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Expression from the tyrosine aminotransferase promoter (nt −350 to +1) is liver-specific and dependent on the binding of both liver-enriched and ubiquitous *trans*-acting factors

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ABSTRACT

The rat tyrosine aminotransferase (TAT) gene promoter (nucleotides −350 to +1; $TAT_{0.35}$) was able to sustain liver-specific expression both *ex vivo* in transient transfection (TAT-expressing H4IIEC3 hepatoma cells vs. TAT non-expressing CCL1.2 fibroblasts) and in *in vitro* transcription (rat liver vs. spleen crude nuclear extracts). In either case, the index of tissue specificity (6.2 and 6.7 in *ex vivo* and *in vitro* experiments, respectively) was close to that obtained with 10 Kb of TAT gene 5′-flanking sequences in transient transfection. Using computer-assisted search of homologies, DNase I footprinting, gel retardation and methylation interference assays, we showed that $TAT_{0.35}$ sequences spanning nt −156 to −175 and nt −268 to −281 interacted with the liver enriched $NF\text{-}1_{Liver}$ (a member of the NF1 gene family) and HNF1 respectively, whereas those encompassing nt −57 to −85 and nt −283 to −288 interacted with the ubiquitous NF-Y and with ubiquitous 'CCAAT'-box binding factor(s), respectively. Competition studies in *in vitro* transcription carried out with wild type and mutated oligonucleotides, demonstrated that NF-Y *cis*-elements were crucial for basal TAT promoter activity, both in liver and spleen whereas $NF1_{Liver}$ and HNF1 were only efficient in the liver (supported ~60% and 30% of basal $TAT_{0.35}$ activity respectively). Altogether, these results support the conclusion that $TAT_{0.35}$ was able to sustain at least part of the liver specificity of TAT gene expression.

INTRODUCTION

Constitutive expression of several liver-specific genes has been shown to be driven by a few hundred nucleotides (up to 500) of 5′-flanking sequences, this basal promoter activity being often enhanced or repressed (extinguished) by either constitutive or hormone-dependent far upstream sequences (1–11).

In the liver, the tyrosine aminotransferase (TAT) gene belongs to the genes' cluster that is activated in the neonatal period (reviewed in 12). Thereafter, TAT gene expression is modulated by hormones such as glucagon (via the cAMP signaling pathway; 13, 14) and glucocorticoids (15; review in 16).

Constitutive and hormone-induced TAT gene expression is dependent on at least two distinct genetic loci (17–19; see 20 for review). One of these loci (*Tse-1*) extinguishes basal TAT gene expression, but leaves the gene partially responsive to glucocorticoids (19), consistent with previous studies of the extinction phenomenon in intertypic somatic cell hybrids (18,21). Making use of *Tse-1*-containing or non-containing and of FTO2B hepatoma cells, Boshart and coworkers (22) have shown that one motif (BI), which maps −3.6 Kb 5′ to the TAT cap site and encompasses a cAMP response element (TAT-CRE), was the primary target for *Tse-1*-mediated gene extinction. A nearby motif, BIII, which binds the liver-enriched *trans*-acting factor HNF4, cooperates with the TAT-CRE, and confers a strong liver-specificity to the −3.6 Kb enhancer (23).

The −3.6 Kb enhancer lies within a DNAse I hypersensitive site (HS). Seven such HSs have been mapped within 11 kb of TAT gene 5′-flanking sequences both in liver and in hepatoma cells (24–26). Most of these HSs are constitutive, and behave as liver-specific enhancers acting on either basal (26) or glucocorticoid-induced (25) transcription. A single HS is hormone-dependent and is responsible for primary glucocorticoid induction (27).

One of these HSs, the promoter-HS was different (absence of the distal cleavage at −200) in *Tse-1* positive versus *Tse-1* negative hepatoma cell lines (23,26). This observation suggested that the TAT promoter itself might be involved in the liver-specific control of TAT gene expression and prompted us to address the following questions: (a) are proximal TAT gene

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5′-flanking sequences able to drive basal liver-specific gene expression and (b) is this tissue-specificity due to the presence of *trans*-acting factors in TAT-producing cells or alternatively to that of *trans*-acting repressor(s) in TAT non expressing cells?

In the present paper, we provide evidence that TAT promoter sequences (nt −350 to +1) are able to sustain liver-specific expression, both in transient transfection and in *in vitro* transcription, and that two liver-enriched *trans*-acting factors, $NF1_{Liver}$ and HNF1 and the ubiquitous NF-Y contribute to liver-specific transcription from the TAT promoter. Their respective role in the tissue-specificity is discussed.

MATERIALS AND METHODS

Transient expression and transcription vectors

pSV_E CAT, the promoterless pSB1 and pTC_{10} expression vectors have been described elsewhere (28,29).

$pTC_{3.3}$ was obtained by HindIII digestion and subsequent recircularization of pTC_{10}. Conversion of the unique KpnI site of pTC_{10} into a HindIII site, followed by complete HindIII digestion and recircularization, yielded $pTC_{0.35}$. In pTC_{10}, $pTC_{3.3}$ and $pTC_{0.35}$, transient CAT expression was driven by TAT gene 5′-flanking sequences spanning from nt −10,095, −3,336 and −350 to nt +3 respectively (Fig.1A). The additional pTC_9, $pTC_{3.9}$, pTC_3, $pTC_{1.3}$ were generated from pTC_{10}, making use of SmaI (−8,923), XhoI (−3,918), EcoRI (−3,046) and BamHI (−1,299) restriction sites, respectively.

The DNA templates used in *in vitro* transcription $p(C_2AT)_{19}$, $pML(C_2AT)_{19}$ (referred to as $pAdML_{370}$) (30) or $pAdML_{270}$ (9), and pTAT generated G-free transcripts of 385bp ($pAdML_{370}$), 375bp (pTAT) or 270bp ($pAdML_{270}$). pTAT and $pTAT_{inv}$ corresponded to insertions of a 394bp SstI fragment excised from $pTC_{0.35}$ either in the correct or reverse orientation relative to the 'G-free cassette', respectively (Fig.1B).

Plasmid DNA was prepared and purified according to (31) and (32).

Cell culture and transient transfection

Differentiated rat hepatoma cells H4IIEC3 (H4II herein) (33) and mouse fibroblasts L−M (ATCC CCL1.2) were cultured in Coon's modified Ham's F-12 medium supplemented with 5% fetal calf serum (34) and in Dulbecco's modified Minimum Essential Medium supplemented with 10% fetal calf serum, respectively.

Transfection (20 μg of plasmid DNA/dish) was carried out using the calcium phosphate-DNA co-precipitation procedure (35), as described (1, 36) and cells were harvested 72 h post-transfection. For each construct, a minimum of 3 independent transfection experiments were performed with at least 2 different plasmid preparations.

