Neuron Specificity of the Neurofilament Light Promoter in Transgenic Mice Requires the Presence of DNA Unwinding Elements*

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Three reporter genes, the chloramphenicol acetyltransferase (CAT), the lacZ, and the intronless NF-L DNA, were used to test the activity of the proximal promoter region (−292 bp) of the human neurofilament light (hnF-L) gene in transgenic mice. Surprisingly, the hnF-L/CAT construct was highly sensitive to position effect, and its expression was found at low levels in several tissues of adult transgenic mice (Beaudet, L., Charron, G., Houle, D., Tretjakoff, I. Peterson, A., and J ulien, J.-P. (1992) Gene (Amst.) 116, 205-214). In contrast, the hNF-L/lacZ or the hNF-L/intronless constructs were expressed exclusively in the nervous system during embryonic development and in adult animals. The DNA sequences analysis of the different reporter genes revealed the presence of matrix attachment regions (MARs) within the 3′-untranslated regions of all three transgenes. DNA unwinding elements were found within the MARs of lacZ and hNF-L gene constructs but not in the CAT gene construct. When this element was removed from the lacZ construct, expression of the hNF-L/lacZ transgene became susceptible to position effect and was no longer tissue-specific. These results indicate that DNA unwinding elements are essential for position effect independence conferred by MARs to the hNF-L basal promoter.

A variety of genes such as those coding for luciferase (2), β-galactosidase (lacZ) (3), chloramphenicol acetyltransferase (CAT) (4),1 growth hormone (5), or alkaline phosphatase (6) have been extensively used as reporter genes, because they possess enzymatic activities that are easily detectable either in vitro or in vivo. With the availability of transgenic mouse technology, reporter-containing vectors have been developed for applications as diverse as analysis of enhancer and promoter function (7-10) and cell lineage studies (11). Transgenic mice have been found to be powerful tools to delimitate regions responsible for tissue-specific (12) developmental (13) or stimulatory (14) pattern of expression.

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1 CAT, chloramphenicol acetyltransferase; MAR, matrix attachment region; bp, base pair(s); kb, kilobase pair(s); UTR, untranslated region; PCR, polymerase chain reaction; X-gal, 5-bromo-4-chloro-3-indolyloβ-d-galactoside.

When a transgene integrates into a host genome, its expression pattern might be affected by elements localized near the insertion site (15). This position effect has been related to the absence of insulator sequences responsible for the establishment of a higher order of chromatin structure called the loop domain (16). Loop domains may represent independent transcription units, with genes located within a loop being subject to inside regulatory environment and insulated from outside environment (17, 18). Loops are maintained on the nuclear matrix by MARs located at both extremities and called domain boundaries (19). Even if domain boundaries do not seem to have transcriptional regulatory property of their own, their insulating effect on expression has been demonstrated as new domain boundaries have been characterized (20-25). It has been shown that not only a position-independent expression but also a finer regulation of a transgene developmental and stimulatory pattern of expression could be achieved when transgenes are flanked by MARs (26). Recently, compilation and comparison of MAR sequences have allowed the assessment of many MAR characteristics (27). MARs range from 250 bp to several kb in length, they are enriched in A/T nucleotides, they bind to nuclear matrix in vitro and in vivo, and they often contain consensus topoisomerase II cleavage sites, single-stranded, kinked, or curved DNA and potential binding sites for homebox containing DNA-binding proteins. Sometimes MARs colocalize with replication origins (28, 29), regions implicated in transcriptional regulation (30, 31), and regions of nuclease hypersensitivity (32). Considerable effort has been made to delimitate the minimal requirement for MAR to have an effect on the transcription process. Although binding to nuclear matrix seems to be a prerequisite, this criterion is insufficient by itself to assure an effect on transcription. Using stable transfection and mutagenesis experiments, sequences presenting unwinding capability have been shown to play an important role for transcriptional activation (32). However, their influence on tissue-specific and developmental transcriptional activation has yet to be determined.

