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Research Article

Multiple isoenzymes of the Na+,K+-ATPase (alpha, alpha+, and alpha 3) have been identified by molecular cloning (Shull, G. E., J. Greeb, and J. B. Lingrel. 1986. Biochemistry. 25:8125-8132; and Schneider, J. W., R. W. Mercer, and E. J. Benz, Jr. 1987. Clin. Res. 35:585A. [Abstr.]). At least one of these, the alpha 3 chain, represents a novel form for which protein products and enzymatic activities are just beginning to be defined in rodents. We have recently demonstrated that expression of alpha 3 is largely confined to neuromuscular tissues of fetal and adult rats (Schneider, J. W., R. W. Mercer, M. Gilmore-Hebert, M. F. Utset, C. Lai, A. Greene, and E. J. Benz, Jr. 1988. Proc. Natl. Acad. Sci. USA. 85:284-288). We now report that certain human leukemia cell lines including HL60, HEL, and Molt 4 express mRNA for both alpha and alpha 3 isoforms of Na+,K+-ATPase; mRNA was not detected in several other cell lines, including K562 and U937; no cell lines expressed alpha+ mRNA. In uninduced HL60 cells, alpha 3 mRNA comprised 20-30% of total Na+,K+-ATPase mRNA. Furthermore, in HL60 and HEL cells, both alpha and alpha 3 mRNA declined after induction of maturation by DMSO, retinoic acid, or hemin. However, the reduction in alpha 3 mRNA was far more dramatic. alpha 3 mRNA virtually disappeared, but alpha mRNA […]

Expression of Multiple Na^+,K^+-Adenosine Triphosphatase Isoform Genes in Human Hematopoietic Cells

Behavior of the Novel A3 Isoform during Induced Maturation of HL60 Cells

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Abstract

Multiple isoenzymes of the Na^+,K^+-ATPase (α, α^+, and $\alpha 3$) have been identified by molecular cloning (Shull, G. E., J. Greeb, and J. B. Lingrel. 1986. *Biochemistry.* 25:8125–8132; and Schneider, J. W., R. W. Mercer, and E. J. Benz, Jr. 1987. *Clin. Res.* 35:585A. [Abstr.]). At least one of these, the $\alpha 3$ chain, represents a novel form for which protein products and enzymatic activities are just beginning to be defined in rodents. We have recently demonstrated that expression of $\alpha 3$ is largely confined to neuromuscular tissues of fetal and adult rats (Schneider, J. W., R. W. Mercer, M. Gilmore-Hebert, M. F. Utset, C. Lai, A. Greene, and E. J. Benz, Jr. 1988. *Proc. Natl. Acad. Sci. USA.* 85:284–288). We now report that certain human leukemia cell lines including HL60, HEL, and Molt 4 express mRNA for both α and $\alpha 3$ isoforms of Na^+,K^+-ATPase; mRNA was not detected in several other cell lines, including K562 and U937; no cell lines expressed α^+ mRNA. In uninduced HL60 cells, $\alpha 3$ mRNA comprised 20–30% of total Na^+,K^+-ATPase mRNA. Furthermore, in HL60 and HEL cells, both α and $\alpha 3$ mRNA declined after induction of maturation by DMSO, retinoic acid, or hemin. However, the reduction in $\alpha 3$ mRNA was far more dramatic. $\alpha 3$ mRNA virtually disappeared, but α mRNA declined by only $\sim$ 50%. In contrast, when maturation of HL60 cells along the monocyte/macrophage lineage was induced by exposure to phorbol esters, $\alpha 3$ mRNA remained abundant. Moreover, mRNA for the β subunit of the Na^+,K^+-ATPase increased dramatically. Our results demonstrate that the $\alpha 3$ isoform, formerly thought to be confined to neuromuscular tissues, is expressed in restricted lineages of hematopoietic origin. These leukemia cell lines should provide a useful model for analyzing regulation of the $\alpha 3$ isoform gene and characterization of $\alpha 3$ isoform activities.