CAT assays

CAT activity (37) was assayed as in (1) (100 μg total protein/assay; 37°C for 1−3 h) and normalized as follows:

$$\frac{\text{CAT activity expressed from the test plasmid per pmol of transfected test plasmid}}{\text{CAT activity expressed from } pSV_E\text{-CAT per pmol of transfected } pSV_E\text{-CAT}}$$

Tissue-specific index was calculated as follows:

$$\frac{\text{normalized CAT activity in H4II}}{\text{normalized CAT activity in L−M}}$$

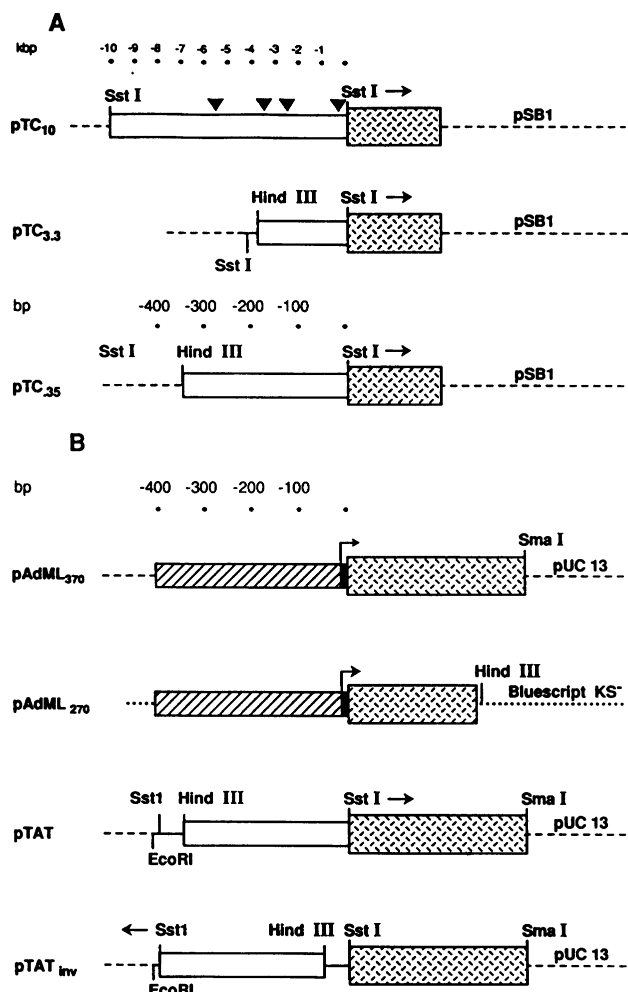

Figure 1. Schematic diagram of transfection and *in vitro* transcription vectors. Open weave boxes indicate CAT (A) and G-free cassette (B) sequences. Open, hatched and black boxes represent TAT 5′-flanking, AdML 5′-flanking and AdML+1 to +10 sequences, respectively. Dashed, thin and dotted lines correspond to pSB1 or pUC13, lambda Charon 35 polylinker and Bluescript DNA, respectively. Arrowheads point to the positions of liver-specific and hormone-dependent DNase I hypersensitive sites.

Nuclear extract preparation and *in vitro* transcription reaction

Rat (10−11 week-old male Sprague−Dawley) liver and spleen crude nuclear extracts (CNEs) (2) were always prepared in the presence of protease inhibitors (38,39).

Transcription was carried out according to Gorski *et al.* (2). The transcription mixtures (20 μl) contained 2.4 mg/ml of liver or spleen nuclear proteins, 0.4 mM of 3′-O-methyl GTP (Pharmacia), and template DNA: 5−40 μg/ml of $pAdML_{370}$ or pTAT in dose-response experiments; 30 μg/ml of pTAT and 10 μg/ml of linearised $pAdML_{270}$ in competition experiments.

Following transcription, the reaction mixture was treated with proteinase K, extracted with phenol-chloroform, precipitated with ethanol and the radioactive transcripts were analysed on sequencing gels (7 M urea−4% acrylamide). *Bona fide* transcripts were quantitated either by liquid scintillation counting of the dried gel, or by laser densitometry scanning of the gels' autoradiograms, within the linear range of the film's response (GelScan; LKB).

Table I. Promoter elements spanning nt −350 to +1 mimic the *in vivo* tissue specificity of basal TAT expression

Plasmid	GRU (HSs)	DEX	Normalized CAT activity		
			H4II	L-M	TS_{index}
pTC_{10}	-5.4 & -2.5	-	398±68$(5)#	59.7±16.5 (8)	6.7
		+	5244±903 (5)	133±53.2 (8)	39.4
$pTC_{3.3}$	-2.5	-	278±118 (5)	31.5±17.5 (7)	8.8
		+	641±243 (5)	34.4±7.4 (7)	18.6
$pTC_{0.35}$	-	-	88.9±38.9 (3)	16.8±3.0 (8)	5.3
		+	137±76.8 (3)	19.2±3.6 (7)	7.1

Differentiated hepatoma (H4II) and fibroblast (L−M) cells were transfected with 20 μg of the indicated expression vectors, then cultured for an additional 72 h and harvested. When indicated (+, column 3) 1 μM dexamethasone was added 24 h prior to cell harvesting. CAT activity was assayed and normalized as described in Materials and Methods.
GRU (Glucocorticoid Responsive Unit; numbers in column 2, indicate that the TAT 5′-flanking sequences of the transfected plasmid encompassed the corresponding DNAse I HSs (27). Computation of the TS_{index} is defined in Materials and Methods.
$, mean ± SEM; #( ), number of independent transfections.

Protein−DNA binding assays

DNAse I protection assays (40) were performed as described (38). ^{32}P-end-labeled $TAT_{0.35}$ (50ng/ml) was mixed with double stranded poly (dI−dC) (50 μg/ml) and incubated at 0°C for 15 min either with BSA (1 mg/ml; control) or with liver or spleen CNEs (0.5−1 mg protein/ml) in a final volume of 20 μl. DNase I (Cooper Biomedical) was then added at either 1.25 μg/ml (control) or 2.5 μg/ml (CNEs) and digestion was allowed to proceed at 0°C for 1−3 min. When digestion was completed, the samples were processed as described (38) and analyzed on sequencing gels (7 M urea−5% acrylamide). The gels were dried and autoradiographed at −70°C.

When gel mobility shift assays (41) were to be performed, the 20 μl incubation mixture contained 50ng/ml of ^{32}P-end-labeled synthetic ds-oligonucleotides, 50μg/ml of double stranded poly(dI−dC) and 0.3mg/ml of liver or spleen CNEs. After a 10 min incubation at 0°C, the reaction mixture was analyzed on 6% non-denaturating polyacrylamide gels. The gels were then dried and autoradiographed at −70°C.

