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The 5′ flanking region of the rat LAP (C/EBPβ) gene can direct high-level, position-independent, copy number-dependent expression in multiple tissues in transgenic mice

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ABSTRACT

The efficiency and tissue-specificity of transgene expression in animals is usually subject to the position of integration into the host chromatin. We have discovered that a 2.8kbp fragment flanking the rat gene encoding the transcription factor LAP (C/EBPβ) directs position-independent, copy number-dependent expression in transgenic-mouse livers. Concomitantly, the DNAse I hypersensitivity pattern normally observed in the liver is established in the integrated transgene construct demonstrating that this region is capable of creating chromatin structures equivalent to the endogenous situation. These observations are reminiscent of the locus control regions (LCR) described for several genes. Additionally, this LAP element functions with both intron-less and intron-containing genes. The tissue specificity of this element, however, is not restricted to liver. The 2.8kbp region is capable of allowing position-independent, copy number-dependent expression in brain, kidney, heart, spleen, and lung, but in a construct-dependent manner. This is, to our knowledge, the first transcription factor gene with which a cis-linked LCR-like element has been associated.

INTRODUCTION

One method that is widely used to examine important regulatory regions of genes is the generation of transgenic animals. In this system, the gene of interest is stably and relatively randomly integrated into its host genome and is present in all cells (see 1). Furthermore, the transgene is subjected to developmental processes, such as DNA methylation and folding into higher order chromatin structures, which may strongly influence its expression (2). A frequently encountered complication of the transgenic animal approach is that micro-injected DNA becomes integrated into the proximity of dominant positive or negative regulatory elements. This may result in inappropriately higher or lower transgene expression (3, reviewed in 4). Such an observation is usually a strong indication that essential regulatory elements are absent from the transgene.

During the course of analysis using transgenic animals it was seen that a few genes' expression patterns were not substantially influenced by position effects. The cis-acting elements responsible for this position-independent and copy number-dependent expression has been termed the Locus Control Region (see 5 for review). The most extensively studied LCR is that of the human β-globin gene cluster discovered by Grosveld and his colleagues (6). Other genes with associated LCRs include those encoding human CD2 (7,8), chicken lysozyme (9), human α-globin (10), human MHC class 1 (11), human adenosine deaminase (12), human keratin 18 (13,14), murine class II MHC Eα (15), and mouse metallothionein (16).

The rat Liver-enriched Activator Protein (LAP) is a transcription factor that was cloned by virtue of its affinity to the mouse albumin D binding site (17). This factor, also known as C/EBPβ, NF-IL6, IL-6DBP, and AGP/EBP (18–21), is a close relative of C/EBPα, being 71% homologous in the basic region and the leucine zipper (17). The LAP and C/EBPα genes are also similar, in that both are intron-less transcription units (17, 22). LAP mRNA accumulates in all rat tissues examined, being most abundant in lung and liver, somewhat sparser in spleen and kidney, and quite rare in testis and brain. Therefore the LAP gene is probably ubiquitously expressed but absolute levels are modulated for each tissue type. In contrast to the wide distribution of LAP mRNA, LAP protein accumulates preferentially in liver (17).

In this paper we have studied the chromatin structure of the rat LAP gene by DNAse I hypersensitivity analysis and observe hypersensitivity in the distal and proximal promoter regions. Based on previous findings, this observation prompted us to test this region for activity in transgenic animals. This study shows that two modified LAP genes and a hybrid construct are expressed in transgenic animals in a position-independent, copy number-dependent manner in 23 out of 24 cases in liver. We suggest

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that we have localized a powerful LCR-like element capable of overriding positional effects. Indeed, in liver, the patterns of DNAse I hypersensitive sites (HSs) established in the transgenes are very similar to the endogenous rat pattern, thereby demonstrating equivalent chromatin structures. In other tissues transgene expression was construct-dependent and did not precisely follow the magnitude of expression of the endogenous LAP gene. Where the transgenes were expressed in multiple tissues, the magnitude of expression per μg of RNA was similar in all tissue types. The cis-acting LAP elements that we describe in this paper may thus be very useful in expressing transgenes in a broad range of tissues.

MATERIALS AND METHODS

Plasmid construction

S1/LIP. The construct S1/LIP was generated from a 4.8kbp genomic rat fragment (containing 2.8kbp 5′ and 200bp 3′ to the LAP gene) cloned in a pBS+ plasmid. The fragment was digested with NcoI and religated removing a 393bp internal NcoI fragment, which encodes the N-terminal region of LAP (17).

LCD. The LCD construct was prepared by insertion of a DBP cDNA fragment (kindly provided by E.Falvey), containing a novel NcoI restriction site at the start codon, into the NcoI site of S1/LIP.

LD. This construct was created by subcloning a blunted BstEII−XhoI fragment of the rat DBP gene into the EcoRV site of pBluescript. The plasmid was then digested with ClaI, blunted with Klenow, and digested with XhoI. The LAP 5′ sequences were prepared as follows. S1/LIP plasmid was cleaved at the NcoI site, incubated with Bal31 (removing 28bp from the region upstream of the NcoI site), then digested with SalI which cleaves in the polylinker. This LAP 5′ flanking region was then gel purified and ligated into the XhoI-blunt DBP vector above.

DNAse I hypersensitivity mapping

Liver and spleen nuclei were prepared from 3 to 6 month old male rats as indicated by Mirkovitch and Darnell (23). Optimal DNAse I concentration (100μgml^{-1}) was determined in a separate experiment using various concentrations of DNAse I (not shown). Under optimal concentrations, a time course of digestion was done from zero to 24 minutes on ice. Reactions were stopped by the addition of EDTA and SDS to 50mM and 1%, respectively. DNA was isolated after digestion overnight with proteinase K (100μgml^{-1}) and extraction with phenol:chloroform (1:1). Approximately 50μg of purified DNA was digested overnight with EcoRI. The digested DNA was re-extracted and the concentration determined. Agarose gels loaded with 10μg of DNA per lane were analyzed by Southern blotting (as below). A 723bp 3′ LAP probe NheI−EcoRI was labelled by the random primer procedure (24).

DNAse I hypersensitivity mapping in nuclei isolated from transgenic animals was essentially done as above, except that a concentration gradient of DNAse I was used instead of a time course. Tissues were initially homogenized in a 0.5M sucrose solution (composition, other than sucrose, is as stated for buffer A, ref. 23), then adjusted to 1.8M sucrose by the addition of 2.4M sucrose/buffer A solution, and nuclei were isolated as previously stated above. DNAse I concentrations used were from 1.5μgml^{-1} to 25μgml^{-1}, and reactions were allowed to proceed for 5 minutes on ice. DNA was isolated as above, and digested with BglII alone (LD transgenics) or with a second enzyme (DraI for S1/LIP or EcoRV for LCD, see Fig. 7). A 3′ random primer radio-labelled probe specific for each construct was used for hybridization.

Southern blotting

Approximately 5−10μg of genomic DNA was digested with restriction enzyme according to manufacturers recommendations (with the addition of BSA to 400 μgml^{-1}). The digested DNAs were electrophoresed on 0.7−1.0% agarose gels and Southern blotted onto Nytran membrane (Schleicher and Scheull) using standard protocols (24). Copy number estimates were made by densitometry.

Transgenic mice

DNA fragments were prepared as described (6) and microinjected (at a concentration of 1ngμl^{-1}) into the pronuclei of fertilized eggs resulting from a cross of B6D2F$_1$ mice (IFFA CREDO, France). Surviving injected eggs were transferred to pseudo-pregnant recipients of either NMRI or B6D2F$_1$ strains essentially as indicated previously (25). All analysis were done on F$_1$ or F$_2$ generations.

