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Regulation of the atherosclerosis candidate gene Aop2

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it retains DNA binding activity and thus might behave as a dominant negative. Therefore, we overexpressed ΔSF-1 in bovine adrenocortical cells to determine its effects on the production of four proteins involved in steroid biosynthesis: P450scc, steroidogenic acute regulatory (StAR) protein, 3β-hydroxysteroid dehydrogenase (3β-HSD), and the transcription factor DAX-1. Cells stably expressing ΔSF-1 were generated, clones isolated, and protein expression verified by Western and gelshift analyses. Western analysis of cellular extracts revealed reduced DAX-1 and P450scc content. The increase in P450scc and StAR expression that resulted from cAMP stimulation for 24 h in wild-type cell lines was markedly attenuated in ΔSF-1 clones. By contrast, DAX-1 production was reduced following cAMP treatment in both control and mutant clones, indicating this downregulation was SF-1 independent. Interestingly, irrespective of treatment, 3β-HSD levels were relatively unaffected by the presence of the mutant protein in most clones. These data are consistent with a role for SF-1 in P450scc and DAX-1 gene regulation and provides evidence for SF-1 involvement in cAMP-stimulated StAR expression. Supported by NIA grants AG12287, AG13663 to PJH; SRK by T32-AG000183-10.

1605

MISEXPRESSION OF *cNSCL2* CAUSED RETINAL PHOTORECEPTOR DEGENERATION

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The purpose of this study was to elucidate the molecular mechanisms underlying retinal development by examining how bHLH genes are involved in the development of various types of retinal cells. Retrovirus expressing *cNSCL2*, a bHLH gene normally expressed in horizontal cells and amacrine cells, was microinjected into the chick subretinal space at embryonic day 2 (E2). Retrovirus expressing GFP was used as a control. Retinas were harvested at different developmental stages and retina cell populations were analyzed. Misexpression of *cNSCL2* in the developing retina reduced the number of cone photoreceptor cells (*visinin* mRNA+), and resulted in alterations of the outer nuclear layer structure. In the outer nuclear layer, *visinin*-expressing cells formed rosette structures and between the rosettes were inner nuclear neurons. The numbers of ganglion cells, amacrine cells, and horizontal cells were not decreased. The experimental embryos had normally-sized eyes that appeared grossly indistinguishable from control eyes. Misexpression of *cNSCL1*, a closely related bHLH gene sharing 96% identity in the bHLH domain and expressed in young ganglion cells, did not result in a decrease in photoreceptor cells when misexpressed in a similar manner. This suggests that reduction of photoreceptor cells was related to an intrinsic property of *cNSCL2*. Our data indicate that *cNSCL2*, a bHLH gene expressed in horizontal and amacrine cells, negatively affects photoreceptor gene expression, and suggest that both positive and negative mechanisms may be employed in retinal development for the production of various types of cells.

1606

Differential Gene Expression Patterns Between human Hippocampus and Other Brain Tissues

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The relative expression levels of genes in human hippocampus versus other brain tissues were determined using Human GeneFilters (GF211, Research Genetics) with ~ 4,000 genes hybridized with complex probes prepared from mRNAs of pooled individual human brain tissues. Under standard hybridization conditions (42o C for hybridization and 50oC for washes), the results showed no significant difference in gene expression patterns between the hippocampus and the amygdala. No genes were expressed more abundantly in the hippocampus than in the thalamus and only 2 genes more abundantly in the thalamus than in the hippocampus. Fourteen genes were expressed more abundantly in the hippocampus than in the cerebellum and 6 genes more abundantly in the cerebellum than in the hippocampus. Two genes were expressed more abundantly in the hippocampus than in the spinal cord and 5 genes more abundantly in the spinal cord than in the hippocampus. In addition, we also tested the effect of hybridization conditions on the output results of the relative gene expression levels of the hippocampus and the spinal cord. When the hybridization and wash stringency was increased from 42o C to 50o C and 50oC to 55 oC, respectively, the results showed 10 genes (involved in the signaling pathway of PKC or in the neuronal activity) were expressed more abundantly in the hippocampus than in the spinal cord and 36 genes (highly associated with immune/inflammatory responses or recruitment of lymphocytes) more abundantly in the spinal cord than in the hippocampus. Most differentially expressed genes between the hippocampus and the spinal cord detected at the low stringency were also found at the high stringency condition. These results reveal part of the differential roles and biological functions between hippocampus and spinal cord as well as the feasibility of using high-density cDNA-arrays for characterization of specific subsets of gene transcripts between two brain tissues.

