jacques, 75014 Paris, France

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ABSTRACT

The rat aldolase B 5′ flanking region (nucleotides − 194 to + 41) contains sufficient information for liver-specific expression. A detailed investigation of factors binding to the rat aldolase B 5′ flanking region has allowed us to identify three distinct factors that filled different sites of this region (A, B, C). The liver-enriched C/EBP or related factors bind to box C, as demonstrated by the specific interaction with bacterially expressed C/EBP protein. Box B bearing the CCAAT sequence binds the ubiquitous factor NFY. Surprisingly, Box A is able to bind two liver enriched factors, namely HNF1 and HNF3. However, in the context of the intact promoter, as shown by footprinting competition experiments, HNF3 binds solely to this sequence. HNF3, but not HNF1 is a transcriptional activator as demonstrated in the *in vitro* transcription assay.

INTRODUCTION

Transcriptional regulation depends on the interplay of various transcriptional factors and regulatory proteins interacting directly or indirectly with cis-acting DNA elements (1). Trans-acting factors, including protein binding to cognate DNA sequences either far upstream or in the vicinity of the cap site, activate the transcriptional preinitiation complex formed close to the initiation site (2). Tissue-specific as well as 'ubiquitous' factors are involved in the tissue-specific expression of some genes. In the last two years, a number of liver-specific or liver-enriched trans-acting factors have been characterized, and their cDNAs cloned. They belong to different families including proteins with homeodomains [e.g., hepatocyte nuclear factor 1: HNF1 (3, 4, 5)]; leucine zippers or related dimerization domains [e.g., CAAT/enhancer binding protein; C/EBP,DBP and related proteins, LAP, AGP/EBP, IL-6DBP, Ig/EBP, NF-IL6, (6, 7, 8, 9, 10, 11, 12)] and proteins of the nuclear receptor superfamily [(e.g., HNF4, probably identical to LFA1, and HNF2 (13, 14, 15)]. Other factors have not been ascribed to these families [e.g., HNF3 (16)].

These liver-specific transcriptional factors, together with ubiquitous factors are variably arranged on the promoters of various liver-specific genes.Indeed, the combination of liver-specific and 'ubiquitous' trans-acting factors for a given liver-specific gene is uniquely arranged. For instance, in the 3′→5′ sense the albumin gene promoter binds the factors C/EBP, HNF1, NFY, C/EBP/DBP or LAP, NF1 and C/EBP (17, 18); the L-PK gene promoter binds HNF1, NF1, HNF4 and MLTF (19); the transthyretin gene promoter binds the factors HNF3, C/EBP, HNF1, HNF3, HNF4, C/EBP (20 and see for review 21).

In fact, as illustrated by binding of different proteins to site D of the albumin gene promoter (C/EBP, DBP, LAP, AGP/EBP, Ig/EBP) (6, 7, 8, 9, 11), a new level of complexity is provided by the fact that several related and unrelated proteins may bind to a unique DNA sequence. Moreover, a given protein may recognizes different DNA sequences [e.g., C/EBP binds to site D of the albumin promoter and to several types of CAAT sequences (6)].

In contrast to other genes for liver-specific metabolism enzymes [e.g., L-PK (22), phosphoenol pyruvate carboxykinase (23, 24), tyrosine aminotransférase (25, 26)], little is known about aldolase B gene regulation.

Aldolase B is a glycolytic enzyme (EC: 4-1-2-13) specifically expressed in liver, kidney and small intestine and controlled at the transcriptional level by diet and hormones (27), positively by glucose and insulin and negatively by glucagon and its messenger, cAMP (28). Regulation of the aldolase B and L-pyruvate kinase (L-PK) genes (29) is similar except that the former is not totally silent in fasted animals whereas the latter is. This difference is not unexpected since, in contrast to L-PK, aldolase B is required not only for glycolysis but also for gluconeogenesis. The transcription of the aldolase B gene is developmentally regulated, mRNA is detected at day 14 of gestation and is maximum at birth (30).

Recently, Tsutsumi et al (31) reported three binding sites on the aldolase B promoter within the 200 bp upstream from the cap site. The proximal motif A was identified as binding HNF1 (32) or a related protein, while a not characterized protein binds to motif B, a CCAAT box. A third, more distal footprinted region (motif C) was not investigated further. Tsutsumi et al (31) showed that the 200 bp aldolase B gene promoter was sufficient to direct a liver-specific transcription in a cell free run-off assay and that motifs A and B were required for this transcription.

However, we recently observed using a transient transfection assay that a 190 bp fragment of the aldolase B promoter, including

motifs A, B, and C, behaves as a very weak liver-specific promoter, and is fully activated by an activator located in the first intron of the gene (33). These results were striking because truncated albumin (34, 35, 36) and L-pyruvate kinase (37) gene promoters, bearing only the HNF1 binding site, were stronger than this 190 bp aldolase B promoter. This paradox prompted us to re-evaluate the nature of transcriptional factors interacting with the aldolase B promoter. In this paper we report that motif C, homologous to site D of the albumin gene promoter, binds members of the C/EBP family (6), motif B binds NFY/CP1 factor (38, 39, 40, 41) and perhaps other related proteins, motif A binds preferentially HNF3 factor (16).HNF3 seems to be a functionally important factor in a liver cell free-transcription system.

MATERIALS AND METHODS

Preparation of nuclear extracts

Nuclear extracts from adult liver, spleen and fetal liver at 17 days of gestation were purified according to Gorski et al (42).

DNase 1 footprinting and gel shift assays

The aldolase B probe (nt −190 to + 41) was 3′end-labeled. The standard footprinting reactions were as described (17). The oligonucleotides used are listed in table 1. Labeling and gel shift assays in 6% polyacrylamide were performed as described (41), in the presence of poly (dI: dC) (2 μg/sample, as a mean) as a nonspecific competitor. The oligonucleotides encompassing the aldolase promoter CPAB, CAB and PAB were kindly provided by F. Tronche from M. Yaniv laboratory.