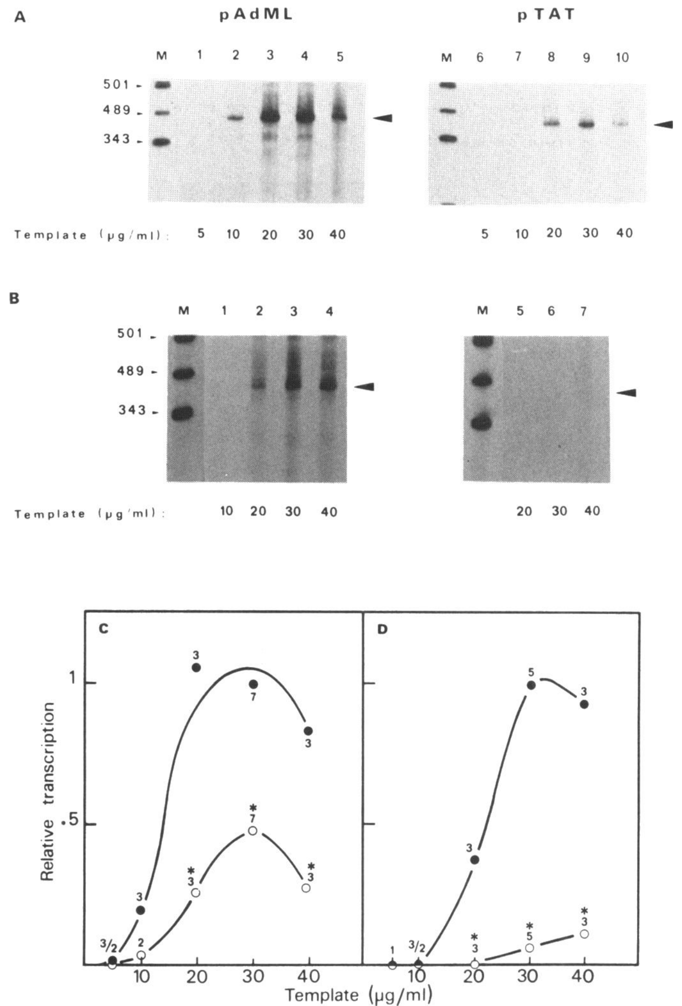

Figure 2. $TAT_{0.35}$ driven *in vitro* transcription is tissue-specific. Liver (**A**) or spleen (**B**) CNEs (2.4 mg/ml of nuclear proteins) were incubated with various concentrations of either $pAdML_{370}$ (A, lanes 1−5; B, lanes 1−4) or pTAT (A, lanes 6−10; B, lanes 5−7) template DNAs (range: 5−40 μg/ml). The concentration of template DNA added in the reaction mixtures is indicated below each lane. M: ^{32}P-labelled HpaII-digested pUC8. Arrowheads point to the position of correctly initiated transcripts. (**C**) and (**D**): compilation of the results obtained in independent dose-response relationship experiments with liver (C) and spleen (D) CNEs. The radioactivity of the transcripts synthesized from either $TAT_{0.35}$ or AdML 5′-flanking sequences was measured at each template concentration, then standardized relative to that of the transcripts synthesized from 30 μg/ml of pAdML in the same experiment, with the homologous nuclear extract. The mean values were calculated and plotted as a function of template concentration. The number of experiments is indicated at each template concentration. (*): The amount of transcripts synthesized from pTAT was significantly different from that generated from pAdML, according to the paired Student's 't' test ($p<0.05$). (●), pAdML; (○), pTAT.

RESULTS

The most proximal 350 bp of TAT gene 5′-flanking sequences ($TAT_{0.35}$) support tissue-specific transient expression of a reporter gene

When controlled by 10 kb of TAT gene 5′-flanking DNA (pT-C_{10}), basal CAT gene expression was significantly higher in differentiated (H4II) rat hepatoma cells than in mouse L−M fibroblasts, the TS_{index} being equal to 6.7 (Table I). Transient transfection of the same cell lines with progressive 5′ deletions of pTC_{10} led to very close basal and dexamethasone-induced CAT activities for 10 and 8.9 kb, for 3.9, 3.3 and 3.0 kb and for 1.3 and 0.35 kb of TAT 5′-flanking sequences with basal normalized CAT activity being progressively decreased (pTC_{10} > $pTC_{3.3}$ > $pTC_{0.35}$) (Table I). However, whatever the length of 5′-flanking sequences, the magnitude of CAT expression was always significantly higher ($p<0.05$) in hepatoma cells than in fibroblasts with $TS_{indexes}$ larger than 5 ($p<0.05$). The slight variations (max. ~35%) in TS_{index} observed between constructs were not significant ($p>0.5$). Moreover, the liver-specificity of basal promoter activity was not dependent on the presence or

