Nucleic Acids Research, 1993, Vol. 21, No. 7 1629–1635

Estrogen-inducible and liver-specific expression of the chicken Very Low Density Apolipoprotein II gene locus in transgenic mice

Jan Wijnholds, Sjaak Philipsen[1], Sara Pruzina[1], Peter Fraser[1], Frank Grosveld[1] and Geert AB*
Laboratory of Biochemistry, Groningen University, Nijenborgh 4, 9747 AG Groningen, The Netherlands and [1]Laboratory of Gene Structure and Expression, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

Received December 1, 1993; Revised and Accepted March 4, 1993

ABSTRACT

We have examined the chicken Very Low Density Apolipoprotein II (apoVLDL II) gene locus in transgenic mice. A DNA fragment composed of the transcribed region, 16 kb of 5′ flanking and 400 bp of 3′ flanking sequences contained all the information sufficient for estrogen-inducible, liver-specific expression of the apoVLDL II gene. The far-upstream region contains a Negative Regulating Element coinciding with a DNaseI-hypersensitive site at −11 kb. In transgenic mice, the NRE at −11 kb is used for downregulating the expression to a lower maximum level. The NRE might be used for modulating apoVLDL II gene expression, and may be involved in the rapid shut-down of the expression after hormone removal.

INTRODUCTION

The ability for stimulus-induced tissue-specific gene expression during development and differentiation in higher organisms is preprogrammed. In this way, different cells can use the same stimulus for different expression programs. The stimulus can have an environmental cause like infection, starvation or stress. Alternatively, it can be a molecule involved in intercellular signaling, for example a hormone (1). The chicken apoVLDL II gene provides an example of a gene which is expressed tissue-specifically and is strictly dependent on a hormone (2). In this case, the liver is the expressing tissue and estradiol is the inducing hormone. In other tissues, estradiol stimulates the transcription of different genes, for example in chicken oviduct the lysozyme gene is stimulated several-fold (3). The regulation of the expression of these genes requires the action of *trans*-acting transcription factors and *cis*-acting DNA elements, like promoters, silencers and enhancers (1,4,5).

Control regions conferring liver-specific expression in transgenic mice have been shown for example with the human α1-antitrypsin (6), α1-glycoprotein (7) and apoCIII VLDL (8) genes, and the mouse abumin (9), α-fetoprotein (10), angiotensinogen (11) and transthyretin (12) genes. The expression of a transgene is often influenced by its chromosomal position, resulting in highly variable expression that is not related to the transgene copy number. Some examples are known in which the transgene is expressed in a position-independent, copy-number dependent manner (13–16). The information for position-independent, copy-number dependent expression is provided by so-called Locus Control Regions.

We have shown previously that the induction of the apoVLDL II gene in liver is marked by the appearance of DNaseI-hypersensitive sites (HSS) in the transcribed and flanking regions (17; see Figure 1). One set of DNaseI HSS is present in the sequence immediately 5′ flanking to the gene. Genomic footprinting experiments demonstrated the tissue-specific and estrogen-dependent interaction of transcription factors with the 300-bp promoter region of the gene (18,19). The function of this region has been examined by transient gene transfer experiments (20,21). The region contains two estrogen response elements (EREs). *In vitro* DNaseI footprinting and band-shift analysis has yielded a detailed picture of the protein binding sites in the promoter region. In addition to the estrogen receptor, proteins binding to these sites (18,19) are the ubiquitous COUP-transcription factor (22) and the liver-enriched transcription factors C/EBP (23), DBP (24) and LF-A1 (25). In addition to the estrogen-inducible sites, constitutive, liver-specific HSS have been mapped, notably at 1.7 kb and 11 kb in front of the gene (17 and unpublished data).

In the current studies with transgenic mice, we attempt to delineate the apoVLDL II gene domain. A DNA construct covering the transcribed region and 16 kb of 5′ flanking DNA exihibited liver-specific and estrogen-inducible expression. Within this gene construct the far-upstream region contains information for silencing transcription in non-liver tissues. A DNA fragment covering a DNaseI hypersensitive site at −11 kb could be operationally defined as a Negative Regulating Element (NRE). This NRE may have an important function in the modulation of the apoVLDL II gene expression, specifically in preventing uncontrolled expression which would result in hyper-triglyceridemia (8).

* To whom correspondence should be addressed

MATERIALS AND METHODS

Transgenic mice

Transgenic mice were generated as described (26–28). Fragments for micro-injection into the pronuclei of fertilized eggs from CBAxC57BL/10 mice were purified from plasmid sequences by gel electrophoresis, glass beads and an Elutip-d column. Southern blot analysis of EcoRI digested tail DNA was used to identify transgenic mice. Three-weeks-old mice were estrogen-stimulated for 2 days by daily s.c. injections of 50 μl 0.4 mg/ml 17β-estradiol in 1,2-propylene glycol unless otherwise indicated.

