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Previously, many neuroblastoma and related somatic hybrid cell lines were shown to acquire voltage-sensitive ion channels and other neuronal properties when intracellular cyclic AMP levels were elevated for a number of days. Cells with elevated cAMP acquire new proteins such as the α-subunit of voltage-sensitive calcium channels and other proteins of unknown function. A cDNA library was constructed from poly A^+ RNA prepared from NG108-15 neuroblastoma-glioma hybrid cells that had been treated for 5 days with 1 mM dibutyryl cAMP. The library was screened and 17 cDNA clones were obtained that correspond to species of RNA that are 3 to 40 times more abundant in cells treated with dibutyryl cAMP than in cells cultured without this compound. Each cloned cDNA was used as a probe with Northern blots to determine the number of species of poly A^+ RNA responsive to dibutyryl cAMP and the chain length of each species of RNA. The results suggest that the 17 cDNA clones correspond to species of RNA transcribed from 10 genes. Partial nucleotide sequences of the cDNA inserts from 3 clones were obtained. Clone NG-32 corresponds to mouse mitochondrial mRNA for ATP synthase subunit 6, a mitochondrial gene. This protein is part of the H^+ channel portion of the mitochondrial ATP synthase complex. Treatment of NG108-15 cells with dibutyryl cAMP results in an 8-fold increase in the abundance of mRNA for this protein. The nucleotide sequence of clone NG-10 cDNA was identified as part of the D-loop region of mouse mitochondrial DNA that contains the origin of replication for the heavy strand of DNA. The 5'-terminal nucleotide sequence of some molecules of heavy strand mitochondrial DNA is known to consist of a short segment of RNA that is complimentary to a short light strand mitochondrial DNA sequence nearby. Hence, NG-10 cDNA may correspond to an RNA transcript of the light strand of mitochondrial DNA that serves as a primer for the initiation of heavy strand mitochondrial DNA synthesis. Treatment of NG108-15 cells with dibutyryl cAMP results in a 40-fold increase in this species of RNA. These results show that treatment of NG108-15 neuroblastoma-glioma cells with dibutyryl cAMP results in marked increases in the abundance of RNA transcripts from heavy and light strands of mitochondrial DNA. Further work is needed to determine whether cAMP regulates mitochondrial biogenesis or the ability to synthesize ATP.

A λgt11 cDNA library was prepared from rat brain poly A^+ RNA and screened with oligodeoxynucleotide probes that correspond to the α-subunit of L-type voltage-sensitive calcium channels. Eleven positive clones were detected that have cDNA inserts 1.6-5.5 Kb in length. Nucleotide sequence analysis reveals strong homology as well as differences in the deduced amino acid sequences of the α-subunits of rat brain and rabbit skeletal muscle L-type

voltage-sensitive calcium channels.

To detect recombinant DNA clones that correspond to novel homeobox genes a Drosophila genomic DNA library was screened with multiple oligodeoxynucleotide probes, each designed to hybridize to multiple homeobox genes. Five clones that gave positive signals with 2 or more oligodeoxynucleotide probes exhibited specificities that could not be explained on the basis of known nucleotide sequences of Drosophila homeobox genes. Nucleotide sequence analysis of the homeobox regions of 4 clones revealed 4 new homeobox genes (NK-1,2,3,4). Two recombinant clones contained identical DNA inserts, each insert contained 2 new homeobox genes (NK-3 and NK-4). The deduced amino acid sequence of the NK-1 homeobox exhibits the highest homology to the homeobox regions of deformed, zen-2, and zen-1 (75, 72, and 71% homology, respectively). The relative homology of the NK-2 homeobox is as follows: NK-4 > NK-3 > NK-1 = IAB-7. The homology of NK-3 is: NK-2 > labial > NK-4 > NK-1; and NK-4 homology is NK-2 > zen 2 = NK-3 > labial. Genomic DNA fragments from the 4 new homeobox genes were used to screen cDNA libraries prepared from poly A^+ RNA from 0-3 hr Drosophila embryos or from 3 - 12 hr embryos. One NK-1 cDNA clone was obtained from the 3-12 hr embryo cDNA library, but none was detected in the 0-3 hour embryo libary. Comparison of the nucleotide sequences of NK-1 cDNA and genomic DNA clones showed that the NK-1 gene has 3 exons. One of the 2 introns detected resides within the homeobox region.

