Review Letter

Advances in Na^+,K^+-ATPase studies: from protein to gene and back to protein

N.E. Broude, N.N. Modyanov, G.S. Monastyrskaya and E.D. Sverdlov

Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, 117871 Moscow, USSR

Received 19 August 1989

Complete primary structures of both subunits of Na^+,K^+-ATPase from various sources have been established by a combination of the methods for molecular cloning and protein chemistry. The gene family homologous to the α-subunit cDNA of animal Na^+,K^+-ATPases has been found in the human genome. Some genes of this family encode the known isoforms (αI and αII) of the Na^+,K^+-ATPase catalytic subunit. The proteins coded by other genes can be either new isoforms of the Na^+,K^+-ATPase catalytic subunit or other ion-transporting ATPases. Expression of the genes of this family proceeds in a tissue-specific manner and changes during the postnatal development and neoplastic transformation. The complete exon-intron structure of one of the genes of this family has been established. This gene codes for the form of the catalytic subunit, the existence of which has been unknown. Apparently, all the genes of the discovered family have a similar intron-exon structure. There is certain correlation between the gene structure and the proposed domain arrangement of the α-subunit. The results obtained have become the basis for the experiments which prove the existence of the earlier unknown αIII isoform of the Na^+,K^+-ATPase catalytic subunit and have made possible the study of its function.

Na^+,K^+-ATPase; Isoform; Gene sequence; Exon-intron structure; Protein spatial organization; Gene evolution

1. INTRODUCTION

Na^+,K^+-ATPase is a universal enzymatic system in animal cell plasma membranes that performs the ATP-driven directed transport of sodium and potassium ions [1]. The enzyme releases sodium ions out of the cell and pumps potassium ions into the cell. Thus it regulates the water-salt composition and maintains the ion concentration gradient which, in turn, serves as the motive force for transporting other substances. Na^+,K^+-ATPase is involved in generation of electric signals and participates in most important biological processes.

That is why, for many years, a lot of effort has been put into the study of this enzyme. Biochemical and kinetic research resulted in postulating the basic ideas of the coupling of conformational changes of this enzyme with its cation-transporting functions [2,3]. There is, however, no direct proof at the molecular level that there exists a relationship between the changes of the protein structure and ion transport. Lack of information on the primary structure of the protein and its membrane arrangement is a major reason why there occurs a gap between the structural and functional studies.

For the last 2–3 years our conception of the Na^+,K^+-ATPase structure has been deepened mainly due to the application of molecular cloning and genetic engineering techniques. Due to these new approaches a lot of structural information has been gained rapidly. Furthermore, it has made it possible to study not only the structural elements of the protein which are indispensable for its functioning but also the biosynthesis of the enzyme subunits and its regulation. The results of this research have contributed to the discovery of numerous forms of the Na^+,K^+-ATPase catalytic subunit. This has posed new problems concerning the regulation of ion transport.

Many reviews on different aspects of the Na^+,K^+-ATPase structure and function have appeared for the past two years [4–7]. Our paper summarizes the data on the structure of genes encoding Na^+,K^+-ATPase and considers whether there exists a correlation between the structure of these genes and that of the protein.

2. cDNA CLONING AND PROTEIN STRUCTURE

Na^+,K^+-ATPase belongs to the E1E2 type of

Correspondence address: N.E. Broude, Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, ul. Miklukho-Maklaya 16/10, 117871 GSP Moscow V-437, USSR

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

transport ATPases which form phospho-derivatives of aspartic acid as an intermediate. However, contrary to other transport ATPases of the E1E2 type the sodium pump consists of two subunits. The catalytic subunit (110 kDa) contains all the functional sites of the enzyme. Binding sites for ATP and Na^+ and the phosphorylation site reside in the cytoplasmic region of the α-subunit, binding sites for the enzyme inhibitor, ouabain, and for potassium ions are located in the regions exposed on the other side of the cytoplasmic membrane. The β-subunit (relative molecular mass of the protein portion 35 kDa) is a glycosylated peptide with unknown functions.

Biochemical studies unravelled two molecular forms of the Na^+,K^+-ATPase catalytic subunit found in mammals and arthropods [8–10]. An exhaustive review on this subject can be found in the recent paper by K. Sweadner [6]. Here we will discuss this question only briefly.

Two forms of the α-subunit can be distinguished by their electrophoretic mobility and sensitivity to cardiac glycosides [8]. They were isolated from kidney and axolemma [8,12]. Besides, the two forms differ in sensitivity to proteases, cross-linking agents and *N*-ethylmaleimide [8]. The differences in the primary structure of the N-terminal fragments [12] and in other antigenic determinants of both isoforms have helped to isolate the isoform-specific antibodies [13]. Yet it was not clear, why differences occur in the primary structure: they might arise either because of the existence of several genes for the α-subunit, or because of the post-transcriptional modification of the product of the only gene (alternative splicing of mRNA), or because of the posttranslational modification of the protein product.

In 1985–1986, cDNAs of the α-subunit of Na^+,K^+-ATPase were cloned from the electric organ of *Torpedo* [14] and from the kidney of sheep [15] and pig [16]. Soon afterwards, this was followed by the cloning of the β-subunits from the above mentioned sources [17–19]. Later some articles have been published on the structures of both subunits or only of catalytic subunits from some mammals, birds and insects [20–24]. These structures show a remarkable homology. Even the *Torpedo* and the mammals, which are phylogenetically very distant, have homologous α-subunits (85%). Homology of α-subunits of different mammals exceeds 90%. The β-subunits from different sources also have a high level of homology (65%), but lower than the α-subunits.

Evidently, the folding of α- and β-subunits in the membrane is conserved in evolution, as follows from the similarity of hydropathy profiles for the subunits from different sources.