RNA isolation

Cytoplasmic RNA was prepared using a modified citric acid protocol (26). Briefly, up to 1.5g of tissue was homogenized in 2ml of 5% citric acid and centrifuged (Sorvall HB4 rotor, 3krpm, 4°C) for 5 minutes, to remove nuclei. The supernatant was collected and centrifuged at 10krpm (Sorvall HB4 rotor, 30 minutes, 4°C). The resulting pellet containing precipitated cytoplasm was dissolved in 3ml of 200mM Tris, pH8, 2% SDS, extracted three times with phenol:chloroform (1:1) and precipitated with 2.5 vol of ethanol. The RNA precipitate was collected by centrifugation, resuspended in 2ml of TES (10mM Tris, pH8, 1mM EDTA, 0.5% SDS) and precipitated by addition of LiCl to 2M (overnight at 4°C). The precipitate was collected by centrifugation, resuspended in 0.5ml of TES, and reprecipitated with ethanol. Total RNA was prepared from mouse tissues by LiCl/Urea extraction as described (27).

S1 nuclease protection analysis

Probes were prepared as follows. For the S1/LIP analysis, a RsaI digested plasmid containing the rat LAP gene was kinased with [gamma-^{32}P]ATP at the RsaI site, digested with BamHI, and gel purified. This 1120bp probe gave a 221nt protected fragment for the S1/LIP transgene mRNA and a series of protected fragments for the endogenous transcript due to mismatches in sequence between the rat and mouse genes. Probes for the LCD analysis were prepared by PCR, using the cloned rat LAP gene or the LCD construct and oligonucleotides from the 5′ upstream region of LAP (starting at position −396, 5′ GGGATCCGAC GGTTGGGGTC 3′) and from the 3′ downstream sequences within either LAP (ending at position +257, 5′ AGCGGCTCC-A GGTAGGGGCT 3′) or DBP (ending at position 476, 5′ CC-TGCAGAAG GCTCCTCAGC 3′). The 3′ downstream oligonucleotides were kinased with [gamma-^{32}P]ATP prior to PCR (28). PCR probes were purified on denaturing polyacrylamide gels prior to use. Using the 653bp LAP probe, protection by the 5′ end of the endogenous LAP transcript gave a fragment of 257nt. The 618bp LCD probe gave a protected fragment of 222nt when hybridized to the 5′ end of the transcript from the

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transgene. An LD specific PCR probe was generated from the LD construct using the 5′ LAP oligonucleotide and the 3′ DBP oligonucleotide. This 837bp probe generated a 441nt protected fragment mapping the 5′ end of the transcript from the LD transgene. The endogenous DBP messages gave a protected fragment of 355nt resulting from hybridization to the region of homology with the probe. Quantitation was performed by densitometry, and values adjusted for specific activities in the experiments using the LCD and LD probes.

Specific activities of probes were determined by Cerenkov counting and OD_{260} measurements. 15 to 30ng (single stranded equivalent) of each probe was coprecipitated together with 20−50μg of cytoplasmic RNA. S1 nuclease assay condition were as stated (24) with the hybridization temperature for the S1/LIP assay being 52°C and the LCD and LD assays being 60°C. '3x' controls using 75−150μg of RNA were done to confirm that the probe was in excess.

Ribonuclease protection assay

Assays were preformed as described (29) using 10μg of total RNA and a radio-labelled ~750nt RNA transgene-specific probe generated with T7 RNA polymerase from a linearized plasmid containing PstI subclone of the LD transgene construct (containing the LAP promoter from ~ −600bp to +85bp and 62bp of the DBP gene). Samples were hybridized overnight at 52°C. A specific protected fragment of ~145nt was expected, mapping the 5′ end of transcript, and analyzed by 6% polyacrylamide/urea gel electrophoresis.

Run-on transcription in isolated nuclei

Radio-labelled RNA was prepared and analyzed as described (26).

RESULTS

Multiple DNAse I hypersensitive sites are located within the 5′ region of the rat LAP gene

One classical method for the localization of gene regulatory regions has been DNAse I hypersensitivity mapping (see 30 for review). This assay provides a means to examine large regions of DNA for perturbations of the normal chromatin structure. To date, most DNAse I HSs have been found to mark important

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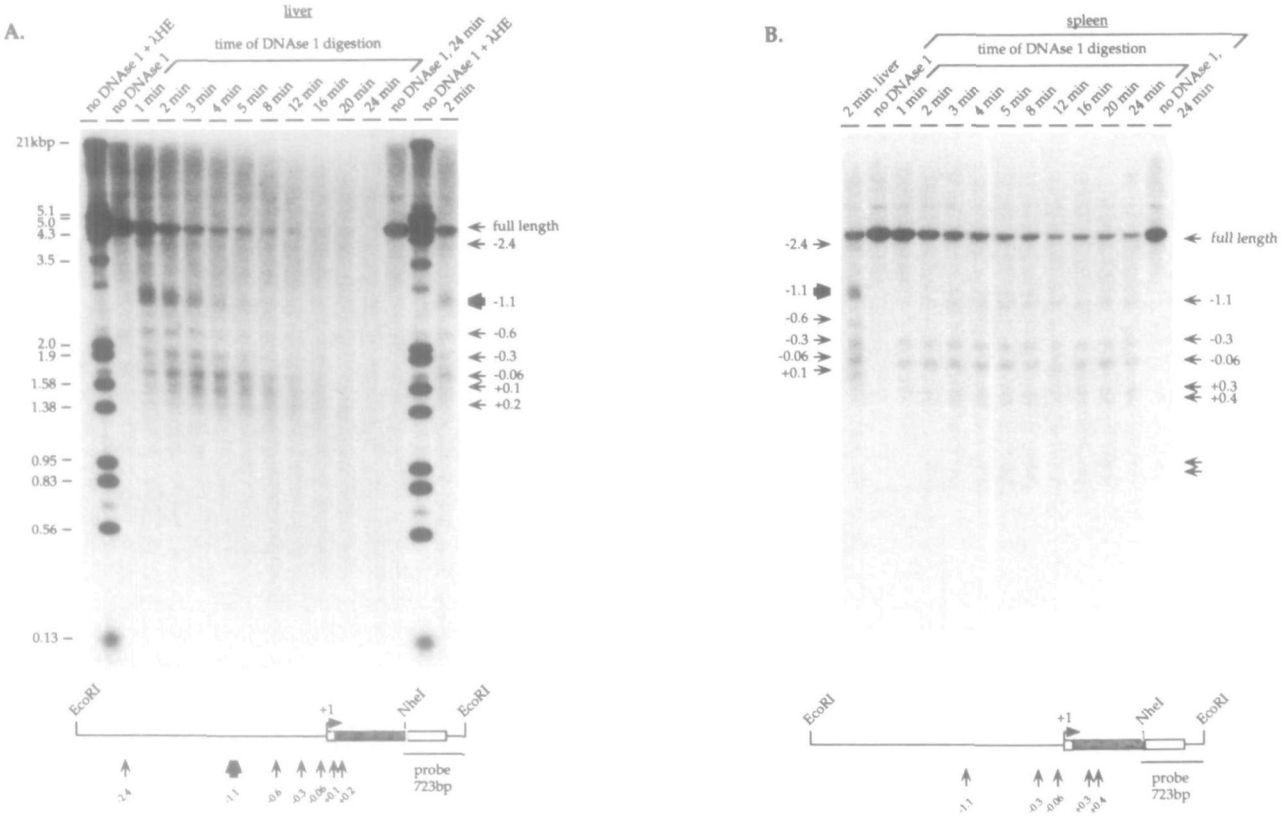