Introduction

Na^+,K^+-ATPase (Na^+ pump) is a membrane-bound heterodimeric enzyme that uses energy derived from the hydrolysis of ATP to exchange intracellular Na^+ ions for extracellular K^+. We and others have recently discovered that Na^+,K^+-ATPase in different tissues exhibits unexpected diversity and heterogeneity of expression (1–4). Three α subunit mRNAs, named A1 (α), A2 (α^+), and A3 ($\alpha 3$) have been characterized. Each α isoform mRNA is the product of a different gene located on a different chromosome arm (5, 6); each is expressed with a distinctive pattern of tissue and developmental regulation (3, 4). Previous work with rat α isoform cDNAs and tissue mRNAs has shown that the Na^+,K^+-ATPase A1 mRNA corresponds to the ubiquitous Na^+ pump especially abundant in transport epithelia and neuromuscular cells; the Na^+,K^+-ATPase A2 mRNA corresponds to the previously described α^+ protein isoform in that it predominates in neurons and muscular tissues, with lower levels of expression in cardiac cells. Na^+,K^+-ATPase A3 mRNA encodes a novel isoform that, before this report, appeared to be expressed only in large neuron groups in brain, photoreceptor cells of the retina, fetal heart, and at very low levels in stomach and lung (4). Based on its extremely high nucleotide sequence homology with the A1 and A2 mRNAs, we (4) and Schull and co-workers (1–3) have proposed that the A3 isoform encodes a third, previously unappreciated isoenzyme of the Na^+,K^+-ATPase.

The protein isoform encoded by Na^+,K^+-ATPase A3 mRNA is not yet characterized in neural tissues, even though we had previously demonstrated that the A3 mRNA does encode a stable protein capable of membrane insertion by cell-free translation of synthetic mRNA templates (3). Recent gene transfection studies (7; unpublished data) suggest that the A3 form from rodents encodes a ouabain-inhibited Na^+,K^+-ATPase activity. The ion transport and cardiac glycoside binding activities of the human A3 isoform, however, remain largely unknown, as do its intracellular trafficking and localization. Further understanding of the properties and role of the A3 isoform and the regulation of A3 gene expression will require a more convenient and manipulable source of both the mRNA and enzyme than is available from selected cells within the central nervous system or fetal heart.

To facilitate further exploration of the A3 isoform genes and its products, we have surveyed a number of cell lines for the presence of the A3 mRNA. We have encountered expression of A3 mRNA in a select subset of cell lines of hematopoietic lineage within the granulocyte/macrophage and lymphoid lineages. In this report we show that expression of the genes encoding the A1 and A3 isoforms of the Na^+,K^+-ATPase is modulated within these cell lines. Moreover, production of A3 mRNA is sufficiently abundant in HL60 and Molt 4 cells to

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allow these cells to serve as a source for studies of A3 gene regulation and, potentially, for characterization of the function of the $\alpha 3$ protein isoform.

Altered cation fluxes across the plasma membrane, generated in part by changes in Na^+,K^+-ATPase activity, are thought to be important for regulating cellular growth and differentiation (8–11). In this regard, it is interesting that our recent studies have found that A3 isoform is expressed in fetal but not adult rat heart, whereas the A2 isoform increases dramatically after birth in the rat central nervous system. Thus, there may be a role for differential expression of Na^+ pump isoforms as part of the normal sequence of development and/or differentiation of specific tissues. In keeping with this hypothesis, we have now found that the A3 isoform is more sensitive to the changes accompanying induced maturation of hematopoietic cell lines than the A1 isoform gene.

Methods

DNA probes. Human Na^+,K^+-ATPase α and β cDNAs were isolated from a human kidney cDNA library (unpublished data); human α^+ and $\alpha 3$ cDNAs were isolated from a human brain cDNA library in collaboration with John Gilbert, Richard Bartlett, and Alan Roses (Duke University, Durham, NC). Additional DNA probes used in this study include human genomic c-*myc* (12), human genomic fragments containing "alu" repetitive sequences (13), and human γ-actin cDNA (14).