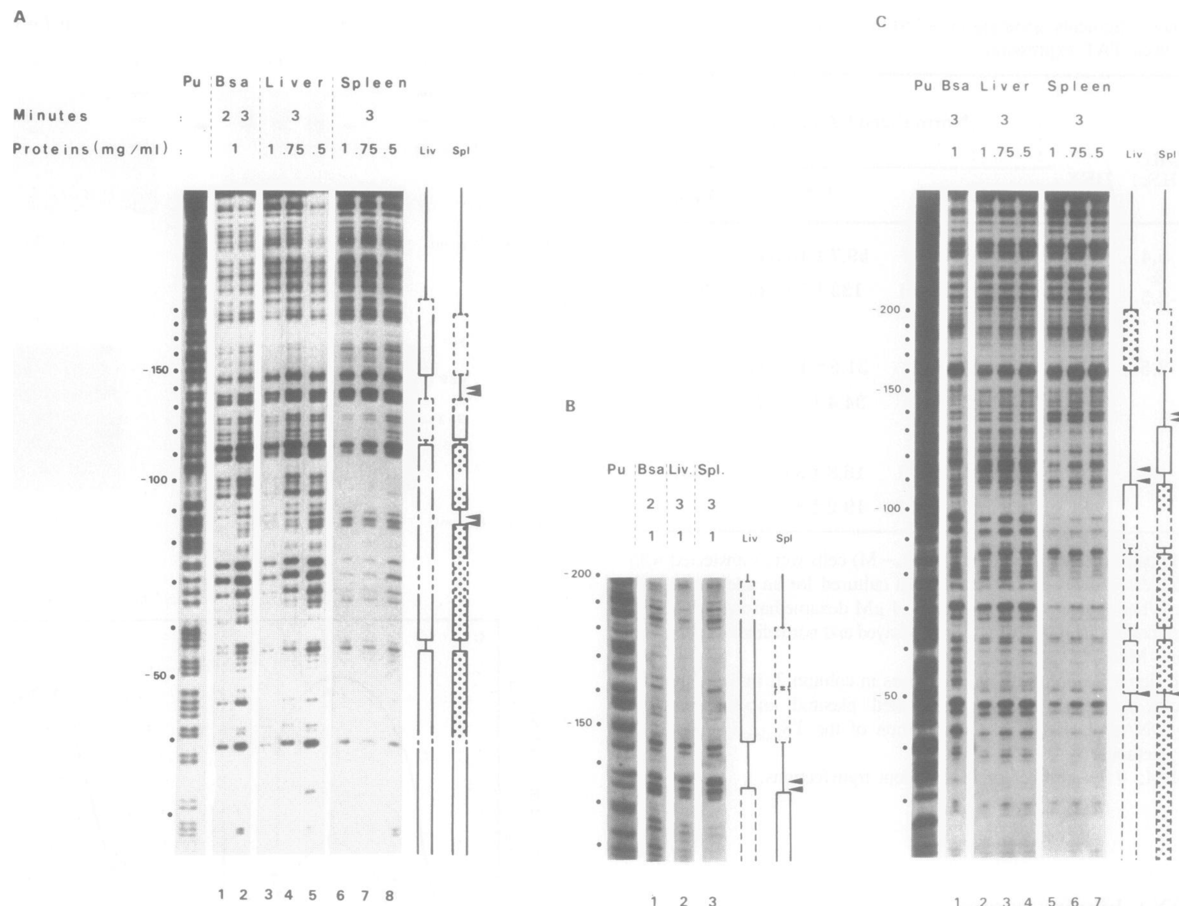

Figure 3. *In vitro* footprinting analysis of $TAT_{0.35}$. The EcoRI–HindIII restriction fragment excised from $pTAT_{inv}$ was ^{32}P end-labeled at the EcoRI site on the lower strand (T4 polynucleotide kinase) (**A**, **B**) or on the upper strand (filling-in with Klenow) (**C**), then DNAse I protection assays were performed as outlined in Materials and Methods. (B) Some of the samples analyzed in (A) were re-submitted to electrophoresis so that nucleotide sequences contained within nt −120 and −180 were expanded. The origin of the CNE, the duration of DNAse I digestion and the concentration of nuclear proteins added are indicated above each lane. Pu, G+A chemical cleavage ladder used as size marker. Boxes alongside the autoradiograph indicate the regions protected from DNAse I digestion. Dashed drawings indicate that the corresponding stretches of nucleotides are not completely protected in the presence of 1 mg/ml of nuclear proteins. Stippled areas indicate that the facing stretch of nucleotides is more efficiently protected with the corresponding crude nuclear extract. Arrowheads point to enhanced DNAse I cleavages.

absence of functional GREs (−2.5 HS). Such was not the case of glucocorticoid-induced CAT expression which was both highly liver-specific and dependent on the presence of −2.5HS (Table I).

$TAT_{0.35}$ drives efficient liver-specific *in vitro* transcription

Since $TAT_{0.35}$ was able to drive tissue-specific CAT gene expression in differentiated hepatoma cells, we checked whether these promoter elements were also able to drive liver-specific *in vitro* transcription. Transcription assays (2) were performed as a function of template concentration in the presence of a constant amount of liver or spleen CNEs (Fig. 2A and 2B) and the results of 2−7 independent experiments were compiled (Fig. 2C and 2D).

With rat liver CNEs, the amount of transcripts synthetized from the AdML and $TAT_{0.35}$ promoters were almost undetectable when plasmid concentration was 5 μg/ml (Fig. 2A, lanes 1, 6). Similar patterns of transcription efficiency were obtained from both promoters as a function of template concentration, with an optimum at ~30 μg/ml of template DNA (Fig. 2A). At optimal template concentration, the average amount of *bona fide* transcripts synthetized from pTAT represented ~48% of that synthetized from pAdML (Fig. 2C). The decrease observed at 40μg/ml was probably related to exhaustion of ubiquitous transcription factor(s) (Fig. 2C). Transcripts were never obtained when incubation was carried out in thepresence of 2 μg/ml α-amanitin or of $pTAT_{inv}$ (not shown).

With rat spleen CNEs, the bell-shaped pattern of transcription (similar to those obtained with either $pAdML_{370}$ or pTAT in the presence of liver CNEs) was only obtained from the AdML promoter (Fig. 2D). In contrast, $TAT_{0.35}$ driven transcription was very weak; very low amounts of transcripts accounting for 7.2% and 13.6% of those synthesized from pAdML at identical template concentrations were generated only at 30 and 40 μg/ml of template, respectively (Fig. 2B and D).

Under optimal *in vitro* transcription conditions with liver and spleen CNEs (30 μg/ml of template), the tissue specific index equalled 6.7. Although still significant it declined sharply (2.9-fold) at 40 μg/ml (at DNA concentrations < 30 μg/ml, the tissue specific index was not computable, due to the lack of $TAT_{0.35}$ driven transcription in the presence of spleen CNEs).

The results obtained both in transient transfection and in *in vitro* transcription experiments led us to propose that the 350 bp of TAT gene 5′-flanking sequences closest to the cap site were able to drive liver-specific gene expression.

Table II. Sequences of the oligonucleotides used in this study

Sequence	Oligonucleotide
-85 TCACTCTCAACCAATAGCACGAAGGCTTC AGTGAGAGTTGGTTATCGTGCTTCCGAAG -57	II
-109 AGGTCCGGGGGAGGGACTTAGTT AGGCCCCCTCCCTGAATCAAGAG -84	III
-135 CAGAGTCGGGGGTGGGGTATGG CGTCTCAGCCCCCACCCCATACCCCCA -110	IV
-163 GGTGGCCCAGGGTTGGGGTGAGAA CCACCGGGTCCCAACCCCACTCTT -140	V
-200 CCAGATACTTGATGTAAGGACAAATCCCAGATTGGAAG GGTCTATGAACTACATTCCTGTTTAGGGTCTAACCTTC -163	VI
-176 TCCCAGATTGGAAGGTGGCCC AGGGTCTAACCTTCCACCGGG -155	VII
-290 AGACCAATAAAGTTAATCACTGTCA TCTGGTTATTTCAATTAGTGACAGT -265	VIII
-85 TCACTCTCCTGGTGCAGCACG AGTGAGAGGACCACGTCGTGC -63	IIm
-176 TCCCAGATATCGTGGTGGCCC AGGGTCTATAGCACCACCGGG -155	VIIm
-290 TCTGGTGCAAAGTTAATCACTGTCAC AGACCACGTTTCAATTAGTGACAGTG -265	VIIIm
CCTCCATGACTCCAGAACTAA GGAGGTACTGAGGTCTTGATT	random
231 TGCTGGGGACTTTCCACACCCTAA ACGACCCCTGAAAGGTGTGGGATT 254	SV40 Core (1)
20 TATTTTGGATTGAAGCCAATATGATAATGA ATAAAACCTAACTTCGGTTATACTATTACT 49	Adenovirus (1) CTF/NF1
-93 GGGGTAGGAACCAATGAAATGAAAGGTTA CCCCATCCTTGGTTACTTTACTTTCCAAT -65	Albumin CCAAT (1) = NF-Y
-93 GGGGTAGGCTGGTGCGAAATGAAAGGTTA CCCCATCCGACCACGCTTTACTTTCCAAT -65	$NF\text{-}Y_m$
-140 TAGTCAAACAACTTTTTGGCAAAGATGGTATG ATCAGTTTGTTGAAAAACCGTTTCTACCATAC -109	$NF1_L$
-140 TAGTCAAACAACGAGCACCAGATCTTGGTATG ATCAGTTTGTTGCTCGTGGTCTAGAACCATAC -109	$NF1_{Lm}$
TCGATGGTTAATGATCTACAGT ACCAATTACTAGATGTCAAGCT	PE56
TCGATGGTGTATGAGGTACAGT ACCACATACTCCATGTCAAGCT	ΔS34
-128 CAGCCAGTGGACTTAGCCCCTGTTTG GTCGGTCACCTGAATCGGGGACAAAC -103	HNF2 (2)

Oligonucleotides are numbered relative to either the cap site (+1) for cellular promoter elements (II, III, IV, V, VI, VII et VIII, NFY, NF1, HNF1 and HNF2), or to the residue 1 of the viral genome (Adenovirus CTF/NF1). CCAAT, $NF1_{Liver}$ consensus sequences are underlined. Solid and open circles indicate strong and weak protein–DNA contact points deduced from methylation interference data (ds-oligos V, VI and VII: our work and G.S-G and A.G, unpublished observations)
(1) data from (60); (2) data from (43).