Figure 1. Analysis of DNAse I hypersensitivity in the endogenous rat LAP gene. (**A**) Rat liver nuclei were treated for indicated times with DNAse I and analyzed as stated in Materials and Methods. The LAP NheI−EcoRI probe is indicated below. Size in kbp of radio-labelled lambda HindIII/EcoRI markers (mixed with EcoRI digested mouse genomic DNA) are given on the left; positions of DNAse I HSs, relative to the major LAP transcriptional start site, are indicated on the right. Sample '2 min' was run along side the lambda marker to achieve more accurate sizing. Shown below is a schematic representation of the LAP gene (open box with coding sequence shown as stippled box) and observed DNAse I HSs in liver nuclei (vertical arrows) with relative positions to the LAP major transcriptional start site (horizontal arrow). (**B**) Rat spleen nuclei were analyzed as above. Lane labelled '2 min, liver' is a sample from the liver analysis (relative positions for liver given on the left) for comparison with splenic HSs (relative positions given on the right). The two unlabelled arrows denotes two HSs at position ~ +800 (position determined in a separate experiment). Below is a schematic representation of the LAP gene with DNAse I HSs in spleen nuclei (symbols as above).

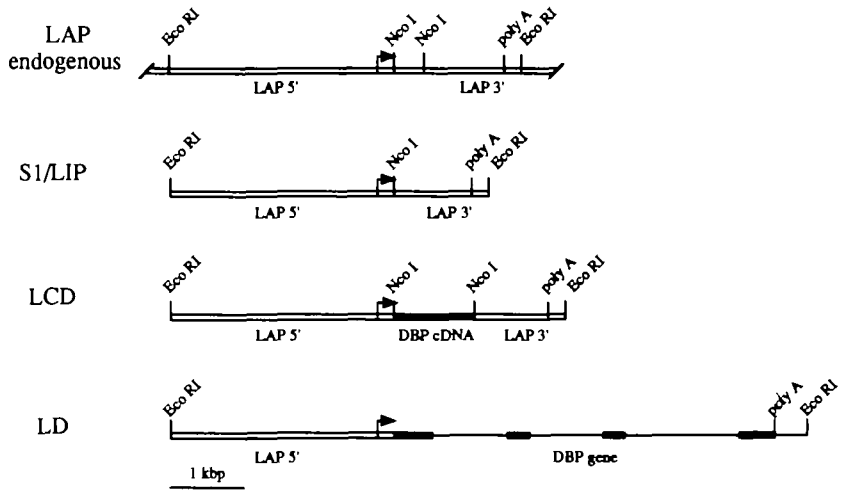

Figure 2. Schematic representations of the LAP gene and constructs used for transgenic mouse analysis. Open boxes denote LAP sequence, black boxes coding sequence for DBP, and thin lines DBP intronic sequences. DNA fragments, as shown, were used for generation of transgenic animals.

Table 1. Transgene expression in liver

line		estimated copy no.	transgene expression	expression per copy
S1/LIP	128	1–2	0.07	0.04–0 07
	121	1–2	0.04	0.02–0.04
	102	1–2	0.05	0.02–0.05
	9	1–2	0.02	0.01–0.02
	63	2–3	0 01	<0.01
	72	2–3	0 73	0.24–0.37
	142	3–5	3.64	0.73–1 21
	36	4–6	4.04	0.81–1.01
	107	6–8	3 55	0.44–0.59
	26	8–10	3.11	0.31–0.38
	45	6–8	1 81	0.22–0.30
LCD	43	1–2	0	0
	58	1–2	0.18	0.09–0.18
	49	2–3	0 42	0.14–0 21
	18	5–6	1.14	0.19–0.22
	57	10–14	1.94	0.14–0.19
	25	13–17	2.40	0.14–0.18
LD	6*	1–2	0.20	0.10–0.20
	60	1–2	0.10	0.05–0.10
	48	2–3	0.30	0.10–0.15
	12	2–4	1.28	0.32–0.64
	6	6–8	2 10	0.28–0.35
	61	6–8	1 73	0.23–0.30
	66	8–12	4 20	0.35–0.53

Transgene copy numbers were estimated by Southern blot analysis and densitometry on the signal resulting from the transgene and using either the endogenous mouse lap or dbp genes as internal controls. Relative 'transgene expression' levels were determined by S1 nuclease expression analysis (as shown) using densitometry of the transgene compared to the uppermost band of the endogenous lap signal (in S1/LIP analysis) or the endogenous lap signal in analysis of LCD and LD transgenics. In the LCD and LD analysis, expression values were corrected for differences in specific activities of the respective probes.

'Expression per copy' was determined by dividing the relative level of transgene expression by the estimated copy number. Given that different mRNA sequences are generated from the three transgene constructs, S1/LIP, LCD, and LD, the accumulation levels should not be compared between different constructs.

cis-acting regulatory elements. To search for such regions regulating the LAP gene, the rat liver was chosen as the source of nuclei since this is an abundant tissue in which LAP is strongly transcribed. DNA was isolated from nuclei treated with DNAse I and subjected to Southern blot analysis over a 4.6kbp region of the LAP gene (~2.8kbp 5′ flanking and 200bp 3′ flanking, see Fig. 1A). Using EcoRI digested DNA and the NheI–EcoRI 3′ probe, DNAse I HSs are seen at positions −2.4kbp, −1.1kbp, −0.6kbp, −0.3kbp, and −0.06kbp within the region 5′ of the gene. Two HSs are also observed downstream of the

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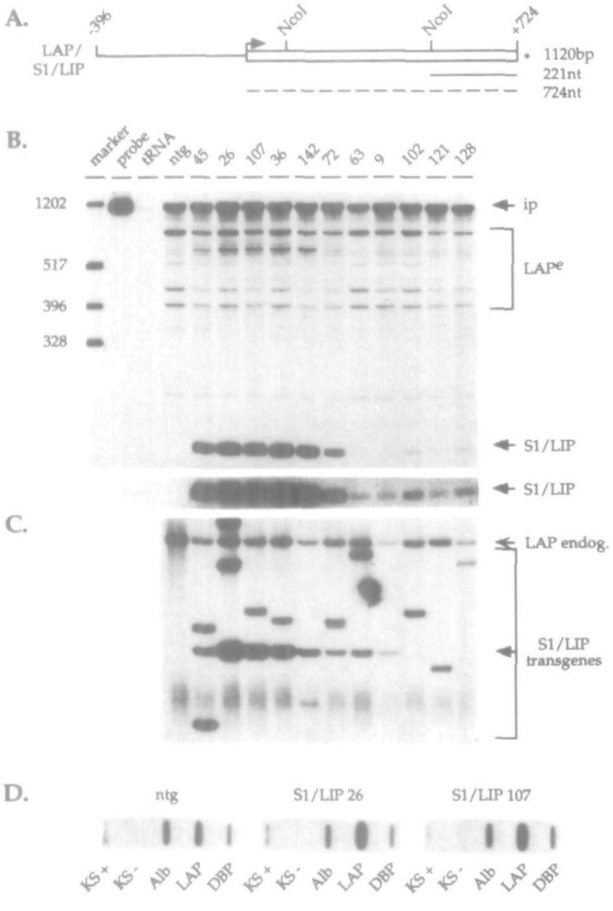