1607

Conditional Knock-In mice Harboring An Amino Acid Substitution of NMDA Receptor

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To establish an amino acid substitution of the relevant gene in a cell-type specific manner in mice, we developed a new method by generation of two types of mice by utilizing the splicing mechanism and the Cre-loxP recombination, and applied our method to substitution of the amino acid critical to the voltage-dependent Mg^{2+}-block of the NMDA receptor (NMDAR). One of the two mice (NMDAR-flox mouse) carries both normal and mutant exons of NMDAR gene in tandem arrangement, an artificial intron in between and two loxP sites on both sides of normal exon, and has an expression of only normal NMDAR before Cre-loxP recombination. The other mouse is a transgenic mouse (Cre mouse) expressing Cre recombinase under the control of nestin promoter, which is activated in CNS. To induce an amino acid substitution in cell-type specific manner, NMDAR flox-Cre mice were genarated by mating NMDAR-flox mice with Cre mice, and analyzed molecular biological, histological and electrophysiological methods. The recombination of NMDAR gene was confirmed in CNS and germ cells. RT-PCR analysis revealed that mutant NMDAR was not expressed at all before Cre-loxP recombination and detected only in the NMDAR-flox-Cre mice. There was no difference in cell architecture of brain between in the wild-type and NMDAR-flox-Cre mice by histochemical analysis. On the other hand, whole-cell electrophysiological recordings in hippocampal slices indicated that mutant NMDAR exhibited altered property of voltage-dependent Mg^{2+} block in CA1 pyramidal cells. These data clearly indicate that we succeeded in developing a new method for an amino acid substitution in mice in cell-type specific manner.

1608

Keratinocyte growth factor (KGF) is expressed in the hippocampal neurons and attenuates ischemia-induced delayed neuronal death

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Fibroblast growth factors are polypeptides with various biological activities in vivo and in vitro, and their receptors are expressed in the widespread and specific neuronal populations of the adult brain. In the present study, we asked whether KGF would express in the brain, and have protective against ischemic brain injury. In situ hybridization analysis revealed that intense silver grains for KGF mRNA are observed in the neuronal cells of the cerebral cortex, hippocampus and amygdala in rat brain. Continuous cerebroventricular infusion of KGF for a 7 day period to gerbils starting 2 days before temporary right carotid artery occlusion (20 min) caused higher survival rate compared to vehicle-treated ishemic animals. In situ detection of DNA fragmentation (TUNEL) revealed that ischemic animals infused with KGF contained fewer TUNEL-positive neurons in the hippocampal CA1 field than those infused with vehicle alone at the 4th and 7th day after ischemia. These results suggest that KGF has a protective effect against ischemic hippocampal neuronal damage in vivo, which may provide new therapeutic strategy in the survival of neurons in response to cerebral injury. Supported by Mitsui Life Social Welfare Foundation, and grants (11877270, 12470322), Japan.

1609

Regulation of the Atherosclerosis Candidate Gene Aop2

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We previously have identified Aop2 (Antioxidant Protein 2) as a candidate gene for atherosclerosis susceptibility differences in female mice. AOP2 is a member of the thiol-specific antioxidant (TSA) family of proteins that are capable of reducing hydroperoxides in the presence of a thiol-containing electron donor. TSA proteins are highly evolutionarily conserved, although little is known about their biological role in mammalian cells. We thus sought to investigate the regulation of Aop2 in the liver, where it is most highly expressed. Since male mice are resistant to atherosclerosis, we first compared Aop2 expression between males and females and found higher levels in males. We next examined the effect of oxidative stress on Aop2 expression in the H2.35 mouse hepatocyte line. While H202 had no effect, Aop2 levels were significantly elevated in response to glucose oxidase-induced H202 production. This induction occurred within 15 minutes, remained elevated at three hours, and returned to basal levels by five hours.