In vitro transcription assays

The DNA templates used in the run off assays were derived from p(C2AT) and pML(C2AT) plasmids, kindly provided by SAWADOGO and ROEDER (43).The aldolase B promoter cloned in front of the G-free cassette was obtained by polymerase chain reaction using as primers a 27-mer oligonucleotide complementary to sequence +8 to −19, 5′ AATAGGATGGA-TtTGCTTGCAGAGCAC (the t is a substitution for the wild type C), and a 24-mer oligonucleotide, 5′ ttgtcgaCTCTGACATTC-TACGAG, identical to sequence −194 to −178 of the coding strand, plus a SalI restriction site at its 5′ end. The lengths of the G-free cassettes used were 380 bp for the L-PK and aldolase B constructs and 270 bp for the AdML promoter. The generated transcripts were of different lengths: 394 b for L-PK, 388 b for aldolase B and 280 b for AdML. 600 ng of the L-PK and aldolase B constructs and 100 ng of the AdML construct were generally

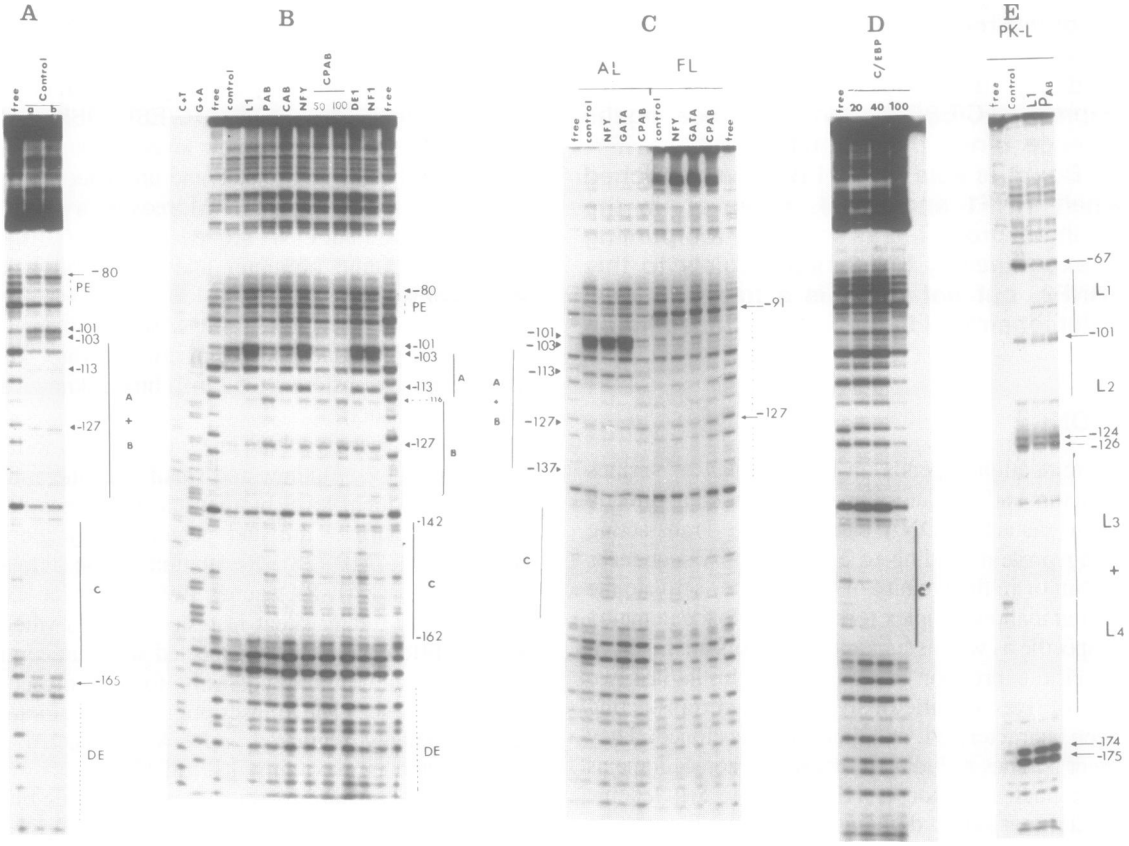


Figure 1. DNase 1 footprinting pattern using extracts from adult liver, fetal liver and purified recombinant C/EBP protein. The non-coding 3′end labeled fragment, extending from −190 to +41 for aldolase B (part A to D) and from −186 to +77 for L-PK promoter (part E) were incubated with 45 μg of adult liver extract and 80 μg fetal liver extracts in the presence of 0.1 to 0.15 μg of DNase 1. Lanes 'C+T', 'G+A' are sequence ladders respectively and lanes: control, represent the footprinting reaction in absence of competitor oligonucleotide. Lanes: free, indicate digestion of the probe without protein. Arrowheads indicate DNase 1 hypersensitive sites. Part A: Characterization of the protected regions. PE: proximal element; A+B: corresponds to a large protected region (Box A and B); C: Box C; DE: distal element. Lanes: a and b, correspond to 45 and 90 μg protein added, respectively. Part B: Footprinting competition experiments with 50 ng of L1, PAB, CAB, NFY, DE1, NF1, and 50 and 100 ng CPAB, oligonucleotides listed in table 1. Part C: Footprinting patterns in presence of adult (AL) or fetal (FL) liver nuclear extracts with competitor oligonucleotides listed in table 1. Part D: Footprinting occuring after addition of 20, 40, 100 ng of recombinant C/EBP protein (88 aminoacids). Part E: Footprinting experiment using the L-PK promoter as a probe. 50 ng of L1 and PAB oligonucleotides were added.

used in the assays. *In vitro* transcription reactions were performed as described (37).

RESULTS

DNAse I footprinting analysis of the aldolase B gene promoter

Nuclease S1 mapping and primer extension analysis allowed us to define the transcription start site and to assign it to a consensus 'initiator' sequence Py Py C A Py Py Py Py (44): we found the 5′ TCCAAGCAGATCCATCCTA 3′ sequence (data not shown).

Upstream from the cap site, DNAse I footprinting analysis using adult rat liver nuclear extracts revealed 5 protected regions termed PE (proximal element), boxes A+B and C and DE (distal element) [figure 1A]. This nomenclature was chosen to permit an easy comparison with boxes A, B and C described by Tsutsumi et al (31).

Region A+B spans from −139 to −101, with hypersensitive sites at nt −101, −103, −113 and −127. It could be divided into boxes A and B by binding competition experiments (figure 1 B). Excess of oligonucleotides (listed in table 1) CPAB (nt −142 to−103) and PAB (nt −126 to −104) suppressed.the hypersensitive sites at nt −101, −103 and −113, but induced appearance of a novel hypersensitive site at nt −116. Excess of oligonucleotides CAB (nt −140 to −116) and CPAB attenuated or suppressed protection from nt −139 to −116, but only slightly reduced the hypersensitive site at nt −127. Surprisingly, oligonucleotide PAB (and to a lesser extend, CPAB) also displaced protection on box C, from −162 to −142. Since regions A and C share no clear homology, this result rather suggests a concerted interaction between factors binding to boxes A and C. This interaction could be mediated by proteins bound to box B. All the oligonucleotides encompassing a CAAT motif (NFY, NF1, DE1, table 1) displaced protection on box C, DEI being the most effective.