Cell culture and chemical induction of maturation. Cells were grown in suspension in RPMI medium with 10% FCS and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Inductions were carried out with 1.25% DMSO for 5 d, 1 μM cis-retinoic acid for 5 d, tumor promoting agent for 2 d, or 100 μM hemin for 4 d.

RNA isolation and blot analysis. Total RNA was prepared from cells or tissues by the guanidinium isothiocyanate/CsCl gradient method (15). Total RNA was enriched for mRNA by oligo-dT cellulose column chromatography. RNAs were separated by 1% agarose/formaldehyde gel electrophoresis, transferred to (or spotted directly onto) Gene Screen Plus (New England Nuclear, Boston, MA), and hybridized as recommended by the membrane supplier. DNA probes were labeled with α-[^{32}P]dCTP using the multipriming kit (Amersham Corp., Arlington Heights, IL).

Run-off transcription analysis. Isolation of nuclei from induced and uninduced HL60 cells was exactly as described by Groudine et al. (16). The nuclear run-off reactions, isolation of ^{32}P-labeled RNA, hybridization conditions, and filter washing conditions were as described by Linial et al. (17).

Results

Expression of Na^+,K^+-ATPase α isoform mRNAs in human leukemia cell lines. We analyzed a panel of total cellular RNAs isolated from human cell lines for the presence of α isoform mRNAs. Cell lines studied included HeLa, HL60, K562, Molt 4, HEL, hemin-induced HEL (see results described below), and U937 cells (lanes *1–7*, Fig. 1). RNA blots were probed with either human kidney Na^+,K^+-ATPase A1, human brain A2, or human brain A3 cDNAs using conditions previously shown (3) to eliminate crosshybridization among highly homologous α isoform cDNAs and mRNAs. A human γ-actin cDNA probe was used to control for the amount of RNA loaded in each lane (~ 35 μg). The hybridization signal obtained with each isoform probe when annealed to ~ 25 μg of human fetal heart and fetal brain RNA is shown at the far right of Fig. 1. Each result was obtained in at least two independent blotting experiments.