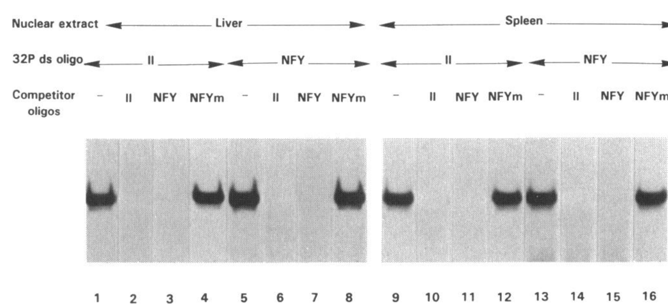

Figure 4. Ds-oligo II interacts with the ubiquitous nuclear factor NF-Y. End-labeled ds-oligo II (lanes 1–4 and 9–12) and NF-Y recognition sequence from the albumin promoter (lanes 5–8 and 13–16) were incubated with liver (lanes 1–8) or spleen (lanes 9–16) CNEs, in the absence of competitor (lanes 1, 5, 9, 13) or in the presence of a 90-fold molar excess of unlabelled ds-oligos II or of either wild type or mutated NF-Y (NF-Y and NF-Ym, respectively) as indicated above the lanes.

The $TAT_{0.35}$ promoter binds ubiquitous and tissue-specific *trans*-acting factors

In vitro footprinting experiments showed that a series of closely spaced $TAT_{0.35}$ sequences were protected from DNAse I digestion. In most cases the patterns of protection displayed in the presence of spleen or liver CNEs were superimposable, although at a given protein concentration, the magnitude of liver-mediated protection was often weaker (Fig. 3). In contrast, the protection of the sequence spanning nt −147 to −200 was more efficient in the presence of liver than of spleen CNEs (Fig. 3A, lanes 3–8; Fig. 3B, lanes 2, 3), and was more obvious on the lower than on the upper strand (compare protections in Fig. 3A and 3C).

Localisation of putative *cis*-acting elements for ubiquitous and liver-specific *trans*-acting factors within $TAT_{0.35}$

Ds-oligos encompassing the $TAT_{0.35}$ sequences that had been shown to be protected in *in vitro* DNase I footprinting (ds-oligos II–VIII in Table II) were designed. Gel shift assays showed that only the retardation patterns displayed by ds-oligos V, VI, VII and VIII were dependent on the origin of the CNE (not shown), suggesting that the corresponding sequences interacted with at least some tissue-specific factor(s). As a matter of fact, search of homologies pointed out that the $TAT_{0.35}$ sequences spanning nt −147 to −168 encompassed putative recognition sequences for liver-enriched $NF1_{Liver}$ (a liver-enriched member of the NF1gene family, nt −163 to −168, 100% similarity with the $TGG^{C}/_{A}A$ consensus) (38, 42), and HNF2/HNF4/LFA1 (nt −156 to −161, 100% similarity with the $TG^{G}/_{A}{}^{A}/_{C}CC$ consensus) (43). Moreover, this search localized putative recognition sequences for the liver-enriched HNF1/LFB1 (nt −266 to −281, 85% similarity with the $T^{G}/_{A}GTTAATN^{A}/_{T}T^{C}/_{T}NNCA$ consensus) (43, 44, reviewed in 45) and for the ubiquitous NF-Y (nt −70 to −77).

The ubiquitous NF-Y binds to and is a potent transcriptional activator of $TAT_{0.35}$

The single retarded complex generated by radiolabeled ds-oligo II was specific and identical with both liver or spleen CNEs, arguing for the binding of a ubiquitous *trans*-acting factor (Fig. 4; lanes 1,2,9,10). As expected from the presence of a 'AACC-AAT' homology within ds-oligo II, a *bona fide* NF-Y ds-oligo

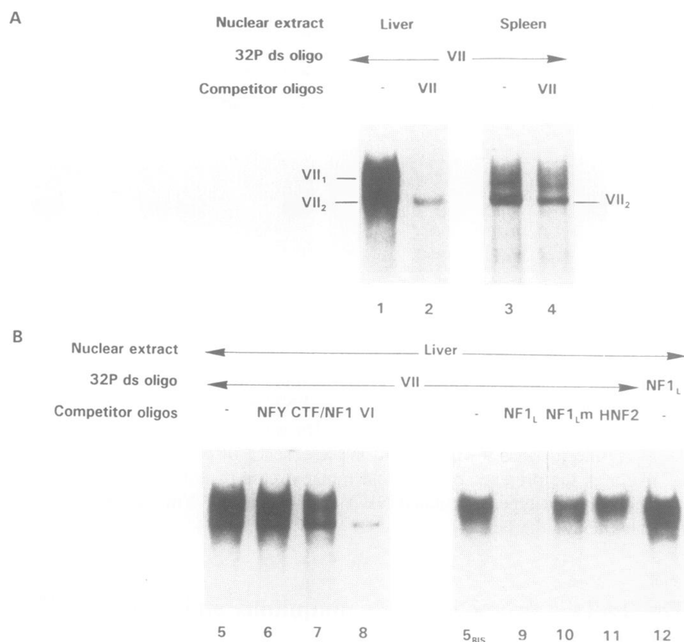

Figure 5. Ds-oligo VII interacts with at least one liver-specific nuclear factor, $NF1_{Liver}$. End-labeled ds-oligo VII (A: lanes 1–4; B: lanes 5–11), $NF1_{Liver}$ recognition sequence from albumin promoter (B, lane 12) were incubated with liver (A: lanes 1, 2; B) or spleen (A: lanes 3, 4) CNEs, in the absence of competitor (A: lanes 1,3; B: lanes 5, 5bis and 12) or in the presence of a 90-fold molar excess of unlabelled ds-oligos VII (A, lanes 2,4), VI, NF-Y, adenoviral CTF/NF1, $NF1_{Liver}$, mutated $NF1_{Liver}$ ($NF1_{Lm}$) or HNF2, as indicated above the lanes in (B).