Regulation of rat neuropeptide Y gene expression:

Untreated PC12 rat pheochromocytoma cells and N18TG-2 mouse neuroblastoma cells possess relatively low basal levels of neuropeptide NPY (0.25 and 0.13 pg/μg total RNA), while NG108-15 mouse neuroblastoma x rat glioma hybrid cells contain remarkably high amounts (11 pg/μg RNA). Untreated human neuroblastoma lines SK-BN-SH and SK-N-MC also contain relatively high amounts of NPY mRNA.

During the past year we studied the regulation of NPY mRNA abundance in PC12 cells by cAMP, phorbol esters, glucocorticoids, and calcium ionophore. The results are as follows:

Cyclic AMP elevation by forskolin or 8-bromo-cAMP elicts moderate elevation (4-10-fold) over 12-48 hr of treatment and synergizes with phorbol ester.

Phorbol esters such as phorbol 12-myristate 13-acetate (PMA) that activate protein kinase C elicit little or no effect alone but synergize with cAMP to produce large elevations (20-200-fold) over 12-48 hr of treatment. Responses to phorbol ester are enhanced by A23187, a calcium ionophore, which increases the cytoplasmic Ca^{++} ion concentration.

Nerve growth factor (NGF (2.5 S) strongly elevates NPY mRNA (40-100-fold) during 1-6 days of treatment. The increase is evident by as early as 3 hr of NGF exposure and is sensitive to cycloheximide, indicating a requirement for protein synthesis. Phorbol ester synergizes with NGF to produce 300-fold elevations in NPY mRNA.

Glucocorticoids such as dexamethasone (Dex) elicit 2-3-fold potentiations of the cAMP and phorbol-ester elicited elevations of NPY mRNA. Dex biphasically modulates the stimulations by NGF, potentiating early (3-10 hr) effects of NGF but profoundly inhibiting the large stimulations at later times (1-6 days). The latter inhibitory phase resembles previously described antagonisms by glucocorticoids of NGF inductions of specific mRNAs.

We have looked for an effect of NGF treatment on the stability of NPY mRNA. Upon addition of actinomycin D to inhibit RNA synthesis, NPY mRNA in control and NGF-treated cells decayed with half-lives of 5 and 9 hr, respectively. We conclude that part of the increase in NPY mRNA due to NGF is a consequence of increased NPY mRNA stability, but that transcriptional activation must also take place to account for the 40-100-fold elevation of NPY mRNA. Regulation of NPY mRNA levels also was observed in SK-N-MC human neuroblastoma cells, which have a high constitutive expression of the gene. Dex, forskolin, or PMA have no effect alone, but Dex + forskolin and forskolin + PMA double the NPY mRNA level.

Regulation of proenkephalin (pEnk) gene expression.

Glucocorticoids and cAMP synergistically increase the abundance of pEnk mRNA in C6 rat glioma cells. We examined the mechanism of this increase by run-on transcription experiments involving nuclei isolated from C6 cells treated with or without Dex and/or forskolin for 1-24 hr. Dex alone had no effect on the pEnk transcription rate, and forskolin alone elicited a brief stimulation that reached 6-fold at 1 hr. Dex + forskolin elicited a more sustained stimulation of 5-6 fold at 2-6 hr. These results suggest that cAMP elevates pEnk mRNA in C6 cells by stimulating transcription and that glucocorticoids exert a permissive effect by sustaining the stimulation by cAMP.

We are presently searching for a putative glucocorticoid regulatory element of the pEnk gene by transient expression assays of plasmids having portions of the pEnk gene linked to a reporter gene, chloramphenicol acetyltransferase (CAT). Rat proenkephalin genomic clones were isolated and mapped. A fragment containing 2500 bases of the 5' upstream region and 47 bases of the first exon was ligated in both orientations in front of the CAT gene in a promoterless vector, and this construct was transfected into C6 rat glioma cells. CAT expression was found to respond as expected to forskolin but did not respond, either with or without forskolin, to Dex. Thus the putative element is not within the sequence tested. Since regulatory elements are sometimes found in introns, another construct that contains the above sequence plus the rest of the first exon and all of the first intron has been constructed and is being tested.

