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Two different liver-specific factors stimulate *in vitro* transcription from the human α1-antitrypsin promoter

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The region from −137 to −2 of the human α1-antitrypsin (α1AT) promoter directs liver-specific *in vitro* transcription. Two *cis*-acting elements, A and B, have been identified within this segment by site-directed mutagenesis. Competition with synthetic oligonucleotides corresponding either to the A or to the B sequence inhibits transcription from the wild-type promoter *in vitro*. *Cis*-linked A and B elements mediate liver-specific transcription from a truncated HSV-TK promoter *in vitro*. Five different proteins, LF-A1, LF-A2, LF-B1, LF-B2 and LF-C, bind to the α1AT promoter in liver extracts. LF-A1 and LF-B1 are positive transcriptional factors which bind to the A and B elements respectively. Their absence in spleen provides an explanation for the liver specificity of transcription. A protein similar to LF-B2 is present in spleen. Binding of LF-B1 and LF-B2 to the α1AT promoter is mutually exclusive, suggesting that LF-B2 might be a repressor.

Key words: human α1-antitrypsin promoter/mutually exclusive binding/liver specific *in vitro* transcription/transcriptional activators

Introduction

In most cases differentiated cells specifically transcribe the appropriate genes when these are introduced into them by transfection (Banerji *et al.*, 1983; Neuberger, 1983; Queen and Baltimore, 1983; Walker *et al.*, 1983; Ott *et al.*, 1984; Ciliberto *et al.*, 1985). This implies that the information for selective expression is a constitutive property of the transcriptional apparatus of the cell. Therefore, many questions related to the molecular mechanisms responsible for tissue-specific gene expression can be addressed by identifying cell-specific *trans*-acting factors and studying how they are in turn differentially regulated.

Several proteins possibly required for tissue-specific gene transcription have been identified and characterized on the basis of DNA-binding assays (for review see McKnight and Tjian, 1986; Maniatis *et al.*, 1987). However, only in a few cases have their functional properties been established, exploiting the recent development of *in vitro* transcriptional systems which reproduce the selectivity of transcription observed *in vivo* (Gorski *et al.*, 1986; Bodner and Karin, 1987; Scheidereit *et al.*, 1987; Heberlein and Tjian, 1988).

We have been involved in the study of the transcriptional regulation of several hepatocyte-specific genes. By transfection into human hepatoma cell lines we have shown that for most of them the information for cell-type-specific expression resides in the 5′ flanking region (Ciliberto *et al.*, 1985; D'Onofrio *et al.*, 1985; Colantuoni *et al.*, 1987; Oliviero *et al.*, 1987). In the case of human α-1-antitrypsin gene (α1AT), we have found that there are two different promoters, one active in macrophages and the other in hepatocytes (Perlino *et al.*, 1987). The liver-specific promoter contains multiple *cis*-acting elements spread over several hundred base pairs in the 5′ flanking region. *In vivo* the minimal DNA segment capable of conferring cell-type specific expression consists of the 137 base pairs immediately upstream of the cap site. Within this sequence we have defined two regions which are essential for transcription and have identified two proteins from rat liver nuclear extract which specifically bind to them (De Simone *et al.*, 1987; Hardon *et al.*, 1988).

In order to acquire a deeper understanding of the functional role of these and other liver factors, we have analyzed transcription from the α1AT promoter *in vitro* using rat liver and spleen nuclear extracts. We show that transcription from the α1AT promoter is dependent on the presence of at least two positive *trans*-acting factors which are absent in spleen extracts.

Results

The region from −137 to −2 of the α1AT promoter is sufficient for liver-specific transcription in vitro

We have studied the specificity of transcription from the α1AT promoter *in vitro* using rat liver and spleen nuclear extracts, prepared according to the procedure described by Gorski *et al.* (1986). Nuclear extracts were also prepared from HeLa cells, where both the endogenous and the transfected α1AT promoter are inactive (Ciliberto *et al.*, 1985).

All the constructs used as *in vitro* transcription templates contained the G-less reporter cassette (Sawadogo and Roeder, 1985a). The largest segment of the α1AT promoter used in this study extends from −640 to −2 relative to the transcriptional start site. This segment was cloned in front of a 380-bp-long G-less cassette to yield the plasmid α1AT640[380]. The adenovirus-2 major late (AdML) promoter (from −404 to +10), which is known to be active in most cell types, was cloned in front of a shorter G-less cassette (AdML404[180]), and used as an internal control (Figure 1A).

AdML404[180] is transcribed with equal efficiency in rat liver, rat spleen and HeLa cell nuclear extracts (Figure 1B). In contrast, specific transcription from the α1AT promoter is at least 10 times higher in rat liver than in rat spleen and HeLa cell nuclear extracts (Figure 1B).

To identify the shortest DNA segment of the α1AT promoter still able to direct efficient *in vitro* transcription, we generated a set of 5′ deletions between positions −640 and −36. Each mutant was tested for its transcriptional