Figure 3. Analysis of transgene expression in the livers of S1/LIP transgenic animals. (**A**) Schematic representation of the S1 nuclease protection analysis probe used below. Positions are relative to the major transcriptional start site (horizontal arrow). Open box denotes transcribed sequences of the LAP gene within this region and asterisk the site of kinased ^{32}P label. Solid line below demonstrates the size of the expected protected fragment resulting from hybridization with the mRNA from the S1/LIP transgene (221nt). Dashed line represents region of hybridization of the rat probe with the mouse endogenous LAP gene, which results in protected fragments of up to 724nt (see Materials and Methods). (**B**) S1 nuclease protection analysis of 50μg of cytoplasmic RNA isolated from livers of non-transgenic (ntg) and transgenic mice harbouring the S1/LIP transgene (numbers correspond to each different mouse line). A double stranded end-labelled DNA fragment (indicated above, 10ng per sample) was used as a probe. 'ip' indicates the size of the intact probe, 'LAP^e' and the open bracket indicate major protected fragments attributed to endogenous mouse LAP mRNA, and 'S1/LIP' represents the protected fragment resulting from the transgene encoded mRNA. Size of marker fragments are shown on left. Lane 'probe' contained a small quantity of undigested probe to control for probe integrity. Lane 'tRNA' was a non-specific hybridization control with 50μg of yeast tRNA. Small panel below shows S1/LIP protected fragments of the same autoradiograph above, but with increased photographic contrast. (**C**) Southern blot analysis of BamHI digested tail DNA (samples corresponding to the lanes directly above in panel B) probed with a radio-labelled EcoRI–BamHI 5′ fragment. S1/LIP transgenes are in the area indicated by the open bracket with the arrow representing tandem head-to-tail or head-to-head repeat fragments (both ~4.2kbp). Tail-to-tail repeat fragments are not detected with this probe. The endogenous mouse LAP gene fragment is indicated by 'LAP endog.'. (**D**) Run-on transcription analysis in liver nuclei isolated from non-transgenic (ntg) and two homozygous lines of S1/LIP transgenic mice, 26 and 107. KS+ and KS− are control vector plasmids; Alb denotes mouse albumin coding sequence (26); LAP (26) and DBP (32) denote DNAs for coding sequences of the two genes respectively. All nuclei were isolated in the evening to obtain maximal signals for DBP (26).

transcriptional start site at positions +0.1kbp and +0.2kbp (Fig. 1A). The most intense HSs are seen in the region most distal 5′ to the transcriptional start site, even when using a probe from the 3′ end of the fragment. This suggests that the more distal 5′ HSs are more sensitive than the more proximal HSs. In keeping with this notion, when a probe from the 5′ region is used, the broad HS at −1.1kbp is observed, but not the downstream HSs (data not shown).

To examine whether these HSs are liver specific or whether they follow the general pattern of LAP gene expression, the spleen was chosen as a second source of nuclei to look for DNAse I HSs (Fig. 1B). Several of the HSs seen in the spleen (at −0.06kbp, −0.3kbp, and a portion of −1.1kbp) are at positions also observed in liver. Additional HSs at +0.3kbp, +0.4kbp, and two weak HSs at approximately +0.8kbp appear specific to the spleen. Accordingly, the HSs seen at +0.1kbp, −0.6kbp, distal portion of −1.1kbp, and −2.4kbp appear to be liver specific. The finding of ubiquitous, liver specific, and spleen specific HSs within this gene suggests that at least in part, the broad expression pattern of LAP mRNA may come about by overlapping repertoires of tissue-enriched and ubiquitous transcription factors and different cis-acting elements.

Expression from the LAP 5′ flanking region in transgenic mice is copy number-dependent in liver

The fact that the HSs associated with the LAP 5′ flanking sequences are found in both liver and spleen suggests that this region may be important for the expression of the LAP gene. Transgenic mice lines were established which harbour constructs containing 2.8kbp of the LAP 5′ flanking region. The first transgene was created by the deletion of an internal NcoI fragment. This transgene encodes a N-terminal truncated version of LAP (construct S1/LIP, Fig. 2; ref. 31). A second transgene was created by replacement of the internal NcoI fragment with the cDNA encoding another related transcription factor, DBP (ref. 32; construct LAP cDNA DBP or LCD, Fig. 2). Previous studies did not reveal any HSs within the transcribed regions of the DBP gene in rat liver (Talbot and Schibler, unpublished result); therefore transcriptional control elements are probably not residing within these sequences.

Sixteen founder transgenic animals for the S1/LIP construct were generated of which 11 lines passed on the transgene to their offspring. The copy numbers range between one and approximately nine, as estimated by Southern blot analysis of DNA isolated from transgenic animals and densitometry (Fig. 3C; Table 1). For the LCD construct, seven founder transgenic animals were generated of which six passed on the transgene to their offspring. The copy numbers, estimated as above, range from one to approximately 15 (see Table 1).

To analyze transgene expression, cytoplasmic RNA was prepared from the livers of adult (two to six months of age) transgenic animals, and subjected to S1 nuclease protection analysis (Fig. 3 and 4). Only animals of either the F1 or F2 generation were used for further analysis as this prevents ambiguities resulting from mosaic animals. A BamHI–RsaI probe (Fig. 3A) that covers the 5′ portion of the LAP gene gives rise to a specific 221nt fragment for the S1/LIP transgene transcript (resulting from hybridization of the probe only to sequences downstream of the 3′ NcoI fragment) and a series of fragments from the endogenous LAP gene (resulting from the LAP mRNA 5′ end and regions of non-homology between the

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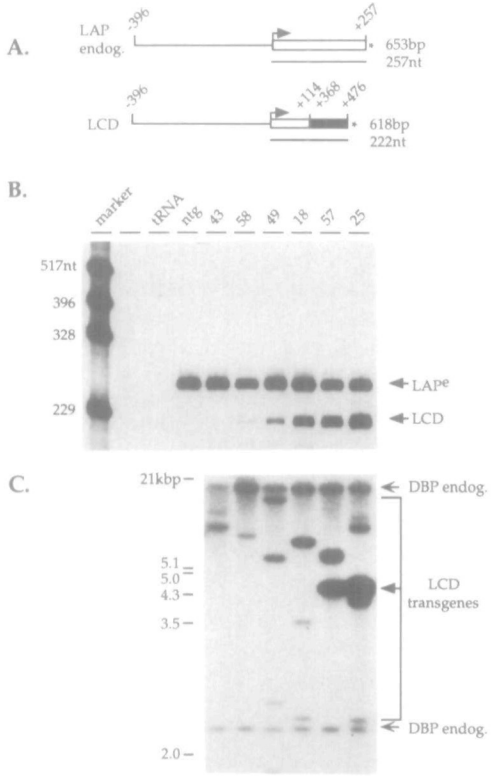

Figure 4. Analysis of transgene expression in the liver of LCD transgenic animals (**A**) Schematic representation of S1 nuclease protection analysis probes for detection of LCD transgene encoded message. Double stranded probes specific for mRNAs from the endogenous mouse LAP gene (653bp) or LCD transgene (618bp) were used (see Materials and Methods). Numbered positions correspond to LAP sequences (line and white box) or DBP sequences (black box) relative to their respective transcription start sites. Asterisk denotes position of kinased ^{32}P label. Protected fragments of 257nt and 222nt (indicated beneath probe diagram) are observed for correctly initiated transcription products from the mouse LAP gene and LCD transgene, respectively. (**B**) S1 nuclease protection analysis of non-transgenic (ntg) and LCD transgenic lines (indicated by numbers). 25μg of cytoplasmic RNA isolated from liver and a mixture of the LAP endogenous and LCD probes (20ng of each) was used in the analysis as described in Materials and Methods. The ratio of probe specific activities was LCD/LAP=0.5. 'tRNA' lane is as in Fig. 3, except 25μg was used. Marker sizes are indicated on the left. Protected fragments corresponding to LAP endogenous (LAP^e) and LCD (LCD) transgene encoded transcripts are shown on the right. (**C**) Southern blot analysis of EcoRV digested tail DNA (single site per construct) from transgenic mice (corresponding to the mouse line indicated directly above) using a DBP cDNA probe. Mouse endogenous DBP fragments are indicated by 'DBP endog.'. LCD transgenes are indicated by the open bracket; arrow denotes expected head-to-tail repeat fragment (~5.1kbp). Head-to-head and tail-to-tail tandem repeat fragments are ~7.5kbp and ~2.7kbp, respectively. Marker sizes are shown on the left.

