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Different factors bind to the regulatory region of the Na^+,K^+-ATPase α1-subunit gene during the cell cycle

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Three factors that bind to the positive regulatory region (ARE) of the Na^+,K^+-ATPase α1-subunit gene were shown to be present in growing BALB/c-3T3 cells as shown by the gel retardation assay pattern in which three specific complexes (C1, C2 and C3) were identified. The complexes are similar to those observed in MDCK cell nuclear extracts in which linker substitution mutations in the competitor gave parallel specific effects in both cells. During the process of the cell growth cycle, the relative mobility of C3 was altered, and the amount of C1 decreased in the G_0 state. All three complexes (C1, C2 and C3) disappeared and other specific complexes with higher mobilities were alternatively observed at 6 h after serum stimulation and thereafter. The expression of the mRNA for the α1-subunit gene was repressed at G_0 and gradually increased after serum stimulation. These results suggest that different sets of factors are responsible for the transcription of the gene at different stages of the cell cycle.

Na^+,K^+-ATPase; Cell cycle; Transcription factor; Gel retardation assay; Nuclear extract; Serum stimulation

1. INTRODUCTION

Na^+,K^+-ATPase is the enzyme responsible for maintaining the Na^+ and K^+ gradient across the plasma membrane through its ion transporting ability, the energy for which is supplied by its hydrolysis of ATP. The enzyme is composed of two subunits, the α subunit that contains the catalytic site and the β subunit whose function is still unclear. Both the α and β subunits exist in three isoforms: α1, α2, and α3 and β1, β2, and β3, respectively [1–4]. The isoforms are encoded by different genes [5]. The α1 subunit gene is one of the housekeeping genes and is expressed in virtually all tissues so far examined. The positive regulatory element of the gene (ARE) is common among various types of cells and resides in the region encompassing positions −102 to −61 in the 5′-flanking region of the gene [6]. The transacting factors that bind to this element have been identified by gel retardation analysis [6] and direct Southwestern cloning [7]. At least six different factors interact with the ARE region. The repertoire of factors is different among various cell types and tissues. In this report, we analyzed the transacting factors binding to the ARE region by gel retardation analysis of nuclear extracts from synchronized BALB/c-3T3 cells and revealed that the composition of the repertoire of binding factors changes depending on the cell cycle.

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Abbreviations: ARE, ATP1A1 regulatory element; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; Na^+,K^+-ATPase, sodium- and potassium-dependent adenosine triphosphatase; PMSF, phenylmethylsulfonyl fluoride.

2. MATERIALS AND METHODS

2.1. *Cell culture and media*

BALB/c-3T3 cells were cultured as described [8].

2.2. *Preparation of nuclear extracts*

Nuclear extracts were prepared as described by Schreiber et al. [9]. BALB/c-3T3 cells were grown in 15-cm dishes; and cells were harvested at the G_0 state and at 2, 4, 6, 8, 10, 12, 24 and 36 h after serum stimulation. The cells were washed twice with ice-cold Tris-buffered saline (TBS: 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl), harvested with a cell-scraper, and then centrifuged at 3,000 rpm for 7 min at 4°C. The cell pellet was resuspended in 1 ml of TBS and centrifuged at 15,000 rpm for 15 s. The washed pellet was suspended in 900 μl of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF), and the suspension was allowed to stand on ice for 15 min. After addition of 58.5 μl of 10% (w/v) NP-40 the suspension was mixed well, and then centrifuged at 15,000 rpm for 30 s. The pellet was resuspended in 30 μl of buffer B (20 mM Tris-HCl (pH 7.3), 0.2 mM EDTA, 1.5 mM $MgCl_2$, 25% (v/v) glycerol, 2 mM DTT, 0.5 mM PMSF) containing 0.02 M KCl. After addition of 120 μl of buffer B containing 0.6 M KCl the suspension was incubated at 0°C for 30 min. Then the suspension was centrifuged at 15,000 rpm for 15 min. The supernatant was collected and stored at −70°C until use. The protein concentrations of the nuclear extracts were determined by the method of Bradford.