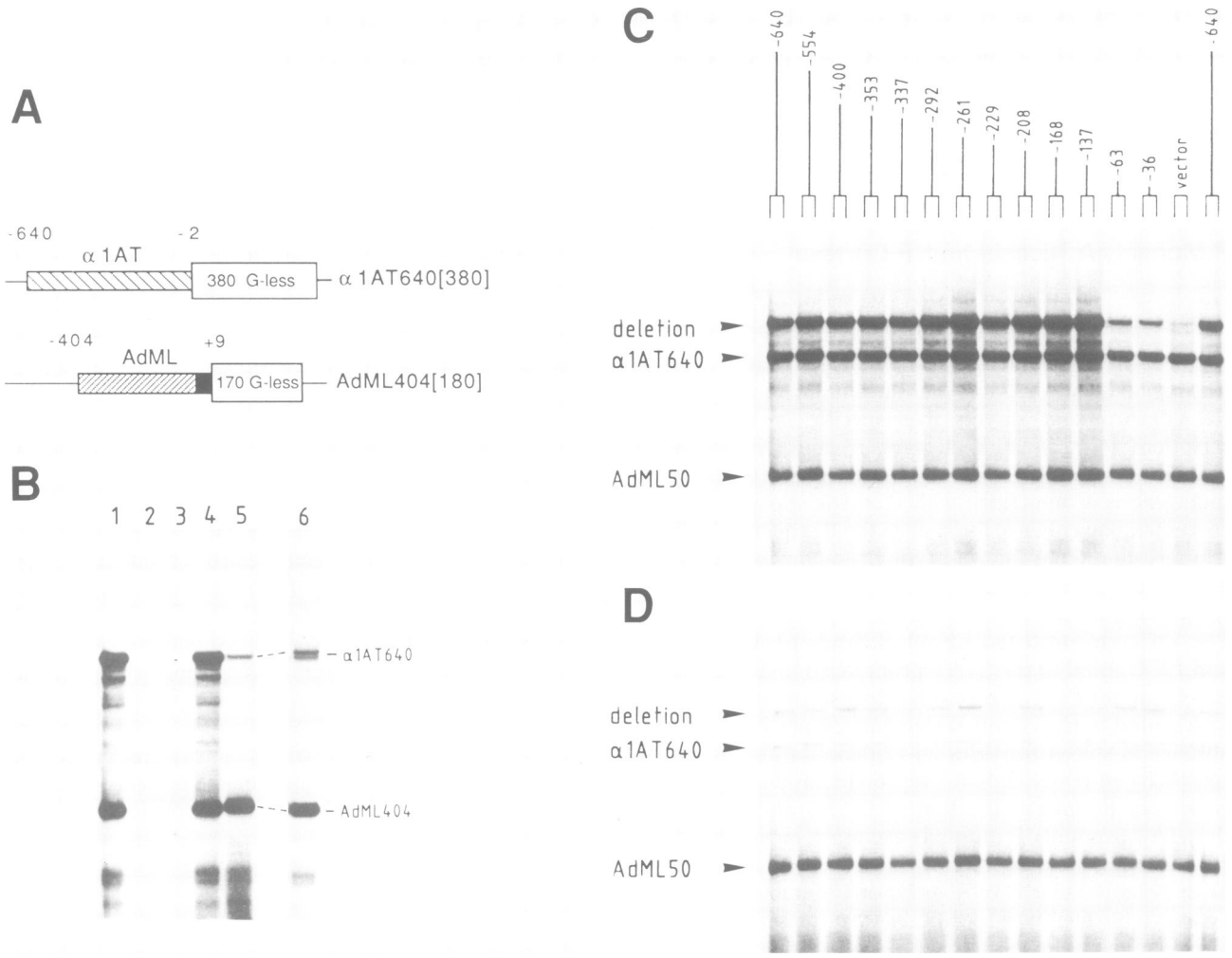

Fig. 1. The region from −137 to −2 of the α1AT promoter is sufficient for liver-specific *in vitro* transcription. (**A**) Diagram of the G-less cassette constructs containing the α1AT and the AdML promoters. The 5′ flanking region of each gene is shown as a hatched box. AdML sequences downstream of the transcription start site are represented by a black box. The G-less cassette is represented as a dotted box. (**B**) Tissue-specific *in vitro* transcription from the α1AT promoter. 375 ng of α1AT640[380] and 25 ng of AdML404[180] were transcribed *in vitro* with rat liver (**lanes 1–4**) and rat spleen (**lane 5**) nuclear extracts. The same concentration of templates was used with HeLa cell nuclear extract (**lane 6**; Materials and methods). 20 μg/ml (**lane 2**) and 2 μg/ml (**lane 3**) of α-amanitin were added to the transcription reaction. The products of transcription were resolved by 6% polyacrylamide–urea gel electrophoresis. (**C,D**) *In vitro* transcription from α1AT deletion mutants. Plasmids containing progressive 5′ deletions of the α1AT promoter were assayed *in vitro* for their transcriptional activity with rat liver (**C**) and rat spleen (**D**) nuclear extracts. In each transcription reaction equal amounts (130 ng) of α1AT640[250] and 30 ng of AdML50[180] were included as internal controls. *In vitro* transcription from the vector p(C_2AT)[380] is shown as a negative control.

activity in liver nuclear extract (Figure 1C). Plasmid containing the region from −640 to −2 of the α1AT promoter in a 250-bp G-less cassette vector (α1AT640[250]) and the −50 AdML promoter deletion in front a 170-bp G-less cassette (AdML50[180]) were used as internal controls.

Densitometric quantitation of the data from several experiments shows that deletion up to position −137 does not significantly alter the level of transcription in the liver extract. Further deletion to −63 results in a 5-fold reduction in the activity of the wild-type promoter. Even with only 36 base pairs, transcription from the α1AT promoter is above background levels. All promoter deletions directed essentially equivalent low levels of transcription in the spleen extract (Figure 1D) indicating that the −137/−63 region is involved in tissue-sepcific *in vitro* transcription from the α1AT promoter.

Three cis-acting elements are required for efficient in vitro transcription from the α1AT promoter

To investigate the internal organization of the minimal proximal element of the α1AT promoter we utilized several base pair substitution mutants whose transcriptional activity has been assayed *in vivo* by transfection into human hepatoma cell lines (De Simone *et al.*, 1987). All mutants (Figure 2B) were introduced in the plasmid α1AT261[380] and tested *in vitro* with rat liver extract (Figure 2A). A plasmid containing the wild-type α1AT −261/−2 promoter fragment fused to a 250-bp G-less cassette (AT261[250]) was used as an internal control.

Three domains are necessary for efficient *in vitro* transcription from the α1AT promoter: the A element from −119 to −104) defined by mutants EM3 and EM4, the B element (from −77 to −64) corresponding to mutants PM1

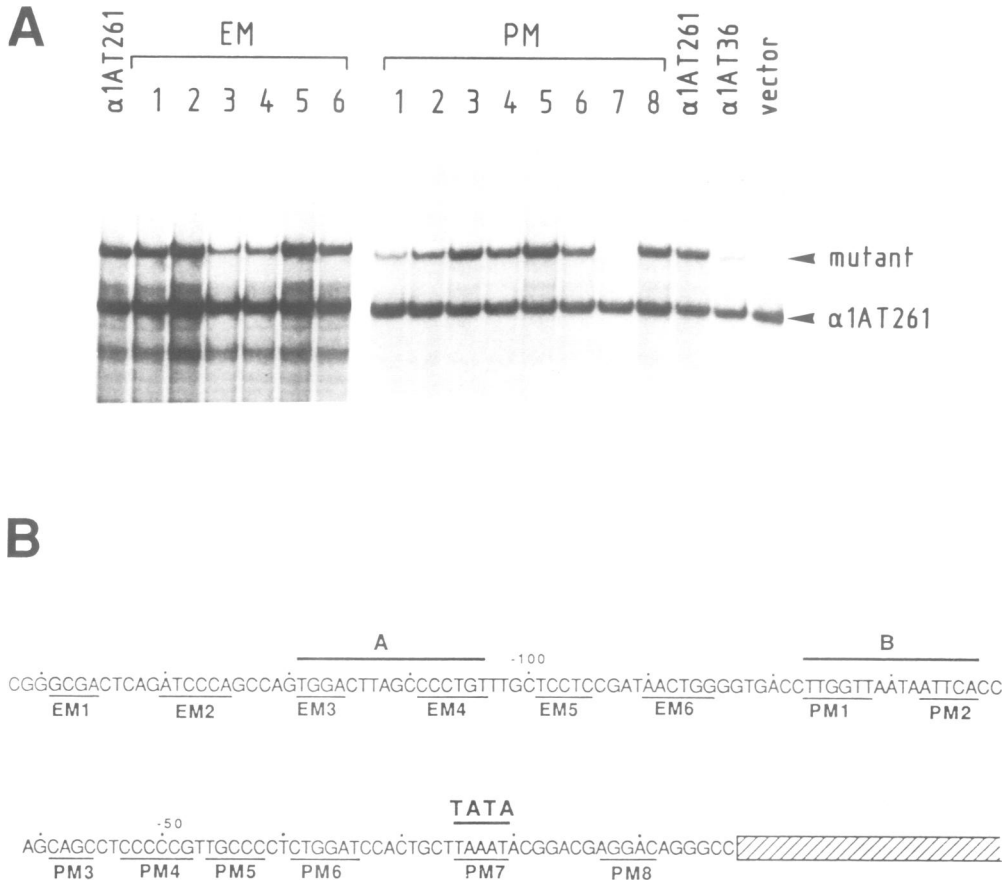

Fig. 2. Mutant analysis of the α1AT promoter by *in vitro* transcription. (**A**) *In vitro* transcription from mutant constructions. Equimolar amounts (400 ng) of the plasmid α1AT261[250] and of each mutant were tested for transcriptional activity with rat liver nuclear extract. α1AT261[380], α1AT36[380] and the vector p(C_2AT)[380] were used as positive and negative controls. (**B**) Sequence of the proximal region of the α1AT promoter. Numbers represent the distance in base pairs upstream from the transcription start site. Mutated sequences (see Materials and methods) are underlined. TATA box, A and B elements are indicated with thick lines.

and PM2 and the TATA box. The TATA box mutant is the only one that completely abolishes transcription from the α1AT promoter *in vitro*, confirming the absolute requirement for this sequence in the initiation of transcription.

The differential *in vitro* expression from the α1AT promoter in liver, spleen and HeLa nuclear extracts and the transcriptional activity of the mutants parallels, at least qualitatively, the results obtained *in vivo* (De Simone *et al.*, 1987).

Different proteins bind to the α1AT promoter proximal region in liver nuclear extract

The functional identification of the A and B domains suggests that these elements are recognized by positive *trans*-acting factors present in rat liver nuclear extract.

Eight distinct regions of protection from DNase I digestion have been detected on a −640/−2 fragment of the α1AT promoter, using transcriptionally active nuclear extracts (data not shown).

Since the first 137 bp upstream of the transcription start site are sufficient for tissue-specific *in vitro* transcription from the α1AT promoter, we focused on this region. Within this minimal element sequences from −134 to −66 and from −46 to −23 on the upper strand are protected from DNase I digestion (Figure 3A). On the lower strand corresponding footprints are detected from −138 to −65 and from −45 to −25 (Figure 8a).

At least four distinct proteins account for the observed protections. We will refer to these factors as LF-A1, LF-A2, LF-B and LF-C. This conclusion stems from several independent observations.

The binding to the B domain (from −85 to −66 on the upper strand) is resistant to heat treatment for 5 min at 65°C, whereas the other footprints disappear at this temperature (Figure 3B). This result allows LF-B to be defined as a protein distinct from those interacting with the other regions. Bindings over sites A and C have been further dissected through footprint competition experiments. Synthetic double-stranded oligonucleotides, A-oligo, B-oligo and C-oligo (Figure 3F), were used as specific competitors. An unrelated double-stranded oligonucleotide, L-oligo (see Materials and methods), was included as a control.