Constructs

A 20-kb SalI fragment containing the complete 2.9-kb apoVLDL II gene, 400 bp of 3′ flanking and 16 kb of 5′ flanking DNA was purified from a lambda EMBL3 VLDL clone (Philipsen, S., Meinsma, D. and AB, G., unpublished) and cloned into the SalI site of pUC18 forming clone 20VLDLpUC18. This clone was cut with BamHI and religated, thereby deleting the −16 kb to −5 kb region and forming clone 9VLDLpUC18. The 9-kb BamHI/SalI apoVLDL II fragment from 9VLDLpUC18 was subcloned in the BamHI/SalI sites of the Bluescript vector, forming 9VLDLpBlue. A 2-kb BamHI/EcoRI 5′ fragment from −12.5 kb to −10.5 kb was blunted and cloned in the correct orientation into the blunted BamHI site of 9VLDLpBlue, forming 11VLDLpBlue.

A 20-kb SalI fragment from 20VLDLpUC18, a blunted 9-kb BamHI/SalI fragment from 9VLDLpUC18 and a 11-kb NotI/SalI fragment from 11VLDLpBlue were used to generate transgenic mice.

DNA analysis

DNA from different tissues was digested with EcoRI, TaqI or BamHI, Southern blotted (29) onto nylon or nitrocellulose membranes and probed with apoVLDL II and Thy-1 specific probes. The probes used were a 674-bp BstEII/EcoRI fragment from apoVLDL II intron A, a 1.1-kb EcoRI/BamHI fragment from the apoVLDL II 5′ flanking region, and a 1413-bp ApaI fragment from Thy-1 exon 4. Copy-numbers were estimated on a Phospho-imager using Thy-1 as an internal control.

RNA analysis

Total RNA was isolated from tissues homogenized in 3 M LiCl 6 M Urea (30).

Northern blotting was performed as described by Krumlauf (31). Northern blots were hybridized with a 506-bp apoVLDL II cDNA probe excised from pGEMVLDL-18A with SacI (Wijnholds, unpublished data). The blot was rehybridized with a 460-bp Eco-RI/TthIII1 mouse histone H4 probe as an internal control to correct for loading differences.

S1 nuclease protection analysis was carried out as described by Kollias *et al.* (28). Probes used were a 3′ end-labeled 670-bp SpeI/SalI apoVLDL II-fragment from 9VLDL-pUC18 and a 430-bp β-actin XhoI/AvaI-fragment from pHFβA-1 (32). Total RNA (5 μg) and labeled DNA probes (10–30 ng) were co-precipitated, resuspended in 20 μl S_1-hybridization buffer (40 mM PIPES, pH 6.4; 400 mM NaCl, 1 mM EDTA, 80% formamide) and incubated at 51°C for 16 h. Samples were digested with 200 units of S_1 nuclease (Boehringer Mannheim) at 25°C for 2 h after adding 200 μl of S_1 digestion buffer (280 mM NaCl, 30 mM NaAc, pH 4.8, 2 mM $ZnSO_4$). The reaction was stopped by adding 20 μl 50 mM EDTA, 1% SDS and 10 μg tRNA. After phenol-chloroform extraction, ethanol precipitation, and resuspension in 5 μl S_1 loading buffer (7 M urea, 5 mM Tris-borate, 1 mM EDTA, 0.1% xylenecyanol, 0.1% bromophenolblue) the samples were analyzed on a 5% polyacrylamide, 7 M urea sequencing gel.

Primer extension analysis was performed using a 26-nucleotide apoVLDL II pimer, 5′-CTGTCCTGCTTTCTCTCTCCC-AGGCT-3′, from exon 1. Total liver RNA (5 μg) and 10^5cpm [α-^{32}P]-labelled primer (10^8 cpm/μg) were co-precipitated, resuspended in 8 μl H_2O, and 2 μl annealing buffer (1.25 M KCl, 50 mM Tris–HCl, pH 8.0, 5 mM EDTA) was added. The samples were incubated for 1 hr at 65°C, cooled on ice and supplemented with 23 μl extension mix (20 mM Tris–HCl, pH 8.3, 10 mM $MgCl_2$, 10 μg/ml actinomycin D, 5 mM DTT and 1 mM dNTP's) and 1 μl 10 U/μl AMV reverse transcriptase. The reaction was incubated for 1 h at 42°C, stopped by the addition of phenol/chloroform and extracted in the presence of 10 μg carrier tRNA. The samples were ethanol precipitated, resuspended in 5 μl of S_1 loading buffer and analyzed on a 8% sequencing gel.

DNAaseI HSS mapping

Nuclei were isolated according to Gorski *et al.* (34) and resuspended at a DNA concentration of 1 mg/ml in 15 mM Tris pH 7.5, 60 mM KCl, 15 mM NaCl, 0.2 mM EDTA, 0.2 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine. Incubation of the nuclei was according to Philipsen et al (36). DNAaseI (Worthington) was added to concentrations of 1–8 μg/ml, and the reaction was started by the addition of $MgCl_2$ to 10 mM and $CaCl_2$ to 1 mM final concentration. The reaction was allowed to proceed for 5 min on ice, and stopped by the addition of 1 vol 50 mM EDTA, 1% SDS. DNA was isolated, digested with appropriate restriction enzymes and analyzed by Southern blotting.