The proximal promoter region (positions −292 to +15 bp) of the human neurofilament light gene (hnF-L) is not sufficient to drive high level and neuron-specific expression of a CAT reporter gene in transgenic mice (1). However, nervous system-specific expression is obtained with the same promoter linked to a lacZ reporter gene or an hnF-L/intronless gene.2 These results prompted us to look for functional similarities between the lacZ and the hnF-L/intronless reporter genes that could explain their clear tissue-specific expression. Our results demonstrate that unwinding elements colocalize with MARs found at the 3′-untranslated region (UTR) of the lacZ and the hnF-L/intronless reporter genes. Deletion of a DNA segment

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containing these unpaired DNA regions abolishes tissue-specific expression of hNF-L-lacZ transgene in transgenic mice. We propose that unwinding elements are one of the important features required for MAR to isolate the hNF-L basal promoter from position effect.

**EXPERIMENTAL PROCEDURES**

Construction of Plasmid—The hNF-L basal promoter (positions -292 to +15 bp) was ligated to a HindIII linker. To obtain the plasmid pNF-L-CAT, the hNF-L basal promoter ligated to the HindIII linker was inserted in the HindIII site of pUC-9 containing the CAT gene. The β-galactosidase gene (lacZ) was isolated from the plasmid pCH110 (Pharmacia) with a HindIII digestion followed by an EcoRI digestion. The 4,486-bp fragment containing lacZ was subcloned in the Bluescript pSK + vector linearized with a HindIII and EcoRI digestion creating the plasmid pSKlacZ. The hNF-L basal promoter ligated to the HindIII linker was inserted in the HindIII site of pSKlacZ to create the plasmid pH-LacZ.

To delete all three introns of the hNF-L gene, we took advantage of the presence of a conserved BglII restriction site in the middle of exon 1 between the human gene and the mouse gene and of another conserved EcoRI restriction site near the polyadenylation signal of both genes. First, a 4.9-kb BambI-XbaI fragment that includes the hNF-L gene (1) was subcloned in a Bluescript pSK + vector to create the plasmid pNF-LlacZ. The pH-LacZ plasmid was digested to completion by BglII and EcoRI. From the mouse CDO clone (33), a 1,220-bp BglII-EcoRI fragment was isolated and ligated to the open pSKHNF-L vector, creating the plasmid pHN-LIntronless.

The pHN-LlacZ promotor was directly isolated by complete BamH I digestion of the plasmid pHN-LlacZ and microinjected. Only the basal sequences of SV40 that allow correct polyadenylation were present in this 4,035-bp fragment. This fragment includes no plasmid sequences.

Microinjection into Mouse Eggs—The hNF-LlacZpromotor construct was isolated as described above and diluted to a concentration of 1 μg/ml in 10 mM Tris-HCl (pH 7.5), 0.15 mM Na+ EDTA prior to microinjection in male pronuclei of C57Bl/6J mice and with 0.25 mg/ml bovine serum albumin by centrifugation for 10 min at 750 × g, pelletized supernatants were resuspended in RSB with 0.25% sucrose, and an equal volume of solution containing 20 mM Tris-HCl (pH 7.4), 4 mM NaCl, and 20 mM EDTA was added. After a 10-min incubation, the nuclei were centrifuged at 1,500 × g for 15 min. Pellets were extracted twice by suspension in 10 mM Tris-HCl (pH 7.4), 2 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.25 mg/ml bovine serum albumin and centrifugation at 4,500 × g for 15 min. The resulting nuclear matrices were washed three times with 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM MgCl2, and 0.25 mg/ml bovine serum albumin by centrifugation for 30 s at 10,000 × g.

Probe Labeling—Supercoiled containing plasmids (1 μg) were digested with restriction enzymes: hNF-L-lacZ with EcoRI, BamHI, and HindIII; hNF-L-CAT with BamHI, HindIII, and Scel; and hNF-LIntronless with BamHI, BglII, XbaI, and EcoRI. After complete digestion, 20 units of calf intestine phosphatase were added, and the mixture was incubated for 1 h at 37°C. Phosphates were inactivated by incubation at 65°C for 15 min, and the reaction mixture was then extracted twice with phenol-chloroform:isoamyl alcohol (25:24:1) prior to ethanol precipitation. DNA pellets were resuspended in water and end-labeled with [-γ-32P]ATP using T4 polynucleotide kinase according to Sambrook et al. (37).