Competition with the C-oligo only removes the footprint in the C domain (from −46 to −23 on the upper strand, Figure 3C), defining LF-C. Competition with the A-oligo eliminates not only the A1 footprint (from −134 to −98 on the upper strand) but also the C footprint (Figure 3D). Similarly an excess of B-oligo concomitantly removes the footprints over the B and C regions (Figure 3E). These results suggest that efficient binding of LF-C might depend

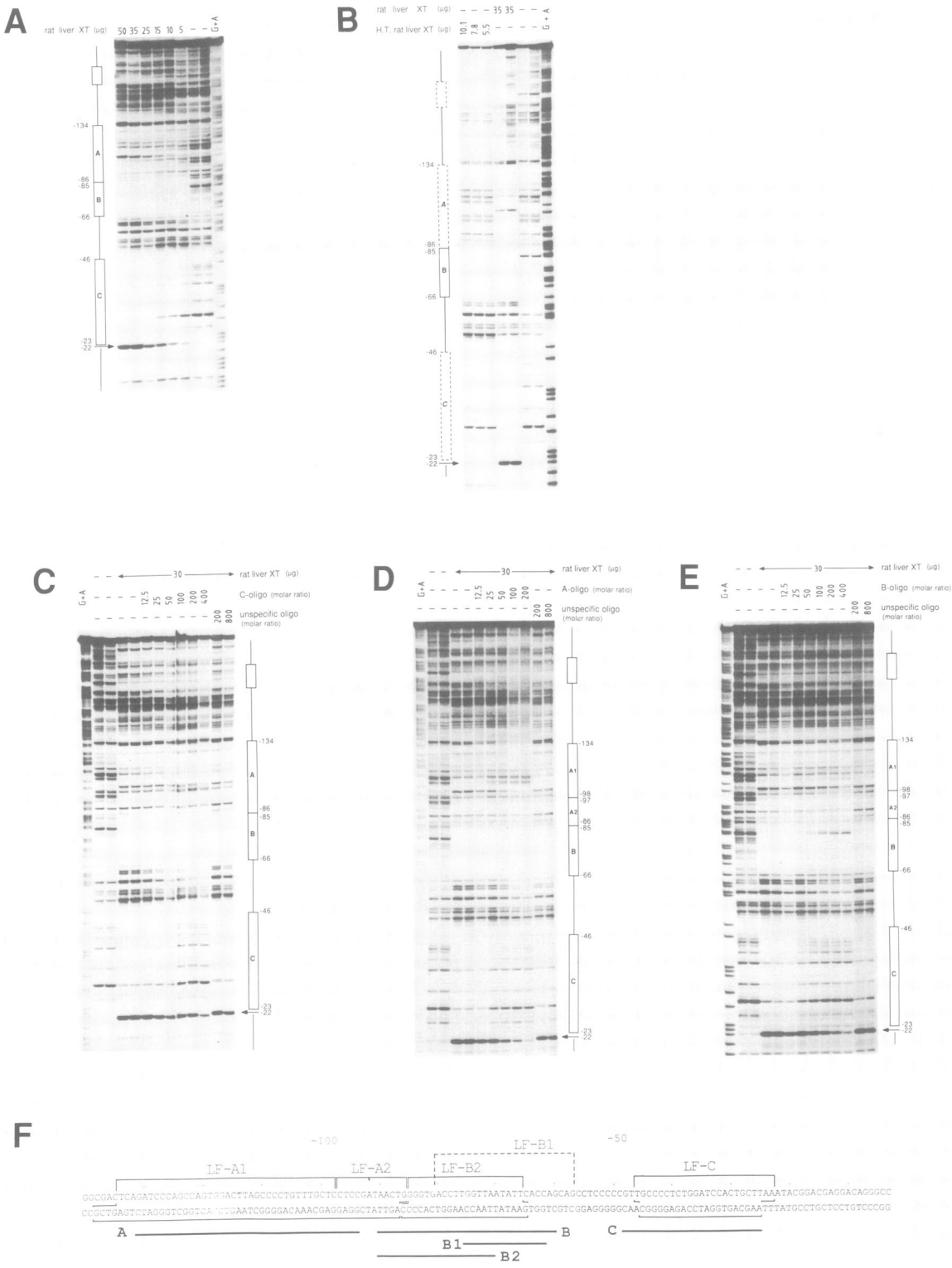

Fig. 3. DNase I footprinting analysis with rat liver nuclear extract. Binding reactions were carried out as described in Materials and methods on the −261/−2 fragment of the α1AT promoter (upper strand). G + A chemical cleavage was used as sequence marker. Control reactions on naked DNA are shown. Boxes alongside the autoradiograph indicate the regions protected from DNase I digestion; the arrow shows a DNase I hypersensitive site. Numbers refer to the positions relative to the start site of transcription. The amount of proteins used is reported on top of each lane. Digestion products of the footprinting reactions were electrophoresed on a denaturing 8% polyacrylamide−urea gel. (**A**) DNase I footprint with crude rat liver extract. (**B**) Heat-resistant binding activity detected on the α1AT promoter. Crude rat liver extract was heated for 5 min at 65°C and cooled on ice. Denatured proteins were removed by centrifugaton (5 min in an Eppendorf centrifuge) and the supernatant was used in a DNase I footprint assay. Dashed boxes indicate protections due to heat-sensitive factors. (**C, D, E**) DNase I footprint competition experiments. About 1 ng of end-labelled probe was mixed with increasing amounts of cold competitor C-oligo in (**C**), A-oligo in (**D**) and B-oligo in (**E**), and incubated with liver nuclear proteins. Excess of an unrelated oligonucleotide L (see Materials and methods) was used as a negative control. Control reactions in absence of competitor oligo are shown. (**F**) Summary of DNA−protein interactions on the α1AT promoter. The regions of DNase I protections are boxed. The sequences of the oligonucleotides used in binding and competition experiments are indicated by thick lines.

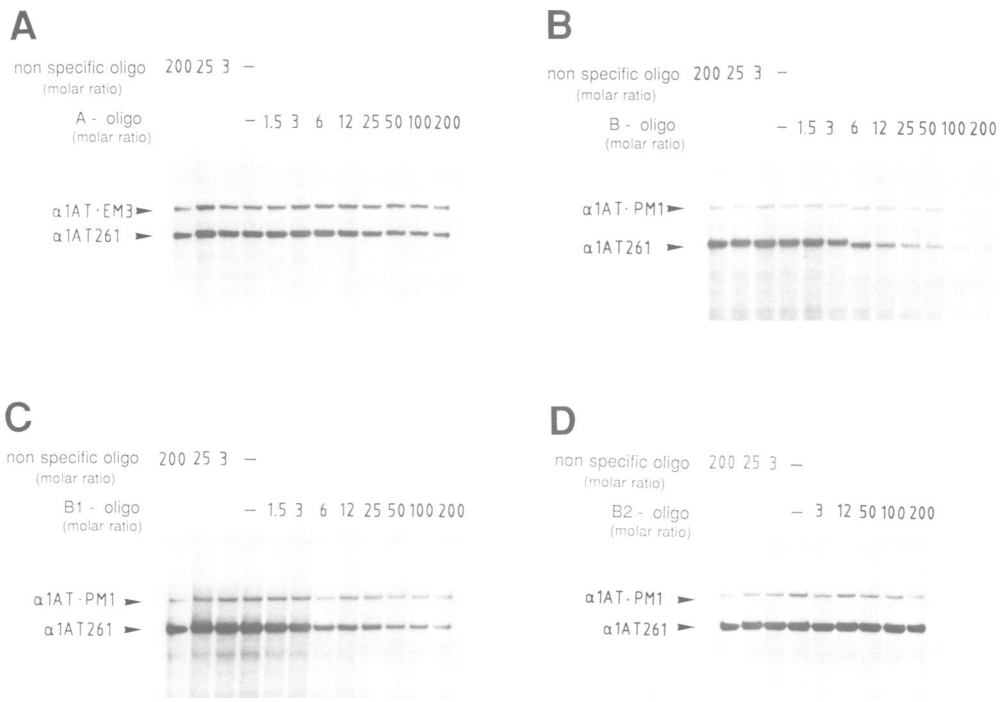

Fig. 4. Competition with synthetic oligonucleotides in *in vitro* transcription. (**A, B, C, D**) Equal amounts (100 ng) of mutant and wild-type promoters were used in *in vitro* transcription reactions with rat liver nuclear extract. Carrier DNA (200 ng of sonicated salmon sperm DNA) was kept constant in all reactions. Increasing amounts of specific oligo were added as shown at the top of the lanes. L-oligo was used as negative control.