RESULTS

Transgenic mice containing the apoVLDL II locus

Transgenic mice containing the chicken apoVLDL II gene locus were generated. The DNA fragments used for micro-injection contained the transcribed region, 400 bp of 3′ flanking region and either 16 kb (construct 20V) or 5 kb (construct 9V) of 5′ flanking DNA (Figure 1). An additional DNA construct (construct 11V) was used in which the BamHI/EcoRI fragment between coordinates −12.5 to −10.5, covering the DNaseI-hypersensitive site at −11 kb, was cloned in front of construct 9V. The hypersensitive site may represents a site of protein-DNA interaction involved in the regulation of the apoVLDL II gene.

Newborn mice were screened by Southern blotting of EcoRI-digested tail DNA, using an apoVLDL II-specific probe. Three founder transgenic mice (20V17, 20V20 and 20V22) were obtained with construct 20V, two (9V7 and 9V8) with construct 9V and five (11V5, 11V6, 11V9, 11V13, 11V15) with construct 11V. Mice 20V20 and 20V22 were used to generate F1 offspring. For further analysis of the founder mice, EcoRI-digested DNA from at least four tissues (liver, brain, kidney and lung) was hybridized with a chicken apoVLDL II probe and a Thy-1 probe as a loading control. Only mouse 11V15 showed a different ratio in the transgene versus Thy-1 control signal in the various tissues and this is therefore the only mosaic animal in these experiments.

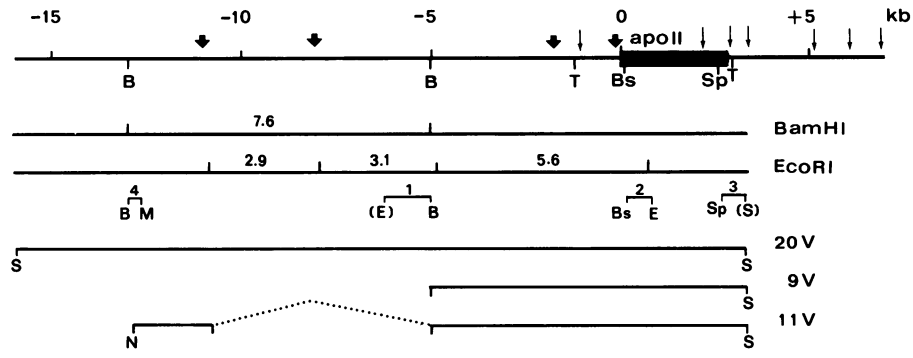

Figure 1. Map of the chicken apoVLDL II gene locus. The upper line shows the apoVLDL-II(apoII) locus. The bold and thin arrows indicate strong and weak DNAaseI hypersensitive sites, respectively, in chicken. The transcribed region is indicated as a bold block. The coordinates are given in kb relative to the cap site. The next three lines show the BamHI and EcoRI restriction maps and the apoVLDL II probes (1, 2, 3 and 4) used for Southern blotting and S_1-nuclease analysis. The length of the internal restriction fragments is indicated in kb. B, BamHI; Bs, BstEII; E, EcoRI; M, MboI; N, NotI; S, SalI; Sp, SpeI; T, TaqI. The constructs 20V, 9V and 11V used to generate transgenic mice are indicated on the lower four lines.

Table I. Expression levels of chicken apoVLDLII mRNA in transgenic mice

Mouse	Copy number	Sex	ApoII mRNA cpm[a]	β-actin mRNA cpm	ApoII/β-actin mRNA ratio
20V17	14	F	122,259	23,059	5.30
20V20a	6	F	19,607	14,783	1.33
20V20b	6	F	52,455	20,632	2.54
20V20b(−)	6	F	0	10,368	0
20V22	2	M	28,270	9,477	2.98
20V22(−)	2	M	7,914	11,382	0.70
11V5	8	F	96,295	20,898	4.61
11V6	15	M	107,771	27,670	3.89
11V9	4	F	100,050	25,878	3.87
11V13	27	F	84,616	54,317	1.56
11V15	20[b]	F	12,177	6,714	1.81
9V7	7	F	84,625	5.631	15.03
9V8	3	M	66,138	4,570	14.47

[a]Corrected for the signal (1,466 cpm) in non transgenic mice.
[b]Mouse 11V15 is mosaic.

The integrity of the integrated DNA was checked by Southern blotting; no rearrangements had occurred during chromosomal integration (data not shown). The constructs were inserted as multimers in a head-to-tail configuration into single locations of the mouse genome. The sites of chromosomal integration differed between the mice, as could also be concluded from restriction analysis. VLDL copy-numbers estimated with a Phosphor-Imager using the Thy-1 single-copy gene as a reference varied from 2 to 27 and are compiled in Table I.