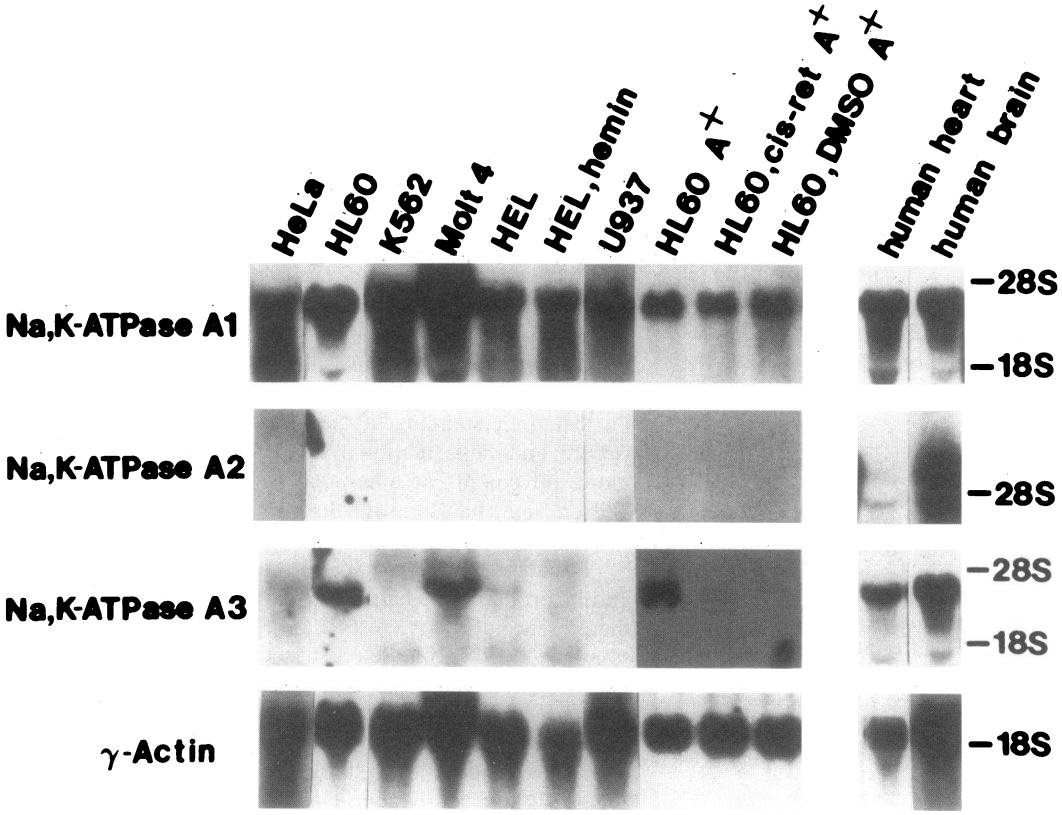

Figure 1. Blot hybridization of Na^+,K^+-ATPase A1, A2, and A3, and γ-actin cDNAs to total (~ 35 μg) or A^+ (~ 1 μg, A1 panel only, or ~ 5 μg) RNAs isolated from various cell lines and tissues. The positions of 18S and 28S rRNAs are shown at the right.

All six of the cell lines examined express Na^+,K^+-ATPase A1 mRNA in approximately equivalent abundance (Fig. 1). This result is consistent with previous characterization of A1 as a ubiquitous enzyme in rat tissues. The high abundance of A1 mRNA in these cells is striking. Na^+,K^+-ATPase A2 mRNA was not found in any of the human cell lines examined, even though we found abundant A2 isoform with this cDNA probe in human neuromuscular tissues, especially brain (Fig. 1). In contrast, mRNA encoding the A3 isoenzyme of Na^+,K^+-ATPase was expressed in only some of these human cell lines; HL60, Molt 4, and HEL cells contained A3 mRNA, whereas Hela, K562, and U937 did not.

Fig. 2 shows the hybridization signals obtained when A1 and A3 cDNA probes (of nearly identical length and specific activity) were annealed to slot blots containing defined amounts of uninduced HL60, HeLa cells, and human fetal brain total RNAs. This result shows that Na^+,K^+-ATPase A1 mRNA is more abundant in total RNA isolated from HL60 cells than in an equivalent amount of RNA from fetal brain tissue, and suggests that A3 represents ~ 20–30% of the total Na^+,K^+-ATPase α isoform mRNA found in HL60 cells.

Na^+,K^+-ATPase α isoform mRNA expression during induced maturation of HL60 cells. Fig. 3 illustrates the substantial differences in the relative content of A1 and A3 mRNA observed after DMSO induction as a function of time. A1 mRNA and actin mRNA both decline by 50–70% ~ 24 h after induction. We routinely observe this degree of mRNA reduction for many HL60 mRNA species after DMSO treatment. In contrast, there is a more dramatic decline of A3 mRNA to virtually undetectable levels. Densitometric scanning of comparable Northern blots is illustrated in Fig. 4. The decline in A3 mRNA activity is ~ 50%, whereas A3 mRNA essentially disappears.

Induction of monocyte maturation in HL60 cells is associated with retention of A3 mRNA and increased expression of the mRNA for the β subunit. Fig. 3 also illustrates the substantially different effects of induction along the monocyte/macrophage pathway of HL60 cells on Na^+,K^+-ATPase mRNA accumulation. A3 mRNA and A1 mRNA are both present at approximately the same levels observed before induction. β mRNA, which is detectable only at low levels in uninduced and early DMSO-induced cells, was noted to increase substantially in a mouse in cells induced with phorbolesters for 48 h. Similarly, β mRNA increased late after DMSO induction between 48 and 72 h. At the present time we have not examined β mRNA production in further detail. However, Fig. 3 clearly shows the differential effects of granulocytic and monocyte/macrophage maturation on the relative accumulation of A1 and A3 mRNAs.