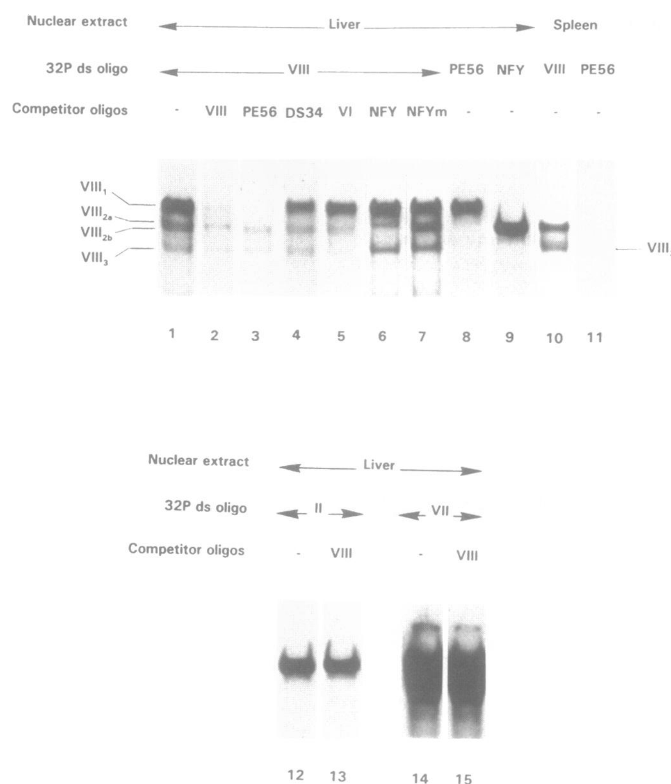

Figure 6. Ds-oligo VIII interacts with one liver-specific nuclear factor, HNF1 and ubiquitous 'CAAT-box' binding factor(s). End-labeled ds-oligo VIII (lanes 1–7, 10), PE56 (lane 8, 11), NF-Y (lanes 9, 12, 13) or ds-oligo VII (lanes 14,15) were incubated with liver (lanes 1–9, 12–15) or spleen (lane 10, 11) CNEs, in the absence (lanes 1, 8–12, 14) or in the presence of a 90-fold molar excess of ds-oligos VI, VIII, PE56, DS34 (the mutated counterpart of PE56), NF-Y or NFYm as indicated above the lanes.

('albumin CCAAT') but not its mutated counterpart (NF-Y_m in Table II) was also an efficient competitor (Fig. 4, lanes 3,4 and 11,12). Ds-oligos VI and CTF/NF1 which possess CAAT homologies were poor competitors, whereas unrelated $TAT_{0.35}$ ds-oligos (III, IV, V) did not compete at all (not shown). Conversely, labeled NF-Y generated the same retardation pattern than ds-oligo II in the presence of liver and spleen CNEs and this gel shift was competed by 90-fold excesses of unlabelled NF-Y and ds-oligo II, but not by NF-Y_m (Fig. 4, lanes 5–8 and 13–16).

In the presence of liver CNEs, the transcriptional activity of $TAT_{0.35}$ was reduced by about 80% by 25 fold excesses of ds-oligos II or NF-Y but not by IIm or NF-Ym (Fig. 7A). Similar results were obtained in the presence of spleen CNEs: transcription from $TAT_{0.35}$ was inhibited by 64% by a 25-fold excess of ds-oligo II, but remained unchanged in the presence of IIm (Fig.7A)

Altogether, these results demonstrate that NF-Y (a) is a *trans*-acting factor which binds to the $TAT_{0.35}$ sequences spanning nt −57 to −85 and (b) behave as a potent, ubiquitous transcriptional activator of basal $TAT_{0.35}$ promoter activity: it was almost equally potent in the presence of either liver or spleen CNEs.

The liver-enriched $NF1_{Liver}$ binds to and contributes to the transcriptional activity of $TAT_{0.35}$

The DNAse I footprint spanning nt −140 to −200 was initially arbitrarily split into two ds-oligos (V and VI), and preliminary gel shift and methylation interference experiments (GS-G and AG, unpublished; summarized in Table II) led us to localize the DNA–protein interactions between nt −156 and −176 (ds-oligo VII).

Ds-oligo VII gave rise to tissue-specific retardation patterns (Fig. 5A, lanes 1, 3). Two DNA–protein interactions were generated in the presence of liver CNEs. The broad, heavily labelled VII_1 band represented specific DNA–protein interactions since it was competed by a 90-fold molar excess of unlabelled ds-oligo VII (lane 2). On the contrary, a 90-fold excess of ds-oligo VII did not extinguish the faint, faster migrating gel shift VII_2 which therefore represents non-specific DNA–protein interaction (lane 2). VII_2 was the main retarded band which was observed in the presence of spleen CNEs, and as was the case for liver CNEs, it was due to non-specific DNA–protein interactions (lane 4). Depending on the spleen CNE, an additional faint slower migrating smear was inconsistently observed (lane 3).

Because binding sequences for the liver-enriched $NF1_{Liver}$ and HNF2 were contiguous within ds-oligo VII (cf. supra), we wondered whether one of these *trans*- acting factors was responsible for the liver-specific gel shift VII_1. VII_1 was not competed by unlabelled ds-oligos HNF2 at a 90-fold molar excess (Fig. 5B, lane 11) but was completely extinguished by a 90-fold excess of ds-oligo $NF1_{Liver}$ (site E of albumin promoter, 38) but not by its mutated counterpart (lanes 9, 10). Furthermore, incubation of ^{32}P-labelled ds-oligo $NF1_{Liver}$ with liver CNEs generated a retardation pattern identical to VII_1 (lane 12) and

this gel shift was extinguished by a 30-fold molar excess of unlabelled ds-oligo VII (not shown). As expected, VII_1 was not competed by unlabelled ds-oligos encompassing recognition sequences for the ubiquitous NF-Y and CTF/NF1 (lanes 6, 7).

In the presence of liver CNEs, $TAT_{0.35}$ promoter activity was reduced to the same extent (52 and 59%) by 100-fold excesses of ds-oligo VII and $NF1_{Liver}$ respectively, but remained unchanged in the presence of a 100-fold molar excess of mutated $NF1_{Liver}$. However a slight inhibition of transcription (~20%) was observed in the presence of mutated ds-oligo VII at a 100-fold molar excess (Fig.7B). By contrast, neither ds-oligo VII nor *bona fide* $NF1_{Liver}$ did abolish the slight transcriptional activity generated from $TAT_{0.35}$ in the presence of spleen CNEs (Fig.7B).

Altogether, these results support the conclusion that $NF1_{Liver}$ binds to the $TAT_{0.35}$ sequence spanning nt −156 to −176 and behave as a liver-specific transcriptional activator of promoter activity.

The liver-enriched HNF1 binds to and contributes to the transcriptional activity of $TAT_{0.35}$

Ds-oligo VIII generated a tissue-specific retardation pattern: the slower migrating gelshift ($VIII_1$) was only observed in the presence of liver CNEs (Fig. 6, lanes 1, 10), and encompassed a putative HNF1 recognition sequence. Accordingly, $VIII_1$ was extinguished by PE56 (a *bona fide* HNF1 binding site; 46) at a 3-fold molar excess, but not by its mutated form DS34 (46, Table II) or by ds-oligos which encompass a CCAAT homology (NF-Y, CTF/NF1 and ds-oligo VI from $TAT_{0.35}$) even at 90-fold molar excesses (Fig. 6, lanes 3−6 and data not shown). Moreover, PE56-HNF1 complexes had the same mobility than $VIII_1$ and were competed by unlabelled ds-oligo VIII (lane 8 and data not shown). An additional band ($VIII_3$) was competed by PE56 but not by DS34 (lanes 3, 4). This band was also observed when spleen CNEs were incubated with radiolabeled ds-oligo VIII and PE56 (lanes 10,11) and may represent the interaction of v-HNF1 homodimers with the HNF1 binding site.

In the presence of liver CNEs, $TAT_{0.35}$ promoter activity was reproducibly reduced by ~29% in the presence of a 100-fold molar excess of PE56) but remained unchanged in the presence either of DS34 or of a random ds-oligo (Table II; Fig. 7C). By contrast, a 100-fold molar excess of PE56 did not modify $TAT_{0.35}$ promoter activity in the presence of spleen CNEs (Fig. 7C).