The chicken apoVLDL II mRNA is correctly processed in the transgenic mouse liver

To establish whether the correct transcription initiation sites of the chicken apoVLDL II gene are used in mouse, total RNA from the livers of estrogen-treated transgenic mice was analyzed by primer extension. Chicken liver RNAs from adult females and estrogen-treated males were used as positive controls. Using an exon-1 specific primer, three different extension products (Figure 2) were found corresponding to the major start site (nominated +1) and two minor start sites (−11 and + 7) described earlier (36). The start sites used in chicken (lanes 2 and 3) are also used in the transgenic mice (lanes 1 and 5−7). To investigate whether the proper polyadenylation site is used in the mouse liver, we performed S_1 nuclease protection experiments. The results show that the chicken apoVLDL II transcript has the correct 3′ terminus in transgenic mice (Figure 3). Furthermore, the mature transcripts in transgenic mice and chicken appear to be of the same length on Northern blots (Figure 4A), indicating that the apoVLDL II mRNA is correctly spliced in transgenic mice. Apparently, the avian signals for initation, poly-A addition and processing are faithfully recognized by mammals.

Liver-specific and estrogen-inducible apoVLDL II gene expression in transgenic mice

Quantification of the apoVLDL II gene expression in the livers of transgenic mice by S1 nuclease protection experiments (Figure 3) showed that construct 20V is expressed to similar levels as the mouse β-actin gene, which means that the transgene is expressed at high level. Compared to chicken liver where the apoVLDL II transcript forms about 10 % of the total mRNA (2) apoVLDL II expression in mouse liver is 30-fold lower.

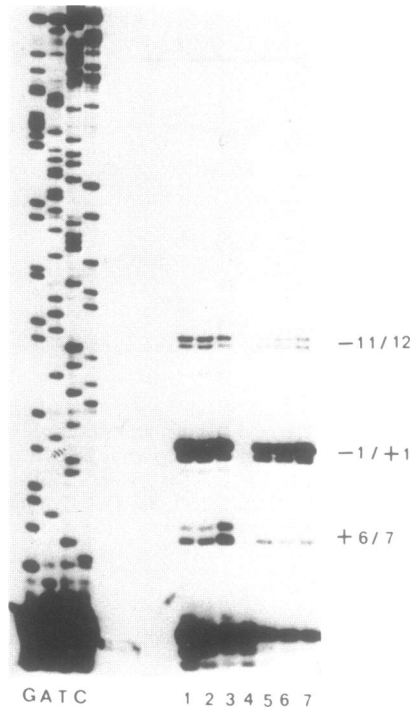

Figure 2. Primer extension analysis of transcription start site. A 26-nt apoVLDL II primer was annealed to total liver RNA, extended with reverse transcriptase and displayed by electrophoresis. Reactions contained 15 μg total RNA of the transgenic mouse 20V17 (lane 1), or 5 μg total RNA from a laying hen (lane 2), an estrogen-treated rooster (lane 3), a hormone-naive rooster (lane 4) and the transgenic mice 20V17 (lane 5), 9V7 (lane 6) and 9V8 (lane 7). A sequence ladder (G, A, T, C) generated with the same primer on an apoVLDL II DNA template was run in parallel. The positions of the three sets of cap sites in chicken and transgenic mice correlating with the length of the extended fragments are indicated.

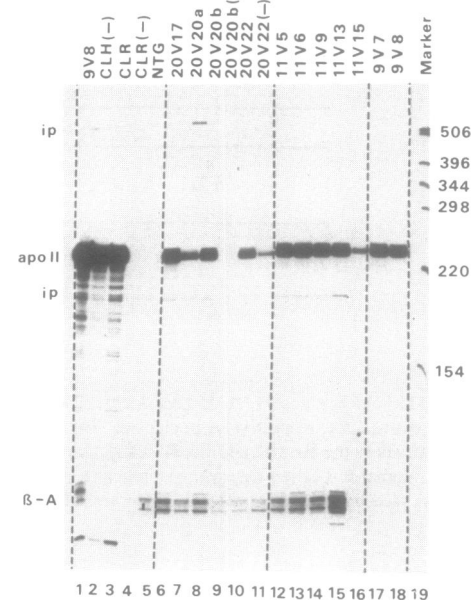

Figure 3. Quantitation of apoVLDL II mRNA levels in transgenic mice. Nuclease protection analysis of total liver RNA and a mixture of 3′ labelled apoVLDL II probe 3 (see Fig. 1) and β-actin probes (see Materials and Methods) is shown. The protected 257-nt 3′ apoVLDL II fragment (apoII) and the 100-nt β-actin (β-A) fragment as well as the input DNA bands (ip) along with the size (nt) of marker DNA fragments are indicated. Lanes 1−5 contained control S1-reactions of: 3×the standard amount of transgenic mouse (9V8) liver RNA (lane 1); 5 μg chicken liver RNA from a mature hen (CLH; lane 2) or mature rooster (CLR; lane 3,4), 5 μg RNA from a non-transgenic mouse (NTG, lane 5). Lanes 6- 23 contained S1-reactions in the presence of 5 μg RNA from transgenic mice with VLDL constructs 20V (lanes 6−11), 11V (lanes 12−16) and 9V (lane 17−18). All experimental animals were treated with estrogen except for those indicated with (−).