The α-subunit polypeptide chain contains eleven hydrophobic regions – potential transmembrane domains. Various models imply the α-subunit polypeptide chain to traverse the membrane bilayer 6–8 times [14–16]. Limited proteolysis of the membrane-bound enzyme [25] and immunochemical analysis of the α-subunit topography in the membrane [26] showed that the α-subunit polypeptide chain spans the lipid bilayer seven times. The location of the N-terminus of the protein in the cytoplasmic region of the cell is generally accepted [5]. Monospecific antibodies were successfully exploited to prove that the C-terminus of the α-subunit protrudes into the extracellular space [25,26]. However,some investigators suggest that the C-terminus has an intracellular location [14,15].

The β-subunit sticks out into the lipid bilayer only once so that the major part of the polypeptide chain and the C-terminus are exposed outside the cell [17,18,27].

3. THE FAMILY OF THE GENES FOR THE α-SUBUNIT

The early works on cloning of structural genes for the α-subunit of mammalian Na^+,K^+-ATPase were carried out with cDNA libraries prepared from kidney – an organ containing the largest amount of ATPase. Only one sequence was found in the kidney of sheep [15] and pigs [16,19]. However, further investigations of chromosomal genes for the α-subunit of human Na^+,K^+-ATPase [28,29], as well as of the screening of cDNA library from rat brain [30] have led to the discovery of the gene family. At present, probably, about seven sequences, highly homologous to the gene for the α-subunit, were cloned in the human genome [29,31]. Out of these genes, three correspond to the three mRNAs (αI, αII, αIII), found in rat brain which code for three isoforms of the enzyme α-subunit [30]. One of the genes appeared to be the gene encoding H^+,K^+-ATPase [29,32]. The other genes are not identified [29,31].

The comparative analysis of amino acid sequences of isoforms of Na^+,K^+-ATPase subunits from different sources served to trace their evolution from a common precursor [33]. The application of the cluster analysis (for algorithm see [34]) allowed construction of a dendrogram (fig.1). The results show that the groups of very close structures are made up of either human and rat αIII isoforms, or mammalian and avian α forms. In other words, the related proteins of one species (e.g. human αI and αIII or rat αI and αII) are less homologous than the analogous proteins of different species. This probably means that the isoforms have a very ancient origin.

The rate of evolutionary substitutions for a site per year in the case of the α-subunits is, on average, 0.18×10^{-9} [28]. The comparison of primary structures of human and rat αIII isoforms allows one to evaluate the rate of evolutionary substitutions of this isoform (0.06×10^{-9}) [33]. Then making use of the number of substitutions between human α and αIII isoforms and

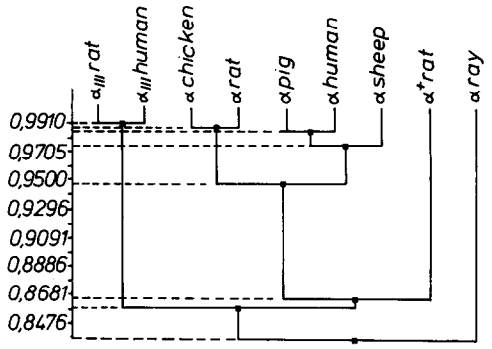

Fig.1. Evolutionary tree of the gene family for the Na^+,K^+-ATPase α-subunit [33].

the mean evolutionary rate of these isoforms (0.12×10^{-9}), we can conclude that α and αIII isoforms diverged about 650 million years ago. Presumably the αII form appeared somewhat later than αIII (fig.1) [33].

A gene for the αI-subunit of Na^+,K^+-ATPase (α1) was mapped in the 3rd murine chromosome [35], on the 2nd mink chromosome [36], and in the short arm of the 1st human chromosome – Ip21-cen [37]. The gene for αII (α2) is located in the long arm of the 1st human chromosome, region cen-q32, and the gene for αIII (α3), in the long arm of the 19th chromosome, 19q12-q13.2 [37,38]. This form is also localized to mouse chromosome 7 [35]. One of the nonidentified genes of this family (ATP1AL1) is located in the 13th human chromosome [37]. The gene for the β-subunit is mapped in the 13th mink chromosome [39], in the 1st mouse chromosome [35] and, presumably, in the long arm of the 1st human chromosome [37]. A homologous sequence representing either a β-related gene or pseudogene is found on human chromosome 4 [37].

Thus, chromosome mapping provides evidence that some genes of the α-subunit family are located in different regions of the first human chromosome, and others are distributed in other chromosomes. It is noteworthy, that genes for the αI and αII forms of the catalytic subunit of the enzyme are located on the first chromosome while the gene for the αIII isoform is found on human chromosome 19.

4. THE STRUCTURE OF THE GENE OF αIII ISOFORM IN CONNECTION WITH THE PROTEIN STRUCTURE

One of the genes belonging to the family of α-subunit genes was isolated in four overlapping clones [40,41]. The analysis of the primary structure of the exons of this gene revealed that it codes for a protein which is 99% homologous with the αIII isoform of the rat brain catalytic subunit [30]. The reconstructed fragment of overall size of 30 kb contains the total coding sequence [40,41]. It consists of 23 exons separated by 22 introns. The comparison of the structure of the chromosomal gene and cDNA of the human Na^+,K^+-ATPase αIII isoform shows that the 1st intron is located between the 2nd and 3rd codons. This location corresponds to the separation of the dipeptide MetGly from the rest of the molecule. It is noteworthy that in the gene coding for αII isoform the first intron is situated between the 4th and 5th codons. This accords with the separation of the tetrapeptide MetGlyArgGly [42] from the remaining part of the protein.

Structural features of the Na^+,K^+-ATPase αIII gene are typical of the majority of eucaryotic genes: exons comprise about 10% of the total gene length, their size varies within 60–269 bp, whereas the introns considerably differ in length (70 bp to 6–8 kbp). The structure of 3′- and 5′-splice sites coincides with the consensus sequence described in [43], many introns of the αIII gene correspond to hydrophilic amino acids (table 1, which, in a different way, presents the data published in [40]).