Differential modulation of A1 and A3 mRNA accumulation in HL60 cells may occur at a posttranscriptional level. As shown in Fig. 5, nuclear runoff analysis of mRNA synthesis before DMSO induction reveals considerably more A1 than A3 mRNA synthesis. The disparity between the rates of mRNA synthesis is considerably greater than the amounts of mRNA accumulation noted in Figs. 1–4. Moreover, there is essentially no change in either A1 and A3 mRNA production after 72 h of induction with DMSO. These results imply that the substantial decline in A3 mRNA accumulation observed after DMSO induction may be accomplished at a posttranscriptional level.

Discussion

Our results show for the first time that mRNA encoding the A3 isozyme of Na^+,K^+-ATPase can be expressed by nonneuromuscular cell lineages. Previous results from our laboratory and others suggest a very narrow tissue specificity for this isoenzyme within neural (including retinal) and fetal cardiac tissues. Our present data show that hematopoietic cells of several different lineages, granulocytic/monocytic (HL60), megakaryocytic (HEL), and lymphoid (Molt 4), express the Na^+,K^+-ATPase A3 mRNA. In addition, our results show that two forms of the Na^+,K^+-ATPase α subunit may be expressed within the same cell type. Furthermore, during induced maturation the expression of these two genes is differentially regulated at the level of mRNA accumulation.

The identification of continuous, conveniently manipulated cell lines that express the A3 isoform gene, and the ability to manipulate expression of this form by chemical induction, should provide a useful system for studying the regulation of the A3 gene, and the promoter, enhancer, and/or silencer gene sequences flanking it. Furthermore, since HL60 cells have been shown to be partially susceptible to manipulation of gene expression by the use of antisense oligonucleotides (18), selective reduction of either A1 or A3 mRNA accumulation should

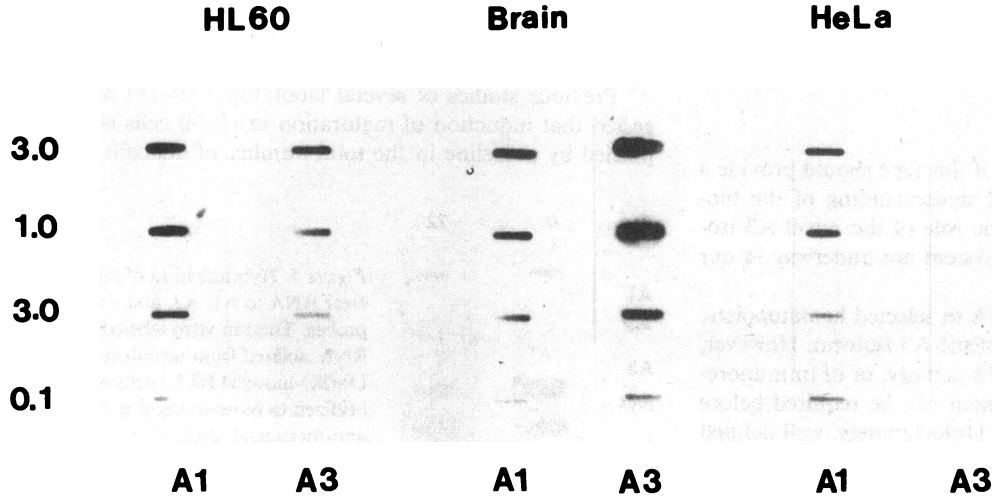

Figure 2. Slot blot hybrization of A1 and A3 cDNA probes to total RNA isolated from uninduced HL60, HeLa cells, and human fetal brain. A1 and A3 cDNA probes were of similar length and specific activity. The amount of RNA in each slot is shown at the right.