The expression of the chicken apoVLDL II gene in mouse liver proved to be dependent on estrogen as is the case in the chicken liver. This became clear when comparing a three-week-old, six-gene-copy transgenic mouse, 20V20b (Figure 3, lane 8), that had been injected with estradiol, to its age-matched hormone-naive sister 20V20b(−) (lane 9) in a S1 nuclease protection experiment. At the age of 3 weeks, female mice are not yet in the estrus-cycle (37). The hormone enhanced the level of apoVLDL transcripts at least 500-fold. Similar results were obtained with male mice. The estrogen-dependent expression was also evident from the Northern analysis (Figure 4B). Whereas the estrogen-treated mouse liver RNA already gave a clear band on a Northern blot after a three-hour exposure, the non-stimulated mouse yielded only a faint signal of the correct size after one-week exposure (data not shown). When the two-gene-copy transgenic male mouse 20V22 was compared with its hormone-naive brother 20V22(−), a four-fold induction was found (Figure 3, lanes 10 and 11). This much lower induction-factor is due to the relatively high expression level of the transgene in the hormone-naive mouse, which is probably caused by positive chromosomal position effects. The gene may have been integrated into a chromatin locus with an open conformation, allowing a substantial level of basal transcription. Steroid receptors are believed to act in two different ways, either by a de-repression mechanism involving chromatin opening or by a direct activation/enhancing mechanism accelerating the formation of the transcription complex at the promoter. The four-fold induction by estrogen of the two-copy transgene may represent the activator/enhancer activity of the estrogen receptor. (18,19,21,38,39).

When total RNA from different transgenic tissues (ovary plus oviduct, uterus, testis, speen, kidney, brain, thymus, heart, lung and small intestine) were examined (Figure 4A and data not shown), no transcripts were detectable even after one week of exposure. In summary, the apoVLDL II transgene expression is liver specific, estrogen-inducible, and occurs at high level.

Sequences required to confine expression to the liver

In all the apoVLDL II transgenic mice analyzed, the transgene was expressed, and expression was always higher in the liver than in the other tissues. The transgenic mice containing the smaller apoVLDL II construct (9V) from which the −16 kb to −5 kb region was lacking, gave a different tissue pattern of expression. Examination of total RNA from various tissues revealed that the tissue-specificity had been lost. Transgene transcripts were, in addition to liver, detectable in kidney, ovary plus oviduct, lung, uterus, thymus and heart (Figure 4C, and data not shown).

Transgenic mice containing construct V11 (carrying the −11 kb HSS), gave similar results. On longer exposures, ectopic expression is found in kidney, uterus (Fig. 4D), thymus (mouse 11V15; not shown) and heart (mice 11V5 and 11V6; not shown). This indicates that the addition of the −11 kb HSS is not sufficient to restore tissue specificity and we conclude that other upstream sequences may be required to restrict expression to the liver.

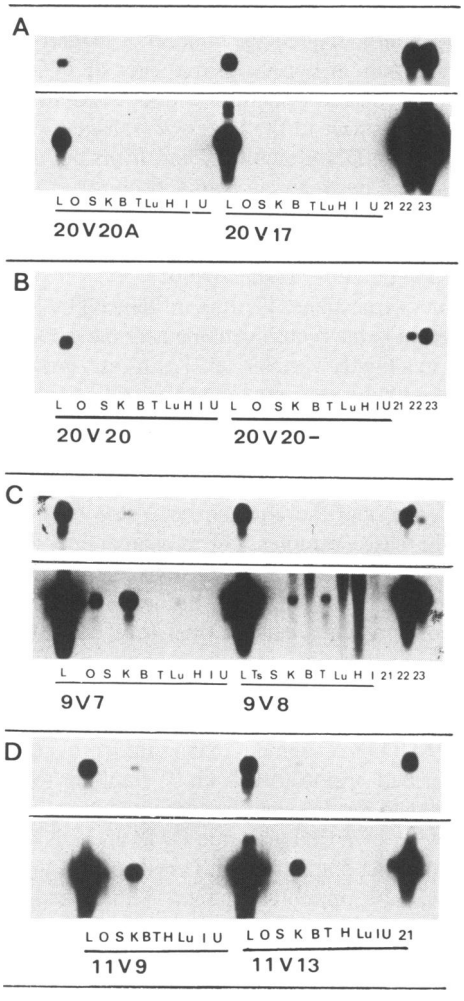

Figure 4. Tissue distribution of expression in transgenic mice. Five μg total RNA from various tissues was Northern blotted after gelelectrophoresis, and hybridized with an apoVLDL II cDNA probe. (**A**) RNA from transgenic mice 20V20 and 20V17. Lanes 21–23: liver RNA from a hormone-naive rooster, estrogen-treated rooster and laying hen, respectively. (**B**) Mice 20V20 and 20V20(−). Lanes 21–23: no RNA, and RNA from mice 20V20 and 20V17, respectively. (**C**) Mice 9V7 and 9V8. Lanes 21–23; no RNA, 5 μg and 0.5 μg liver RNA from mouse 20V17, respectively. (**D**) Mice 11V9 and 11V13. Lane 21: liver RNA from a laying hen. Short (upper) and long (lower) exposures are shown in panel A (3 hr and 7 days), panel C(2 hr and 2 days), panel D (3 hr and 3 days). Panel B shows a 3-hr exposure. L, liver; O, ovary and oviduct; Ts, testis; S, spleen; K, kidney; B, brain; T, thymus; Lu, lung; H, heart; I, small intestine; U, uterus.