It is interesting to discuss the exon-intron structure of the gene for the αIII isoform, taking into consideration the secondary structure of the protein and its folding in the membrane. Though the protein product of this gene has not yet been studied, its calculated secondary structure and folding in the membrane are practically the same as is the case for the αI form of the catalytic subunit from pig kidney. Fig.2 presents the model for the secondary structure of the α-subunit [44]. Arrows indicate the position of introns in the αIII gene (intron I and N-terminal dipeptide not shown) [40].

It was suggested that exons encode transmembrane segments of the membrane proteins, whereby introns fall into hydrophilic loops between transmembrane α-helices [45]. In eight cases the boundaries of transmembrane domains in α3 gene coincide with the position of the introns (introns 3, 4, 5, 8, 16, 17, 20 and 21) (fig.2).

One should note that there is a certain correlation between the location of the introns in the gene and the domain organization of the protein in the cytoplasmic region.

The cluster of alternating α-helical and β-pleated structures within the Na^+,K^+-ATPase catalytic site deserves special attention. Let us compare this enzyme with other proteins, which have different functions but one common feature: they bind nucleotides as cofactors. And furthermore, let us look at those proteins, of which the secondary structure is known. Such a comparison shows certain structural similarities between the nucleotide-binding domains and the genes encoding them. Such proteins as alcohol dehydrogenase [46,47], glyceraldehydephosphate dehydrogenase [48], phosphoglycerate kinase [49], pyruvate kinase [50] contain the nucleotide-binding domains made of six parallel β-strands which are connected by α-helices. These domains are distinctly separated from the catalytic domains. In most cases, the nucleotide-binding domain itself consists of two subdomains. In

Table 1

Splice sites of the αIII gene of the human Na^+,K^+-ATPase [40]

Intron number	Reading frame broken at			Sequences in splice site regions		
				Protein	5′-donor	3′-acceptor
	0	+1	+2			
				▼	⌐intron——⌉	
1	|			M G D K	G G G G)gtg..	..tcgcag(G A
2	|			A M T E	T A T G)gta..	..cctcag(A C
3	|			V Q G L	G C A G)gtg..	..ctgtag(G G
4	|			D N L Y	C A A C)gtg..	..ccacag(C T
5	|			P Q Q A	C C A G)gtg..	..cctcag(C A
6	|			C K V D	C A A G)gtg..	..ccccag(G T
7		|		V E G T A	G A A G)gtg..	..ctgcag(G C
8	|			V T V C	C A C T)gta..	..ctccag(G T
9		|		Q S G T S	T C A G)gtg..	..ccccag(G G
10			|	L K R D V	A G A G)gtg..	..ttgcag(G G
11	|			Y Q L S	C C A G)gta..	..ccccag(C T
12		|		V L G F C	C T T G)gtg..	..cactag(G T
13	|			I L V I	C A A G)gtg..	..ctgcag(G T
14			|	N P R D A	C C C G)gtg..	..tcccag(G G
15	|			R Q G A	A C A G)gtg..	..ccccag(G G
16		|		E E G R L	G A G G)gtg..	..ccacag(G C
17	|			D M V P	C A T G)gtg..	..gcctag(G T
18		|		Q I G M I	A T T G)gtg..	..ttccag(G A
19	|			Q W T Y	G T G G)gtg..	..ctgcag(A C
20			|	G M K N K	T G A A)gtg	ctgcag(G A
21			|	P L K P S	T C A A)gtg..	..ctgcag(G C
22		|		P G G W V	G G G G)gtg..	..ctccag(G T
Splice sites consensus sequence					$^{G}_{A}C^{A}_{C}AG)gtg..$	$.c^{cg}_{tc}cag(G$
Frequence of occurrence of nucleotide (%)					32 32 / 41 32 / 55 36 / 100 82	41 77 / 55 55 / 86 27 / 100 100 / 77

the genes of these proteins the nucleotide-binding domains are encoded by several exons, the introns tend to fall into gaps between certain α/β segments or their pairs. The presence of the intron, which separates the catalytic and nucleotide-binding domains, is characteristic of the genes of this type of proteins.

Fig.2 shows that in the case of Na^+,K^+-ATPase the nucleotide-binding domain identified by affinity modification [53–55] also accommodates two clusters of alternating α/β structures with three β-pleated regions in-between. All of the domain is encoded by six whole exons (10–15) and by the beginning of the seventh (16th) exon. The aspartic acid residue phosphorylated reversibly during the action of the enzyme points to the position of the catalytic domain. The function of the intron located between the catalytic and nucleotide-binding domain can be fulfilled by the intron 9. The introns 12 or 13 can be considered to be analogs of introns which divide the nucleotide-binding domain into two subdomains.

Another approach focuses on revealing the regularities in the primary structure of nucleotide-binding domains [51]. This approach helped to identify the consensus sequences in the case of Na^+,K^+-ATPase [40]. The structure of peptides 242–270 and 710–744 accords well with the sequences of sites A and B, which are involved in the ATP binding, according to Walker et al. [52]. Moreover, in these domains there are the characteristic elements of the secondary structure. These elements are the β/α or $\alpha/\beta/\alpha$ regions [51]. The direct participation of fragment 710–744 of Na^+,K^+-ATPase α-subunit in the ATP binding was shown by affinity labelling [53–55]. Thus, according to these data the nucleotide-binding site of Na^+,K^+-ATPase is formed of two domains, which are separated in the primary structure but spatially proximated.