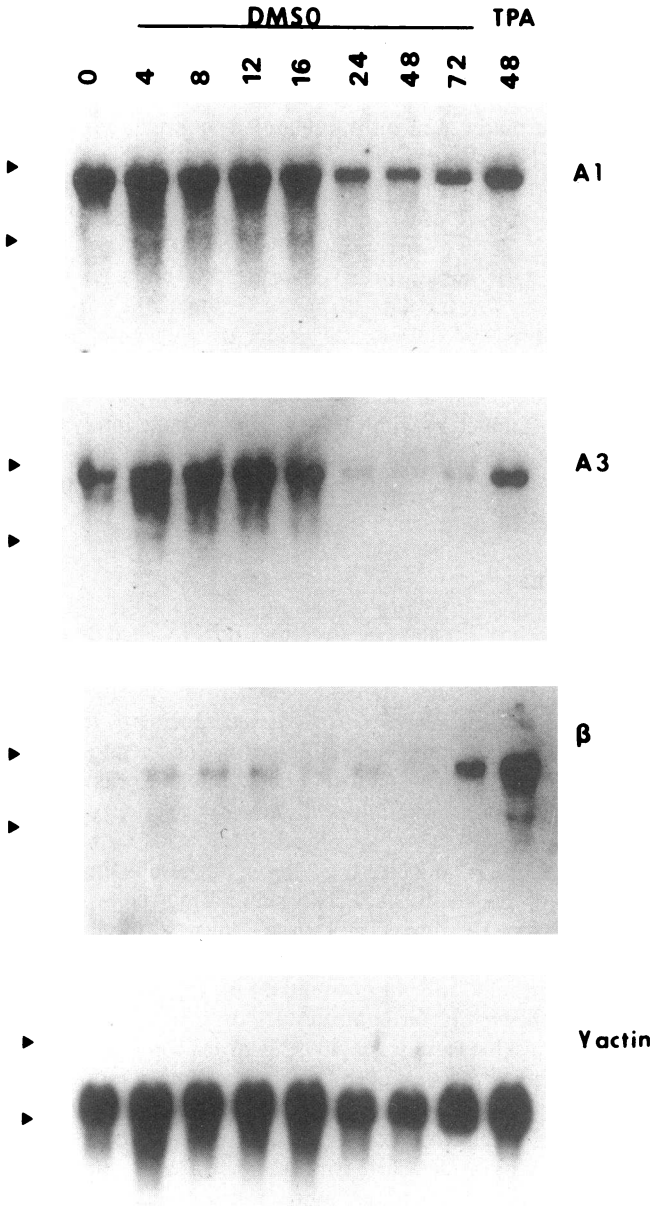

Figure 3. Northern blot hybridization of A1 and A3 mRNA expression during induced maturation of HL60 cells. 30 μg of total RNA from HL60 cells before and at various time points during DMSO or tumor promoting agent induction were hybridized sequentially to A1, A3, β- or γ-actin cDNA probes. Probes were the same size and specific activity. The positions of 18S and 28S rRNAs are shown at the left.

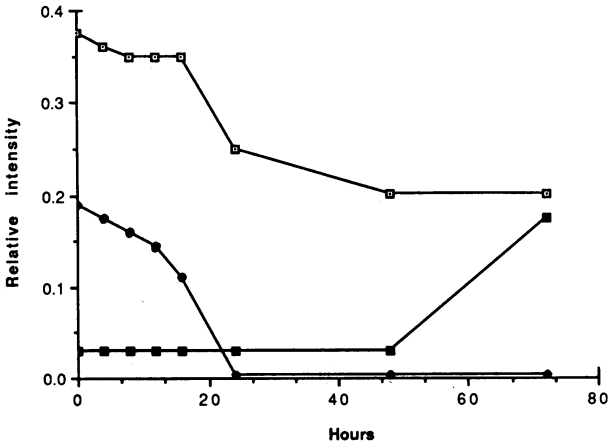

Figure 4. Densitometric scanning of comparable Northern blot. Autoradiographs of 16-h exposures of the hybridization of each cDNA were scanned using a laser densitometer. The intensity of hybridization A1, A3, and β probe for each time was normalized to the hybridization of γ-actin at each point. This value (relative intensity) is plotted versus time during DMSO induction. □, α1; ●, α3; ■, β.

be achievable in culture. Studies of this type should provide a useful system for enhancing our understanding of the biochemical function and physiologic role of the novel A3 isoform. Attempts to establish this system are underway in our laboratory.