2.3. *Gel retardation analysis*

Gel retardation assays were performed as previously described [10]. The probe (containing the ARE sequence) was the *Pvu*II–*Mlu*I fragment (−102 to −58) excised from the rat Na^+,K^+-ATPase α1-subunit gene [11]. The nucleotide sequence of the probe ARE as well as those of competitor fragments containing liker scanning mutations of LS1, LS2 and LS3 is shown in Fig. 1. Sp1 and Oct oligonucleotides (21 bp) were from Promega. The probe was labelled with [α-^{32}P]dCTP, and oligonucleotides were labelled with [γ-^{32}P]ATP.

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        -102                                        -58
ARE:      CTGGAGCCGGTGTCAGGTTGCTCCGGTAACGGTGACGTGCACGCG
          GACCTCGGCCACAGTCCAACGAGGCCATTGCCACTGCACGTGCGC

        -102                                        -58
LS1:      CTGGAGCCGGTGTCAGGTTGCTCCGGTAATCAGATCTGACA
          GACCTCGGCCACAGTCCAACGAGGCCATTAGTCTAGACTGTGCGC

        -102                                        -58
LS2:      CTGGAGCCGGTGTCATCAGATCTGAGTAACGGTGACGTGCA
          GACCTCGGCCACAGTAGTCTAGACTCATTGCCACTGCACGTGCGC

        -102                                        -58
LS3:      CTGGTCAGATCTGAAGGTTGCTCCGGTAACGGTGACGTGCA
          GACCAGTCTAGACTTCCAACGAGGCCATTGCCACTGCACGTGCGC
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Fig. 1. Nucleotide sequence of the probe ARE and competitor LS1, LS2 and LS3 fragments. The nucleotide sequences are shown as double stranded. Positions of *Bgl*II linker substitution are underlined.