Both peptides are highly homologous with the corresponding regions of Ca-ATPase of sarcoplasmic reticulum [56]. The genes for both proteins have the similar exon-intron structure in the regions, which correspond to these fragments. Peptide 242–270 in both proteins is encoded by exon 8, positions of intron boundaries differing only by 19 and 20 nucleotides. The boundaries of the next exon 9, which codes for the most conserved region including the phosphorylation site in both ATPases, is shifted only by 50 nucleotides in Ca-ATPase as compared to Na^+,K^+-ATPase. The second

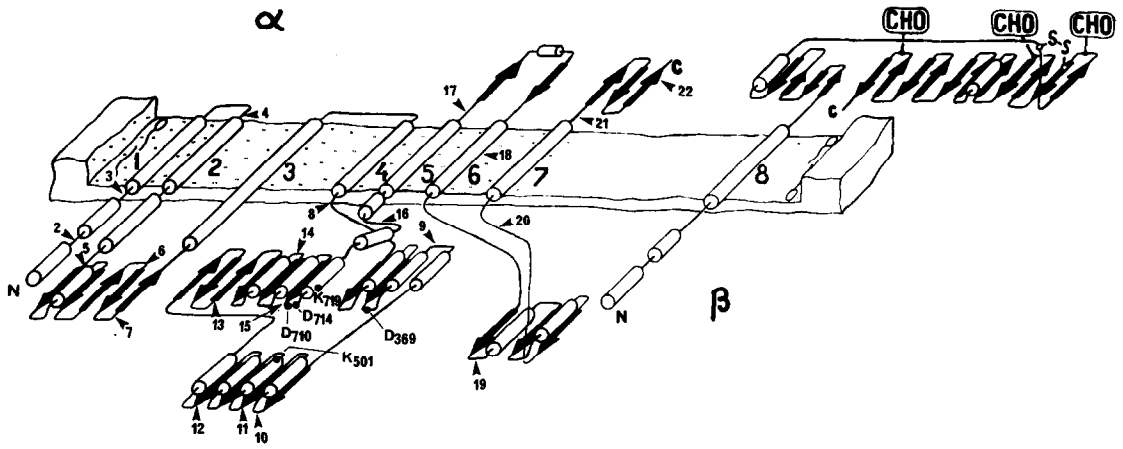

Fig.2. Model for membrane arrangement of the Na^+,K^+-ATPase catalytic subunit. Cylinder and arrows indicate α-helical and β-sheeted regions. Arrows with figures indicate positions and numbers of introns in the gene for the human αIII isoform. Amino acid residues D-369, K-501, D-714, and K-719 enter the enzyme active site. The model of β-subunit is indicated on the right [40,44].

region (peptide 710–714) in both proteins is encoded by the beginning of the 16th exon, positions of the corresponding introns differing by 18 nucleotides.

Thus, it is not only possible to find homologous peptides in ATPases of E1E2-type of different specificity but also to find some analogies in the structure of genes encoding them. The complete exon-intron structure of other genes of Na^+,K^+-ATPase catalytic subunit family is unknown, but the data on the partial structure of some genes of this family [29] imply that all the genes have a similar intron location. These data support the hypothesis on the existence of a common ancestor gene of transport ATPases which duplicated and diverged during evolution into various forms of different specificity. Moreover, some analogies in the organization of nucleotide-binding domains of proteins which fulfill very different functions and analogies in the organization of corresponding genes can be considered to support the Gilbert and Black hypothesis, which pointed out that exons corresponded to units of discreet protein structure and/or function and that proteins had evolved by the combination of domains carrying particular functions [57,58].

5. TISSUE-SPECIFICITY OF EXPRESSION OF VARIOUS ISOFORMS OF Na^+,K^+-ATPase CATALYTIC SUBUNIT

Tissue-specific distribution of two forms of α-subunit of Na^+,K^+-ATPase has been shown earlier, e.g. α-form was found predominantly in mammalian kidney and the mixture of α and α^+ forms, in brain [8,13]. For detailed review see [6]. However, the discovery of the third isoform with the affinities for substrates and inhibitors first unknown complicated the interpretation of the earlier results obtained by immunochemical methods. For instance, the α^+ isoform discovered in rat brain is now believed to be a mixture of αII and αIII isoforms [6,59].

The Northern blotting allows one to study the steady-state concentration of mRNA in different tissues, however it says nothing about the mechanisms operating on translational or posttranslational level. Therefore, the combination of Northern blots with immunochemical methods using isoform-specific antibodies will be most informative.

The contents of mRNA of various forms of the catalytic subunit in mammalian tissues were investigated in several laboratories by means of gene-specific probes [60–63].

The α1 mRNA, 3.7 kb, was discovered in virtually all rat and human tissues investigated [60–63]. The largest amounts were found in kidney, the smallest in liver. Two or even three α2 mRNAs (5.3–6, 3.4–4.5 and 2.4 kb) were found in different amounts in many rat tissues except for liver [61,62,64,65]. This variety could result from the differential use of the polyadenylation signals in the 3′-untranslated region of α2 mRNA [61]. But one cannot rule out some cross-hybridization due to the high level of homology of different isoforms especially when large cDNA probes have been used [64,65]. Both main mRNAs of αII form have been shown to be expressed in brain, skeletal muscles and heart [61].

The α3 mRNA, 3.7 kb, was detected in rat brain, stomach and lung [61]. Hybridization with mRNA from human tissues demonstrated the presence of the αIII isoform in kidney, brain and thyroid gland [60]. The presence of the α3 mRNA in human kidney and its absence in rat kidney can be a result of drug administration in humans or of some other factors. This question deserves special attention and could be solved when the statistically reliable material is available. In situ mapping of rat brain areas suggests that α3 mRNA of Na^+,K^+-ATPase is expressed predominantly by neural cells [66].

Application of gene-specific probes made possible the study of distribution of isoform-specific mRNAs

during ontogenesis. The level of mRNAs of three isozymes of Na^+,K^+-ATPase and β-subunit in rat brain, heart, lung, kidney and skeletal muscles during the development was investigated by Orlowsky and Lingrel [59]. The α forms and β-subunit mRNAs appear to be regulated coordinately during ontogenesis with maximum expression occurring between 15 and 25 days old for brain, heart, kidney and skeletal muscle. The peak of expression in lung was shown to be between 2 and 4 days of neonatal life [59].

Expression of the αI and αIII isoforms in infant and adult human brain and kidney tissues increased as compared with embryonic tissues [60]. These data correlate well with the results on rat brain Na^+,K^+-ATPase activity which also has been shown to increase from pre- to postnatal state [67]. Increasing activity of Na^+,K^+-ATPase during the postnatal development was observed also on rat submaxillary glands [68], rabbit hippocampus [69]. However, only now the study of expression of certain α-subunit isoforms became possible.