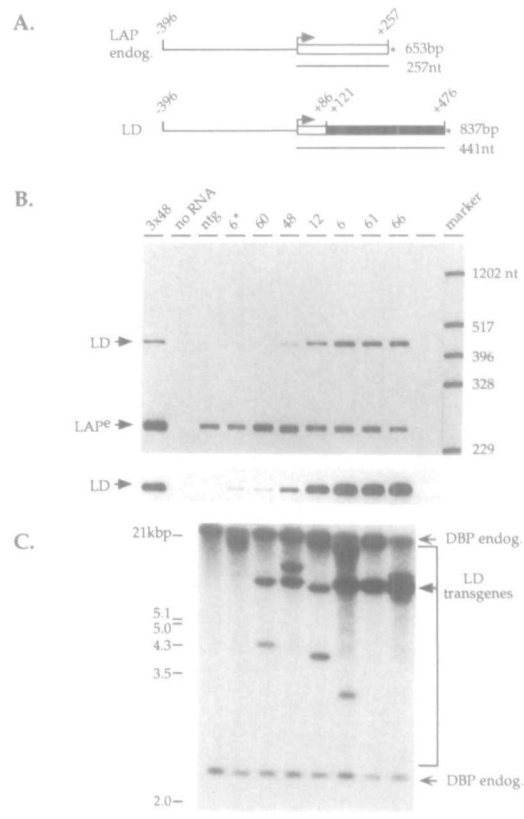

Figure 5. Analysis of transgene expression in the livers of LD transgenic animals. (**A**) Schematic representation of probes used for S1 nuclease protection analysis of LD encoded mRNA. 'LAP endog.' probe (as indicated in Fig. 4) and the LD specific probe (653bp) were used (see Materials and Methods). Numbered positions for the LD probe correspond to LAP sequences (line and white box) or DBP sequences (black box) relative to their respective transcription start sites. Asterisk denotes position of kinased ^{32}P label. The ratio of probe specific activities was LD/LAP=0.4. Protected fragments for correctly initiated endogenous mouse LAP transcripts and LD transcripts are 257nt and 441nt respectively. (**B**) S1 nuclease protection analysis using 25μg of cytoplasmic liver RNA from non-transgenic (ntg) and LD transgenic lines (numbers correspond to different mouse lines) and 20ng of each probe as above (see Materials and Methods). Note that founder LD6 gave rise to two lines, one of approximately 2 copies (left '6*') and one of 7 copies of the transgene (right '6') 'no RNA' control was included to identify non-specific protected probe fragments. '3×48' lane contained 75μg of LD line 48 RNA to demonstrate probe excess. Marker sizes are shown on the right; protected fragments for mouse LAP (LAP^e) and LD (LD) are shown on the left. Small panel below shows a longer exposure of fragments corresponding to LD transcripts. (**C**) Southern blot analysis of EcoRV digested tail DNA (single site per construct) from non-transgenic and transgenic mice (corresponding to the mouse line indicated directly above) using a DBP cDNA probe. Mouse endogenous DBP fragments are indicated by 'DBP endog.'. LD transgenes are indicated by the open bracket; arrow denotes tandem head-to-tail repeat fragment (~8.4kbp). Head-to-head and tail-to-tail tandem repeat fragments are ~15.2kbp and ~1.6kbp, respectively. Marker sizes are shown on the left.

rat and mouse genes). For the LCD transgenics, a transgene-specific S1 nuclease probe was used which encompasses the transcriptional start site. An internal control probe specific for the 5′ end of the endogenous LAP mRNA was also added to the S1 nuclease analysis for the LCD transgenes (see Fig. 4A).

Analysis of the autoradiographs of the Southern blots and S1 nuclease protection analysis (Fig. 3) by densitometry showed that the S1/LIP lines had high levels of expression in animals harbouring three to ten copies of the transgene. Direct comparison to the endogenous gene could not be done as the signal resulting from this analysis was distributed over numerous fragments. Expression levels within the copy number range of three to ten have a standard deviation of 74% from the average value (on a per-gene-copy basis). One to two copy-animals show approximately a ten-fold lower expression (with an internal standard deviation of 49% from the average value for these animals). This finding suggests that the high level of expression in the higher copy animals is due to synergistic effects, and one or more elements are lacking in the single copy animals. However, all lines containing this transgene are expressed at detectable levels, and therefore appear to provide position-independent expression even at lower transcription levels. It has

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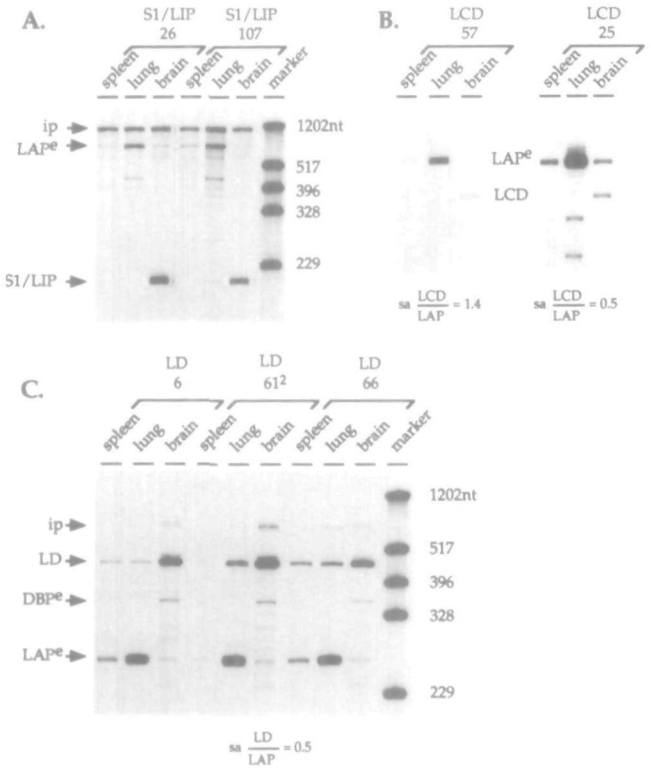

Figure 6. Analysis of transgene expression in spleen, lung, and brain. (**A**) S1 nuclease protection analysis using 20μg of cytoplasmic RNA isolated from spleen, lung, and brain of S1/LIP transgenic animals. Probe and conditions are as indicated in Fig. 3. (**B**) Analysis as above, using 25μg of cytoplasmic RNA isolated from LCD transgenic animals. Probes and conditions are as in Fig. 4. The ratios of probe specific activities for each analysis are shown below respective panel. (**C**) Analysis as above, using 25μg of cytoplasmic RNA isolated from LD transgenic animals. Probes and conditions are as in Fig. 5. The ratio of probe specific activities is shown below panel C. A protected fragment corresponding to the endogenous DBP can be seen in the brain using the LD probe (indicated DBP^e). LD61^2 indicates a mouse that is homozygous for the transgene and therefore has twice the copy number of the heterozygous line 61.