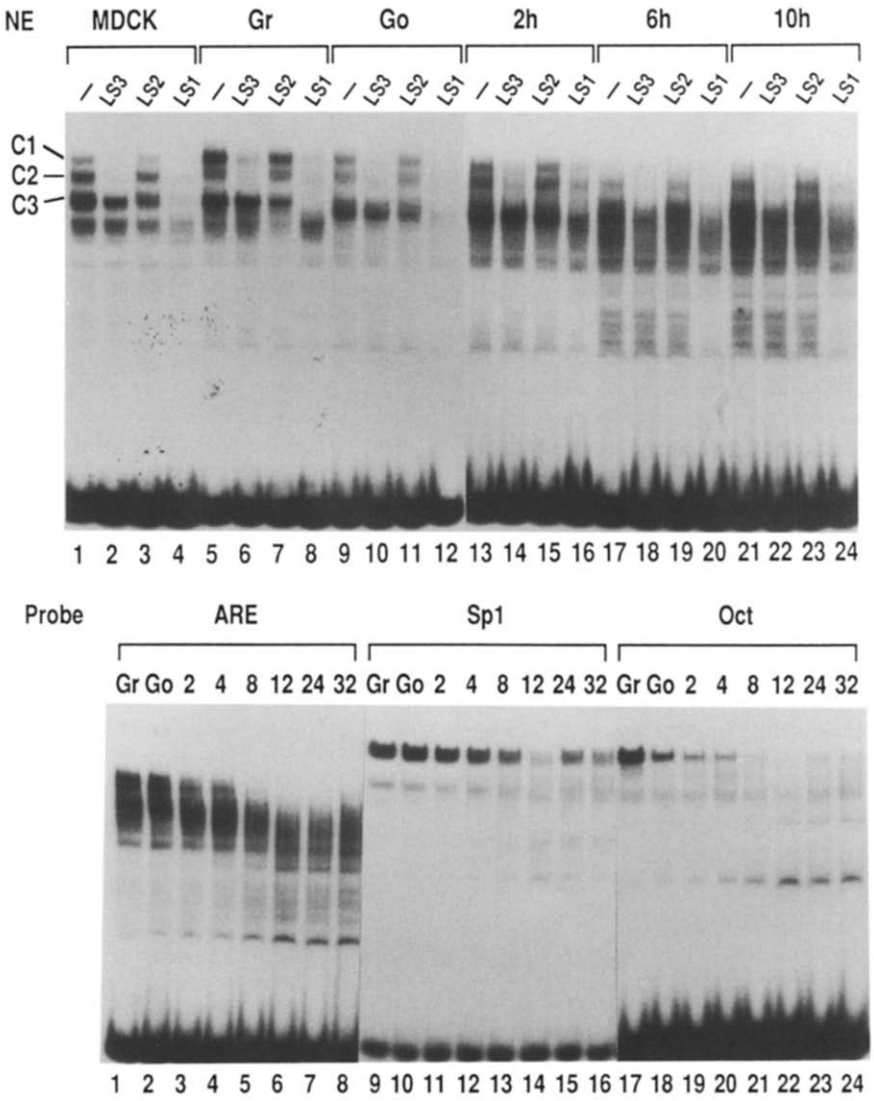
2.4. *Northern blot analysis*

Total RNA was isolated from various stages of BALB/c-3T3 cells according to the method of Chirgwin [12]. A 10-μg sample of the total RNA extracted from BALB/c-3T3 cells was subjected to 1% agarose gel electrophoresis. The probe for hybridization was a 2.2-kb *Nco*I fragment excised from rat Na^+,K^+-ATPase α1 cDNA [13]. Hybridization was performed by using QuikHyb (Stratagene) according to the manufacturer's protocol, except that the final wash was performed with 0.2× SSC, 0.1% SDS at room temperature.

3. RESULTS AND DISCUSSION

3.1. *Gel retardation analysis of 3T3 cell nuclear extracts*

To identify the binding factors of the positive regulatory element in the Na^+,K^+-ATPase α1-subunit gene during the cell cycle, we prepared nuclear extracts from BALB/c-3T3 cells at various cell growth stages. In growing cells, the major gel retardation complexes identified with the ARE probe were similar to those previously identified in the MDCK cell nuclear extract [6] (compare C1, C2 and C3 in Fig. 2A, lanes 1 and 5). The properties of the complexes were similar to those from MDCK cells on the basis of specific competition by three linker substitution mutations, which are the *Bgl*II



linker substitution at −73 to −64 for LS1, at −87 to −78 for LS2, and −98 to −89 for LS3 (lanes 2–4 and lanes 6–8). When the cells entered into the G_0 state, the relative quantity of C1 decreased and the relative mobility of C3 increased to become C3* (compare lanes 5 and 9). Specific antibody to the C3 factor interfered with the formation of the C3 in the growing cells but not in the G_0 state (Kawakami, K. unpublished observations). This suggests that C3* contains immunologically distinct proteins from those in C3 of the growing cells. The specific competition pattern was not different from that of growing cells (lanes 10–12). After 2 h of serum stimulation, the competition pattern of gel retardation complexes was similar to that of the G_0 stage (lanes 13–16). However at 6 h, C1 disappeared and the complex corresponding to C3* became vulnerable to competition by LS3 (lanes 17–20). Furthermore, several faster migrating complexes susceptible to competion by LS1 were observed (lanes 17 and 20). The results were essentially the same as those at 10 h (lanes 21–24). The binding proteins were analyzed in different batches of nuclear extracts. The complexes corresponding to C1, C2, C3 and C3* disappeared at 12 h and thereafter (Fig. 2B, lanes 6–8). As a control, we analyzed the Sp1 and Oct factors with specific probes. Cells at the growing stage, G_0 stage, and 2 h after serum stimulation showed roughly the same binding activites of Sp1, but the activities had gradually decreased by 4 h and 8 h after serum stimulation. By 12 h after the stimulation, the complex had decreased significantly (the decrease was also significant at 10 h, data not shown). The decrease in the complex might coincide with the entry of synchronized cells into the S phase, since BALB/c-3T3 cells enter the S phase at 12 h [14]. In contrast, a single Oct complex was observed in growing cells (lane 17). After entering into G_0, the binding activity of Oct protein dramatically decreased. This phenomenon has been observed in other contact-inhibited cells of the WI38 or HeLa cell lines [15]. A further reduction was observed after serum stimulation until 12 h.

C1 and C2 were observed in all cell types examined so far, while C3 was only observed in certain cell types such as MDCK or L6 [6]. In this experiment, these complexes were observed only in the nuclear extract from growing cells, because the mobility of C3 was altered in G_0. This implies that different sets of transacting factors become involved in the transcription of the gene in the late G_1, S and M phases.