The presence of the A3 mRNA in selected hematopoietic cell lineages implies production of the A3 isoform. However, direct demonstration of A3 mRNA activity, or of immunoreactive A3 plasma membrane protein will be required before this presumption can be verified. Unfortunately, well-defined immunochemical probes for the human A3 isoform are just now in the process of development. In collaboration with others (19) we have recently achieved partial characterization of antibodies capable of recognizing specific rat isoforms. However, equivalent probes for human isoforms are not yet available.

The results discussed in this report suggest several unexpected features of Na^+,K^+-ATPase gene expression in certain hematopoietic cell lines. First, as noted above, the A3 isoform is expressed. Second, differential suppression of A1 and A3 mRNA occurs during granulocytic but not monocyte/macrophage maturation. Third, there is a substantial discrepancy between the amounts of α and β mRNA in uninduced and DMSO-induced cells, but not in cells induced along the monocyte/macrophage lineage with phorbol esters. The latter observation is intriguing and consistent with previous studies (4, unpublished studies) suggesting discrepancies in the amounts of α and β mRNA despite the fact that mature Na^+,K^+-ATPase consists of a 1:1 molecular complex of α and β subunits. We have previously documented more efficient translation of β mRNA than A1 rat mRNA in a cell-free translation system, suggesting that synthesis of subunit proteins may be more nearly equal than predicted by the quantitative levels of their respective mRNAs (3). Further investigation of the relationships between mRNA accumulation and translation into protein awaits the development of appropriate immunochemical probes.

Previous studies of several laboratories (9–11) have suggested that induction of maturation in HL60 cells is accomplished by a decline in the total number of ouabain binding

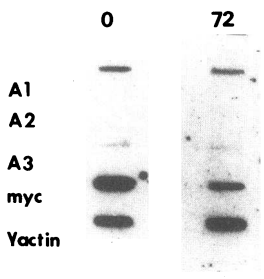

Figure 5. Hybridization of labeled nuclear RNA to A1, A3, and β cDNA probes. Total in vitro labeled nuclear RNA isolated from uninduced or DMSO-induced HL60 cells were hybridized to blots containing 10 μg of anti-messenger sense M13 DNA templates of A1, A2, and A3, as well as 2 μg of γ-actin and *myc* fragment DNA.

sites and Na^+,K^+-ATPase activity. Transient alterations of transport activity have also been reported to occur within the first few hours after induction, although some disagreement exists as to the extent and consistency of these fluctuations. Under all conditions examined, only a single class of high-affinity ouabain binding sites has been detected in HL60 cells; no change in the affinity of these sites has been documented after induction. Our demonstration that two isoforms of the α subunit are expressed within these cells, and that one selectively disappears during DMSO induction from a level sufficiently abundant to have a potential effect on ATPase activity to a virtually undetectable level has several implications regarding these earlier results.

First, we detect no changes in the levels of either A1 or A3 mRNA during the first 24 h after induction. Thus, rapid changes in ion flex detected during the first 12 h must be due to altered translation or posttranslational metabolism and activity of isoforms, rather than altered gene expression.

Second, the presence of only a single class of ouabain sites before and after induction implies that the A3 isoform is either devoid of ouabain binding sites, is less abundant at the protein level than expected on the basis of mRNA accumulation, or is possessed of ouabain binding sites having approximately the same affinity for ouabain as the A1 subunit. Preliminary studies involving transfection of A3 subunit cDNA into surrogate host cells (7, unpublished data) suggest that A1 and A3 mRNA exhibit very similar interactions with ouabain. This observation suggests that two classes of ouabain binding sites should not be a consequence of simultaneous A3 and A1 isoform production. A net decline in ouabain binding sites, however, may ultimately result from reduced A3 isoform production during the later stages of maturation.

In summary, our results suggest that further study of the A3 isoform and its mRNA in human hematopoietic cells should provide additional information about the regulation of the A3 gene and the function of the novel A3 isoform. The superior accessibility of these cells renders them an attractive alternative to studies of select subsets of neuronal or cardiac cells. Conversely, the role of heterogeneous Na^+,K^+-ATPase isoform production in the functioning of mature granulocytes and monocytes should be of interest with regard to the relationships between ion transport and differentiation or function of these cells.

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