previously been seen with the 5'HS2 region of the human β-globin LCR that the prime enhancing element can be removed allowing low-level, copy number-dependent expression (33,34). To assess copy-number dependence, linear regression analysis was done on the entire S1/LIP data set and demonstrated a

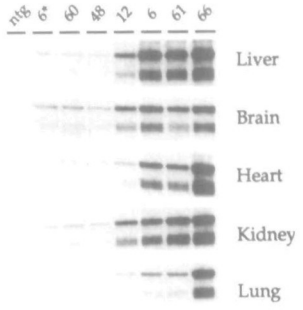

Figure 7. Tissue-expression pattern of the LD transgenic mice RNAse protection analysis of 10μg of total RNA using the RNA probe described in Materials and Methods. The two protected fragments correspond to ~145 and ~155nts. Numbers correspond to transgenic lines and 'ntg' refers to a non-transgenic animal Note that the brain RNA from line LD61 had some smaller protected fragments in this analysis (not shown), and therefore appeared somewhat degraded

correlation coefficient of R=0.77 (a value of R=1 is a perfect linear correlation).

Quantitation analysis of the LCD lines showed a more stable expression level over a wide range of copy numbers (from one to 15) with a standard deviation of 52% from the average value on a per-gene-copy basis (or 15% if LCD 43 is excluded). It should be noted that we have observed an approximately three-fold variation in transgene expression between transgenic littermates (Talbot and Schibler, unpublished result) suggesting an inherent degree of variability from these transgenes. Linear regression analysis of the LCD data set gave correlation coefficient of R=0.99. The results of this and the previous experiment demonstrate that in liver the transgenes are expressed in a copy number-dependent manner (Fig. 3 and 4; Table 1). Only one out of 18 of our breeding transgenic lines (LCD43) examined for these two constructs does not express the transgene. S1/LIP line 63 has a lower level of expression than expected based on its copy number estimation. However, since one band in both S1/LIP 63 and LCD43 clearly represent end fragments (or a separate integration event), we cannot be certain that all copies of the transgene are intact. With the one exception (line LCD43), the transgenes appear to be expressed independently of its position of integration and dependent on the number of transgenes. These constructs therefore carry an element with LCR-like activities.

To determine if the high accumulation of the transgene mRNA is due to high levels of transcription, run-on transcription

Figure 8. DNAse I hypersensitivity analysis in transgenic mice. Nuclei were isolated from liver and spleen of transgenic and non-transgenic mice and treated with increasing concentrations of DNAse I and subsequently analyzed as stated in Materials and Methods. (**A**) S1/LIP line 26 was assayed as above using a double digest of BglII and DraI. The probe was a radio-labelled 576bp PstI–DraI LAP fragment. 'No DNAse I*' is an aliquot of the 'no DNAse I' sample to which ^{32}P labelled lambda HindIII/EcoRI marker was mixed (sizes of fragments given in Fig. 1). The full length transgene fragment (marked 'transgene') and relative positions of DNAse I HSs are shown on the right. Below is a schematic representation of the S1/LIP transgene fragment analyzed above (box indicates transcribed region) with DNAse I HSs indicated as vertical arrows. No clear HSs are observed in spleen. (**B**) LCD line 25 was analyzed as above using a double digest of BglII and EcoRV and a 314bp NaeI–EcoRV DBP probe. Non-transgenic liver is included as a control. Spleen analysis is not shown as no DNAse I HSs are observed Relative positions of DNAse I HSs are indicated on the right. Two HSs indicated by unlabelled horizontal arrows at the bottom of the gel can not be accurately positioned from this analysis. Below is a schematic representation of the LCD transgene (open box indicates LAP transcribed sequences; black box indicates DBP transcribed sequences) with DNAse I HSs indicated as vertical arrows. (**C**) LCD line 57 was analyzed as in Fig. 8B. Spleen HSs positions (relative to transcriptional start site) are indicated on the left; liver HSs are indicated on the right. The two unmarked arrows denote HSs that could not be accurately positioned from this experiment. Below is a schematic diagram of the LCD transgene (see Fig. 8B) with arrows indicating DNAse I HSs observed in this mouse line. The HSs indicated at position −1.8 may be the result of the endogenous mouse DBP sequence as it is only seen with DBP probes (also in Fig. 8D), and a corresponding band can be observed in non-transgenic livers (Fig. 8B and data not shown). (**D**) LD line 61 was analyzed as above using a BglII digest and 291bp PstI–PstI DBP probe. DNAse I HSs observed in spleen are shown on the left; those observed in liver are shown on the right. Below is a schematic representation of the LD transgene (open box indicates LAP transcribed sequence; black box DBP sequences) with arrows indicating DNAse I HSs observed in this mouse line

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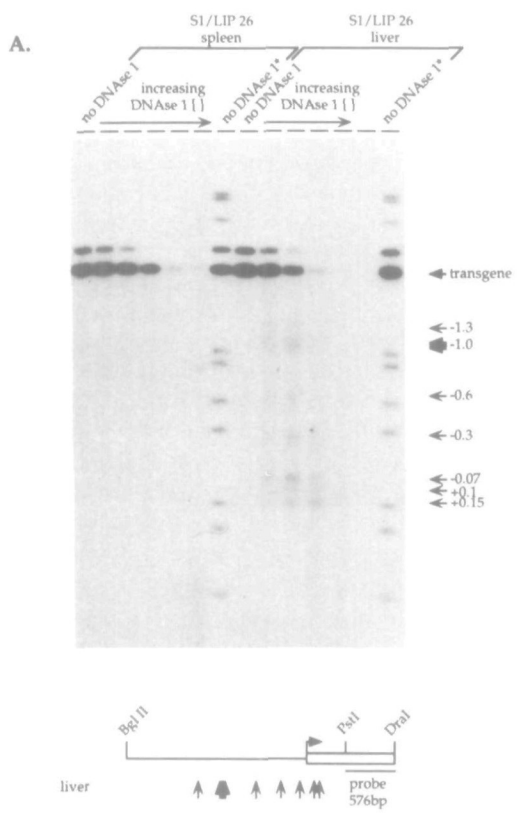