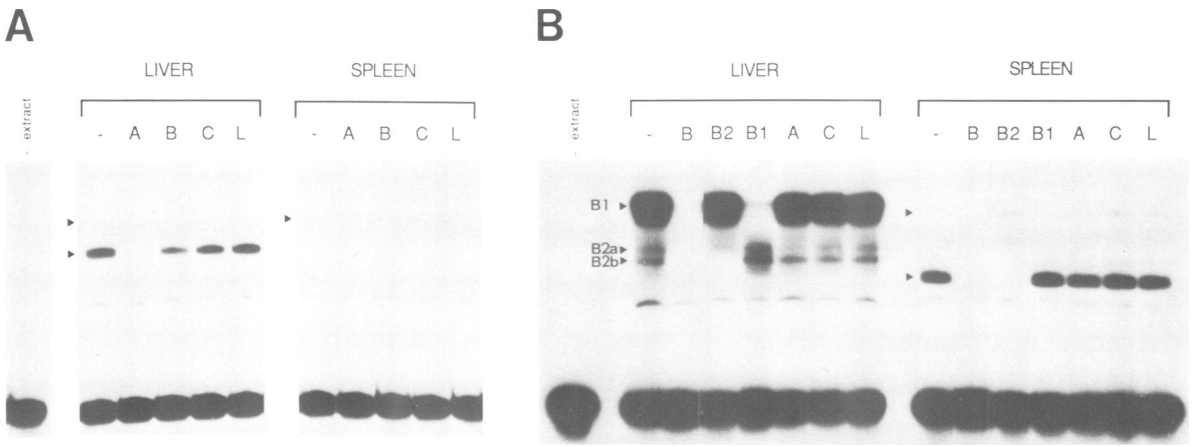

Fig. 5. Gel retardation analysis. (**A, B**) End-labelled, double-stranded oligonucleotides, A-oligo in (**A**) and B-oligo in (**B**), were incubated with crude rat liver or spleen nuclear extracts. The complexes were separated by electrophoresis, as described in Materials and methods. For competition experiments the labelled probe was incubated with a 200-fold molar excess of competitor oligo. Oligonucleotides are abbreviated as follows: A, A-oligo; B, B-oligo; B1, B1-oligo; B2, B2-oligo; C, C-oligo and L, L-oligo. Specific protein–DNA complexes are indicated by arrows.

on the interaction with LF-A1 and/or LF-B. An alternative explanation is that the sequences contained in the A and B regions have a cryptic C-binding site.

In the presence of high concentrations of A- or B-oligo, protection from DNase I digestion still occurs in the A2 domain (from −97 to −86 on the upper strand). Since both oligos only partially cover this region, we believe that the remaining footprint is due to the binding of a different factor (LF-A2).

We note that the LF-A1 footprint is slightly affected by high concentrations of the B-oligo (see below).

Two positive transcriptional factors interact with the A and B domains

The presence of a C region binding protein was not predictable on the basis of the mutant analysis. Mutant PM6, which does not bind LF-C (data not shown), is still transcriptionally active. Moreover, transcription from the wild-type promoter is unaffected by an excess of C-oligo (data not shown), which is sufficient to prevent LF-C binding. This indicates that LF-C, at least in the context of the −261 deletion, does not play an essential role in the transcription from the α1AT promoter *in vitro*. No relevant

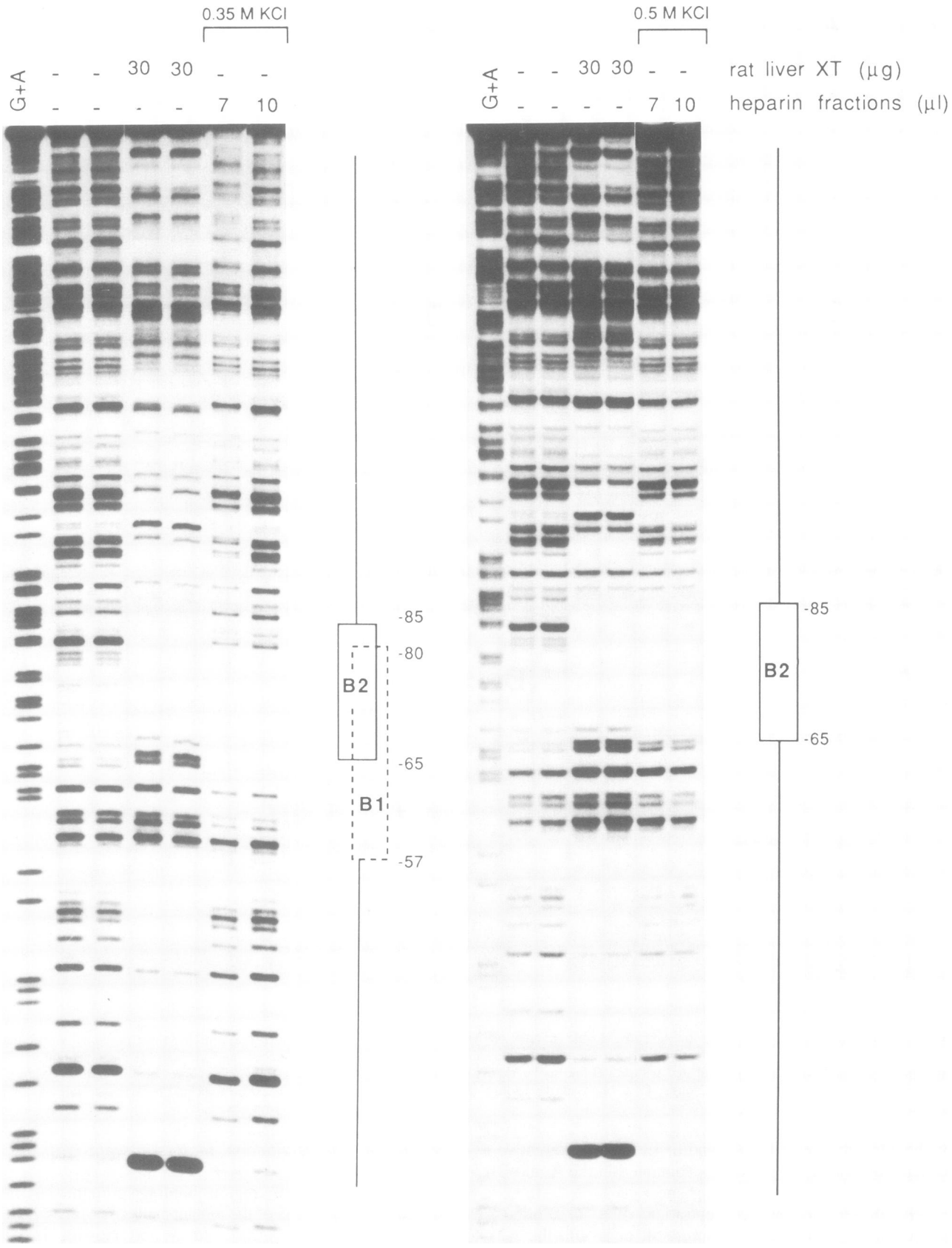

Fig. 6. Footprint analysis of fractionated liver extract. A −261/−2 fragment of the α1AT promoter was incubated with fractions from a heparin–sepharose column. The molar KCl concentration at which the nuclear proteins were eluted is indicated at the top of each lane. DNase I cleavage on naked DNA and footprints with crude liver nuclear extract are shown in parallel. Boxes indicate the protected regions; numbers refer to their boundaries.

phenotype is displayed by mutants within the A2 region (EM5 and EM6). Lacking evidence about the functional role of both LF-A2 and LF-C, we have excluded them from further analysis.

The A1 region corresponds well to the A element which was shown to be essential for *in vitro* (Figure 2B) and *in vivo* (De Simone *et al.*, 1987) transcription. Additional evidence for the functional role of LF-A1 is provided by competition experiments in *in vitro* transcription. Increasing amounts of A-oligo were added to a reaction mixture containing equimolar amounts of the templates of α1AT261[250] and α1AT-EM3[380]. Specific depletion of LF-A1 results in a reduction of the wild-type promoter activity (Figure 4A). Densitometric quantitation from several experiments shows that competition is already effective at a 12-fold molar excess of competitor over template. A 100-fold excess of A-oligo reduces the level of transcription from the wild-type promoter to that of the mutant EM3. We conclude that LF-A1 is a positive transcriptional factor.