**RESULTS**

**Detection of DNA Single-stranded Region**—The reaction with pHN-L-CAT, pHN-LIntronless, and pHN-LlacZ plasmids was performed by resuspending 25 μg of each supercoiled plasmid in 50 μl of 50 mM NaOAc (pH 5.0) containing from 0 to 10% of chloracetaldehyde. The mixture was incubated for 1 h at 37°C. The modified DNAs were purified by two ETOH precipitations and were resuspended in 50 mM Tris-HCl (pH 8.0), 10 mM MgCl2, and 100 mM NaCl. The plasmid pHN-L-CAT was digested with HindIII, the plasmid pHN-LIntronless was digested with BamHI and, after addition of 10 μg of unlabeled carrier DNA, phenol-extracted and ethanol-precipitated. Resulting purified matrix-bound DNA fragments were electrophoretically resolved on 1% agarose gels in TBE, blotted on nylon membrane (Hybond N +), and detected by autoradiography.

Detection of DNA Single-stranded Region—The reaction with pHN-L-CAT, pHN-LIntronless, and pHN-LlacZ plasmids was performed by resuspending 25 μg of each supercoiled plasmid in 50 μl of 50 mM NaOAc (pH 5.0) containing from 0 to 10% of chloracetaldehyde. The mixture was incubated for 1 h at 37°C. The modified DNAs were purified by two ETOH precipitations and were resuspended in 50 mM Tris-HCl (pH 8.0), 10 mM MgCl2, and 100 mM NaCl. The plasmid pHN-L-CAT was digested with HindIII, the plasmid pHN-LIntronless was digested with BamHI, and the plasmid pHN-LlacZ was digested with HindIII and XhoI to linearize each plasmid at the 5′ end of the promoter region. After ETOH precipitation, 2 μg of each plasmid was treated with 0.1 unit of S1 nuclease in 30 μl of 30 mM NaOAc (pH 4.5), 150 mM NaCl, and 2 mM ZnCl2, for 10 min at 25°C, followed by ETOH precipitation. Purified DNAs were separated by electrophoresis on agarose gel and blotted. For the hNF-LlacZ and the hNF-Lintronless transgenes, a 307-bp hNF-L promoter DNA fragment was labeled with [-γ-32P]ATP using an oligolabeling kit (Pharmacia) and was used as probe for Southern hybridization, whereas a 225-bp HindIII-EcoRI DNA fragment corresponding to the 5′ end of the CAT gene was used for the hNF-L/CAT transgene. The positions of labeled bands was revealed by autoradiography.

β-Galactosidase Assay—β-Galactosidase activity was determined by measuring the rate of formation of 4-methylumbelliferone (MUF) from the fluorogenic substrates 4-methylumbelliferyl-β-D-galactopyranoside (MUF-gal) and 4-methylumbelliferyl-β-D-glucuronide (MUF-gluc).

**RESULTS**

Using three different reporter genes (Fig. 1A), very different conclusions regarding the strength, the tissue-specificity, and...
the developmental expression driven by the basal hNF-L promoter were reached. Fig. 1B summarizes the expression pattern region located in transgenic mice with each hNF-L reporter gene construct. The hNF-L/lacZ and the hNF-L/intronless transgenes were correctly expressed in neuronal cells; examples are shown in Fig. 1, C and D, respectively. However, the same promoter was not sufficient to direct CAT expression in those cells (1). The striking finding from those studies is that the different reporter genes exerted some influence on the activity of the hNF-L promoter.

A/T-Rich Regions in the Three Reporter Gene Constructs—The primary DNA sequence of each reporter gene construct was analyzed for its A/T content, as depicted in Fig. 2. Different patterns are observed for each construct. The CAT gene, for instance, is slightly A/T-rich at 55.4%, the lacZ gene is G/C-rich with an average of 44.1% A/T nucleotides, and the hNF-L/intronless gene is composed of a 5’ A/T-depleted region at 35%, followed by a region nearly equal in A/T versus G/C nucleotides at 52.5%. The three constructs have, at their 3’-UTRs, a DNA region enriched at more than 60% in A/T nucleotides; this ranges from 61.9% in the lacZ construct and 66.5% in CAT to 68.9% in hNF-L/intronless construct. The 849-bp A/T-rich region of the CAT construct is composed of SV40 sequences including the small t-antigen intron. In the lacZ construct, the 450-bp A/T-rich region of the CAT construct is composed of SV40 sequences. For the hNF-L/intronless, the 434-bp A/T-rich region is present in the natural 3’-UTR. In summary, each construct has a single A/T-rich region localized at the 3’-flanking region.