No doubt research into the tissue-specific expression of different forms of Na^+,K^+-ATPases will be extended to tissues in pathology. So, e.g. the expression level of the αII and αIII-isoforms decreased in rats in response to increased intravascular pressure, at the same time the correlated enhancement in expression of the αI form and β-subunit of Na^+,K^+-ATPase was observed [70].

The discovery of a family of catalytic subunit genes suggests that the functional diversity of Na^+,K^+-ATPase activity in different tissues and cells can be attributed to different properties of individual isoforms. The problem becomes especially exciting in connection with tissue specificity and developmental regulation of the expression of these isoforms. Different sensitivity of the known isoforms of Na^+,K^+-ATPase to their inhibitor, ouabain, gives a hint of their possible role. The distinct relative contents of isoforms in the cells of different tissues lead to varied sensitivity of these cells to the inhibitor. Let us assume the existence of endogenic inhibitors and activators of Na^+,K^+-ATPase, which differently act on different isoforms. Then the fine-tuned physiological mechanism of dissimilar response of different tissues to the same changes under the conditions of the increase (or decrease) of the inhibitor (or activator) contents in the intercellular medium should operate. The endogenic inhibitor of Na^+,K^+-ATPase with biological properties analogous to those of ouabain has been recently shown to exist [71]. Other potential endogenic regulators of the enzyme were mentioned (for review see [72]).

The study of functional properties of each isoform should deepen the knowledge already gained. The study of the characteristics of the αI and αII isoforms was started some years ago [6,8]. As to the αIII form one should first of all prove its existence. The knowledge of deduced primary and calculated secondary structures of the protein promotes the choice of potential immunogenic peptides, their synthesis and preparation of the antibodies monospecific for this type of the protein. Use was made of this approach to unravel the αIII isoform of the catalytic subunit in the human brain preparation and in pig and human kidney [73]. Its N-terminal sequence Met-Gly-Asp-Lys-Lys-Asp-Asp completely corresponds to the structure of the α3 gene [40]. Thus, unlike αI and αII, the αIII protein is not subjected to posttranslational processing in the N-terminal region. The αIII isoform has the highest sensitivity to ouabain as compared to other isoforms [74]. This was revealed by the analysis of ouabain sensitivity of the rat brainstem axolemma membrane containing only the αIII isoform. These preparations of the membrane were obtained using limited trypsin digestion of brainstem axolemma. Such treatment selectively removes the αII isoform [74]. A high level of ouabain sensitivity was also shown for the product of expression of cDNA of the rat brain αIII isoform in Balb/c 3T3 cells [75].

Thus, the example of the αIII isoform of the catalytic subunit shows the progress in our knowledge of Na^+,K^+-ATPase. This progress was achieved due to the use of the methods of molecular cloning, which allowed one to isolate a gene for a new isoform of the catalytic subunit. The knowledge of its primary structure helped to prove the existence of a new protein in human brain and pig and human kidney [73]. One cannot exclude that other genes of this gene family will be identified as genes coding for new isoforms or for other ATPases with different specificity.

6. ON REGULATION OF EXPRESSION OF GENES FOR Na^+,K^+-ATPase

The study of mechanisms of tissue-specificity and developmental regulation of gene expression is a major problem of modern molecular biology. The tissue-specific regulation at the transcriptional level is best studied. It occurs due to direct or indirect interaction of specific cellular factors with the regulatory *cis*-elements of DNA located in promoters and enhancers. Unfortunately, the regulatory elements of genes for Na^+,K^+-ATPase isoforms are unknown. The only work concerning this problem determines the primary structure of about 1400 bp preceding the gene for the human αII isoform [42].

The hypothetical transcription starting point in the α2 gene is situated at about 100 bp upstream to the initiation ATG codon where the pyrimidine-rich cluster has been found [42]. At a distance of about 30 bp from this site the imperfect TATA box is located, its structure resembles that of analogous sites of other genes, e.g. genes for murine kallikrein, for human α-interferon, for Rous sarcoma virus and virus AAV2

(for review see [76]). The TATA box is not the only region homologous to other genes. In the regulatory region of the gene for αII isoform there is a double repeat of oligonucleotide GGGGGAGA. Closely homologous sequences in analogous regions are found in other genes, e.g. gene for Ca-ATPase [56], gene for serine protease from human bone marrow [77]. A homologous sequence in opposite orientation is discovered in the enhancer of SV-40 and its significance for the enhancer function is shown [78].

Some eukaryotic promoters contain the CCAAT conserved sequence in position −80. The α2 gene contains in this region the CAACAAAC structure flanked by tetranucleotides GTTT [42]. The region −120 involves hexanucleotide GGGCCG, which binds factor Sp1 [79]. The putative regulatory region of the α2 gene contains many oligo-G blocks [42].

The structural analysis of other regulatory elements, their comparison, identification of specific cell factors interacting with them is a way of comprehending the tissue-specific expression of members of a gene family coding for different isoforms of the Na^+,K^+-ATPase catalytic subunit.

7. THE FAMILY OF β-SUBUNIT GENES

Biochemical investigations on Na^+,K^+-ATPase gave birth to the hypothesis that the β-subunit is an integral component of the enzyme. Several lines of evidence show that Na^+,K^+-ATPase α- and β-subunit synthesis is coordinately regulated and both subunits are expressed at approximately equal levels [64,65,80–82].

Several questions arise from the discovery of multiple forms of the α-subunit. First, whether heterogeneity of the β-subunits exists at the protein level, apart from the heterogeneity of glycosylation. β-Subunit mRNAs from different sources (*Torpedo* [17], sheep, pig, dog and chicken [18,19,22,23], human HeLa cells [20], rat [21,83]) are cloned. In each case only the coding sequence was found. The mammalian β-subunit genes show high level of homology (90%). However, more detailed analysis of rat kidney and brain unravelled several species of mRNA with similar coding regions and differing in length of 3′- and 5′-untranslated regions [83]. Size variations at the 3′-end occur due to the use of different polyadenylation sites. Differential utilization of the sites of transcription initiation, which is specific for certain tissues, is a characteristic feature of the β-subunit mRNA. In kidney, muscles and stomach the longer mRNA is synthesized, and in brain the shorter one [83].