We found that untreated SK-N-MC human neuroblastoma cells contain a surprisingly high abundance of pEnk mRNA. Treatment of the cells with forskolin elevated the level 2-fold. PMA + A23187 markedly reduced the level. Dex did not affect basal or forskolin-stimulated levels but did increase the levels in the presence of the inhibitory combination forskolin + PMA + A23187. Thus, cooperative regulation of the human pEnk gene by cAMP and glucocorticoids exists, as with the rat gene, but is

quantitatively less significant in cells having a high constitutive expression of the gene.

Cytoplasmic components of ACh receptor aggregates - We previously showed that vinculin, α-actinin and filamin, all of which are cell adhesion associated proteins, are concentrated at the sites of newly formed nictonic acetylcholine (ACh) receptor aggregates. However, the resolution of our techniques was not adequate to determine the localization of proteins within the aggregates. We have devised a novel technique to overcome this problem. Using a selective replating of myoblasts, large myotubes are grown with very few fibroblasts. A coverslip coated with a purified polypeptide adhesive from shellfish is attached to the upper cell surface, where the ACh receptor aggregates have formed. This coverslip is then lifted, with plasma membranes and partially disrupted myotubes attached, allowing access of antibodies to the membrane cytoskeleton, a high degree of spacial resolution in the plane of the membrane, and minimal cytoplasmic background staining.

With this technique, we have found that vinculin and associated actin filament bundles are adjacent to, but not superimposed upon the Ah receptor enriched domains of the aggregates, while a 43 kilodalton protein (the "43K protein", closely associated with ACh receptors in other systems) and actin in another form are precisely colocalized with the receptors. These results suggest the existence of at least 2 distinct membrane cytoskeleton domains within the newly formed ACh receptor aggregates.

These membrane preparations also facilitated the precise localization of clathrin, the major protein of the coated vesicle "basket". Clathrin is not obviously concentrated in ACh receptor aggregates and is excluded from the ACh receptor enriched domains, but most aggregates contain clathrin in the form of tiny speckles, possibly corresponding to coated pits involved in exocytosis or endocytosis.

Immunogold localization of ACh receptors, the 43K protein and sodium channels. We have succeeded in labeling 2 integral membrane proteins (ACh receptors and sodium channels) and the 43K protein at the electron microscopic level by a postembedding immunogold technique. At both the neuromuscular junction (NMJ) and the ACh receptor aggregates formed in culture, the ACh receptors and the 43K protein are precisely colocalized. Sodium channels are concentrated at the NMJ at a lower apparent density than ACh receptors or 43K protein. However, unlike the ACh receptors, which are concentrated at the crests of the postsynaptic membrane folds close to the nerve ending, the sodium channels are distributed throughout the folds. This result confirms, with higher resolution, results previously obtained with the immunoperoxidase technique.

In cultured myotubes, ACh receptors and the 43K protein are also colocalized in apparently intracellular membrane compartments which remain to be characterized.

Escherichia coli the interaction of cAMP with the cAMP receptor protein (CRP) induces a conformational change in the structure of the protein thereby converting it to a form that is active in regulating gene expression. This regulatory protein has been mutated to a form that functions in gene transcription in the absence of added cAMP. It was the purpose of this study to isolate one of these mutant proteins, the NCR91 protein, crystallize it, and determine the X-ray crystallographic structure of the protein for comparison with that of the structure of the wild-type protein.

Since the cAMP synthesizing system in Escherichia coli is regulated by the sugar transport system known as the phosphoenolpyruvate:sugar phosphotransferase system (the PTS), an interest in our laboratory has been to understand the various mechanisms by which the process of sugar transport is regulated. One of the mechanisms that has been described in gram-positive bacteria for regulation of sugar transport is the process of inducer expulsion. When Streptococcus pyogenes are grown under conditions for the induction of the lactose transport system and then allowed to take up a nonmetabolizable substrate for this system (thiomethylgalactoside, TMG), the subsequent addition of glucose elicits the rapid release into the medium of the accumulated TMG. In this organism, the uptake of TMG is via the PTS and it is presumed that the PTS is involved in the expulsion mechanism. It was the purpose of this study to examine the mechanism of sugar transport regulation in an organism that transports sugars by a mechanism other than the PTS. A representative heterofermentative lactobacillus, Lactobacillus brevis was chosen for the study.