Similarly, competition with the B-oligo reduces wild-type promoter activity almost to the level of mutant PM1 (Figure 4B). While indicating that removal of factors binding to the B domain inhibits transcription from the α1AT promoter, these results do not allow us to conclude that the LF-B binding activity identified by DNase I footprinting with crude liver extract is due to the same positive transcriptional factor defined by mutants PM1 and PM2. The LF-B footprint, in fact, does not cover, on either strand, the sequence mutated in the transcriptionally less active PM2 mutant (compare Figures 2A and 3F).

Several proteins bind to the B region

The discrepancy between our genetic and biochemical data prompted us to undertake a more detailed analysis of the interaction of the B region with nuclear factors present in rat liver. We therefore performed gel retardation experiments using the B-oligo as probe. Three different retarded bands are observed with crude liver extract: B1, B2a and B2b (Figure 5B). All three complexes are specific since they are efficiently competed by an excess of cold B-oligo, while no change in the retardation pattern is observed if an equal amount of C- or L-oligo is added as a competitor (Figure 5A). Competition with A-oligo prevents the formation of complex B2a. Complexes B1 and B2b are not formed when the probe used contains the PM1 mutation (PM1-oligo; data not shown).

To characterize further the factors binding to the B region we fractionated the rat liver nuclear extract on a heparin–sepharose column (see Materials and methods) and assayed all collected fractions by DNase I footprinting. Two distinct activities could be detected, yielding slightly different, although overlapping, footprints (Figure 6). The first activity eluting at ~350 mM KCl, whose footprint extends from −80 to −57 on the upper strand, will be referred to as LF-B1. At ~500 mM KCl a second activity is eluted, LF-B2, which gives a DNase I protection identical to that observed with the unfractionated extract. The two fractions were tested by gel shift with the B-oligo as probe showing that LF-B1 is responsible for the formation of the B1-complex, while the fraction containing LF-B2 is able to give both complexes B2a and B2b (data not shown).

LF-B1 is a transcriptional activator whose binding to the B region is mutually exclusive with that of LF-B2

On the basis of the previous observations we designed two double-stranded oligonucleotides, B1 and B2 (Figure 3F), in order to discriminate between the binding of LF-B1 and LF-B2. The two oligos were used for bandshift competition experiments with crude liver extract and the B-oligo as probe.

The B1-oligo is able to compete for complex B1, but not for complexes B2a and B2b (Figure 5A), while the B2-oligo behaves in the opposite way. These results confirm that LF-B1 has a different sequence specificity from LF-B2. Interestingly the intensity of bands B2a and B2b is considerably increased in the presence of an excess of B1-oligo, indicating that when the formation of the B1 complex is prevented, more complex due to LF-B2 is formed. This in turn suggests that the binding of LF-B1 and LF-B2 to overlapping sites is mutually exclusive. Footprint competition experiments with the B1- and B2-oligos further support this conclusion (Figure 7). The B2-oligo is able to relieve the protection observed in the B region with crude liver extract, but at the same time the appearance of an extended footprint, which corresponds to that obtained with fractionated LF-B1, is induced. A straightforward interpretation of these data is that binding of LF-B2 to the B2-oligo makes more labelled substrate available for LF-B1, consistent with the idea that they bind to the α1AT promoter in a mutually exclusive manner.

The B-oligo is able to bind both factors and competes for transcription of the α1AT promoter. To determine if this effect is due to the interference with the binding of only one or both factors in the α1AT promoter, we carried out competition experiments in *in vitro* transcription with B1- and B2-oligos. Competition with the B1-oligo is as efficient as the B-oligo in decreasing the activity of the wild-type promoter down to the level of the mutant PM1 (Figure 4C), while the B2-oligo has no effect (Figure 4D).

Despite its dominant binding in crude liver extract, LF-B2 is apparently not essential for *in vitro* transcription while the LF-B1 binding site exactly correlates with the positive transcriptional activity defined by mutants PM1 and PM2.

Identification of factors which bind to the α1AT promoter in rat spleen nuclear extract

Both functional and binding data are consistent with LF-A1 and LF-B1 being positive *trans*-acting factors. To investigate whether some or all of the rat liver nuclear proteins identified are exclusively present in liver, we carried out DNase I footprinting and gel retardation assays with rat spleen nuclear extract.

The footprint pattern displayed by the spleen extract is different from that obtained with the liver extract (Figure 8). The A and C domains are not protected from DNase I digestion, while a footprint is detectable in the B region. The spleen factor responsible for this footprint must be very similar to LF-B2 as the boundaries of the protected region are identical, and in both cases the activity is thermoresistant (data not shown). Titration experiments show that the B-binding spleen factor is more abundant than LF-B2 in liver (data not shown).

On the basis of the footprinting analysis we conclude that

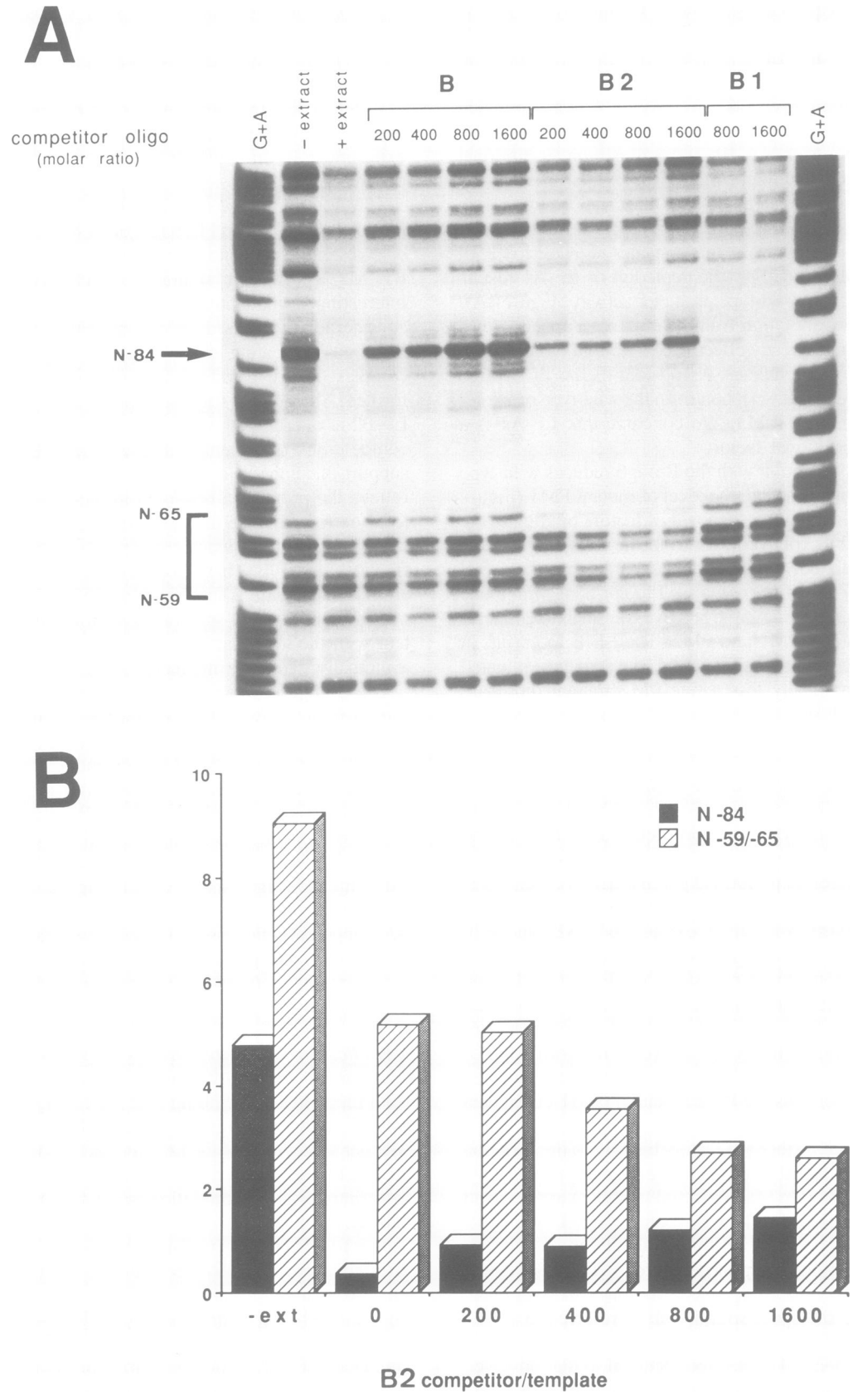

Fig. 7. Footprint competition with B1 and B2 oligos reveals mutually exclusive binding to the B region. (**A**) Footprint competition on the −261/−2 α1AT promoter fragment (lower strand). The end-labelled probe was incubated with 30 μg of total liver nuclear proteins in the presence of increasing amounts of B, B1 or B2 oligonucleotides. In the control lanes DNase I digestions in the absence of competitor, with and without extract, are shown. (**B**) Densitometric quantitation. The intensities of bands at position −84 (B2-band) and from position −65 to −59 (B1-bands) were normalized to an internal reference (band at position −57) and plotted versus different B2-oligo concentrations. Arbitrary units are indicated on the *Y* axis.