Footprints observed with nuclear extracts from 17-day-old rat fetal livers differed from those described above by the absence of protection on box C and by clear changes in the protection pattern of PE and box A. Furthermore, a strong hypersensitive site is detectable at nt −91 (figure 1C).

Identification of C/EBP as a protein binding to box C

Since box DEI/D of the albumin gene promoter has been reported to bind C/EBP and related proteins (7, 8, 45) and since DEI oligonucleotide displaced the footprint observed on the aldolase B box C, we expected that this box C would also bind these factors. Indeed, recombinant C/EBP (a 88 aminoacid fragment retaining the full DNA binding activity, a kind gift of S. Mc KNIGHT) generated a footprint on box C (figure 1D) and bound tightly to the DE1 oligonucleotide from rat albumin promoter (data not shown) at low concentration (1μg/ml). At higher concentration (5μg/ml), nt −139 to −119 on box B were also protected. This confirms the capability of C/EBP to bind to various motifs especially to different CAAT boxes (6).

Footprinting analysis of HNF1 binding to box A

Box A has been reported to bind HNF1 (31) and, indeed obvious analogies exist between the sequences of box A and the HNF1 binding site on the albumin (46) and L-PK (19) promoters (compare the sequences of oligonucleotides PAB, L1 of the L-PK gene and PE 56 of the albumin gene in table 2). Box L1 of the L-PK gene promoter is a very strong palindromic binding site for HNF1 factor. However, an excess of oligonucleotide L1 (up to 200ng), was unable to displace protection of aldolase B box A in DNAse I footprinting experiments (figure 1B) while

Table 1. Sequences of oligonucleotides used in this study. Oligonucleotides are indicated as single stranded sequences and represent cellular and viral adenovirus 2 genomic elements. The position of the nucleotides relative to the cap site (+1) or relative to nucleotide +1 of the adenovirus genome is indicated. CPAB is the sequence corresponding to DNase 1 protection (A+B), CAB to the region B encompassing a CCAAT sequence and PAB to box A.

RAT ALDOLASE B:		
CPAB :	5'CTGTTCACGCGCCAATCAGAGTTATTGAATAAACACCTCC	-142 to -103
CAB :	GTTCACGCGCCAATCAGAGTTATTG	-140 to -116
PAB :	CAGAGTTATTGAATAAACACCTC	-126 to -104
RAT L-PK		
L1 :	AAGAGAGATGCTAGCTGGTTATACTTTAACCAGGACTCATCTCATCT	-106 to -60
RAT ALBUMIN		
PE56 :	TGTGGTTAATGATCTACAGTTA	-63 to -41
NFY :	GGGGTAGGAACCAATGAAATGAAAGGTTA	-93 to -65
DE1 :	GGTATGATTTTGTAATGGGGTAGG	-109 to -86
MOUSE TRANSTHYRETIN		
HNF3 :	GTTGACTAAGTCAATAATCAGA	-111 to -90
MOUSE α GLOBIN		
GATA :	CGGCAACTGATAAGGATTCCCCTG	-211 to -188
ADENOVIRUS 2		
NF1 :	TATTTTGGATTGAAGCCAATATGATAATGA	+20 to +49

Table 2. The HNF3, NFY, and C/EBP recognition sequences of the aldolase B promoter are aligned with various homologous sequences. The deduced consensus sequences recognizing HNF1 and HNF3 binding proteins are compared.

ALIGNMENT OF SEQUENCES HOMOLOGOUS TO A,B,AND C REGIONS

HOMOLOGIES TO HNF3 BINDING ELEMENT

RAT ALDOLASE B	TATTGAATAAA	-120 to -110
HUMAN α1 ANTITRYPSIN	TATTGACTTTG	-376 to -366
	CATTGATTTAG	-195 to -185
MOUSE TTR	TATTGACTTAG	-96 to -106

HOMOLOGIES TO NFY BINDING SITE

ALDOLASE	CGCCAATCAGA	-133 to -123
MLP adenovirus 2	AACCAATCACC	-74 to -84
CCAAT ALBUMIN	AACCAATGAAA	-85 to -75
NFY MHC	AACCAATCAGA	-50 to -60
αCP1 αGLOBIN	AGCCAATGAGT	-58 to -48

HOMOLOGIES TO C/EBP BINDING SITE

ALDOLASE	CTTTGTAATC	-148 to -157
ALBUMIN	TTTTGTAATG	-102 to -93
α1 ANTITRYPSIN	TTAAGCAAAG	-203 to -212

HOMOLOGIES TO HNF1 AND HNF3 BINDING SITES

CONSENSUS	A T C A GGTTAATNNTTAACC	HNF1
HNF1 PK	GGTTATACTTTAACC	-90 to - 76
HNF1 ALBUMIN	GGTTAATGATCTACA	-60 to - 46
SITE A ALDOLASE	AGTTATTGAATAAAC	-123 to - 105
HNF3 TTR	GATTATTGACTTAGT	-93 to -107
CONSENSUS	TATTGACTTAG T T	HNF3