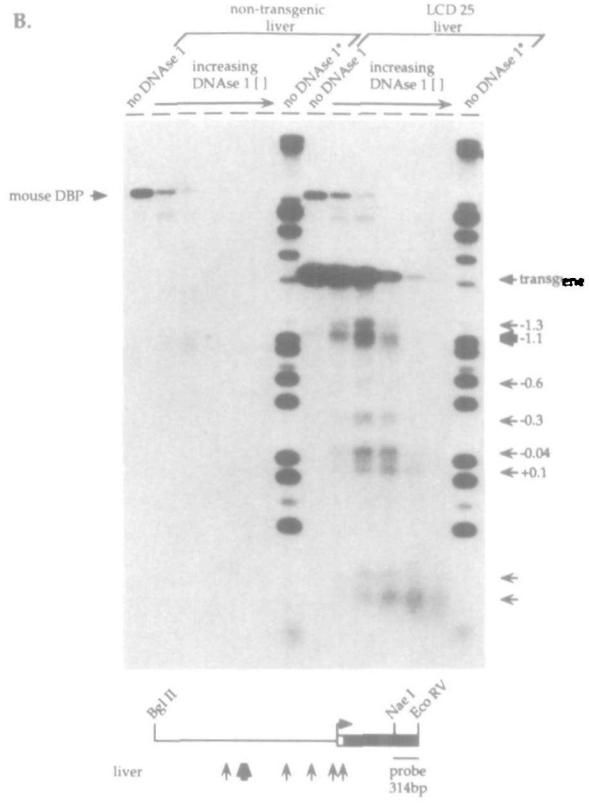

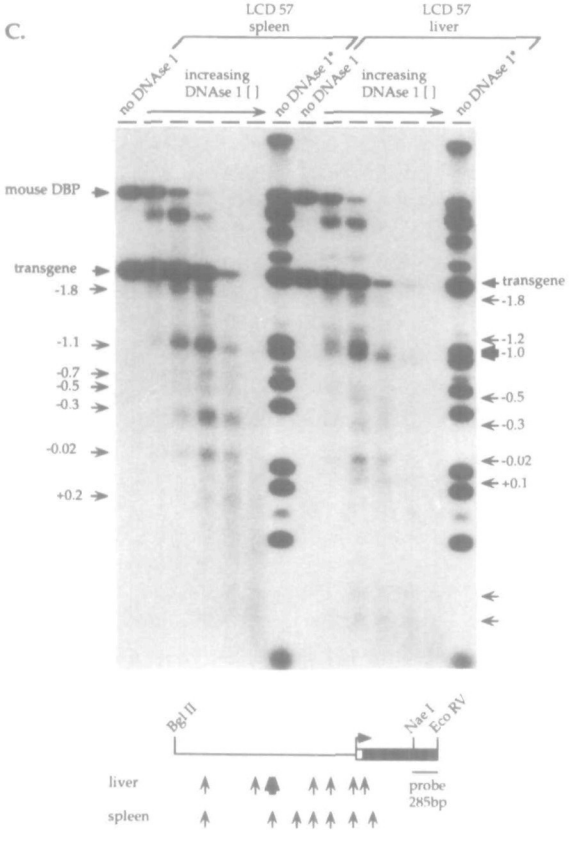

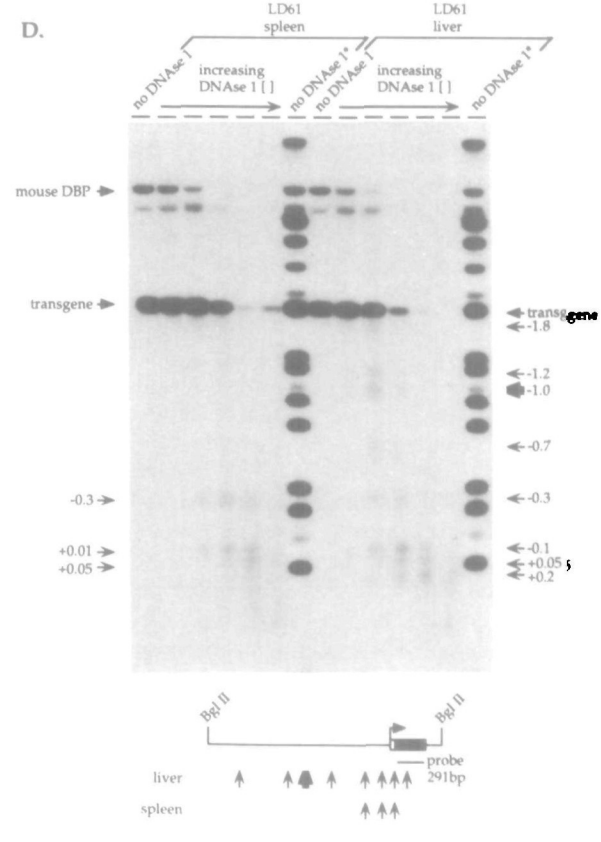

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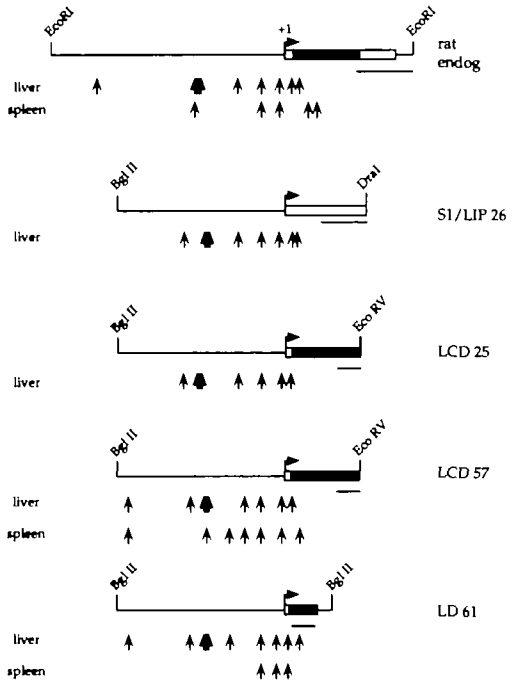

Figure 9. Summary of the DNAse I HSs observed in liver and spleen of the endogenous rat LAP gene, S1/LIP26, LCD25, LCD57, and LD61 lines No DNAse I HSs are seen in the spleens of lines S1/LIP and LCD25 and are therefore not indicated. Restriction sites for the digested fragments are as indicated. The probes used in each analysis are indicated by a line underneath each fragment.

experiments were done with liver nuclei isolated from wild-type and homozygous transgenic animals containing the S1/LIP construct (lines 26 and 107, Fig. 3D). Using a single stranded DNA probe for the entire LAP coding region this experiment clearly shows a strong increase in the LAP/S1/LIP signal (signals due to LAP or S1/LIP mRNA individually could not be distinguished) in transgenic animals compared to the non-transgenic wild-type. We attribute this increase to high levels of transcription from the transgene, and estimate its transcription rate to be greater than 30% of that of the mouse endogenous LAP gene. This value most likely represents its lower limit as the mRNA from the full endogenous LAP gene is approximately 30% longer than the transgene mRNA and should yield a somewhat higher hybridization signal. As it is not known whether the mouse and the rat LAP genes are transcribed with equal efficiency, no firm conclusions can be made whether this level represents 'full' expression.

Since LAP is an intron-less gene, we wished to determine the effect of the 5′ 2.8kbp flanking sequences on an intron-containing gene. It has been previously reported that some transgenes are more efficiently expressed in transgenic mice if they contain intronic sequences (35,36). A third construct analyzed in transgenic mice contains the 5′ 2.8kbp of the LAP promoter fused onto a promoter-less DBP gene fragment (construct LAP-DBP or LD, Fig. 2). Nine transgenic LD founders were generated which produced seven transgenic lines. Copy numbers were estimated as above and range from one to approximately 10 (Fig. 5C). In line LD48, the upper transgene specific fragment observed by Southern blot analysis using a DBP cDNA probe did not hybridize to a 5′ LAP specific probe and therefore this 5′ region appears to be deleted (data not shown). Transgene expression was analyzed by S1 nuclease mapping (Fig. 5B) using cytoplasmic RNA and DNA probes specific for the 5′ end of the LD transgene transcripts and the 5′ end of LAP mRNA (see Fig. 4A). Expression for this data set over a range of copy numbers from one to ten shows a consistent expression level on a per-gene-copy basis with a standard deviation (from the average value) of 59%. Linear regression analysis resulted in a coefficient of correlation of $R=0.95$, and therefore transgene expression appears to be copy number-dependent. Furthermore, we do observe an approximately two fold higher accumulation level for the LD lines versus the LCD lines, which may reflect some function of the introns in transcription. Alternatively, this difference could simply be due to differing stabilities between the two different mRNAs. This experiment supports the idea that the LAP 5′ flanking 2.8kbp region contains a LCR-like element as it is the only sequence common to all constructs.