Analysis of the Aop2 proximal promoter reveals a potential NF-kappa B binding site, suggesting a possible mechanism for this regulation. We found no induction of Aop2 with serum or KGF treatment, despite previous reports that KGF up-regulates Aop2 in serum-deprived keratinocytes. Interestingly, however, we did observe serum and KGF regulation of another gene we believe to be the highly related intronless gene Aop2-rs1. This gene is down-regulated in serum deprived cells, and is induced by serum and KGF after only one hour of treatment. In conclusion, our data strongly support an early role for Aop2 in the oxidative stress response, and suggest a distinct function for the highly related Aop2-rs1 in cell growth. Due to the involvement of oxidative damage in atherosclerosis progression, the study of Aop2 regulation may provide clues to its possible protective role in this process.

1610

The Transcription Regulation of Mouse Trichohyalin Gene in Cultured Mouse Hair Follicle Cells

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Trichohyalin (THH) is a major structural protein of the inner root sheath cells of the hair follicle and participates in the assembly of the cell envelope barrier structure during the late stage of differentiation in a variety of epithelia. Recently we cloned the mouse THH promoter region and studied its transcriptional regulation in cultured mouse hair follicle cells. A series of CAT constructs containing several different size of inserts between –478bp upstream from the transcription start site and the first exon were transfected into mouse hair follicle cells. The results showed as few as 250 bp are sufficient to confer strong promoter activity. Within this region, we found several positive and negative elements including: two Sp1-like motifs (-234, -135), AP1 site (-115), AP2 site (-69), two unidentified positive motifs (-191, –160) named E1 and E2, and one unidentified negative element (-126) named R1. Supershift assays revealed that Sp1, Sp3, c-Jun, AP2-α, AP2-γ each bind to its corresponding motif. In the context of the pCAT (-250/+36) construct, separate mutations of Sp1 (-234), AP1, AP2 and E1 (-191) showed a 40% to 50% reduction of CAT activity, indicating that these motifs are necessary but not sufficient to confer the mTHH promoter activity. In addition, mutation of either Sp1 (-135) or E2 (-160) reduced the promoter activity to the basal level, suggesting that these two elements are essential for the promoter activity. However, mutation of R1 (-126) increased CAT activity 75% higher than the wild type. Therefore complex interactions between several positive and negative elements on the promoter region are important for the regulation of mTHH expression in the cultured mouse hair follicle cells.

1611

Involvement of SP1, SP3, CREB and AP1 in Regulation of the Expression of Human Loricrin.

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Loricrin is the major protein of the cornified cell envelope of the epidermis, which is essential for barrier function. The expression of loricrin is tightly controlled during the terminal differentiation of keratinocytes. Previously we have documented that three functional motifs of Sp1 (-125), CRE-like (-100) and AP1 (-60) and a keratinocyte-specific negative element with sequences of CAGCCAC, named R1 (-110), were located within the first 145 bp upstream of transcription start site. Also supershift data showed that Sp1, Sp3, CREB-1, cJun and cFos bind to their respective motifs. In this study, we have explored the transactivation interplay between these neighboring sites. First, co-transfection of Sp1 and Sp3 expression plasmids with loricrin constructs showed 40% increase and 30% decrease of CAT activity, respectively, in normal human epidermal keratinocytes (NHEK). Forced expression of cJun, p300, CBP overcame the R1 repression and led to a 6, 5 and 3-fold, respectively, increased expression in NHEK cells. Addition of 8-bromo-cAMP or Forskolin after transfection caused 50% and 80% increase, respectively, in the promoter activity. Interesting, cotransfection of CREB-1, CREMa, ATF-1 or ATF-2 expression plasmids caused more than 80% reduction in CAT activity indicating these transcription factors may together sever as a repressor. Furthermore, results from gel-shift data indicated that increases of cAMP levels reduce the binding activity of Sp3 but not Sp1 suggesting that the phosphorylation status of Sp3 might affect its binding on the negative motifs. Together, these data suggest that the ratio of Sp1 and Sp3, dimerization between different CREB family members, and the interplay of between Sp1, CBP, p300 and AP1 might play an important role in the cell-type and differentiation-specific expression of loricrin in epidermal keratinocytes.