Altogether, these results support the conclusion that the liver-enriched *trans-* acting factor HNF1 may play a role in the liver-specific control of $TAT_{0.35}$ promoter activity, but suggested that its effect was moderate. However, since a CCAAT homology lies immediately 5′ to the HNF1 homology (nt −290 to −282, Table II), a functional cooperation between HNF1 and ubiquitous 'CCAAT-box' binding factor(s) cannot be excluded.

This speculation was supported by the following observations. First, ds-oligo VIII was much more efficient than PE56 in inhibiting *in vitro* transcription (~30% and 73% vs. 0% and ~29% at 25- and 100-fold molar excesses, respectively) (Fig. 7C). Second, when ds-oligo VIII was mutated within the CC-AAT homology (VIIIm; Table II), its potency as an inhibitor of transcription (31%) was reduced to that of *bona fide* HNF1 (29%) when added at a 100-fold molar excess in the transcription mixture (Fig. 7C). Third, wild type ds-oligo VIII but not ds-oligo VIIIm (100-fold molar excesses) was able to inhibit transcription from $TAT_{0.35}$ *in vitro* (~40% inhibition) in the presence of spleen CNEs (Fig. 7C), consistant with the ubiquitous nature of the *trans*-acting factor interacting with the CAAT homology 5′ to the HNF1 binding site.

In this connection, we have shown (i) that gel shift $VIII_{2b}$ was completely extinguished by as few as a 3-fold excess of ds-oligo NF-Y, but was not competed by a 90-fold excess of NF-Ym and (ii) that $VIII_{2b}$ and *bona fide* NF-Y complexes displayed identical electrophoreticmobilities (Fig. 6, lanes 6, 7, 9). On the contrary, ds-oligo VIII was unable to extinguish the gel shift obtained with *bona fide* ds-oligo NF-Y or $NF1_{Liver}$ (Fig. 6, lanes 12−15). Therefore, it seems unlikely that the higher efficiency of ds-oligo VIII in inhibiting *in vitro* transcription was due to the prevention of NF-Y and/or $NF1_{Liver}$ interaction with their cognate binding sequences on $TAT_{0.35}$ (nt −57 to −85 and nt −156 to −176, respectively).

DISCUSSION

TAT promoter sequences do sustain part of liver-specific gene expression

Using transient transfection and *in vitro* transcription experiments, we have shown that sequences spanning nt +1 (cap site) to −350 of the rat TAT gene support liver-specific expression. The $TS_{indexes}$ obtained with $pTC_{0.35}$ and pTAT (6.2 and 6.7, respectively) were almost identical to that calculated for pTC_{10} (6.7) (Table I) but were slightly lower than the TS_{index} computed for endogenous gene expression in hepatoma cells versus hepatoma×fibroblast hybrids (7.5−25) (18).

The overall decrease in basal expression, observed in H4II cells when TAT gene 5′-flanking sequences were shortened from −10 to −0.35 kb (~3.5-fold) (Table I), was weaker than that obtained in FTO-2B hepatoma cells (110-fold) (26). Noticeably, the deletion of the −3.6 Kb enhancer produced a >50-fold decrease in activity in FTO-2B (26) but not in H4II cells (Table I and not shown). This may be due to hyperactivity of this enhancer in FTO-2B cells since forskolin treatment leads to weak cAMP induction of TATCAT[4289/+62] (1.5-fold) in these cells (22), whereas cAMP induces TAT mRNA synthesis by 20−30 fold in the liver and in primary cultures of hepatocytes (14).

The weakening of basal expression correlative to progressive deletion of TAT 5′-flanking sequences (Table I) points to a putative functional cooperation between promoter and enhancer elements in the maintenance of a high level of basal gene expression. Accordingly, when the −3.6 HS enhancer (target for *Tse-1* mediated and cAMP reversion of extinction), and the liver-specific −11HS were tested immediately 5′ to tk promoter sequences (ubiquitous) they were more efficient in 7AE27 (*Tse* 1^+) microcell hybrids than in HTC hepatoma cells, although endogenous TAT gene expression was lower in the former cell line (26). As stressed by these authors, this apparent discrepancy suggests the functional relevance of TAT promoter sequences in the liver-specific control of gene expression.

Our results both in transient transfection and in *in vitro* transcription further support the conclusion that $TAT_{0.35}$ controls at least part of the liver-specificity of basal TAT gene expression. Indeed, the lack of chromatin structures and of stable epigenetic modifications (e.g. DNA methylation) (47) in our *in vitro* transcription assays (use of supercoiled plasmid DNA and of dialysed, histone-free CNEs) strengthens the involvement of the promoter sequences *per se*.