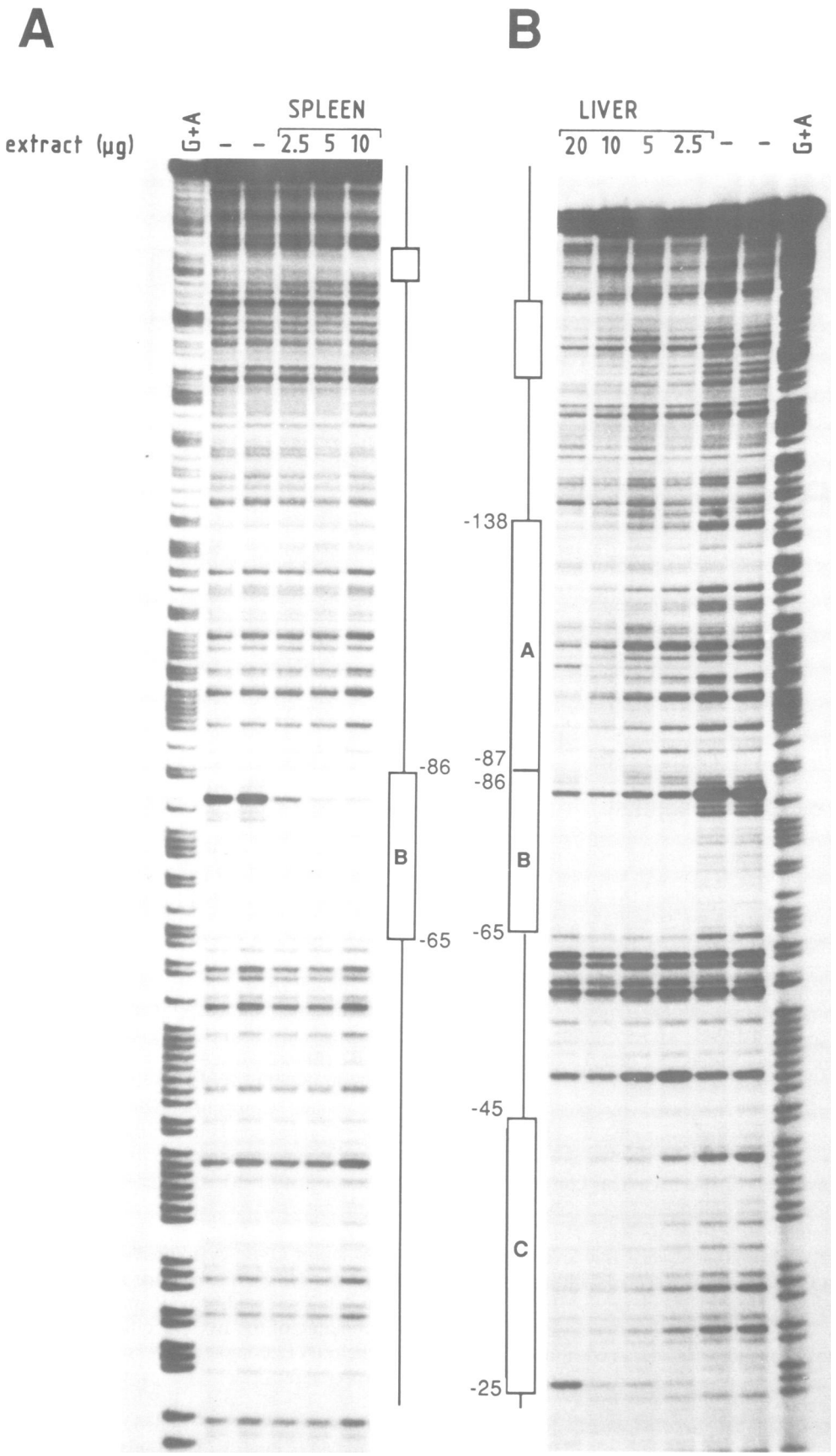

Fig. 8. DNase I protection of the α1AT promoter by factors contained in spleen and liver nuclear extracts. (**A, B**) The end-labelled −261/−2 fragment of the α1AT promoter (lower strand) was used for footprint analysis with increasing amounts of rat spleen (**A**) or rat liver (**B**) nuclear proteins. Boxes indicate the regions protected from DNase I digestion; number specify the limits of the footprints. Control reactions on naked DNA are shown.

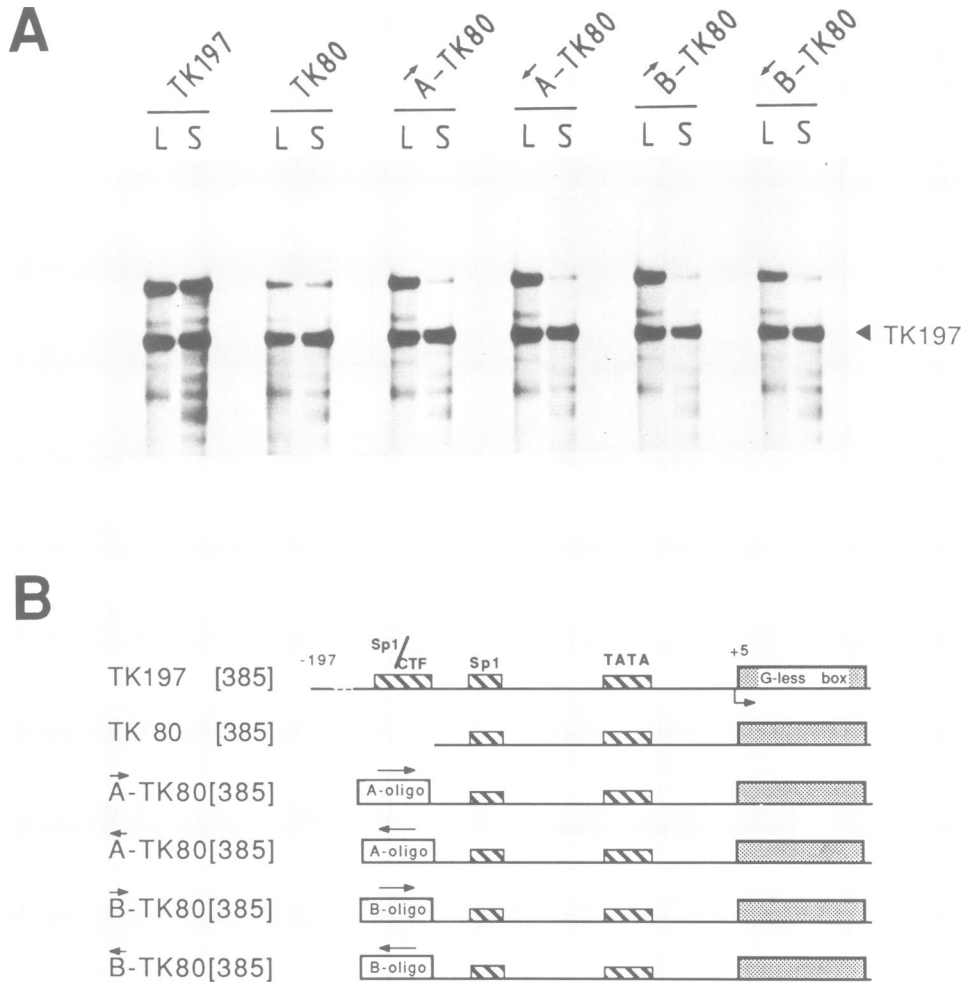

Fig. 9. A and B elements mediate liver-specific *in vitro* transcription from the HSV-TK promoter. (**A**) Equal amounts (130 ng) of TK197[250] and of each construct indicated at the head of each lane were transcribed *in vitro* with 30 μg of liver (L) or spleen (S) nuclear extract as described in Materials and methods. (**B**) Diagram of the α1AT-TK chimeric constructs. The region of HSV-TK promoter is represented by a line. Hatched boxes refer to the DNA segments that interact with Sp1, CTF and TATA binding factor(s). A- and B-oligos are represented as empty boxes; the arrows specify their orientation relative to the direction of transcription. The G-less cassette is shown as a dotted box.