Nuclear Matrix Attachment of the A/T-rich Regions—A/T-rich regions are one of the characteristics of MAR. We thus tested whether the A/T-rich regions present in the constructs were capable of binding to the nuclear matrix. Nuclear matrices were derived from brain and liver tissues of adult mice. The nuclear matrix fraction was prepared by sequential extraction of purified nuclei with low salt buffer, DNase-1, high-salt buffer, and nonionic detergent. DNA from each plasmid-containing construct was digested by appropriate restriction enzymes and end-labeled with [γ-32P]ATP. The binding assay was accomplished by mixing nuclear matrices from either brain or liver with labeled probes in the presence of increasing concentrations of nonspecific competitor DNA. DNA fragments bound to the nuclear matrix were isolated and separated by agarose

struct is derived from plasmid pCH110 (Pharmacia). Its 3’-UTR contains a SV40 DNA fragment that comprises the polyadenylation signal region. The hNF-L/intronless transgene has been generated using conserved restriction sites between the human NF-L gene and the mouse cdNA. We deleted as a block from middle of exon 1 up to the first polyadenylation signal of the human NF-L gene and replaced it by corresponding coding sequences from the mouse cdNA NF-L, making a hybrid NF-L/intronless transgene (detailed under “Experimental Procedures”). No SV40 DNA sequence has been added to this hNF-L/intronless construct and polyadenylation occurs from the first endogenous hNF-L poly(A) signal (71); B, BamHI; BglII; EcoRI; Sc, Scal; XbaI, B. relative expression pattern of the three hNF-L/reporter gene constructs in transgenic mice. Expression was judged to be in the nervous system when it colocalized with nervous system structures. C, X-gal staining on a whole mount hNF-L/lacZ embryo, β-galactosidase (lacZ) staining of one transgenic embryo expressing the transgene. Embryo was analyzed 13.5 days after microinjection, and pictures were taken from side, back, and front views. Positive tissues are identified by an X. D, reverse transcription PCR expression analysis of the hNF-L/intronless transgene. RNAs from various tissues of transgenic mouse line 38 were reverse transcribed prior to the PCR. Two sets of oligonucleotides were used: Set 1 corresponds to oligonucleotides specific for the first exon of the hNF-L gene, and the second set corresponds to the mouse G3PDH gene and is used as an internal control. Upper panel, amplified products from a hNF-L/intronless transgenic mouse mRNA; lower panel, amplified products from G3PDH mRNA.
gel electrophoresis prior to autoradiography. Identification of bound fragment(s) was eased by selection of restriction enzymes digesting the plasmids in fragments of different sizes. Vector sequences present in the assay served as negative control because they possess no A/T-rich region.

Every DNA region of the hNF-L/CAT construct did bind to the brain nuclear matrix but with different affinities (Fig. 3A). The SV40 polyadenylation sequence (band 3) presents the strongest binding signal, followed by the CAT gene itself (band 2) and the hNF-L promoter (band 1), which is only weakly bound. Poly(dA-dT) is a better competitor than unrelated E. coli genomic DNA (Fig. 3A, lanes 8 and 15), indicating that A/T-rich sequences are responsible for specific nuclear matrix DNA attachment as expected (30, 38). Fig. 3C shows the binding pattern of the hNF-L/lacZ construct DNA fragments. Three regions are attached to the nuclear matrix in the presence of nonspecific competitor DNA. These regions correspond respectively to the hNF-L promoter (band 1), the lacZ 3'-UTR (band 2), and the SV40 flanking sequences (band 3). The lacZ gene itself was not attached to the nuclear matrix. The lacZ 3'-UTR has the highest affinity for the nuclear matrix, and its attachment is not competed by high quantity of nonspecific competitor DNA, whereas the promoter region and the SV40 flanking sequences were competed away. For the hNF-L/intronless construct (Fig. 3E), two regions attached to nuclear matrix. The first one corresponds to the promoter and first hNF-L exon (band 1), whereas the second one corresponds to the 3'-UTR (band 2). This latter band has the highest affinity for nuclear matrix, and binding is efficiently competed by poly(dA-dT). For all these constructs, identical results were obtained with brain or liver nuclear matrices. Fig. 3 (B, D, and F) summarize the results for each construct. In conclusion, the 3'-UTR regions of all three reporter genes are bound to the nuclear matrix with high affinity.