The second question is, whether each type of α-subunit is bound to the specific type of β-subunit or different α-subunits can interact with the same β-protein. This question remains to be answered. At present we only know the relative amounts of mRNAs for different α-subunits and the only β-subunit. In some tissues (kidney, brain, heart) the coordinated expression of three α forms and β-subunit can be proposed, in others (muscle, stomach) it seems impossible [83]. Orlowsky and Lingrel found the coordinated regulation of α isoforms and β-subunit during ontogenesis [59].

The recent publication of Martin-Vasallo et al. [84] claimed to discover in rat brain and human liver an mRNA homologous to the mRNA of the β-subunit of Na^+,K^+-ATPase, homology level being 58%. This gene was designated as β2 gene. The expression of this gene proceeds in a tissue-specific manner but distinct from that for the β-subunit gene [84]. Keeping in mind the relatively low level of homology between β1 and β2 genes one cannot exclude that β2 protein is a component of another ion-transporting ATPase. Such a subunit was suggested to be a component of H^+,K^+-ATPase [85].

The discovery of a second type of β-subunit leads to the conclusion, that a family of β-subunit genes exists. In fact, when analysing a human genomic library using pig kidney cDNA of the β-subunit as a hybridization probe Ushkaryiov et al. found at least two different sequences [86]. One sequence presents most probably the gene coding for β-subunit, the isolated fragment contains one exon coding for the putative transmembrane segment and two introns. Their location correlates with boundaries of transmembrane domain of the β-subunit [86]. The second sequence highly homologous to the β1 gene, is intron free and contains many stop codons, and an insert. Therefore it was proposed that this sequence is a pseudogene belonging to the β-subunit family [86].

8. CONCLUSION

Structural analysis of Na^+,K^+-ATPase genes, discovery of multiple isoforms responding differently to the action of inhibitors and determination of tissue-specificity of gene expression open new horizons for investigators involved in problems of physiological ion transport and for those who treat different disorders of water-salt exchange and cardiovascular diseases. Multiplicity of isoforms underlies the strategy of differential action on organs and tissues by means of specific inhibitors or activators of one or another isoform. Further research into the regulation of gene expression, into the role of isoforms, search of new isoforms will provide a deeper understanding of ion transport in the functioning of eucaryotic cell.

Acknowledgements: We wish to thank Dr V.M. Gindilis for help in calculations of evolutionary trees. We are grateful to many colleagues for participation in experiments and discussions.

REFERENCES

[1] Glynn, I.M. (1985) in: The Enzymes of Biological Membranes, vol.3 (Martonosi, A.N. ed.) pp.35–184, Plenum, New York.