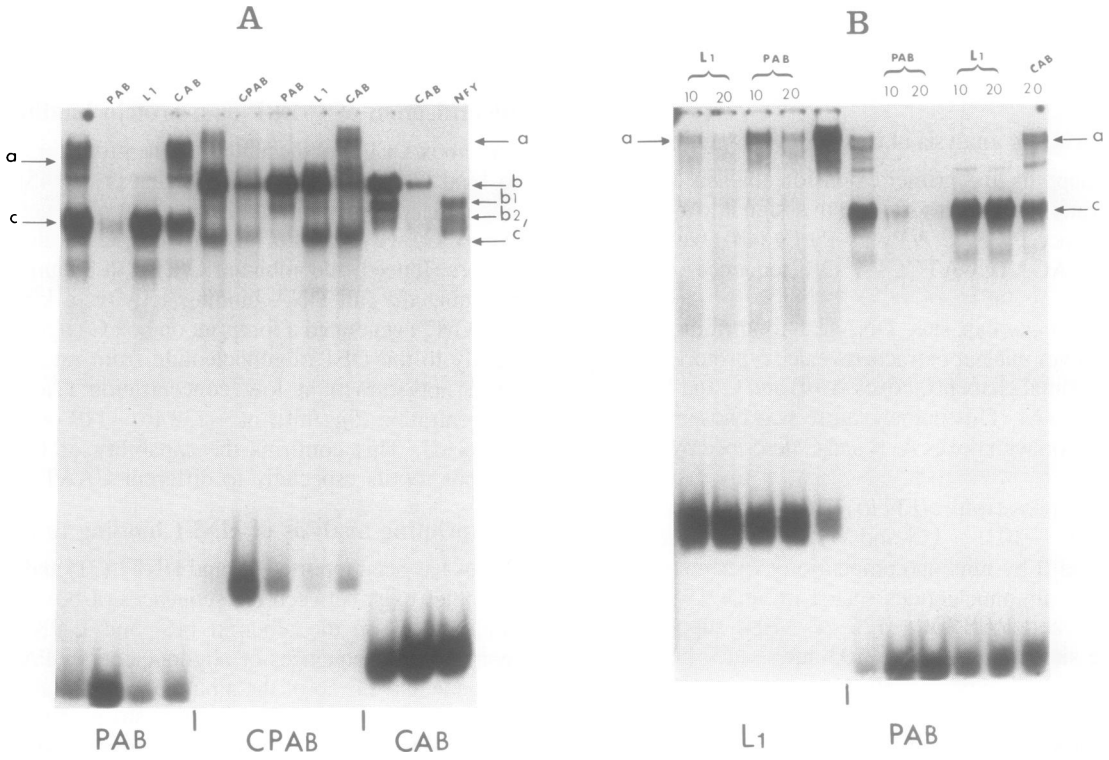


Figure 2. Gel mobility shift assay with labeled oligonucleotides (listed in table 1) in 6% polyacrylamide gels 0.1 to 0.15 ng of kinased oligonucleotides were incubated with 5 μg of adult liver nuclear extracts in the presence of 2 μg poly (dI:dC). Homologous and heterologous competitions were performed with 20 ng oligonucleotides (part A) or as indicated in the upper part of the figure (part B). Arrowheads a, b, b1, b2, c and c' indicate the position of the retarded complexes.

the L1 box protection was efficiently displaced on the L-PK gene promoter by both L1 and PAB oligonucleotides (figure 1E). These results suggest that PAB binds HNF1 efficiently, but that the protein bound to box A in the footprinting experiments is not HNF1. We also note an increased intensity of hypersensitive sites at −101, −103 nt, in the presence of an excess of L1 oligonucleotide

Gel shift assay analysis of proteins binding to region A+B

With adult liver nuclear extracts, oligonucleotide PAB bound two major complexes designated a and c on figure 2; both are displaced by an excess of the same oligonucleotide while only complex a was displaced by an excess of oligonucleotide L1 (figure 2A). The involvement of HNF1 in complex a is confirmed in figure 2B: it comigrated with the complex formed with L1. L1 is a more efficient competitor for displacing complex a formed with PAB than PAB for displacing the complex formed with L1. This means that the affinity of L1 for HNF1 is higher than that of PAB.

Oligonucleotide CAB formed three complexes, the major one being designated b in figure 2A. The three complexes disappeared as a result of homologous competitions, while only complex b was efficiently displaced by oligonucleotide NFY (figure 2A and table 1). It seems therefore that aldolase B box B is able to bind mainly NFY and other minor not identified proteins in liver nuclear extracts.The presence of these three complexes was also observed in fetal liver, brain (figure 3) and spleen nuclear extracts (data not shown), in the same relative proportions as in adult liver nuclear extract.

The results obtained by gel retardation using oligonucleotide CPAB confirmed these findings. Three major retarded complexes were detected, corresponding to a, b and c observed with oligonucleotides PAB and CAB (figure 2A).

We found evidence that box A binds not only the liver enriched factor HNF1 (complex a), but also another protein (complex c) that we demonstrated to be absent in fetal liver and only present in small amount in brain nuclear extracts [ca, cb complexes (figure 3)]. Control experiments using CAB, NF1 and GATA oligonucleotides reinforce this result since the ubiquitous NFY and NF1 factors were present at the same level in adult liver, brain and fetal liver nuclear extracts while the erythroid-specific 'GATA' binding protein was only detected in 17-day-old fetal liver extracts. Since HNF3 has been reported as a liver-specific factor binding to DNA sequences related to HNF1 binding sites (20), we compared the sequences of PAB oligonucleotide to those of the HNF3 binding site on the transthyretin (TTR) gene promoter (table 1 and 2). Only two mismatches occurred in 11 bp (TTATTGAATAA for PAB and TTATTGACTTA for the HNF3 TTR binding site, on the lower strand). The ability of oligonucleotide PAB to bind HNF3 was demonstrated by gel shift assay (figure 4). Liver nuclear extracts incubated with PAB and HNF3 TTR oligonucleotides formed retarded complexes at the same position (band c). These complexes were efficiently displaced by excess of either unlabelled oligonucleotide. In contrast, oligonucleotide L1 (i.e HNF1 binding site) did not compete for the formation of complex c.This is consistent with the footprint data where L 1 did not suppress the box A protection (figure 1).