LF-A1 and LF-A2 are absent in spleen. Nothing can be said about LF-B1, whose footprint could be masked by the LF-B2-like activity, or about LF-C, whose binding, at least in liver, is dependent on the presence of both LF-A1 and LF-B (1 or 2).

Further information is provided by gel retardation experiments. Using the A-oligo as probe we observe that the major complex formed with rat liver nuclear extract is absent in spleen extracts (Figure 5A). This complex is due to LF-A1 because it is not observed if the probe used carries the EM-3 mutation (data not shown). The C- and L-oligos do not affect this complex while low levels of competition for LF-A1 binding were detected with the B-oligo. Different observations indicate a low cross-affinity of the A- and B-oligos for LF-B1 and LF-A1 respectively. We believe that related sequences are contained in both oligos, as suggested by the ability of the PM1-oligo to form the B2a complex (data not shown). In addition to LF-A1, we observe a lower mobility complex (Figure 5A) which appears to be present both in spleen and in liver, and which was not further analysed in this study.

The combined results of the footprint and the gel retardation experiments conclusively demonstrate that LF-A1 is a liver-specific transcriptional factor.

The analysis by gel retardation with the B-oligo shows that a major complex, specifically competed by the B2-oligo, is formed in spleen. Its migration is different from that of LF-B2 (Figure 5B). A much weaker band, whose electrophoretic mobility is slightly different from that of LF-B1, is detectable. Competition with the B1-oligo eliminates this band. Comparison between the relative amounts of the B1 complex in liver and the corresponding activity in spleen indicates that LF-B1 is a liver-specific transcriptional factor.

LF-A1 and LF-B1 specifically enhance transcription from the HSV-TK promoter in liver extract

The data presented provide multiple lines of evidence that the binding of the liver-specific factors LF-A1 and LF-B1 to the α1AT promoter are essential steps in tissue-specific *in vitro* transcription. To test this hypothesis and to verify whether LF-A1 and LF-B1 might function as positive transcriptional factors independently of each other, we attempted to modify the activity of a ubiquitously expressed promoter to make it liver specific.

For this purpose a −197/+5 fragment of the HSV-TK gene promoter was cloned into the G-less cassette vector (TK197[385]) and tested for transcriptional activity. This

construct is efficiently expressed at the same level both in liver and spleen nuclear extracts (Figure 9A).

By using a convenient *Eco*RI restriction site at position −80 we generated a further deletion of this promoter (TK80[385]). Since this deletion eliminates the distal Sp1 and CAAT box elements required for full expression (McKnight and Kingsbury, 1982), the resulting construct is only poorly transcribed in both extracts (Figure 9A). The A- and B-oligos were cloned in both orientations upstream of the truncated TK promoter to yield the plasmids $\vec{A}$-TK80[385], $\overleftarrow{A}$-TK80[385], $\vec{B}$-TK80[385] and $\overleftarrow{B}$-TK80 [385]. Transcription of these chimeric clones is enhanced in liver but not in spleen extracts (Figure 9A). In contrast, the oligo PM-1 does not activate the TK promoter in analogous constructs (data not shown). Similar results have been obtained with the B-oligo cloned in front of a −50 deletion of the AdML promoter (data not shown). The ability of the A and B boxes to confer a liver-specific phenotype on the TK promoter proves that LF-A1 and LF-B1 are not only necessary in the original context to stimulate selective transcription, but that each of them is sufficient, perhaps in conjunction with other ubiquitous factors, to induce tissue-specific expression from heterologous promoters.

Discussion

In recent years considerable information has been accumulated on the role of *cis*-acting elements and *trans*-acting factors that are essential for tissue-specific and developmentally regulated gene expression. In some systems DNA sequences located far away from the transcription initiation site have been revealed to be important. In most cases, however, the promoter region, i.e. the first 100−200 bp upstream of the transcriptional start site, plays a crucial role in the selectivity of expression in different cell-types. This emerges most clearly from recent studies of transcription *in vitro* (Mizushima-Sugano and Roeder, 1986; Gorski *et al.*, 1986; Bodner and Karin, 1987; Heberlein and Tjian, 1988).

In this work we have shown that the proximal region of the α1AT promoter binds several proteins (Figure 3F). We established that LF-A1 and LF-B1 are transcriptional activators since competition with specific oligonucleotides eliminates binding of these factors and results in an inhibition of transcription from the α1AT promoter. Decreased transcriptional efficiency also correlates with mutations that reduce either LF-A1 or LF-B1 binding (see also Hardon *et al.*, 1988). Liver-specific activation of the ubiquitously expressed TK promoter induced by *cis*-linked A and B boxes provides additional evidence for the role of these factors.

Modulation of expression from the TK promoter suggests that selective transcription can be accomplished by the co-operation between transcriptional signals derived from heterologous genes. It is interesting to note that both the A- and B-oligos can stimulate transcription regardless of their orientation. Similarly the B element is also active in the opposite orientation in its original context (P.Monaci and A.Nicosia, unpublished observations). These data indicate that a unique architecture of the transcriptional complex is not essential and that the effect of the various *trans*-acting factors is additive, as if they were independent functional units. Such modular structure has been already proposed to explain the wide assortment of sequence motifs in some viral enhancers which are active in many cell types (Serfling *et al.*, 1985).

Some of the DNA-binding proteins identified in this study—LF-A1, LF-A2, LF-B1 and possibly LF-C—are liver-specific. LF-B2 probably belongs to a class of related proteins which are cell specifically modified: we identified a protein in spleen extract which shares physical and binding properties with LF-B2, but has a significantly different mobility in gel shift experiments. This situation is reminiscent of that of nuclear factor I (NF-I) for which a liver-specific form that binds to the mouse albumin promoter has been observed (Lichtsteiner *et al.*, 1987).

LF-A1 binds to the promoter of other liver-specific genes (human apolipoprotein-A1 and haptoglobin; Hardon *et al.*, 1988). Likewise, LF-B1 binds to rat β-fibrinogen, human α-fetoprotein (P.Monaci and A.Nicosia, unpublished observations) and gives a footprint in the proximal region of the human albumin promoter (Hardon *et al.*, submitted). A synthetic oligonucleotide containing this sequence efficiently binds LF-B1 (P.Monaci and A.Nicosia, unpublished observations). It is likely that the same factor is responsible for the footprint observed in the highly homologous B region of the mouse albumin promoter (Lichtsteiner *et al.*, 1987) and corresponds to the protein which has been identified and partially characterized by Courtois *et al.* (1987).

Several factors have been identified in liver and hepatoma cell nuclear extracts (Babiss *et al.*, 1987; Cereghini *et al.*, 1987; Johnson *et al.*, 1987; Lichtsteiner *et al.*, 1987; Grayson *et al.*, 1988; Raymondjean *et al.*, 1988). Competition in binding experiments (P.Monaci and A.Nicosia, unpublished observations) excludes the identity of LF-A1, LF-B1 and LF-B2 with the previously characterized liver-specific factors ψNF-I and CBP (Lichtsteiner *et al.*, 1987). A pool of different liver-specific binding activities, LF-A1, LF-B1, CBP and ψNF-I, appears to be involved in conferring liver specificity of expression. The analysis of the mouse albumin and α1AT promoters suggests that a variable assortment of these factors governs liver-specific transcription.

We have no information on the role, if any, of LF-C in transcription. A mutant that prevents its binding to DNA, such as PM6, is transcribed at the same rate as the wild-type promoter. Competition with the C-oligo has no effect on transcription, although it effectively removes the footprint from the C region. Redundancy of information in the α1AT promoter might explain these observations, suggesting that the capacity of LF-C to act as a transcriptional activator might be revealed in a different genetic context. Similar considerations apply to LF-A2.