[2] Jorgensen, P.L. (1982) Biochim. Biophys. Acta 694, 27–68.
[3] Karlish, S.J.D. and Stein, W.D. (1982) Ann. NY Acad. Sci. 226–238.
[4] Fambrough, D.M. (1988) Trends Neurosci. 8, 325–328.
[5] Jorgensen, P.L. and Andersen, J.P. (1988) J. Membr. Biol. 103, 95–120.
[6] Sweadner, K.J. (1989) Biochim. Biophys. Acta 988, 185–220.
[7] Rossier, B.C., Geering, K. and Kraehenbuhl, J.P. (1987) Trends Biol. Sci. 12, 483–487.
[8] Sweadner, K.J. (1979) J. Biol. Chem. 254, 6060–6067.
[9] Peterson, G.L., Ewing, R.D., Hootman, S.R. and Conte, F.P. (1978) J. Biol. Chem. 253, 4762–4770.
[10] Peterson, G.L. and Hokin, L.E. (1981) J. Biol. Chem. 256, 3751–3761.
[11] Sweadner, K.J. (1985) J. Biol. Chem. 260, 11508–11513.
[12] Lytton, J. (1985) Biochem. Biophys. Res. Commun. 132, 764–769.
[13] Sweadner, K.J. and Gilkeson, R.C. (1985) J. Biol. Chem. 260, 9016–9022.
[14] Kawakami, K., Noguchi, S., Noda, M., Takahashi, H., Ohta, T., Kawamura, M., Nojima, H., Nagano, K., Hirose, T., Inayama, S., Hayashida, H., Miyata, T. and Numa, S. (1985) Nature 316, 733–736.
[15] Shull, G.E., Schwartz, A. and Lingrel, J.B. (1985) Nature 316, 691–695.
[16] Ovchinnikov, Yu.A., Arsenyan, S.G., Broude, N.E., Petrukhin, K.E., Grishin, A.V., Aldanova, N.A., Arzamazova, N.M., Arystarkhova, E.A., Melkov, A.M., Smirnov, Yu.V., Guryev, S.O., Monastyrskaya, G.S. and Modyanov, N.N. (1985) Dokl. Akad. Nauk SSSR 235, 1490–1495.
[17] Noguchi, S., Noda, N., Takahashi, H., Kawakami, R., Ohta, T., Nagano, K., Hirose, T., Inayama, S., Kawamura, M. and Numa, S. (1986) FEBS Lett. 196, 315–320.
[18] Shull, G.E., Lane, L.K. and Lingrel, J.B. (1986) Nature 321, 429–431.
[19] Ovchinnikov, Yu.A., Modyanov, N.N., Broude, N., Petrukhin, K.E., Grishin, A.V., Arzamazova, N.M., Aldanova, N.A., Monastyrskaya, G.S. and Sverdlov, E.D. (1986) FEBS Lett. 201, 237–245.
[20] Kawakami, K., Nojima, H., Ohta, T. and Nagano, K. (1986) Nucleic Acids Res. 14, 2833–2844.
[21] Mercer, R.W., Schneider, J.W., Savitz, A., Emanuel, J., Benz, E.J. and Levenson, R. (1986) Mol. Cell. Biol. 6, 3884–3890.
[22] Takeyasu, K., Tamkun, M.M., Siegel, N.R. and Fambrough, D.M. (1987) J. Biol. Chem. 262, 10733–10740.
[23] Brown, T.A., Horowitz, B., Miller, R.P., McDonough, A.A. and Farley, R.A. (1987) Biochim. Biophys. Acta 912, 244–253.
[24] Lebovitz, R.M., Takeyasu, K. and Fambrough, D.M. (1989) EMBO J. 8, 193–202.
[25] Ovchinnikov, Yu.A., Arzamazova, M., Arystarkhova, E.A., Gevondyan, N.M., Aldanova, N.A. and Modyanov, N.N. (1987) FEBS Lett. 217, 269–274.
[26] Ovchinnikov, Yu.A., Luneva, N.M., Arystarkhova, E.A., Gevondyan, N.M., Kozhich, A.T., Nesmeyanov, V.A. and Modyanov, N.N. (1988) FEBS Lett. 227, 230–234.
[27] Ovchinnikov, Yu.A. (1987) Trends Biol. Sci. 12, 434–438.
[28] Ovchinnikov, Yu.A., Monastyrskaya, G., Broude, N.E., Allikmets, R.L., Ushkaryov, Yu.A., Melkov, A.M., Smirnov, Yu.V., Malyshev, I.V., Dulubova, I.E., Petrukhin, K.E., Grishin, A.V., Sverdlov, V.E., Kiyatkin, N.I., Kostina, M.B. and Modyanov, N.N. (1987) FEBS Lett. 213, 73–80.
[29] Sverdlov, E.D., Monastyrskaya, G.S., Broude, N.E., Ushkaryov, Yu.A., Allikmets, R., Melkov, A.M., Smirnov, Yu.V., Malyshev, I.V., Dulubova, I.E., Petrukhin, K.E., Grishin, A.V., Kiyatkin, N.I., Kostina, M.B., Sverdlov, V.E., Modyanov, N.N. and Ovchinnikov, Yu.A. (1987) FEBS Lett. 217, 275–278.
[30] Shull, G.E., Greeb, J. and Lingrel, J.B. (1986) Biochemistry 25, 8125–8132.
[31] Shull, M.M. and Lingrel, J.B. (1987) Proc. Natl. Acad. Sci. USA 84, 4039–4043.
[32] Shull, G.E. and Lingrel, J.B. (1986) J. Biol. Chem. 261, 16788–16791.
[33] Broude, N.E., Gindilis, V.M. and Sverdlov, E.D., in preparation.
[34] Alexandrov, V.V. and Gorsky, N.D. (1983) in: Algorithms and Programs for Analysis of Structural Data, p.58, Leningrad, Nauka.
[35] Kent, P.A., Fallows, D., Geissler, E., Glaser, T., Emanuel, J.R., Lalley, P.A., Levenson, R. and Housman, D.E. (1987) Proc. Natl. Acad. Sci. USA 84, 5369–5373.
[36] Matveeva, N.M., Khlebodarova, T.M., Karasik, G.T., Rubtzov, N.B., Serov, O.L., Sverdlov, E.D., Broude, N.E., Modyanov, N.N., Monastyrskaya, G.S. and Ovchinnikov, Yu.A. (1987) FEBS Lett. 217, 42–44.
[37] Yang-Feng, T.L., Schneider, J.W., Lindgren, V., Shull, M.M., Benz, E.J., Lingrel, J.B. and Francke, U. (1988) Genomics 2, 128–138.
[38] Harley, H.G., Brook, J.D., Jackson, C.L., Glaser, T., Walsh, K.V., Sarfarazi, M., Kent, R., Lager, M., Koch, M., Harper, P.S., Levenson, R., Housman, D.E. and Show, D.J. (1988) Genomics 3, 380–384.
[39] Khlebodarova, T.M., Karasik, G.I., Lapteva, S.E., Matveeva, N.M., Serov, O.L., Sverdlov, E.D., Broude, N.E., Modyanov, N.N. and Monastyrskaya, G.S. (1988) FEBS Lett. 236, 240–242.
[40] Ovchinnikov, Yu.A., Monastyrskaya, G.S., Broude, N.E., Ushkaryov, Yu.A., Melkov, A.M., Smirnov, Yu.V., Malyshev, I.V., Allikmets, R.L., Kostina, M.B., Dulubova, I.E., Kiyatkin, N.I., Grishin, A.V., Modyanov, N.N. and Sverdlov, E.D. (1988) FEBS Lett. 233, 87–94.
[41] Sverdlov, E.D., Monastyrskaya, G.S., Broude, N.E., Ushkaryov, Yu.A., Melkov, A.M., Orlova, M.A., Malyshev, I.V., Modyanov, N.N., in preparation.
[42] Sverdlov, E.D., Bessarab, D.A., Malyshev, I.V., Smirnov, Yu.V., Ushkaryov, Yu.A., Monastyrskaya, G.S., Broude, N.E. and Modyanov, N.N. (1989) FEBS Lett. 244, 481–483.
[43] Patget, R.A., Grabovski, P.J., Konarska, M.M., Seiler, S. and Sharp, P.A. (1986) Annu. Rev. Biochem. 55, 1119–1150.
[44] Ovchinnikov, Yu.A., Arystarkhova, E.A., Arzamazova, N.M., Dzhandzhugazyan, K.N., Efremov, R.G., Nabiev, I.R. and Modyanov, N.N. (1988) FEBS Lett. 227, 235–239.
[45] Nathans, J. and Hogness, D.S. (1984) Proc. Natl. Acad. Sci. USA 81, 4851–4855.
[46] Duester, G., Jornvall, H. and Hatfield, G.W. (1986) Nucleic Acids Res. 14, 1931–1941.
[47] Branden, C.-I., Eklund, H., Cambillau and Prior, A.J. (1984) EMBO J. 3, 1307–1310.
[48] Stone, E.M., Rothblum, R.N. and Schwartz, R.J. (1985) Nature 313, 498–500.
[49] Michelson, A.M., Blake, C.C.F., Evans, S.T. and Orkin, S.H. (1985) Proc. Natl. Acad. Sci. USA 82, 6965–6969.
[50] Lonberg, N. and Gilbert, W. (1985) Cell 40, 81–90.
[51] Fothergill-Gilmore, L.A. (1986) in: Multidomain Proteins – Structure and Evolution (Hardie, D.G. and Coggins, J.R. eds) pp.85–174, Elsevier, Amsterdam.
[52] Walker, J.E., Saraste, M., Ruswick, M.Y. and Gay, M.J. (1982) EMBO J. 1, 945–951.
[53] Dzhandzhugazyan, K.N., Lutsenko, S.V. and Modyanov, N.N. (1986) Biol. Membr. 3, 858–868.
[54] Ovchinnikov, Yu.A., Dzhandzhugazyan, K.N., Lutsenko, S.V., Mustaev, A.A. and Modyanov, N.N. (1987) FEBS Lett. 217, 111–116.
[55] Nagano, K., Ohta, T. and Yoshida, M. (1986) Proc. Natl. Acad. Sci. USA 83, 2071–2075.
[56] Korczak, B., Zarain-Herzberg, A., Brandl, C.J., Ingles, C.J., Green, N.M. and MacLennan, D.H. (1988) J. Biol. Chem. 263, 4813–4819.
[57] Gilbert, W. (1978) Nature 271, 501.