Detection of Unwinding Elements in Constructs Expressed in the Nervous System—Some DNA sequences like those from bacterial and yeast origin of replication have the capability to be stably base-unpaired (39–42). The presence of sequences with unwinding properties can be detected by treating supercoiled plasmids containing sequences of interest with chloroacetalddehyde (43). This chemical reacts with unpaired bases and blocks reannealing once the plasmid is relaxed by restriction enzymatic digestion. The reaction leaves single-stranded regions that can be digested with S1 nuclease (44). To detect single-stranded regions in all three hNF-L/reporter gene constructs, the plasmids phNF-L/intronless, phNF-L/lacZ, and phNF-L/CAT were incubated with chloroacetalddehyde. In this experiment, the bacterial replication origin CoE1 present in the Bluescript vector served as internal positive control for the reaction, this sequence being known to unpair easily under stress caused by supercoiling (39). With this mapping procedure, all three plasmids’ replication origins have reacted with the chemical agent (Fig. 4). For the plasmid phNF-L/CAT, the unpaired sequences visualized as a band of 1.6 kb are localized outside the CAT construct region used to generate transgenic mice. No band is visible within the construct region (Fig. 4, left panel). Within the plasmid phNF-L/lacZ, the presence of another single-stranded region was revealed by a clear band migrating at 4.1 kb (Fig. 4, central panel). The single-stranded region is localized in the SV40 sequences not present in the CAT reporter gene construct (Fig. 1). The plasmid phNF-L/intronless also possess unpaired sequences, because bands migrating at 2.2 and 2.4 kb are observed. Those sequences are localized 300 bp before the end of the intronless NF-L gene. We conclude from these results that the lacZ and hNF-L/intronless reporter constructs do contain unwinding elements, unlike hNF-L/CAT, and that for both, these elements are localized within the associated MAR.

Deletion of lacZ Reporter Gene Unwinding Element Abolishes Tissue-specific Expression in Transgenic Mice—We were interested in testing if the DNA unwinding element present in MAR had any influence on the tissue-specific expression of the reporter gene driven by the hNF-L basal promoter. A new lacZ construct was generated by isolating a 4,035-bp BamHI fragment from the hNF-L/lacZ construct (see map in Fig. 1) generating construct hNF-L/lacZunw. The BamHI digestion allowed deletion of the SV40 flanking sequences harboring the unpaired sequences but maintained the 3'-UTR of lacZ and the minimum fragment ensuring an efficient polyadenylation. With the hNF-L/lacZunw construct, only two out of seven transgenic embryos expressed the transgene 13.5 days postfertilization. This proportion is quite different from the hNF-L/lacZ construct where six out of seven transgenic embryos correctly expressed the transgene (Fig. 1B). In the first transgenic embryo, lacZ staining occurred in the liver, limbs, and somites (Fig. 5, a, b, and c). The second transgenic embryo presents punctuated lacZ staining of tissues surrounding the telencephalon and myelencephalon; some nervous tissues are also faintly stained (Fig. 5, d, e, and f). Results indicate that deletion of the unwinding element of the MAR abolishes the tissue-specific expression of the hNF-L/lacZ fusion gene.