The LD transgene also provides copy number-dependent expression in spleen, lung, brain, heart, and kidney

In order to assess the tissue specificity of these transgenes, we analyzed mRNA accumulation arising from the transgenes in higher copy animals in spleen, lung, and brain (Fig. 6). We observe that with all constructs there are high levels of expression in the brain which appear to follow copy numbers. In spleen and lung, however, significant expression is only detected in the LD constructs with no clear expression observed for the S1/LIP or LCD constructs.

To examine the LD transgenic mice further, we analyzed expression levels in the full copy number range for expression in liver, kidney, heart, lung, and brain on total RNA (Fig. 7). Using a transgene specific RNA probe and RNAse protection analysis it is clear that the transgenes are being expressed in these tissues in a copy number-dependent, position-independent manner. Furthermore, the absolute expression levels per μg of RNA appear rather consistent between tissues for each line. As the absolute magnitude of transgene mRNA accumulation is different from that of the endogenous LAP gene encoded mRNA (see introduction), this provides further evidence that the accurate regulation of the LAP gene requires multiple elements, some of which are absent from the transgenes. Nevertheless, the core of activity resulting in position-independent, copy number-dependent expression lies within the 5′ 2.8kbp region.

DNAse I hypersensitive sites in transgenic mouse livers appear at the same positions as observed in the rat liver

To compare chromatin structure of the transgenes with that of the endogenous rat LAP gene, nuclei from transgenic mice were isolated and subjected to analysis of DNAse I hypersensitivity. Liver was chosen as a representative tissue as constant, high levels of expression are observed with all constructs. Spleen was chosen as a second representative tissue as expression in this tissue appears to vary depending on the construct used. Single or double restriction enzyme digests were performed on DNA isolated from DNAse I treated nuclei and resolved by Southern blot analysis. As shown in Fig. 8, the overall HS patterns in the liver are essentially identical for transgenes and the endogenous rat LAP gene in all examined transgenic lines (see summary in Fig. 9).

Considerable variation in the pattern of DNAse I hypersensitivity is observed in the spleens of transgenic animals, ranging from no detectable HSs (line LCD25, not shown, and

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S1/LIP 26) to a pattern similar to that seen in the liver (line LCD57, Fig. 8C). Unexpectedly, the hypersensitivity in the spleen does not appear to correlate with the transgene expression in any straightforward fashion. For example, the transgenes are inefficiently expressed in the spleen of line LCD57 animals despite the strong DNAse I HSs observed. Conversely, the S1/LIP 26 spleen shows no clear HSs within the transgene despite perhaps some (albeit very weak) expression (Fig. 8A). One interpretation of these results is that in the spleen the conspicuous pattern of DNAse I hypersensitivity observed in the transgenes of line LCD 57 is the result of position effects. As integration is random, it would be expected that the transgene would occasionally insert proximal to endogenous promoters and other cis-elements which might utilize the transgene elements and influence DNAse I patterns. The LD61 line, in which expression in the spleen is clearly detected, shows three HSs located at −0.3kbp, +0.01kbp, and +0.05kbp, which are in the same regions in which two HSs are observed in spleen in the endogenous rat gene.

DISCUSSION

Here we show that 2.8kbp of the LAP 5′ flanking sequences contains cis-acting regulatory elements that are sufficient for position-independent, copy number-dependent transcription. Of 24 transgenic breeding lines tested, 23 express their transgenes in liver. Expression in animals with single copy transgenes appears to be at lower levels per gene copy than in those with multiple copies of the transgene, thereby suggesting some synergistic effects in the latter. The synergy observed from multiple copy animals is reminiscent of the observations of the human β-globin LCR in which individual elements are not capable of full expression (37). In the case of human β-globin 5′HS2, the LCR-like properties appear to only be observed in multiple, concatenated transgene insertions (38). The LAP element appears different from this in that we observe position-independent expression in single copy animals, albeit at low levels, and concatenated forms do not seem to be a requirement for expression (despite this being the most common configuration). From this we interpret that the region around the LAP gene contains sufficient information to allow expression, and that multiple integrated copies enhance the level of expression, rather that create a *de novo* LCR-like element.

Supporting our observations of position-independent expression, we observed that the chromatin structure of the transgenes in liver, as assessed by DNAse I hypersensitivity mapping, is found to be in a highly similar, if not identical, state compared to that seen with the endogenous LAP gene in rat. This observation demonstrates that the information required to establish the authentic chromatin state in liver resides within our transgenes. We furthermore suspect that it is the 2.8kbp 5′ region that contains this information as this region allows position-independent expression of the promoter-less DBP gene. Additionally, we have observed that a tissue-specific methylation pattern is reproduced in the transgenes used in this study (Talbot and Schibler, unpublished result), again supporting the notion that the essential elements for establishment of authentic chromatin are contained within our constructs.

Expression levels in spleen and lung is construct dependent. Constructs containing the 3′ end of the LAP gene (S1/LIP and LCD) are not readily detected in spleen and lung, whereas the LD construct, lacking the LAP 3′ end, is expressed in a copy number-dependent manner in spleen, lung, brain, heart, and kidney. This construct-dependent expression must be due either to activation by the intronic sequences of DBP or repression by the 3′ flanking sequences of LAP. Consequently, the non-activated or repressed promoters in the spleen of the S1/LIP and LCD mice may result in the loss of consistent DNAse I HS pattern in this tissue. This interpretation is similar to that of Reitman and colleagues (39) who demonstrated that the enhancer/LCR of the chicken β^A-globin gene is hypersensitive only if a functional promoter is attached or if the transgene is integrated near a promoter. These are intriguing findings as they suggest that the LCR-effect may be a functional-promoter-dependent entity. Absence or repression of a promoter may result in the loss or disruption of an LCR-like effect. This idea is also in agreement with the observations in the human β-globin cluster, where repression of the embryonic and fetal genes results in switching to adult expression (reviewed in 5).

Despite the complexities of LAP gene expression, we believe that we have localized an important regulatory element residing within the 2.8kbp region of the rat LAP gene. This element is capable of directing position-independent expression, copy number-dependent expression in multiple tissues. Furthermore the transcription levels attainable from this element have been estimated to be comparable to that of the endogenous mouse albumin gene. This high level of transcription is somewhat surprising for a transcription factor gene and considering that the LAP mRNA accumulates to only about 100 copies per cell (17), a hundred fold lower level than albumin accumulation. However, robust LAP transcription may be required for the rapid accumulation of mRNA during the acute phase response (19; Descombes and Schibler, unpublished result). Because of this high level of transcription from the 2.8kbp 5′ flanking sequences, we anticipate that the 5′ 2.8kbp region will be valuable in the expression of foreign transgenes both for analysis of the effect of various genes in a broad expression pattern or possibly for the production of medically important products. Additionally, this region appears to function well with both intron-containing and intron-deficient genes, thereby facilitating the use of cDNAs for expression. Lastly, as expression is possible in all tissues examined in this study but is construct dependent, it is conceivable that expression from the LAP 5′ element could be fine-tuned by the inclusion of enhancer or silencer elements into the promoter or 3′ flanking sequences to restrict expression to various tissue types. This would allow expression of transgenes in tissues for which appropriate regulatory elements are not yet available.

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