[58] Blake, C.C.F. (1983) Nature 306, 535–537.
[59] Orlowsky, J. and Lingrel, J.B. (1988) J. Biol. Chem. 263, 10436–10442.
[60] Sverdlov, E.D., Broude, N.E., Sverdlov, V.E., Monastyrskaya, G.S., Petrukhin, K.E., Akopyanz, N.S., Modyanov, N.N. and Ovchinnikov, Yu.A. (1987) FEBS Lett. 221, 129–133.
[61] Young, R.M. and Lingrel, J.B. (1987) Biochem. Biophys. Res. Commun. 145, 52–58.
[62] Herrera, V.L., Emanuel, J.R., Ruiz-Opazo, N., Levenson, R. and Nadal-Ginard (1987) J. Cell. Biol. 105, 1855–1864.
[63] Sverdlov, E.D., Akopyanz, N.S., Petrukhin, E.R., Broude, N.E., Monastyrskaya, G.S. and Modyanov, N.N. (1988) FEBS Lett. 239, 65–68.
[64] Emanuel, J.R., Garetz, S., Schneider, J., Ash, J.F., Benz, E.J.J. and Levenson, R. (1986) Mol. Cell. Biol. 62, 2476–2481.
[65] Emanuel, J.R., Garetz, S., Stone, L. and Levenson, R. (1987) Proc. Natl. Acad. Sci. USA 84, 9030–9034.
[66] Schneider, J.W., Mercer, R.W., Gilmore-Hebert, M., Utset, M.F., Lai, C., Greene, A. and Benz, E.J., jr (1988) Proc. Natl. Acad. Sci. USA 85, 284–288.
[67] Schmitt, C.A. and McDonough (1986) J. Biol. Chem. 261, 10439–10444.
[68] Camden, J. and Martinez, J.R. (1987) Experientia 43, 570–572.
[69] Haglund, M.M., Stahl, W.L., Kunkel, D.D. and Schwartzkroin, P.A. (1985) Brain Res. 343, 198–203.
[70] Herrera, V.L.M., Chobanian, A.V. and Ruiz-Opazo, N. (1988) Science 4862, 222–223.
[71] Tamura, M., Tim-Tak Lam and Inagami, T. (1988) Biochemistry 27, 4244–4253.
[72] Haupert, G.T., jr in: The Na^+,K^+-Pump, Part B: Cellular Aspects (Skou, J.C. et al. eds) pp.297–320, Alan R. Liss, New York.
[73] Arystarkhova, E.A., Lakhtina, O.E. and Modyanov, N.N. (1989) FEBS Lett., in press.
[74] Urayama, O. and Sweadner, K.J. (1988) Biochem. Biophys. Res. Commun. 156, 796–800.
[75] Hara, Y., Nikamoto, A., Kojima, T., Matsuboto, A. and Nakao, M. (1988) FEBS Lett. 238, 27–30.
[76] Bucher, P. and Trifonov, E.N. (1986) Nucleic Acids Res. 14, 10009–10020.
[77] Nakamura, H., Okano, K., Aoki, V., Shimizu, H. and Naruto, M. (1987) Nucleic Acids Res. 15, 9601–9602.
[78] Zenke, M., Grundstrom, T., Mattes, H., Wintzerith, M., Schatz, C., Wildeman, A. and Chambon, P. (1986) EMBO J. 5, 387–397.
[79] Maniatis, T., Goodbourn, S. and Fischer, J.A. (1987) Science 236, 1237–1245.
[80] Lo, C.S. and Edelman, I.S. (1976) J. Biol. Chem. 251, 7834–7840.
[81] Geering, K., Girardet, M., Bron, C., Kraehenbuhl, J.-P. and Rossier, B.C. (1982) J. Biol. Chem. 257, 10338–10343.
[82] Tamkun, M.M. and Fambrough, D.M. (1986) J. Biol. Chem. 261, 1009–1019.
[83] Young, R.M., Shull, G.E. and Lingrel, J.B. (1987) J. Biol. Chem. 262, 4905–4910.
[84] Martin-Vassallo, P., Dackowsky, W., Emanuel, J.R. and Levenson, R. (1989) J. Biol. Chem. 264, 4613–4618.
[85] Okamoto, C., Reenstra, W.W., Li, W. and Forte, J.G. (1989) J. Cell. Biol. 107, 125a.
[86] Ushkaryov, Yu.A., Monastyrskaya, G.S., Broude, N.E., Bessarab, D.A., Modyanov, N.N. and Sverdlov, E.D. (1989) FEBS Lett., in press.