**1879**

**EXPRESSION AND PURIFICATION OF MITOCHONDRIAL Na'/H' EXCHANGER**

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Recently, a human mitochondrial Na'/H' exchanger (NHE6) was cloned. This protein plays an important role in maintaining the mitochondrial Ca²⁺ homeostasis. This is achieved by an integrated cycle of Ca²⁺ uniport and Na'/H', and Na'/Ca²⁺ antiporter pathways located in the inner membrane of mitochondria. Until now, the Na'/H' antiporter was only characterised after partial purification and reconstitution into proteoliposomes. Cloning of NHE6 gives a unique opportunity to study structure-function relationship of the homogenous mitochondrial exchanger in a fully controlled lipid environment. The NHE6 gene was cloned into pET29 expression vector and will be expressed as fusion protein with 6x Histidine residues and S-Tag in Escherichia coli. Expression of NHE6 in E. coli will be achieved under the transcriptional regulation of the T7 promoter. Expressed protein will be purified from inclusion bodies using divalent cations (Ni²⁺). NHE6 after reconstitution into asolectin proteoliposomes will form a homogenous mitochondrial exchanger in a fully controlled lipid environment. The NHE6 gene was cloned into pET29 expression vector and will be expressed as fusion protein with 6x Histidine residues and S-Tag in Escherichia coli. Expression of NHE6 in E. coli will be achieved under the transcriptional regulation of the T7 promoter. Expressed protein will be purified from inclusion bodies using divalent cations (Ni²⁺) immobilized on the His-Bind Resin (Novagen). Activity of NHE6 after reconstitution into asolectin proteoliposomes will be studied with pH-sensitive fluorescent probe BCECF (2',7'-bis-(2-carboxyethyl)-5-(and)-6-carboxyfluorescein, acetoxyxymethyl ester).

References

**1880**

**The role of the MSX1 gene in the formation of tooth buds.**

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It has been reported that the knockout mice devoid of MSX1 gene lack tooth buds. (1) Another report stated that a missense mutation in the human MSX1 gene, resulting in substitution of Arg → Pro within the homeodomain protein MSX1, causes selective tooth agenesis. (2) However, our investigations of the MSX1 gene in the patients with familial or sporadic tooth agenesis have shown in 7 out of 12 patients only a C → T transition (3). This mutation is located in the region encoding 3'UTR and is situated 6 bp downstream beyond the amino acid coding sequence. In the entire coding sequence of the gene clearly there were no mutations found. In the culture of lymphocytes derived from the patients carrying this mutation we found a decreased stability of the MSX1 gene transcript. We concluded that the mutation in the 3'UTR might change stability of mRNA coding for the MSX1 protein (4).

In order to verify our hypothesis, the region of the MSX1 gene encoding 3'UTR, derived from the patient homozygous for this mutation, was cloned in the expression vector, downstream of the reporter gene (CAT). Subsequently, the lymphocytes maintained in culture were transfected with the recombinant vector and the CAT protein was estimated by ELISA, with the use of specific antibodies. The results revealed that the amount of the CAT protein correlated with the stability of the MSX1 gene transcript, providing further support to the notion that tooth agenesis is due to the decreased stability of MSX1 mRNA.


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**1881**

**The influence of the transcription factor LEF-1 on the expression of the ED4 gene**

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Anhydroptic ectodermal dysplasia (MIM305100) mostly results from mutations in the ED4 gene located on the X chromosome. However, in about 5% of the patients no mutations were found in the entire gene. We described a mutation in the regulatory region of this gene in the patient, who showed typical symptoms of anhydroptic ectodermal dysplasia and no mutations were evidenced in all the exons of the ED4 gene. The mutation was located 32bp upstream from the sequence 5'-CTTGAAGA-3', known as the HK-1 motif. This sequence has been found in the regulatory regions of all keratin genes expressed in human keratinocytes and hair follicles and serves as a target for the transcription factor LEF-1. In order to investigate in detail the influence of the LEF-1 (via HK-1 motif) on the expression of the ED4 gene we cloned human LEF-1 cDNA in the plasmid vector carrying six consecutive His codons. Expression of the recombinant vector in E. coli, followed by affinity purification, yielded a 45 kD protein of the predicted length. The regulatory region, comprising of HK-1 motif from the normal individual and the patient carrying the mutation was inserted upstream of the reporter gene (CAT). The two vectors were used to transfect keratinocytes. Weaker expression of the reporter gene was evidenced in the cells transfected with the vector expressing LEF-1 and cotransfected with the vector carrying the mutated fragment, than with the vector containing normal regulatory region of the ED4 gene. Interaction of the LEF-1 protein with the regulatory region of the ED4 gene was investigated by mobility shift assay. This revealed differential interaction with normal and mutant DNA fragments.

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**1882**

**The barrier function of membrane-bound proteins during the H⁺-transport process in mitochondria**

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Although the idea of proton coupling in mitochondria during the oxidative phosphorylation process is widely accepted, the detailed mechanism of proton translocation through the membrane still is not clear. We have previously reported a method for detection of the local H⁺-gradients on the membrane surface (FITC).

In experiments with mitochondria we have shown that uncoupler additions enhance the respiration initiated by substrates. It is observed in this case, that proton transfer through the membrane proteins reduces after uncoupling. This is indicated by changes in attached probe protonation state. However, substrate addition to previously uncoupled mitochondria leads to weak increase in the respiration rate, and no protonation or deprotonation is detected, indicating that no proton transfer occurs in this case. It is suggested from the kinetics of these processes, that the limiting stage of proton transpor through the membrane during respiration is associated with membrane-bound protein area. It is discussed that this area has the features of immobilized buffer systems.

Since the localization of the gradients discussed was unknown previously, some advantages were made to the method, so that it could enable to locate positions of the probe. Several protein subunits, to which the probe is attached, were determined, including some ATP-synthase subunits.

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