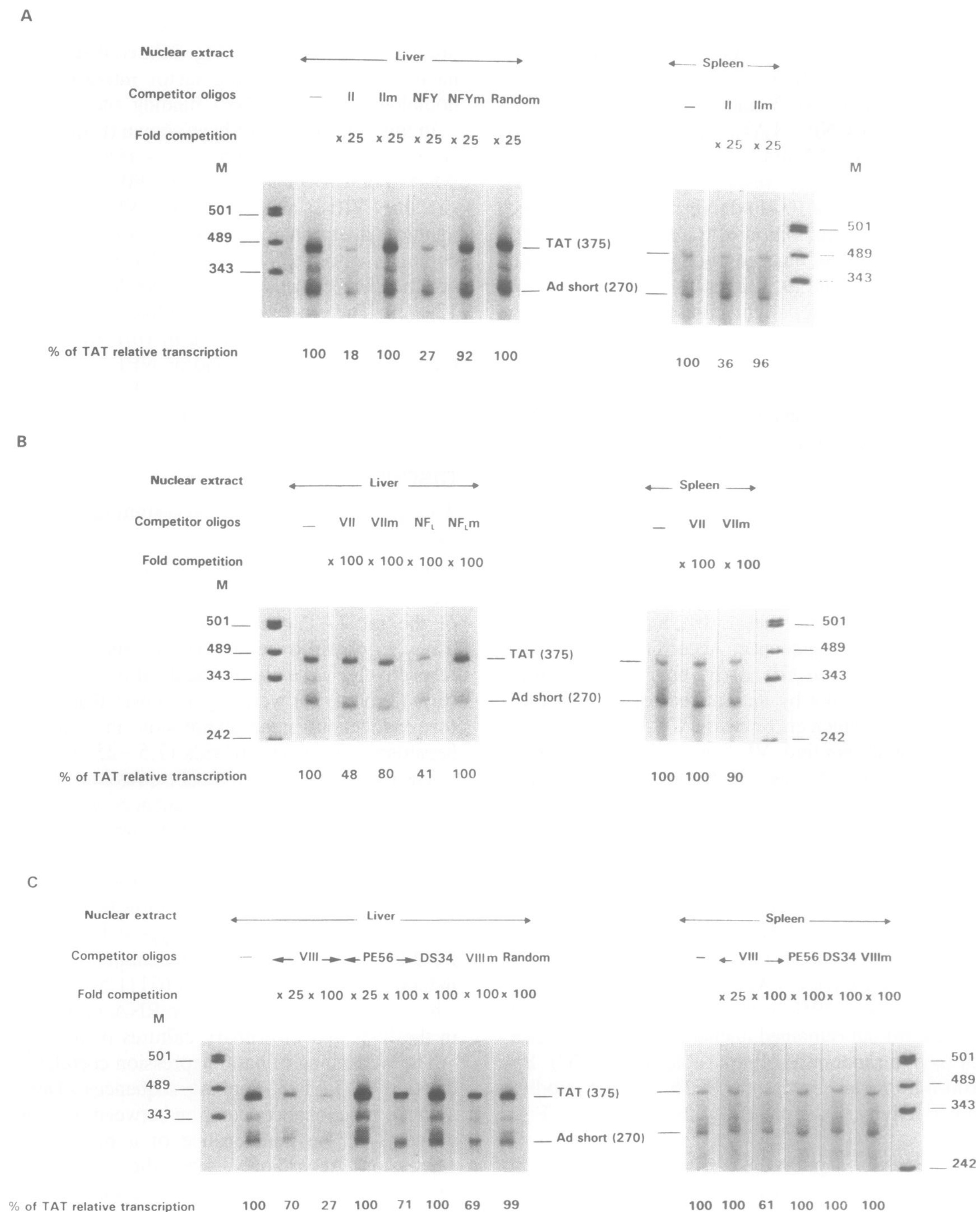

Figure 7. Factors binding to ds-oligos II, VII and VIII behave as transcriptional activators. Liver or spleen nuclear proteins (2.4 mg/ml) were preincubated at 0°C for 10 min in the absence or presence of a 25- or 100-fold molar excess of either wild type ds-oligos II, VII, VIII, NF-Y, $NF1_{Liver}$ ($NF1_L$), HNF1 (PE56), or of mutated IIm, VIIm, $NF1_Lm$, HNF1m (DS34), or of a random ds-oligo, as indicated above the lanes. pTAT and $pAdML_{270}$ (30 and 10 μg/ml final concentration, respectively) were then added to each reaction mixture and transcription was allowed to proceed at 30°C for 45 min. Titration studies with: A, ds-oligo II and related ds-oligos; B, ds-oligo VII and related ds-oligos; C, ds-oligo VIII and related ds-oligos. In each case, the $TAT_{0.35}$ transcriptional activity was computed relative to that of the ubiquitous $AdML_{270}$ internal control. In many instances, the transcriptional activity of the reference promoter was slightly decreased in the presence of a 100-fold excess of competitor, probably owing to non-specific transcriptional inhibition (even observed with a randomly designed ds-oligo). % of TAT relative transcription = % of the $TAT_{0.35}$ transcriptional activity remaining in the presence of competitor ds-oligo (mean of 2 to 6 independent experiments; average SEM for all experiments: 7.54% of mean values; range: 3–9%, with the single exception of one experiment where it was 20%). M: molecular weight marker.

Thus, the liver-specific regulation of TAT gene expression turns out to be regulatedthrough promoter as well as enhancer sequences, as it is the case for other liver-specific genes such as albumin (1,48), α-foetoprotein (49), α_1-antitrypsin and transthyretin (4, 50), and numerous constitutive and inducible tissue-specific genes (see (51) and (52) for reviews).

Tissue-specific transcription from $TAT_{0.35}$ is dependent on *cis*- recognition element(s) for liver-enriched $NF1_{Liver}$ and HNF1

Using CNEs from both TAT-expressing (FTO-2B) and non-expressing (XC) cells, Becker *et al.* (47) obtained identical *in vitro* footprints over the TAT promoter. However, as already mentioned by these authors, such an analysis does not rule out the possibility that the protected sequences could be contacted by different *trans*-acting factors. Accordingly, our less severe conditions allowed us to pinpoint one sequence (nt −147 to −200), the protection of which depended on the origin of the CNE. Computer-assisted search of sequence similarity showed that this sequence and a further upstream one (nt −266 to −281) were putative binding sites for the liver-enriched *trans*-acting factors $NF1_{Liver}$ and HNF1, respectively.

In fact, gel shifts allowed us to show that $NF1_{Liver}$ (38, 42) and HNF1 (44, 46) bound to the $TAT_{0.35}$ sequences spanning nt −161 to −147 and nt −281 to −266, respectively. No other region of the promoter was potentially able to interact with these two liver-enriched *trans*-acting factors (not shown).

The titration of $NF1_{Liver}$ and HNF1 in the *in vitro* transcription mixture (competition experiments) demonstrated that they supported ~60% and ~30%, respectively, of the basal transcription sustained by $TAT_{0.35}$ in the liver. Neither $NF1_{Liver}$ nor HNF1 recognition sequences did inhibit the slight transcription sustained by spleen CNEs, suggesting that these *trans*-acting factors act as positive transcriptional activators in TAT producing cells.

Nevertheless, the functional role of HNF1 *per se* seems moderate, consistent with previous observations that in C2 dedifferentiated hepatoma cells (a FaO derivative which does not express HNF1; 53), basal TAT gene expression was only slightly decreased and still inducible by glucocorticoids (54). However, competition experiments in *in vitro* transcription suggested that *in vivo*, HNF1 may cooperate functionally with ubiquitous 'CCAAT-box' binding factor(s) which bind to promoter sequences immediately 5'- to the HNF1 *cis*-element. Such functional interactions between *trans*-acting factors have already been reported at the level of composite hormone response elements and in some instances, non-receptor factors drive the distinct physiologic effects exhibited by the same receptor molecule which bind to the same HRE (55−58; reviewed in 59).

Are $NF1_{Liver}$ recognition sequences putative targets for *Tse-1*-mediated repression of TAT gene promoter elements?

Tse-1-induced alterations of promoter-HS in 7AE27 cells (26), suggest that *Tse-1* could exert indirect effects [e.g. extinction of expression or alteration of the post-transcriptional modification—of the phosphorylation status for example—of liver-enriched *trans*-acting factor(s)]. In this connection, our observation (AG, unpublished) that identical gel shift patterns were obtained with ds-oligo VI in the presence of CNEs prepared either from hepatoma×fibroblast hybrid cells, which do not express the TAT gene, or from spleen supports this assumption. Since ds-oligo VI interacted with $NF1_{Liver}$ we speculate that extinction of $NF1_{Liver}$ expression might explain *Tse-1*-induced alteration of promoter-HS in 7AE27 cells.

TAT promoter sequences interact with ubiquitous *trans*-acting factors, one of which is NF-Y

Among the ubiquitous *trans*-acting factors binding to TAT promoter sequences, we have identified NF-Y (60,61) which binds to the 'AACCAAT' sequence localized between nt −71 and −77. It behaves as a ubiquitous transcriptional activator, equally active in the presence of liver and spleen extracts (62), as in the mouse albumin promoter (39). NF-Y interacts also, although weakly, with sequences localized 5'- to the HNF1 recognition sequence.

The TAT promoter thus appears as a modular transcriptional control unit being composed of an array of recognition sequences for both tissue-specific and ubiquitous *trans*-acting factors clustered around the initiation site. It is able to confer a liver-specific pattern of transcription, and thus appears to be involved in the overall liver-specific control of TAT gene expression.

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