The binding sites of LF-B1 and LF-B2 are overlapping but not coincident. As in crude liver extract the LF-B2 footprint is dominant and gel shift experiments indicate that LF-B1 is more abundant (compare Figure 6 and 5B), we imagine that the former has a higher affinity for DNA. Nevertheless, by competition with specific oligonucleotides we could show that in our experimental conditions LF-B1, but not LF-B2, is a transcriptional activator. This implies that the binding of LF-B1 to the α1AT promoter is not completely prevented by LF-B2. Alternatively, LF-B1 only transiently binds to the promoter thereby enabling the initiation of liver-specific transcription.

In spleen there is a small amount of a protein with

electrophoretic mobility slightly different to that of LF-B1, and a relatively larger amount of a protein similar, but not identical to LF-B2. These findings raise the possibility that LF-B2 in liver, or its counterpart in spleen, might exert a negative effect on transcription by interfering with the binding of LF-B1. Considering the relative concentrations of the two proteins, in liver the interplay between LF-B1 and LF-B2 could act to control quantitatively the level of transcription, whereas in spleen it would result in a total repression of α1AT transcription. This hypothesis provides a simple molecular model to explain the results of somatic cell fusion experiments. In hybrids between hepatoma and other cell types one observes either the extinction or the activation of liver-specific gene expression in the genome of the non-liver cell (Mével-Ninio and Weiss, 1981). Weiss and co-workers (M.Weiss, personal communication) have established a correlation of these phenomena with the ploidy of the parental genomes: extinction is always detected when diploid cell lines are hybridized, whereas *trans*-activation is obtained when tetraploid hepatoma cell lines are used. These results could be explained by assuming that gene expression is modulated by a fine balance between positive and negative factors; LF-B1 and LF-B2 might perform this role for the α1AT gene. At the moment this hypothesis is only based on the mutually exclusive binding of the two proteins to the α1AT promoter, on their relative abundance in liver and spleen, and on the proven role of LF-B1 as a positive, liver-specific, transcriptional factor. Attempts to ascertain directly the role of LF-B2 in transcription are being carried out. The occurrence of two proteins competing for the same or overlapping sites has been observed in other systems, but evidence on their precise role in transcription is lacking. For instance, in the rat and mouse albumin promoters, Cereghini *et al.* (1987), Lichtsteiner *et al.* (1987) and Raymondjean *et al.* (1988) have concluded that CBP and a protein, presumably belonging to the CAAT box family of binding proteins, exhibit mutually exclusive binding. In this case, genetic evidence points to the possibility that both proteins might be transcriptional activators, even though alternative explanations are possible. Two proteins, present at different stages of development, have the capacity to bind to the promoter of the sea urchin histone gene H2B in a mutually exclusive fashion (Barberis *et al.*, 1987). Finally, the immunoglobulin promoter contains the octamer motif to which either a lymphocyte-specific or a ubiquitous protein can bind (Landolfi *et al.*, 1986; Singh *et al.*, 1986; Staudt *et al.*, 1986), most probably in a mutually exclusive fashion since the contact points of the two proteins on the DNA are indistinguishable.

Clearly only part of the phenomenology relating to the mechanism of developmental and tissue-specific control of gene expression can be studied *in vitro*. Several observations, including the differential behaviour of transfected and endogenous gene (Charnay *et al.*, 1984; Becker *et al.*, 1987; Dente *et al.*, 1988), point to more complex events, perhaps related to an accumulation *in cis* of relevant information during development (DNA methylation, attachment to the nuclear cage, etc.) which restrict or facilitate the accessibility of *trans*-acting factors to the genes. Nevertheless, the *in vitro* approach, of which the present study is an example, has revealed itself to be remarkably informative. The presence of the liver-specific *trans* acting factors LF-A1 and LF-B1 undoubtedly plays a role in the mechanism of regulation responsible for liver-specific expression from the α1AT promoter. How the expression of these regulatory molecules is in turn regulated constitutes the challenging task of future studies.

Materials and methods

Plasmid constructions

Plasmid p(C_2AT)19 was received from M.Sawadogo (Sawadogo and Roeder, 1985a). To improve the efficiency of transcription termination a stretch of 10 G-residues was introduced into the *Sma*I site immediately downstream of the G-less cassette, to yield the plasmid p(C_2AT)[380].

Two shorter G-less cassettes, ~170 bp and ~250 bp long, were generated by *Bal*31 deletion to provide suitable vectors for internal control constructions.

AdML404[180] and AdML50[180] were obtained by *Bal*31 treatment of the plasmids pML(C_2AT)19 and pML50(C_2AT)19 (Sawadogo and Roeder, 1985b).

The −197/+5 region of HSV-TK gene promoter is a *Pvu*II/*Mnl*I restriction fragment from the plasmid pFG5 (Colbere-Garapin *et al.*, 1979).

All α1AT promoter fragments were fused to the G-less cassette at position −2, locating the new start point within the G-less sequence, at a position virtually equivalent to the transcription start site used *in vivo*.

Fusions of several promoters from which we either deleted the cap site or removed by site-directed mutagenesis all the G residues downstream of the transcriptional start point, allow specific, accurate and efficient transcription *in vitro* (P.Nicosia and A.Monaci, unpublished observations).

5′ progressive deletions of the α1AT promoter were generated either by *Bal*31 digestion or by exploiting convenient restriction sites.

Base pair substitution mutants EM and PM from 1 to 6, which acquire a site for the restriction enzyme *Eco*RV, were subcloned in the context of α1AT261[380] from pEMBLα1-CAT mutant series (De Simone *et al.*, 1987). Mutants PM7 and PM8 have been generated by replacing the *Bam*HI−*Apa*I α1AT promoter fragment (−34/−5) with synthetic double-stranded oligonucleotides carrying a mutated sequence (GCCCG for PM7, CTTCA for PM8) in the region indicated in Figure 2A.

Nuclear extract preparation and in vitro transcription reactions

Liver and spleen nuclear extracts from 3-month-old Sprague−Dawley rats were prepared according to Gorski *et al.* (1986) and Lichtsteiner *et al.* (1987).

In vitro transcription reactions were performed as described by Gorski *et al.* (1986). Optimal transcription efficiency was obtained using 30 μg of nuclear extract and 800 ng of template. If less template had to be used, the total amount of DNA was kept constant by addition of sonicated salmon sperm DNA.

Nuclear extract from HeLa cells were prepared according to Dignam *et al.* (1983). *In vitro* transcription was carried out as described (Wildeman *et al.*, 1984): in 10 μl of final reaction volume, 40 μg of extract, 200 ng of circular template and 800 ng of sonicated salmon sperm DNA were used. rGTP was replaced by 800 μM of the chain terminator 3′O-MeGTP and 15 units of RNase T1 were included in the reaction mixture.

Liver nuclear extract fractionation

Rat liver nuclear extract was loaded onto a heparin−sepharose column, equilibrated with nuclear dialysis buffer (NDB: 25 mM Hepes, pH 7.6, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT and 10% glycerol). Proteins were collected by step elution, using NDB of increasing KCl concentration. Fractions containing the peak of each binding activity were pooled, dialysed against NDB and kept frozen in liquid nitrogen.

Binding assays

Gel retardation experiments were carried out by incubating a labelled double-stranded oligonucleotide (~2−3 ng) with 4−6 μg of nuclear proteins in a buffer containing 25 mM Hepes, pH 7.6, 60 mM KCl, 7.5% v/v glycerol, 0.1 mM EDTA, 0.75 mM DTT, 5 mM $MgCl_2$, 3 μg poly(dI-dC) and 100 ng of sonicated salmon sperm DNA as carrier. After 30 min at room temperature, 5 μl of 20% Ficoll was added and the samples were loaded onto a 4% acrylamide gel in 0.25 × TBE buffer and electrophoresed at 10 V/cm for 2−3 h at 4°C.

For competition experiments, 200 ng of a double-stranded oligonucleotide were added to the reaction mixture prior to the addition of the extract. The sequences of the A-, B-, B1-, B2- and C-oligos are indicated in Figure 3F.

The sequence of the L-oligo, used as a negative control, is shown below:

5′ GGTGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGAC
CTTAAGCTCGAGCCATGGGCCCCTAGGAGATCTCAGCTGCCA 5′

DNase I footprinting assays were performed according to Lichtsteiner *et al.* (1987) on a −261/−2 fragment from the α1AT promoter, cloned into pEMBL19+.

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