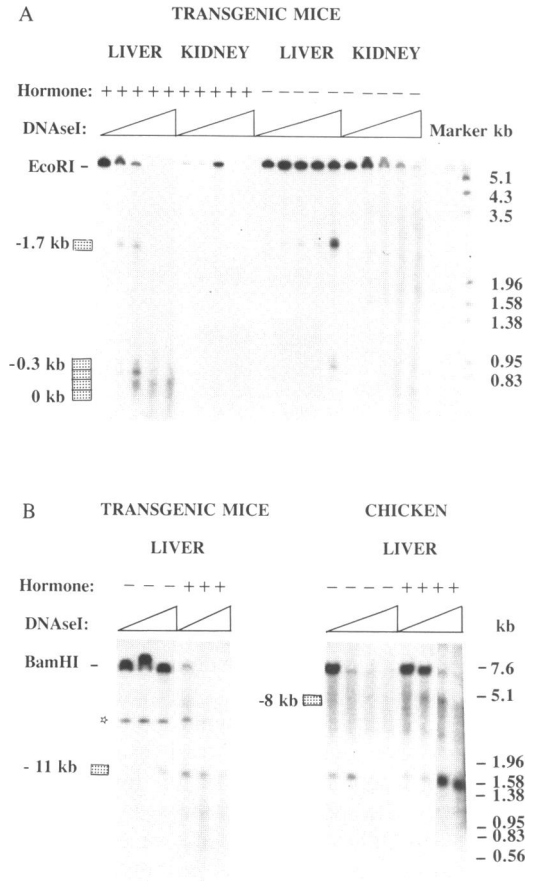

Figure 5. DNAaseI-hypersensitive site mapping at the apoVLDL II gene. Nuclei were isolated and treated with DNAaseI (see Materials and Methods). DNA was purified and restricted with EcoRI (**A**) or BamHI (**B**) and Southern blotted. Liver RNA was from nuclei from estrogen treated (+) or from hormone-naive (−) three-week old male transgenic (20V20) mice and rooster. The wedges indicate an increasing amount of DNAseI. The DNaseI hypersensitive regions are indicated by dotted blocks and their map position relative to the apoVLDL II cap site. The band indicated by a star is DNaseI-independent. Positions and sizes of marker DNA fragments are indicated to the right of each panel. (**A**) EcoRI blot hybridized with probe 2 (Fig. 1), (**B**) BamHI blot hybridized with probe 4 (Fig. 1).

DNAseI HSS mapping of the apoVLDL II transgene in mice

In chicken, constitutive DNaseI HSS were found at −11 kb and −1,7 kb and estrogen-inducible HSS at −8 kb and in the promoter region (17 and unpublished data). These HSS are present in liver but not in kidney. In the present study, we examined the HSS in the livers of hormone-treated and hormone-naive transgenic mice 20V20 containing the larger VLDL fragment. EcoR1- or BamHI-fragments of DNA from DNaseI-treated nuclei were Southern blotted and hybridized with probes abutting suitable restriction sites.

Viewing from the EcoR1 site within the transcribed region (see Fig. 1) upstream into the 5′ flanking DNA, we detect hypersensitive sites at the promoter and at 1.7 kb in front of the gene (Figure 5A). These sites co-localize with the hypersensitive sites in chicken (17). The hypersensitivity of the −1.7 region in mice was constitutive as it is in chicken. However, for the promoter region we observe a notable difference between chicken and mice. In non-stimulated rooster liver this region did not contain hypersensitive sites, implying that protein−DNA interactions at the promoter do not occur prior to hormone administration, a conclusion which has been directly corroborated by genomic footprinting (18,19). However, in non-stimulated transgenic mice hypersensitive sites were found indicating that interaction of DNA-binding proteins with the promoter can take place prior to hormone administration. It is possible that the high gene copy number of transgenic mice has facilitated the detection of unstable protein-promoter complexes. The presence of very low levels of apoVLDL II transcripts in non-stimulated transgenic mice indicates that at least some of these interactions may reach the stage of productive transcription complexes. However, the absence of transcripts in rooster, implying that the promoter is completely closed, indicates that the chromatin conformation in chicken and transgenic mice may differ more fundamentally for example in the precise positioning of the nucleosomes.

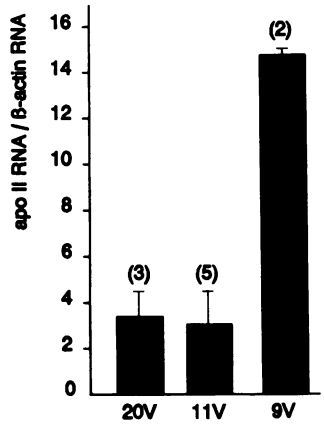

Figure 6. ApoVLDL II mRNA levels in transgenic mice. For each transgene construct, the average apoVLDL mRNA levels relative to the β-actin mRNA levels listed in table I are depicted. The number of mice is indicated above the bars.