DISCUSSION

The hNF-L basal promoter does contain regulatory elements to confer neurone specificity. As demonstrated in transgenic mice, a region extending at the very 5’ end of the promoter between −292 and −190 bp is necessary for neuronal expres-
Reported Gene Interference on Transcription

(45). This –292-bp basal promoter was found to be sufficient to allow expression of the lacZ reporter gene and the hNF-L/intronless gene in a correct tissue-specific and developmental manner (Fig. 1B). However, the CAT reporter gene expression under the same basal hNF-L promoter is highly susceptible to insertion site influence and is not expressed in a neuron-specific fashion (1).

All three transgenes have the same basal hNF-L promoter, and therefore their pattern of expression cannot be totally explained by the influence of this promoter. Clearly, other unknown elements within the reporter genes themselves do interfere with the expression of these transgenes. The presence of regulatory elements such as enhancers and silencers in the CAT or the lacZ reporter genes is unlikely because a neutrality on transcriptional activation of these genes has been inferred from numerous studies. In fact, both CAT and lacZ genes have been extensively used as enhancer and silencer trap vectors (review in Refs. 46 and 47). MARs represent likely candidates to explain apparent discrepancies in our analysis of NF-L promoter (23, 48).

The chromatin of interphase nuclei is organized into topological constrained loops averaging 80–90 kb in length that are attached to the nuclear matrix (19, 49). This DNA organization seems to be important not only in the compaction of the chromatin fiber but also for the utilization of genetic information. Each domain can define an independent unit of gene activity insulated from the regulatory influences of adjacent domains (18). MARs have been found in the 5′- and 3′-flanking regions of fushi-tarazu, sgs-4, and alcohol dehydrogenase genes of Drosophila (19, 21, 50), the J-C intron of the mouse κ immunoglobulin gene (30, 51), the first intron of the human HPRT gene, the chicken lysozyme gene, the human interferon-β gene, the human and murine β-globin gene, the chicken α- and β-globin gene, and the apolipoprotein B gene (reviewed in Ref. 27). MARs enhance general promoter functions in an orientation- and partially distance-independent manner, and their effect is restricted to the integrated state of transfected templates (23). These findings strongly suggest that some MARs might serve as a crucial control point for gene regulation.

Our results are indicative of nuclear matrix attachment through A/T stretches as described for many well characterized MAR sequences (30). The hNF-L/CAT transgene, the MAR coincide to SV40 sequences already reported by Pommier et al. (52) as having the highest in vitro affinity for nuclear matrix in the entire SV40 genomic DNA. The SV40 sequences present in the hNF-L/lacZ transgene bind to nuclear matrix with a lower affinity (52). In this construct, the region having the highest affinity for nuclear matrix is localized at the 3′-UTR of the lacZ

20% of input DNA probe (lanes 1) and autoradiographed (A, C, E). Under each panel, a schematic representation of the constructs summarizes the binding pattern. The thickness of the band underlying the construct region is indicative of the relative attachment in the binding assays (B, D, F). A, the plasmid pNF-L/CAT construct was digested with HindIII, Scal, and BamHI restriction enzymes. DNA fragments were labeled and served as probes. This digestion pattern generated bands at 320 bp (band 1), 657 bp (band 2), and 972 bp (band 3) and 2.8 kb corresponding respectively to the hNF-L promoter, the CAT gene, the SV40 polyadenylation signal, and the pUC-9 plasmid. C, the plasmid pNF-L/lacZ was digested with HindIII, EcoRI, and BamHI restriction enzymes prior to end-labeling. Bands at 320 bp (band 1), 3.7 kb, 450 bp (band 2), 750 bp (band 3), and 2.95 kb corresponding respectively to the hNF-L promoter, the lacZ gene, the 5′-segment of the 3′-UTR, the 3′-segment of the 3′-UTR, and the Bluescript plasmid. E, the plasmid pNF-L/intronless was digested with EcoRI, BglII, and XbaI restriction enzymes prior to end-labeling. This generate bands at 1,120 bp (band 1), 1,220 bp, 485 bp (band 2), and 2.95 kb corresponding respectively to the hNF-L promoter and first exon, mNF-L exons 2, 3, and 4, the hNF-L 3′-UTR, and the Bluescript plasmid.
reporter gene. In addition, these experiments enabled us to identify a novel MAR element in the 3′-UTR of the hNF-L gene. By sequence comparison between the hNF-L/lacZ and hNF-L/intronless MARs, we have tried to highlight similarities that could not be found in the hNF-L/CAT transgene. No highly conserved sequence motif was found that could explain their common neuronal expression pattern, except for their A/T-rich sequences.