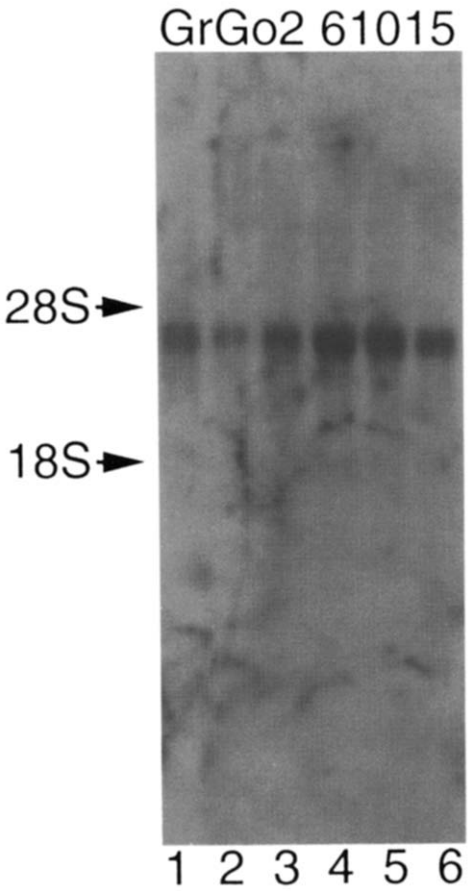


Fig. 3. Northern blot analysis of BALB/c-3T3 cells at various growth stages. Total RNA from the growing stage (lane 1), G_0 (lane 2), 2 h after stimulation (lanes 3), 6 h after stimulation (lane 4), 10 h after stimulation (lane 5) and 15 h after stimulation (lane 6) were analyzed. Positions of 28 S and 18 S ribosomal RNA are indicated.

3.2. *Northern blot analysis of Na^+,K^+-ATPase α1-subunit gene expression*

To determine if the expression of the α1 subunit gene is modulated during the cell cycle, we performed Northern blot analysis. Total RNAs from synchronized cells were hybridized with the rat Na^+,K^+-ATPase α1-subunit cDNA probe. mRNA in the G_0 state was repressed compared to the level of growing cells, while after serum addition, the mRNA levels were gradually increased (Fig. 3, lanes 3–6) and remained constant until 30 h (data not shown). This raised the possibility that the change of ARE binding factors correlates with the change in transcription level of the α1-subunit gene,

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Fig. 2. Gel retardation analysis of BALB/c-3T3 cell nuclear extracts. (A) Nuclear extracts from MDCK cells (lanes 1–4), BALB/c-3T3 growing cells (G_r) (lanes 5–8), cells in the G_0 state (lanes 9–12), 2 h after serum stimulation (lanes 13–16), 6 h after stimulation (lanes 17–20) and 10 h after stimulation (lanes 21–24) were analyzed by the ARE probe (described in section 2). Competitors added are shown above the lanes; −, in the absence of competitors. The positions of specific complexes C1, C2 and C3 are indicated. The protein amount of the nuclear extracts used are 3.7 μg for MDCK, 5.8 μg for the growing stage, 5.2 μg for G_0, 5.9 μg for 2 h, 5.8 μg for 6 h and 6.9 μg for 10 h. (B) Nuclear extracts from various stages, growing (G_r) (lanes 1, 9 and 17), G_0 (lanes 2, 10 and 18), 2 h after stimulation (lanes 3, 11 and 19), 4 h after stimulation (lanes 4, 12, and 20), 8 h after stimulation (lanes 5, 13, and 21), 12 h after stimulation (lanes 6, 14 and 22), 24 h after stimulation (lanes 7, 15 and 23) and 32 h after stimulation (lanes 8, 16 and 24) were analyzed by ARE (lanes 1–8), Sp1 (lanes 9–16) and Oct (lanes 17–24) probes.

although we have not excluded the possiblity that the stability of the mRNA may have changed. We had observed that the ST2 gene, which was expressed only after the serum stimulation, was induced in different time course in the serum stimulated BALB/c-3T3 cells compared with that of the Na^+,K^+-ATPasc $\alpha 1$ subunit gene [16]. Na^+,K^+-ATPase $\alpha 1$- and $\beta 1$-subunit messenger RNAs increased rapidly after folic acid treatment of the kidney in vivo (folic acid induced quiescent renal cells to proliferate), with a peak at 6 h, followed by a decline up to 24 h, but remained above the non-treated control level [17]. The time course of induction was somewhat different from those we observed in 3T3 cells. This may be due to the different system or the different time course of cell cycle progression.

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