The organisms used in the studies were Escherichia coli, Lactobacillus brevis and Lactobacillus buchneri. Cloning the gene for the CRP protein from strain NCR91 of Escherichia coli provided a basis for hyperexpression of the protein. The hyperexpressed protein was purified by a conventional method to produce homogeneous CRP91. Crystals of the protein were grown in the presence of cAMP under the same conditions as for the wild-type protein (in the presence of phosphate buffer at room temperature). Diffraction data on a single crystal were collected on a Nicolet imaging proportional counter and then processed on a VAX computer. A difference Fourier map of the CRP91 and wild-type CRP proteins was calculated using the program PROTEIN. Uptake of thiomethylgalactoside into Lactobacilli was measured using the radioactive sugar analog. Pools of free or phosphorylated thiomethylgalactoside were measured in boiled extracts of cells which were then fractionated by anion exchange chromatography. Cell-free extracts of Lactobacilli were prepared by sonication of cell suspensions. Sugar phosphorylation in cell extracts was measured by incubating the extracts with radioactive thiomethylgalactoside or 2-deoxyglucose.

The Crystal Structure of a cAMP-independent Mutant of the cAMP

Receptor Protein: Escherichia coli NCR91 synthesizes a mutant form of the cAMP receptor protein in which alanine 144 is replaced by threonine. This mutated form of CRP can, in the absence of adenylate cyclase, confer on cells the CRP* phenotype which is due to the ability of the mutated CRP to function as a transcription regultor in the absence of cAMP. CRP91 has been purified and crystallized with cAMP under the same conditions as used to crystallize the wild-type CRP-cAMP complex. X-ray diffraction data were measured to 2.4-Angstrom resolution and the CRP91 structure was determined using initial model phases from the previously determined wild-type structure. A difference Fourier map calculated between CRP91 and the wild-type CRP showed the two alanine to threonine sequence changes in the dimer and also a change in the orientation of cysteine 178 in one of the subunits. Refinement of the structure indicated that there were small differences in the CRP91 structure compared to that of the wild-type protein that included concerted motions in the small domains, in the hinge region between the two domains and in an adjacent loop between beta-strands 4 and 5. These findings indicate that the mutation at residue 144 causes changes in the position of some protein atoms that are distal to the mutation site.

Regulation of beta-Galactoside Transport and Accumulation in Heterofermentative Lactic Acid Bacteria. Lactobacillus brevis and Lactobacillus buchneri are examples of heterofermentative organisms. They do not have a functional phosphoenolpyruvate:sugar phosphotransferase system and transport thiomethylgalactoside by an active transport mechanism that results in the accumulation of intracellular free thiomethylgalactoside. When cells were preloaded with thiomethylgalactoside and then exposed to glucose, there was a rapid efflux of the intracellular galactoside. When the glucose was depleted from the medium by uptake into the cells, then there was a restoration of the uptake and accumulation of thiomethylgalactoside. The glucose-promoted efflux of thiomethylgalactoside required the intracellular phosphorylation of glucose. The glucose-promoted efflux was not inhibited by iodoacetate. These results were interpreted to indicate that a phosphorylated metabolite of glucose at or above the level of glyceraldehyde-3-phosphate was required to evoke displacement of intracellular thiomethylgalactoside from these cells. Experiments using the counterflow technique indicated that exposure of cells to glucose converted the uptake of thiomethylgalactoside from an active uptake mechanism to a facilitated diffusion mechanism that allowed equilibration of thiomethylgalactoside between the intracellular and extracellular spaces. Since this phenomenon in the heterofermentative Lactobacilli had similar characteristics to that of inducer expulsion that takes place in the homofermentative Streptococcus and Lactobacillus species, a possible similarity in the mechanism was explored. The inducer expulsion mechanism in the homofermentative bacteria involves HPr, a protein component of the phosphoenolpyruvate:sugar phosphotransferase system which appears to serve also as a transport regulator. Using complementation assays with strains of Staphylococcus that are

deficient in HPr, it was established that Lactobacillus brevis extracts have HPr activity although this organism lacks a functional phosphoenolpyruvate:sugar phosphotransferase system. This study is consistent with the idea that HPr can function as a sugar transport regulator independently of its role as a phosphocarrier in sugar transport.