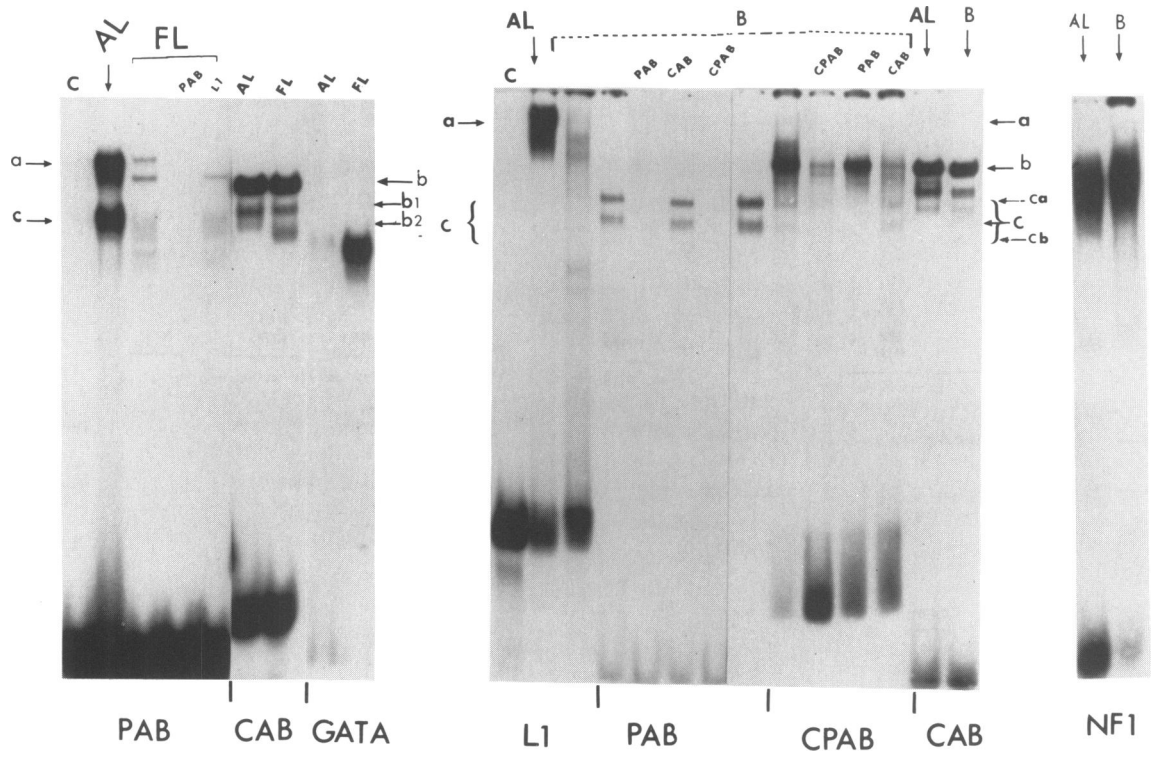


Figure 3. Gel retardation assays using 0.1 to 0.5 ng of kinased oligonucleotides incubated in the presence of 2 μg poly (dI: dC) with 5 μg of nuclear extracts prepared from: adult liver (AL), 17-day-old fetal liver (FL), or brain (B). Homologous and heterologous competitions were performed with 20 ng of each oligonucleotide. Arrowheads a, b, b1, b2, c, c_a, c_b, indicate the positions of the complexes. The ladder c indicates incubation in the absence of protein.

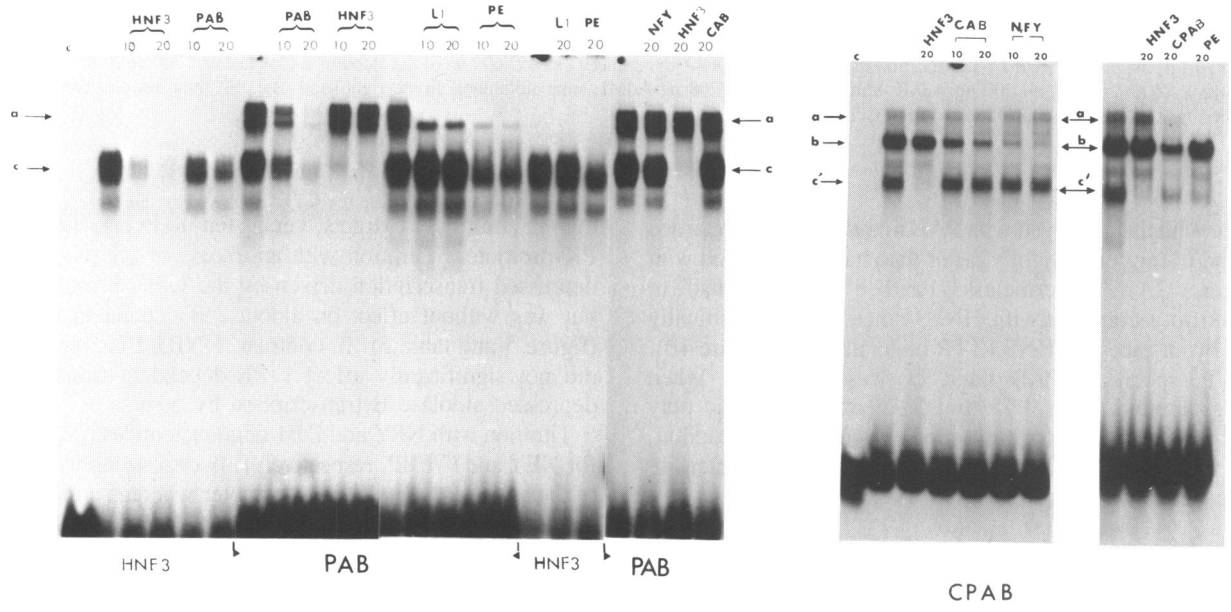


Figure 4. *Identification of HNF3 binding protein* by homologous and heterologous competition in band shift assay experiments. 5 μg of adult liver nuclear extract were used in the presence of 2 μg poly (dI: dC). The amount of unlabeled oligonucleotide competitors (ng) is indicated in the upper part of the figure.

From these experiments we can also conclude that factor HNF3 has a greater affinity for the TTR motif than for the aldolase B site.

While L1 oligonucleotide displaced only the HNF1-specific retarded band a (figure 2B), the albumin HNF1 binding site (site B or PE) displaced both bands a and c that correspond to HNF1- and HNF3-specific complexes, respectively. Therefore the albumin binding site PE seems to have affinity for both HNF1 and HNF3 factors, like the aldolase B site (PAB). However, the latter seems to have more affinity for HNF3 while the former has more affinity for HNF1.