1612

Dual role for the proximal promoter of the SP-10 gene: enhancer in spermatids and insulator in all other cell-types.

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Transcription of the SP-10 gene, which encodes an acrosomal protein, is initiated postmeiotically in the haploid round spermatids during mammalian spermatogenesis. Promoter analysis in transgenic mice indicated that either the -408/+28 (436 bp) or the -266/+28 (294 bp) proximal promoter of the SP-10 gene was sufficient to activate spermatid-specific gene expression (Reddi et al., 1999, Biol Reprod 61: 1256-1266). No position effects were evident in any mouse line that expressed the transgene, in that no ectopic expression was observed. The objective of the present study was to test the hypothesis that SP-10 promoter fragments shielded the transgene from position effects and prevented transcription in somatic cells. To address this, functional assays were performed using reporter gene constructs in which a heterologous CMV enhancer was placed upstream of the SP-10 promoter fragments to mimic the integration of the transgene adjacent to a pan-active enhancer. In transiently transfected COS-7 cells, CMV enhancer upstream of SP-10 core promoter (-91/+28) yielded high level of transcriptional activity. However, insertion of the -408/-92 SP-10 promoter (but not a stuffer fragment) between the core promoter and the enhancer resulted in a 70-fold inhibition of transcription. This enhancer-blocking activity was directional and position-dependent, characteristic features of an insulator. Next, the insulator function was tested in the context of chromatin by generating transgenic mice. Previous reports showed that CMV enhancer activated transcription in 24 different tissues in transgenic mice. The present study showed transgene expression in spermatids but not in somatic tissues by northern and RT-PCR indicating that the SP-10 promoter blocked the CMV enhancer activity. These data provide evidence for both activator and insulator function of the SP-10 promoter in vivo and offer a possible explanation for the integration site-independent promoter specificity in transgenic mice. Supported by NIH HD36239 (PPR) NIH HD29099 (JCH).

1613

Hypermethylation of the Testis-specific Histone H1t Gene May Contribute To Transcriptional Repression in Nongerminal Cells

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The testis-specific histone H1t gene is actively transcribed in pachytene primary spermatocytes. Previous work using transgenic mice demonstrated that the proximal promoter of H1t was sufficient for testis-specific transcription of the H1t gene. Additionally, replacing the TE element of the H1t promoter with heterologous DNA abolished H1t expression in all tissue types tested. The H1t proximal promoter contains five CpG dinucleotides, two of which are found within the essential TE element. Two additional CpG dinucleotides are found immediately downstream of the message start site. An in vivo analysis of the methylation status of the H1t proximal promoter, using genomic DNA derived from primary spermatocytes, revealed that all seven CpG dinucleotides contain unmethylated cytosines. When using genomic DNA derived from liver cells, where the H1t gene is repressed, all seven of these CpG dinucleotides are methylated. We have also shown using transient transfection assays that in vitro methylation of an H1t promoter-driven Luciferase reporter plasmid reduced expression by more than 30 fold compared to an unmethylated reporter plasmid. Our findings suggest that methylation of cytosines within CpG dinucleotides in the H1t proximal promoter may contribute to the transcriptional repression of the H1t gene in non-expressing cell types. This work was supported by the Department of Veterans Affairs and NIH grant HD29381.

1614

Binding of nuclear proteins to an upstream element within the histone H1t proximal promoter correlates with transcription.

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The testis-specific histone gene is expressed only in the testis in the pachytene spermatocyte stage of spermatogenesis. Testis-specific expression of the rat H1t histone gene in transgenic mice is regulated by 141 base pairs of the proximal promoter. The H1t proximal promoter contains two testis-specific elements, TE1 and TE2 along with all of the consensus elements common to the somatic promoters, an AC box, a GC box, a CCAAT box, and a TATA box . We have identified another potential regulatory element positioned from 134 to 106 nucleotides upstream of the histone H1t transcriptional initiation site by in vitro DNase I footprinting. Nuclear proteins from testis bind to the corresponding 28 bp element and produce a unique low mobility complex as observed in electrophoretic mobility shift assays. Upon examination of nuclear proteins derived from enriched populations of germinal cells, the binding activity is abundant in pachytene spermatocytes but reduced in early spermatids. The presence of