With another probe specific for the 5′ end of the far-upstream region from −12,5 kb to −5 kb, we detected one HSS at −10.8 kb in induced as well as non-induced mouse liver (Figure 5B). The [−8 kb] HSS that has been found in chicken is however absent in the transgenic mice. The same results were obtained with a probe that labels the 3′ end of this far-upstream region (data not shown). The presence of the −11 kb HSS and the absence of the −8 kb HSS in transgenic mice suggest that only the former site is involved in modulation of the apoVLDL II gene transcription.

The upstream sequence contains a negative regulating element at −11 kb

The expression data of three different constructs (20V, 11V and 9V) are depicted in Table I and Figure 6. Within each group of transgenes generated with a particular construct, the expression showed no correlation with the gene-copy number. However, the gross apoVLDL II expression appears to reach a certain level for each group. The largest construct (20V) gave a 5-fold lower expression than the shortest construct (9V). This operationally defines the region extending from −5 to −16 kb 5′ flanking to the gene as a negative regulating DNA fragment. To further localize the responsible Negative Regulating Element(s) (NREs) within this fragment, we have investigated the effect of a 2-kb subfragment, containing the −11 kb HSS, linked to the 9V construct on the transcription of the apoVLDL II transgene. This construct, 11V, showed a 5-fold reduced activity when compared to the 9V construct. This identifies the fragment covering the −11 kb hypersensitive sites as a NRE.

DISCUSSION

In this report, we show that the chicken apoVLDL II gene can be correctly transcribed in transgenic mice and that the expression is tissue-specific and estrogen-inducible. The expression in liver was of high-level and varied moderately between mice carrying the same gene construct, indicating that position effects did not play a dominant role. Expression did not follow gene copy number implying that the apoVLDL II fragment tranferred to the mice did not comprise a locus control region. Sequences far-upstream of the gene were shown to repress transcription in tissues where the gene is normally not transcribed. In addition, these far-upstream sequences contain a negative regulating element which map at hypersensitive sites at 11 kb in front of the gene. The correct initiation and poly-adenylation of the apoVLDL II transcript in the livers of transgenic mice implies that the *cis*-acting DNA elements and *trans*-acting factors are largely conserved between chicken and mouse. The result is in agreement with the proper expression in transgenic mice of the chicken lysozyme (13) and β-globin genes (16) in macrophages and erythropoietic cells, respectively.

Expression of transgenes is often influenced by the regulatory elements present in the mouse genome near the site of integration. This results in a highly variable level of transcription which does not correlate with the gene copy number and often shows a more or less promiscuous tissue pattern of expression. Tissue-specific, position-independent, copy-number dependent expression of transgenes has been obtained with the human β-globin (15) and CD2 genes (14), and the chicken lysozyme (13) and β-globin genes (16). In these examples, chromosomal position effects are overruled by Locus Control Regions. ApoVLDL II gene expression in our transgenic mice did not follow copy number, but appeared to reach a certain final level for each particular construct. From this we conclude that the position effects of mouse sequences at the site of integration are relatively minor. Our results show that the information for high expression in liver resides in a 9-kb DNA segment extending from 5 kb 5′ flanking to the transcribed region to 0.4 kb 3′ flanking to the gene. A putative candidate for this specific activity is the constitutive HSS at −1.7 kb where we find genomic DMS and DNaseI footprints in liver and oviduct compared with erythrocytes around −1935 and −1831 (18,19; unpublished data). Other candidates are the hormone-inducible DNaseI HSS at the promoter region and in the third intron (17).

In this report, it is shown that the far-upstream region contains a Negative Regulating Element at −11 kb. This situation is somewhat resembling the situation in the 5′ flanking region of the chicken lysozyme gene, where upstream NRE's downregulate the expression of the gene (39). The following question arises: What physiological role may the NRE have? High expression levels of the gene might, like the human apoCIII VLDL gene in transgenic mice, give rise to a form of hypertriglyceridemia (8). The apoVLDL gene would need some kind of modulation to prevent overexpression, and the NRE at −11 kb might be used to reduce the expression after the removal of the inducing stimulus.

ACKNOWLEDGMENTS

J.W. thanks the members of the Laboratory for Gene Structure and Expression for helpful suggestions and probes. J.W. was the recipient of an EMBO short-term fellowship. P.F. was supported by an NIH postdoctoral fellowship. This work was supported in part by the Medical Research Council (UK).

REFERENCES

1. Beato,M. (1989) *Cell* **56**, 335−344.
2. Wiskocil,R., Bensky,P., Dower,W., Goldberger,R.F., Gordon,J.I. and Deeley,R.G. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 4474−4478.
3. Schütz,G., Nguyen,M.C., Giesecke,K., Hynes,N.E., Groner,B., Wurtz,T. and Sippel,A.E. (1978) *Cold Spring Harbor Symp. Quant. Biol.*, **42**, 617−634.
4. Mitchell,P.J. and Tjian,R. (1989) *Nature*, **245**, 371−378.