The gel shift pattern obtained with oligonucleotide CPAB was

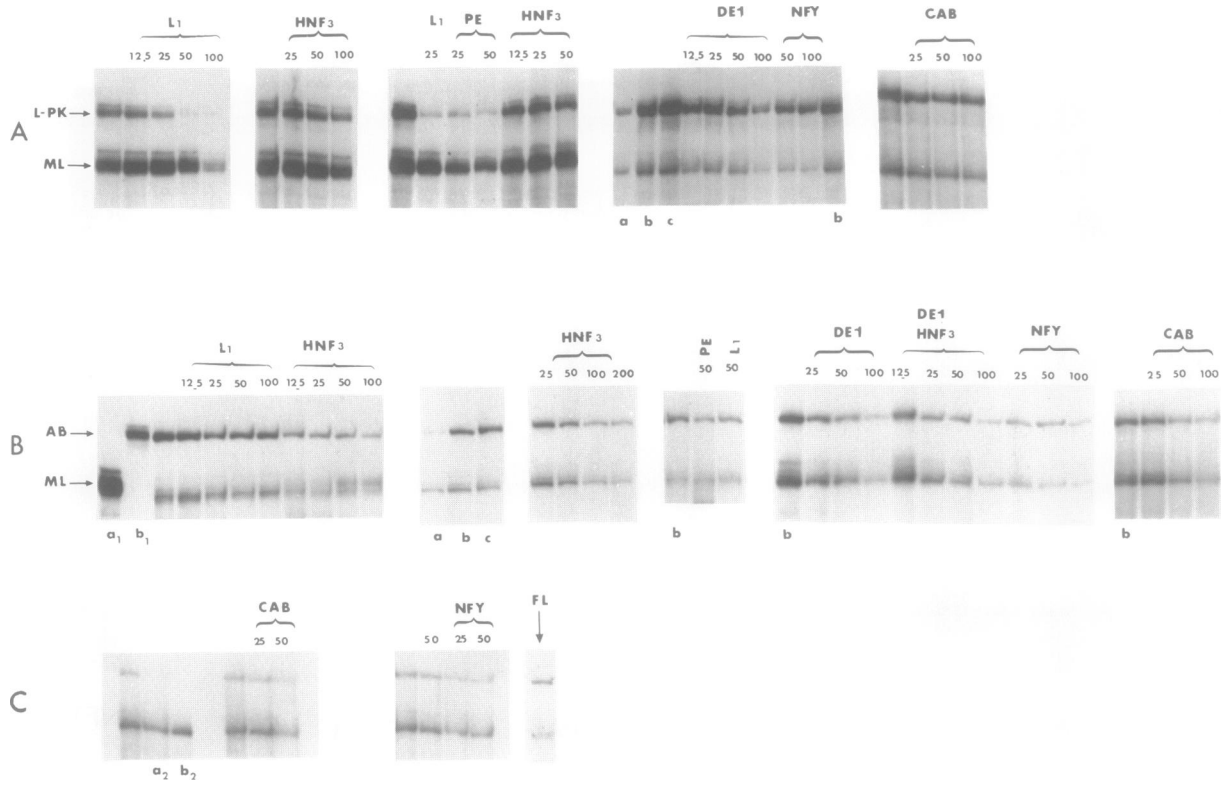


Figure 5. Cell-free transcription analysis of L-PK (part A) and aldolase B (part B) promoters. The reaction mixtures contained 22 μg of adult liver nuclear extracts, 600 ng of each liver-specific template and 200 ng of the adenovirus major late promoter template (ML), except in lanes a and b (where 7, 14 μg of nuclear extracts were used) and in lanes a_1 and b_1 (where the run-off assays were performed with 600ng of either aldolase B or AdML templates). The intensity of the radioactive bands was scanned with a Shimadzu densitometer. The results are expressed as percentages of the values obtained for the AdML template in the titration experiments with the L1, PE, and HNF3 oligonucleotides, or as a ratio of the values for aldolase B over that obtained for L-PK templates in the titration experiments with NFY, CAB and DE1 oligonucleotides. Titrations were performed by adding to the reaction medium increasing amounts of different competitor oligonucleotides, as indicated in the upper part of the picture. *Part C*: The reaction mixtures contained 40 μg of spleen extract, 600 ng of the aldolase B template and 200 ng of the AdML template except in lane a_2 (600 ng L-PK + 200 ng AdML templates) and b_2 (200 ng AdML template alone). In the right-hand lane, FL indicates that the spleen extracts were replaced by the same amount of fetal liver extract.

at variance with that observed with PAB in that the fastest retarded major band c′ migrated slightly faster than band c observed with PAB.(figure 2A). Nevertheless, band c′ seemed also to correspond to a complex with HNF3 since it was specifically displaced by an excess of HNF3 TTR oligonucleotide (figure 4B). This HNF3-specific CPAB band c′ was reinforced when increasing amounts from 0.75 to 1.5 μg of non-specific poly (dI:dC) competitor DNA were added to the binding reaction, while, as expected, PAB band c gradually decreased in intensity under the same conditions (data not shown).

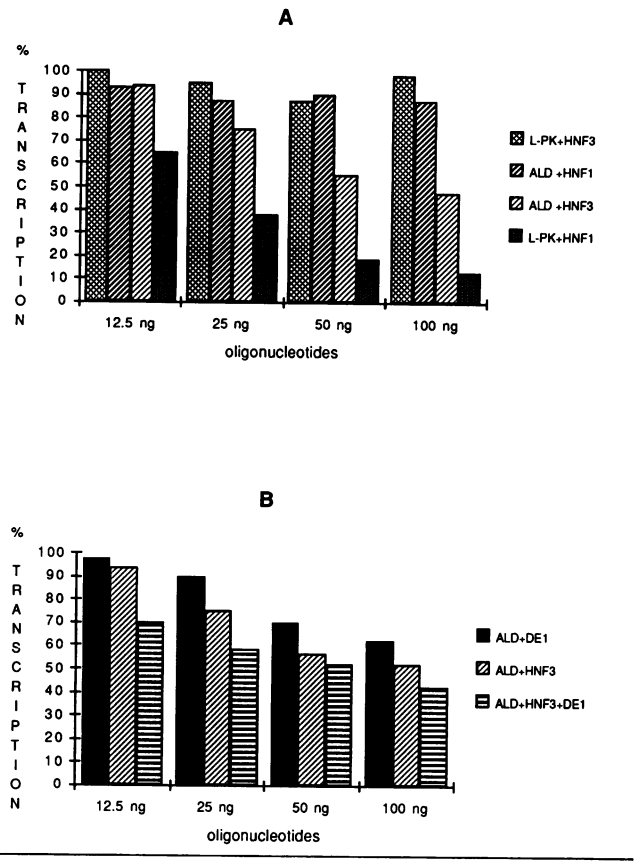
Cell free transcription analysis of the aldolase B gene promoter

Run off transcription assays were performed using a G-free cassette as a reporter gene, as described elsewhere (43). Transcription efficiency of the aldolase B construct (192 bp upstream from the cap site) was compared to that of the 183 bp L-pyruvate kinase construct already investigated (37). We used as internal standard the adenovirus major late promoter (AdML) driving a shortened version of the G-free cassette (37) (figure 5).