One intrinsic property of A/T-rich sequences is that they unfold easily (53). DNA single-stranded regions are formed by opening of the double helix under torsional stress in different paranemic structures (reviewed in Ref. 54). Sequences composed of a minimum of 15–20 consecutive purines on one strand have a tendency to present that state (55–61). In vivo, unpaired sequences can be identified by digestion of nuclei with S1 nuclease. This generates a pattern of single-strand nuclease-sensitive sites. The presence of single-strand nuclease-sensitive sites has revealed that single-strand nuclease-sensitive sites are generally found in promoter regions (62, 63), within genes (59, 64), or at the 3′ end (65). Their occurrence on chromatin coincides with a region of transcriptional activity (66–68). It has been demonstrated that DNA strand unpairing could be induced by supercoiling and that this phenomenon is independent of external protein factor for its initiation (66). This characteristic has allowed us to directly test the presence of unpaired sequences in supercoiled plasmids containing the different MARs. Sequences with unwinding properties were present only in plasmids containing NF-L fusion constructs correctly expressed in the nervous system of transgenic mice. It was found within the associated MAR and colocalized with polyuridine sequences.

Position Effect—Position effect is characterized by the influence on transgene expression of regulatory elements placed at the vicinity of the insertion site. Using an enhancer trap vector, it has been demonstrated that insertion near such elements is a rare event because only a weak proportion of transgenic mice lines expressed the reporter gene (69). Recently there has been much evidence supporting the idea that MARs can establish a domain of independent gene activity and insulate a reporter gene from chromosomal position effects in transgenic animals (18, 26).

The hNF-L/intronless and the hNF-L/lacZ transgenes are basically not susceptible to insertion site interference for their tissue-specific expression, whereas the hNF-L/CAT construct is highly sensitive to position effect (1). A conceivable explanation for the different expression patterns of these hNF-L/reporter gene constructs in transgenic mice is that the different transgenes are in different chromatin configurations, the hNF-L/intronless and the hNF-L/lacZ transgenes being in independent loop domains while transcription of the hNF-L/CAT transgene is dependent on the integration site because it does not carry the information for self-modulation of chromatin structure.

Unwinding elements within the MARs were found only in the two NF-L transgenes correctly expressed in the nervous system. Our results show that when this element is deleted from the lacZ reporter gene, only two out of seven transgenic embryos did express the transgene but in a nonspecific fashion in contrast to 6 out of 7 transgenic embryos expressing correctly the complete hNF-L/lacZ construct. This indicates that removal of the unwinding element made expression of the lacZ transgene insertion-dependent. Using a lacZ transgene with a deleted 3′ end, Allen et al. (69) have shown that one out of six transformed embryonal stem cells expressed a hsp/lacZ transgene, this weak proportion of expressing versus unexpressing cells being caused by rare insertion events near activating sequences.

Tissue Specificity—Expression patterns observed with the hNF-L/lacZ and hNF-L/intronless transgenes were characteristic of the endogenous NF-L pattern of expression. Without the unwinding element of an MAR, the hNF-L promoter is probably too weak to override chromatin-induced repression as sug-
suggested by the expression of the hNF-L/CAT transgene. We suggest that MARs with unwinding elements can function as domain boundary and generate an independent domain in which a weak promoter, such as the hNF-L basal promoter, is excluded from the influence of MARs with unwinding elements. In this chromosomal context, a promoter is able to drive its reporter gene expression according to regulatory elements contained within this domain.

The same phenomenon is likely to be observed with other basal tissue-specific promoters. A good strategy in assessment of a promoter transcriptional characteristic would be to test this promoter with and without MAR with unwinding elements. The lacZ reporter gene from pCH110 is a good choice because the unwinding element influence can be removed easily by a BamHI digestion. The addition of MARs with DNA unwinding elements should be considered when designing transgene or viral vectors because it might circumvent some of the expression problems usually encountered.

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