5. Ptashne,M. (1986) *Nature*, **322**, 697–701.
6. Kesley,G.D.,Povey,S., Bygrave,A.E. and Lovell-Badge,R.H. (1987) *Genes Dev.*, **1**, 161–171.
7. Dente,L., Ruther,U., Tripodi,M., Wagner,E.F. and Cortese,R. (1988) *Genes Dev.*, **2** 259–266.
8. Ito,Y., Arzolan,N., O'Connell,A., Walsha,A. and Breslow,J.L. (1990) *Science*, **240**, 790–793.
9. Pinkert,C.A., Ornitz,D.M., Brinster,R.L. and Palmitter,R.D. (1987) *Genes Dev.*, **1**, 268–276.
10. Hammer,R.E., Krumlauf,R., Camper,S.A., Brinster,R.L. and Tilghman,S.M. (1987) *Science*, **235**, 53–58.
11. Clouston,W.M., Lyons,I.G. and Richards,R.I. (1989) *EMBO J.*, **8**, 337–3343.
12. Yan,C., Costa,R.H., Darnell,J.E.,Jr., Cehn,J. and Van Dyke,T.A. (1990) *EMBO J.*, **9**, 869–878.
13. Bonifer,C, Vidal, M., Grosveld, F. and Sippel A.E. (1990) EMBO J., **9**, 2843–4848.
14. Greaves,D.R., Wilson,F.D., Lang,G. and Kioussis,D. (1989) *Cell*, **56**, 979–986.
15. Grosveld,F., Blom van Assendelft,G., Greaves,D.R. and Kollias,G. (1987) *Cell*, **51**, 975–985.
16. Reitman,M., Lee,E., Westphal,H. and Felsenfeld,G. (1990) *Nature*, **348**, 749–752.
17. Kok,K., Snippe,L., AB,G. and Gruber,M. (1985) *Nucl. Acids Res.*, **13**, 5189–5202.
18. Wijnholds,J., Philipsen,J.N.J. and AB,G. (1988) *EMBO J.*, **7**, 2757–2763.
19. Wijnholds,J., Muller,E. and AB,G. (1991) *Nucl. Acids Res.*, **19**, 33–41.
20. Beekman,J.M. (1990) Thesis, Groningen University.
21. Beekman,J.M., Wijnholds,J., Schippers,I.J., Pot,W. and AB,G. (1991) *Nucleic Acids Res.*, **19**, 5371–5377.
22. Tsai,S.Y., Carlstedt-Duke,J., Weigel,N.L., Dahlman,K., Gustafsson,J.-A., Tsai,M.-J. and O'Malley,B.W. (1988) *Cell*, **55**, 361–369.
23. Landschulz,W.H., Johnson,P.F. and MCKnight,S.L. (1988) *Science*, **241**, 1681–1688.
24. Mueller,C.R., Maire,P. and Schibler,U. (1990) *Cell*, **61**, 279–291.
25. Monaci,P., Nicosia,A. and Cortese,R. (1988) *EMBO J.*, **7**, 2075–2085.
26. Brinster,R.L., Chan, H.Y., Trumbauer,M.E., Yagle,M.K. and Palmiter,R.D. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4438–4442.
27. Hogan,B., Constantini,F. and Lacy,E. (1986) *Manipulating the mouse embryo. A laboratory manual.* Cold Spring Harbor Laboratory.
28. Kollias,G., Wrighton,N. Hurst,J. and Grosveld,F. (1986) *Cell*, **46**, 89–94.
29. Southern,E.M. (1975) *J. Mol. Biol.* **98**, 503–517.
30. Auffray,C. and Rougeon,F. (1980) *Eur. J. Biol.*, **107**, 303–314.
31. Krumlauf,R., Holland,P., McVey,J. and Hogan,B. (1987) *Development*, **99**, 603–617.
32. Gunning,P., Ponte,P., Okayama,H., Engel,J., Blau,H. and Kedes,L. (1983) *Mol. Cell. Biol.*, **3**, 787–795.
33. Gorski,K., Carneiro,M. and Schibler,U. (1986) Cell, **47**, 767–776.
34. Philipsen,S., Talbot,D., Fraser, P. and Grosveld,F. (1990) *EMBO.J.*, **9**, 2159–2167.
35. Van het Schip,A.D., Meijlink,F.C.P.W., Strijker,R., Gruber,M., van Vliet,A.J., van der Klundert,A.A.M. and AB,G. (1983) *Nucl. Acids Res.*, **11**, 2529–2540.
36. Theiler K., 1989. The house mouse. Atlas of embryonic development. Springer-Verlag New York Inc.
37. Martinez,E., Givel,F. and Wahli,W. (1987) *EMBO J.*, **6**, 3719–3727.
38. Metzger,D, White, J.H. and Chambon, P. (1988) Nature, **334**, 31–36.
39. Baniahmad,A., Muller,M., Steiner,C. and Renkawitz,R. (1987) *EMBO J.*, **6**, 2297–2303.