Aldolase B and L-PK promoters were of similar transcriptional efficiency with adult liver nuclear extracts. In extracts prepared from livers of 17- to 18-day-old rat fetuses or from adult rat spleen, the aldolase B promoter was only two-fold less active than in adult liver extracts, versus ten- to twenty-fold for the L-PK promoter. Titration with an excess of L1 oligonucleotides depressed transcription driven by the L-PK promoter by 90% but was without effect on aldolase B dependent transcription (figure 5 and table 3). In contrast, HNF3 TTR oligonucleotide did not significantly affect L-PK-dependent transcription but depressed aldolase B transcription by 50%.

Titration with NFY and DE1 oligonucleotides (i.e, binding site for NFY and C/EBP, respectively) affected strongly the efficiency of the adenovirus major late promoter whereas it did not change the efficiency of the L-PK promoter. Therefore, the results of this titration on the activity of the aldolase B promoter were expressed with respect to the invariant L-PK promoter activity. Titration with either NFY or CAB oligonucleotides reduced similarly the activity of the aldolase B promoter by about 40%, while in adult liver nuclear extracts NFY seemed to be a better inhibitor of the adenovirus major late promoter than CAB (figure 5). This later result is consistent with the albumin NFY binding site having a stronger affinity for the NFY factor than the aldolase B site. Reduction obtained by titration with DE1 oligonucleotide in liver extracts [i.e, the albumin C/EBP binding site D (45)] was about of 55%. A mixture of both DE1 and HNF3 or NFY and HNF3 oligonucleotides did not reduced the activity of the aldolase B promoter more than each of the oligonucleotides

Table 3. Competition experiments: the relative transcription as a function of increasing amount of oligonucleotides HNF1, HNF3, DE1 is expressed as a percentage of the transcriptional level in the absence of competitors. Part A: transcriptional efficiency from the L-PK (L-PK) and aldolase B (ALD) promoters and part B aldolase B promoter alone. The conditions are described under the figure 5. Numerical values are to the average of three independent experiments.



individually (figure 5, table 3 and data not shown). Consistently with the reported absence of HNF3 and C/EBP in spleen nuclear extracts (21), the non-specific aldolase B promoter activity detected in these extracts was insensitive to titration with the oligonucleotides specific to HNF3 or C/EBP factors and slighly affected by the oligonucleotides NFY or CAB. This non-specific activity is at the same level as the activity of the aldolase B promoter in the liver extracts in presence of titrating oligonucleotides in excess, that is to say of about 50% of the maximal specific activity.

DISCUSSION

Tissue-specific control of transcription by RNA polymerase II is likely to depend on a limited number of tissue-enriched factors acting in a combinatory fashion with ubiquitous transactivators near the start site of transcription. Analysis of the DNA/protein interactions in the 200 bp upstream from the cap site constitutes a first insight into the mechanisms responsible for the liver-specific expression of the genes. Genes that are expressed in the same tissues, in a more or less coordinated fashion, are expected to be controlled by sets of identical or functionally similar trans-acting factors.

Promoters of various liver-specific genes, extensively investigated since about 5 years are for instance controlled by various combinations of half a dozen liver-enriched factors and ubiquitous factors [e.g., rat and mouse albumin (18, 47); human α1 antitrypsin (21, 48); mouse transthyretin (21); α1-inhibitor III (49); human transferrin (50); rat L-pyruvate kinase (37)]. Since L-pyruvate kinase and aldolase B genes are regulated in a closely related manner in the same tissues and in response to the same stimuli, it was especially interesting to determine whether this expression pattern was due to analogous arrangements of cis-acting elements recognizing the same transcription factors.

Tsutsumi et al (31) recently described three DNAse I footprints (A, B, C) on a 200 bp upstream aldolase B fragment generated by liver nuclear extracts. These authors tentatively identified the factor binding to box A as HNF1 which could be separated into two active fractions by affinity chromatography on wheat germ agglutinin sepharose column (32). In fact, our gel shift assay data demonstrates that box A binds both HNF1 and HNF3, a different liver-enriched factor first described by COSTA et al (20) on the transthyretin gene promoter. In the context of the complete 200 bp aldolase B promoter fragment, interaction with HNF3 seems to be functionally dominant over interaction with HNF1, as suggested by the absence of inhibition in the cell-free transcriptional system of the aldolase B promoter by an excess of L1 oligonucleotide, whereas excess TTR HNF3 oligonucleotide inhibits the transcriptional activity by half. A similar inhibition was observed by Tsutsumi et al (31) by titration with an oligonucleotide similar to PAB. HNF3 trans-acting factors have been reported, using transient transfection assays, to be important in transcriptional activation of the mouse transthyretin promoter (16). Moreover, multimerized HNF3 binding motifs are able to confer liver-specific expression on a heterologous promoter (20).

Aldolase box B contains a typical CCAAT motif that interacts with the CCAAT binding factor NFY and also with different 'ubiquitous' proteins. NFY has been demonstrated to bind to the albumin gene promoter around nt −80 (41, 51) and also to box Y of the major histocompatibility class II genes (38, 52).

In contrast to the albumin NFY motif, binding specificity of aldolase B box B displays unique features, namely the presence of clearly detectable retarded bands with faster mobility, well competed by oligonucleotide CAB but not by oligonucleotide NFY. These fast migrating extra bands observed with box B could correspond to different heterodimers. NFY and the two fast-migrating extra bands observed with oligonucleotide CAB are ubiquitous which is in disagreement with the report by Tsutsumi et al (31) that box B bound a liver-enriched factor. However since these authors did not checked their brain nuclear extracts for the presence of other ubiquitous factors, it could be that their extracts were depleted in DNA binding activities for artefactual reasons. Titration with excess NFY oligonucleotide reduced the cell-free transcriptional activity of the AdML promoter very strongly, as expected (53). In contrast, efficiency of the L-PK promoter was not affected, which allowed us to use it as standard. As compared to the L-PK promoter, the aldolase B promoter was inhibited by about 40% when excess NFY oligonucleotide was added to the reaction. Tsutsumi et al reported a more marked inhibition using titration with a box B oligonucleotide, but did not detect inhibition of the AdML promoter. Using the same oligonucleotide, we also observed a decrease in the transcriptional efficiency of the aldolase B promoter which reached the same plateau as with the NFY oligonucleotide without affecting the activity of the AdML promoter probably because the CCAAT motif of the adenovirus major late promoter has a better affinity for NFY than the aldolase B CCAAT motif.

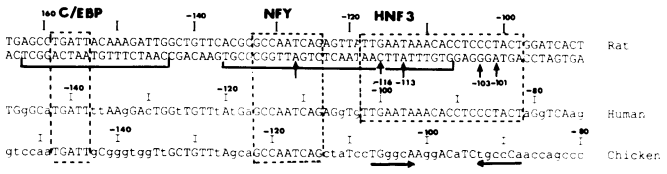


Figure 6. Sequence alignment with human, rat and chicken aldolase B promoters. Arrows indicate hypersensitive sites and brackets the protected areas. The conserved sequences are boxed.

Box C also contains a CCAAT box on the lower strand that binds specifically the C/EBP factor or related proteins, as demonstrated by DNAse I footprinting experiments: the footprinting on box C was displaced by oligonucleotide DE1, the binding site for C/EBP (6), DBP (8), LAP (7) and related proteins on the albumin gene promoter. Furthermore, box C binds a recombinant fragment of C/EBP including the DNA binding domain (45, 6). The presence of a C/EBP binding site on the aldolase B gene promoter reinforces the concept that this trans-acting factor could play an important role in the regulation of genes involved in energy metabolism (54–55). However, if such C/EBP binding sites are indeed present on the promoters of the genes for phosphoenolpyruvate carboxykinase [a gluconeogenic enzyme (56)] and for lipogenic enzymes (55), they are absent from the L-PK promoter whose expression is closely coordinated with that of the aldolase B gene. Titration with oligonucleotide DE1 reduces the cell-free transcriptional activity of the aldolase B promoter by about 50%, which confirms the functional importance of the proteins binding to box C. A gel shift assay does not allow easy characterization of the actual proteins interacting with DNA elements such as albumin DE1 or aldolase B box C, because the multiple possible interactions result in the formation of a broad, poorly defined retarded band. It is therefore difficult to determine which of these proteins (C/EBP, DBP, LAP...) is functionally relevant. Transactivation of aldolase B constructs by C/EBP and DBP expression vectors in transient transfection seems to indicate that both trans-acting factors are active on the aldolase B promoter, according to the cellular context (in preparation).

Maximal inhibitions obtained by the titration experiments in run off transcription assays (55% with either oligonucleotide HNF3 TTR, NFY or DEI) might seem relatively modest compared, for instance, to the strong inhibition of the L-PK promoter by excess HNF1 binding sites. However, we demonstrate here that the aldolase B promoter is half as active in non-specific spleen nuclear extracts as in liver extracts, while spleen is devoid of HNF3 and very poor in C/EBP factors and while in the same conditions the transcription from the L-PK promoter is barely detectable. Consistent with this, titration experiments with HNF3 and DE1 oligonucleotides are inefficient in these spleen extracts. Interestingly, excess NFY oligonucleotide is also unable to reduce the non-specific aldolase B promoter activity. This high background activity of the same level as the promoter activity in liver extracts containing an excess of HNF3 and/or DE1 oligonucleotides, could be due to the high efficiency in a cell-free transcription system of the proximal promoter region, including PE, TATA box and 'initiator'. Our aldolase B-G free cassette differs indeed, from that reported by Tsutsumi et al (31) by the presence of the first nucleotides of the aldolase B gene first exon generating a putative initiator site (44), while this fragment was not included in the Tsutsumi's construct (31). We verified that the non-specific activity of the aldolase B promoter was observed for different ratios of template/nuclear extracts and therefore does not seem to be explained by artefactual titration of some factors in our experimental conditions.In any case, our result suggests that, in the absence of HNF3 and/or C/EBP, NFY is unable to stimulate the aldolase B promoter, as already deduced from the inefficient titration with NFY oligonucleotide in liver extracts containing excess HNF3 and/or DE1 oligonucleotides (data not shown).

In contrast to the results reported here *in vitro*, the promoter is absolutely tissue-specific (33) *In vivo* or *ex vivo*, and the influence of the trans-acting factors is likely to be more important, as proven, by a 50-fold transactivation by C/EBP in transient transfection assays (in preparation). In addition, the aldolase B gene is normally controlled by interactions between its tissue-specific promoter and an activator located in the first intron (33). A correct interplay between the different transcriptional factors acting on promoters could be a requirement not only for the basal activity of these promoters, but also for their ability to be fully activated by remote control regions.

The sequence alignment of the 5′ flanking regions among rat, human and chicken aldolase B genes is instructive (figure 6). Human and rat sequences display 83% homology from −90 to −102 nt. NFY and C/EBP recognition sequences are located at about the same distance from the cap site in the three genes. Chicken box C fits the high affinity consensus sequence for C/EBP, 5′ GCAATC. The box C sequence is conserved on 5 nucleotides only between the three species, which is not surprising for a C/EBP recognition site that is known to be rather degenerated. Human and rat HNF3 binding recognition element A share an identical sequence of 20 nucleotides which is lacking in the chicken promoter where it is replaced by a palindromic TGGGCA sequence. As a common feature, all these promoters bear a long poly A stretch that could induce a bend of the DNA structure bringing trans-activators nearer the TATA box (57).

Finally, it is interesting to compare the arrangement of cis-acting DNA elements binding transcriptional factors in the promoters of different liver-specific genes. The first striking observation is that although their developmental tissue-specific dietary and hormonal controls are very similar, the promoters of the aldolase B and L-PK genes possesses a very different DNA element arrangement: from 3′ to 5′, binding sites for HNF1, NF1, LFA1, HNF2-4 and MLTF on the L-PK promoter (19); HNF3, NFY and C/EBP on the aldolase B promoter (figure 6, table 2). In contrast, the aldolase B promoter more closely resembles the albumin gene promoter which binds factors HNF1, NFY and C/EBP. Albumin site B or PE and aldolase B site A both bind the two transcriptional factors HNF1 and HNF3 in our gel shift assays. Nevertheless, using the competition experiments, we found a greater affinity of the albumin promoter for HNF1 and, in contrast, of the aldolase B promoter for HNF3. This result, with others, suggests that the albumin gene would be dependent on HNF1 and the aldolase B gene on HNF3 in their transcriptional regulation.

It appears therefore that the regulation characteristics of a gene proceed from an interplay between all its cis-acting DNA regions, close to the cap site or in remote positions, and cannot be predicted only from arrangement of the elementary trans-acting factor binding sites in each region. Moreover, it could be that same sort of molecular synonymy exists, namely that different

combinations or regulatory elements results in the same type of control.

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